CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Exploration of Prevalent Bacterial Gut Microbiome of *Culex quinquefasciatus(Culicidae)*

by

Shahir Bano

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Dedicated to Allah Almighty, Hazrat Muhammad (S.A.W.W) and my father Muhammad Sibtain. My mother prayers have always enlightened my way throughout my life. It's also dedicated to my brother and all other family members who taught me that the best kind of knowledge to have is that which is learnt for its own sake. They taught me that even the largest task can be accomplished if it is done one step at a time.

CERTIFICATE OF APPROVAL

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(Shahir Bano)

Abstract

Mosquitoes are primary vector for transmission of many economic importance diseases like nuisance, systematic and local skin reactions and also for acute death causing disease like dengue, malaria and West Nile Virus. *Cx.quinquefasciatus* is known as southern domestic mosquito and responsible for many allergic and viral diseases like West Nile Virus and lymphatic filariasis. Midgut microbiota of this mosquito consist of many gram negative and positive bacteria. The microbiome of adult *Cx.quinquefasciatus* midgut were isolated on differential culture media and the results indicate the presence of *E. coli, Staphylococus,Klebsiella spp and Lactobacillus spp.* Biochemical characterization of prevalent bacterial specie revealed it a gram positive, Urease negative, Citrate positive and Catalase negative. The results of 16s rRNA sequencing indicates its close association with *Lactobacillus reuteri* specie with 99% similarity. Phylogenetic analysis showed that the sequenced strain seems to independently evolved and revealed ancestral similarity with *Uncultured lactobacillus sp, and lactobacillus reuteri.* The strain antibiotic sensitivity was checked against three antibiotics. The strain showed highly resistance against Imipenem with 93.3% and least with Nalidixic acid with 15.38% and showed 0% insecticide resistance against Allethrin , Resmethrin and Thiacloprid. The computational results of the functional genomic of the whole genome obtained by the NCBI Genome and RAST server, it was observed that *Lactobacillus* species were not involved in any of the aromatic compound metabolic pathways but 15 subsystems were identified to be involved in the antibiotic resistance pathways.

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Abbreviations

WNV	West Nile Virus
BLAST	Basic Local Alignment Search Tool
SLEV	Saint Louis Encephalitis Virus
WEEV	Western Equine Encephalomyelitis Virus
RVF	Rift Valley fever Virus
WHO	World Health Organization
MAC	Mosquito Abatement Coordination
MDR	Multidrug Resistance
AR	Antibiotics Resistance
PBS	Phosphate Buffer Solution
E.coil	Escherichia coli
EMB	Eosin Methylene Blue Agar
MACC	MaCconkey Agar
B.A	Blood Agar
MSA	Mannitol Salt Agar
SCA	Simmon Citrate Agar
UAB	Urea Agar Base
Rrna	Ribosomal RNA
MEGAX	Molecular Evolutionary Genetic Analysis
NCBI	National Center for Biotechnology Information
S.aureus	Staphylococcus aureus
TSB	Tryptic Soy Broth
UPGMA	Unweighted Pairwise Group with Arithmetic Mean

Chapter 1

Introduction

1.1 Background

Mosquitoes are primary vector for transmission and spread of many importance diseases like systematicreaction,nuisance and local skin reaction. Mosquito is also responsible for many acute and death causing diseases like chikungunya, dengue, malaria lymphatic filarasis, yellow fever and other arboviruses [1]. Mosquitoes cause many deaths and cases every year. Culex *(Diptra:Culicidae)* species are the largest species of mosquitoes in the world [2].

It is known as a most blood feeder on human and animals and this behavior increase chance of transmitting many zoontonic diseases and this increase its significance in public health [3].

Among the culex, *Culex quinquefasciatus* is most important species and it is also known as southern domestic mosquito, this mosquito is found in subtropical areas of world and most common in Asia, Africa, Australia, Africa, New Zealand and United states [4].

Cx.quinquefasciatus is a threat to citizens in open environment as in well as inside of the home. *Cx.quinquefasciatus* is responsible for transmission of many

diseases like West Nile Virus and filariasis. *Cx.quinquefasciatus* is very important for spreading of filariasis and very important vector of this disease in tropical forests [5]. Many pathogens of human and wild/domestic animals are carried by *Cx.quinquefasciatus*. Many viruses such as SLEV, WNV and WEEV are also transferred by this species. In South America, SLEV is mostly caused by *Cx.quinquefasciatus*. This species reproduces in the body of bird.When a healthy mosquito feeds a blood meal from diseased bird, it got infected and able to transmit this pathogen in a healthy host. This usually occur in spring the nesting season of bird. This virus is then transfer to human through mosquito, and the aged people are more susceptible to disease than young ones. The symptoms of disease are same as that of flu, including fever and headache. In severe condition, symptoms may include confusion and stiffness. After onset of these symptoms, extreme stress occurs. The brain starts swelling due to drowsiness, sleepiness, excitement or stress. *Cx.quinquefasciatus* is not only important in public health but also causes many severe noctornal allergic reactions and discomforts by biting its host. Not only mosquito cause many diseases in birds and humans but the biting of this mosquito also effect a large proportion of population as it can cause severe reaction and allergy by biting [6]. The pain cause by this biting is sometimes unbearable very high and unbearable. This mosquito also shows resistance to many insecticides.It is fact that the susceptibility of *Cx.quinquefasciatus* to pyrethriodes such as permethrin and deltamethrin to is relatively lower than that of *Anopheles stephensi* [7] [8].

In recent studies during zika virus outbreak some of *Cx.quinquefasciatus* were found in urban areas which predict the participation of *Cx.quinquefasciatus* in a new era of the emerging Arbo virus. According to World Health Organization about 146 million people effected with filariasis every year [9]. In Pakistan, during the summer months, there has been a high level of population growth of *Cx.quinquefasciatus* recorded in different cities [10]. The adult female of mosquito lay eggs in large permanent aquatic habitat that contain high amounts of decomposing organic matter, such as sewage effluents and septic tanks [11]. However, the immature stages of this species are often found in artificial containers filled

with contaminated or organic water, but rarely coexist with Aedes aegypti in the same container [8]. As a result of rapid urbanization and unplanned development in cities, the production of mosquito habitats is increasing the growth of vectors of various diseases and resulting in the spread of diseases. Use of gutters, drainage channels and other physically rich, stable sources of water for elliptical location and larval development [12] [7]. Due to overcrowded cities and unhealthy conditions, Pakistan is at high risk of epidemics. Misuse of pesticides in agriculture and public health programs has created toxic hazards such as pesticide resistance, rehabilitation of pest species, humans and other non-targeted organisms, and environmental pollution [13]. To overcome these risks, the researcher needs to innovate biological plants and genetically modified plants that can produce the products which can be used for alternative synthesis of antibiotics [14]. As synthetic insecticides are responsible of producing resistance in bacteria living in gut of mosquito. Mosquito control is still the primary strategy for controlling mosquito-borne diseases. Resistance to mosquito repellents, resistance to parasites, cost of developing new drugs, limitations of vaccines and environmental risks of pesticides all call for the development of disease eradication strategies [15]. The larva of *Cx.quinquefasciatus*, live in all types of water reservoirs including permanent and temporary water like tanks, drains, ponds, Paddy, marshy, wet pits and other organic contaminated sites [6] and this from these larva most common type of mosquitoes emerged that found in major cities of Pakistan [10]. The immature, larvae of mosquitoes has been challenged by a number of insecticides. The aquatic stage is also where larvae acquire microbes that colonize them and eventually become gut microbiota. This microbial environment, mediated by gut microbiota, is critical for subsequent survival and success, especially during the adult stage [16]. Mosquitoes have symbiotic microorganisms in their gut and also have many bacteria in saliva. These microorganisms contribute in nutrition, reproductive trait and other physiological changes of mosquito, some bacteria also help in detoxification of mosquito diet and their contribution in insecticide resistance is also noticed [4]. Chemical insecticides are used to control mosquitoes and with passage of time mosquitoes are evolving strong resistance against insecticides so there is need to identify the bacteria which

are helping mosquito to evolve resistance against insecticides [5].

1.2 Problem Statement

Cx.quinquefasciatus mosquito is important vector for transmission of many economic and medically important diseases. Keeping in view the importance of microbiome as a supportive instrument in survival of insects particularly in vectors, there is need to target specific methods for vector (mosquito) control strategies.

1.3 Objectives

The study entails following objectives:

1. To isolate and identify most prevalent bacterial specie from gut of mosquito (species *Cx.quinquefasciatus*).
2. To evaluate antibiotic and insecticide resistantance in most prevalent bacterial species in gut of mosquito.

1.4 Scope

The findings of these studies can help the researchers and people working in this field to explore the role or impact of microbiota in mosquito such as physiology, development and insecticidal resistance etc. It can help to design the better mosquito control program to control the vector and vector associated diseases.

Chapter 2

Review of Literature

2.1 Morphology

Mosquitoes are small (up to 15 mm), two-winged insects. Since it is considered the primary vector of the disease, culex mosquito is recognized worldwide as a West Nile Virus spreading mosquito. This mosquito species seem to have preferred their blood meal to birds and mammals, including humans. Adult mosquito is dark brown to black in color and its body consists of three parts, head, thorax and abdomen [17]. Length of adult culex mosquito is about 3.96 to 4.25 mm. The proboscis, thorax and wings are brown in color and tarsi that darken the rest of the body. The head of this mosquito is light brown and in middle it is lightest brown. Antennas and probes are the same length, but in some cases the antenna is slightly shorter than the proboscis. There are thirteen segments in flagellum that have at no scales [18]. The scales of the thorax are narrow and curved. The abdomen has a light, narrow, round band at the base of each side. Bands barely touch the crescent-shaped bandwidth spot [19]. The *Cx.quinquefasciatus* lay their eggs in stagnant water so they fly to stagnant water at night fly to the stagnant water so that they can lay eggs. Their eggs will sink in everything so they lay eggs in old tires, paddies, marshy places, bird baths or any container. If the water dried up before hatching of eggs or completing of life cycle of mosquito, they die. The larvae

of *Cx.quinquefasciatus* takes five to eight days at 30 ^{o} C for their development and during this time take biotic food in water [20]. In larval stage there is series of four instars and when it comes up at its forth instar larvae stop eating further food and grow up into pupil. After 36 hours at 27-36 ^{o} C adults emerge from pupil and now they are ready to fly [20]. The time for development is different for all stages and depends upon natural conditions and temperature variables. Both male and female take sugar from plants as their food but females needs blood meal for eggs development. *Cx.quinquefasciatus* are opportunistic feeders as they find birds and mammals at night for blood but blood is only taken by female mosquito. Eggs are developed when female mosquito digest blood meal and the female find a place to lay eggs in this way another cycle starts. Female mosquito lays four to five rafts in its life and the number of eggs are variable according to environmental conditions [20].

2.2 Habitat

Cx.quinquefasciatus adapted to survive in domestic and peridomestic habitats in urban and semi urban environmental conditions and are most dominant species in these environments [17]. There are generally four type of *Cx.quinquefasciatus* which are paddy, canal, pond and swamp [21]. They prefer paddies that grow in large numbers and cover floating vegetation, and plants with cold dirty water. Canals with deeper climates with less sulfur water, less emerging vegetation and more floating vegetation. Marsh was a deep, clear-water habitat close to human habitation, with a small amount of floating vegetation and a large cover of emerging plants. Ponds with swords, the hottest and most disturbing houses with a small amount of floating and emerging plants. The habitat of *Cx.quinquefasciatus* has a higher water delivery value and an abundance of other water invertebrates. *Cx.quinquefasciatus* is a domestic mosquito and associated with human activities and habitats [22] [23]. The eggs of this specie do not survive in drought and dry condition that's why this is found in remote areas and established in natural environments this one is also confined in tropical to subtropical areas of the world.

Artificial containers and man-made objects like grounds, pits, pounds and stock ponds are also commonly habitats of these mosquitoes [23].

Larvae of these mosquitoes are also found in natural areas like river banks, rock holes, ground pond, coconut husks and tree trunks [24]. These larvae also prefer water with organic contents. Some extraordinary larvae are also found in sewer ponds, cesspools and septic tanks. Adults mosquitoes take blood from many regions where there is natural conditions and wild and domestic birds and mammals are available as well as humans. *Cx.quinquefasciatus* are founds in forms of clutches of European ships and in this way they spread in all around world. The eggs or larvae of mosquitoes spread through ships as they are hidden in many plastic goods and water in ships [25].

2.3 Metamorphosis

Mosquitoes exemplify tremendous environmental flexibility in which the following examples of successful reproduction of mosquitoes can be demonstrated. Similar like Diptera, these mosquitoes can also show complete metamorphosis. *Cx. quinquefasciatus* need aquatic environments for their growth. Most types of mosquitoes are unautogenous (unnatural), which follows copulation these female mosquitos need blood meal for egg development and complete their life cycle. Only a small number of mosquito species have populations that are autogenous [26].

2.3.1 Oviposition

The Oviposition means that the egg is released from the ovaries into the external environment and this is a common phenomenon in vertebrates other than eutherian mammals. After feeding of blood meal female *Cx.quinquefasciatus* lay eggs in the form of oval rafts and there are 100 or more eggs in each raft and these eggs are hatched in 24 to 30 hours after that. These mosquitoes lay eggs on surface of water but the parameters choose by female mosquito for laying eggs are still

unknown. Female choose suitable breeding ground in such key factors like light incidence, water quality, existing rafts of eggs and availability of food [27]. The content of organic matter in water is main key to attract female for laying eggs and this factor is very known for culex species because culex female select site for eggs laying according to quality of water and other food contents of organic matter in water. When gaseous components like methane, ammonia and carbon dioxide are released from water in results of dissolving organic matters, these attract female mosquitoes to lay eggs [28]. *Cx.quinquefasciatus* always lays eggs in oval shaped rafts and each rafts includes more than hundred eggs and lay eggs when it is in standing position on the surface of water. When the development of larvae is complete, the larvae are not admitted to the distress or depots and hatches. The presence of different chemicals and matters included in water also effect on eggs and larvae and may cause change some of its genes position. The developmental stages of culex are often found in permanent species and female choose another breeding season in same water. The number of generations in same water depends upon the biotic and abiotic factors, and most specifically climate temperature that slows down according to stages of development [29]. The presence of different chemicals and matters included in water also effect on eggs and larvae and may cause change some of its genes position. The number of generations in same water depends upon the biotic and abiotic factors, and most specifically climate temperature that slows down according to stages of development [30].

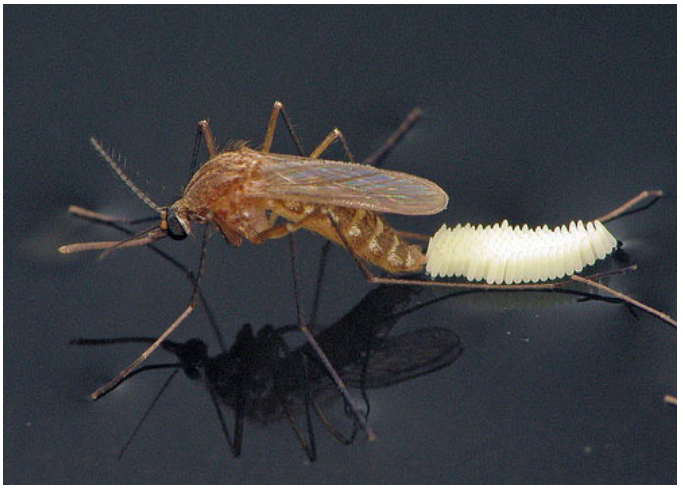


FIGURE 2.1: Oviposition [29]

2.3.2 Adults

After few days' eggs are ready to hatch and produce larvae. The larvae required biotic matters in water to feed, in suitable conditions and temperature 30^o C larvae takes 5-8 days for complete development. There are four instars of larvae and developed gradually when the fourth instar is ready larvae stop eating and further growing. After fourth instar larvae molt to pupal stage and after 36 hour adults emerge from the pupa. The head of pupae is short and pointed that's dark from the base. The mouth brushes have yellow filaments and eight segments on abdomen. The development depends upon temperature and other natural conditions [30]. When adult emerge from pupae pressure increase by adult hemolymph and legs and wings grows up. Pupae come at surface when it is ready to change into adult. They immediately empty their gut and droplets are drain and air is also expelled from gut. After few minutes' adults are ready to fly when thein cuticle is sclerotized. Male need one day and female one and half days to adjust metabolism according to new environment. When they emerge they have difference in sexual maturity both male and female. Male mosquito are not sexually mature when they are emerge because they rotate their hypopigmentation through 180^o before they are ready to develops and they take about 24 hours for this while male in population emerge in 36 to 48 hours earlier tha female for attaining sexual maturity [31]. Size of adult *Cx.quinquefasciatus* is between 3.96 to 4.25 in length. The mosquito is dark brown to black in color and its body consists of three parts, head, thorax and abdomen. Length of adult culex mosquito is about 3.96 to 4.25 mm. The proboscis, thorax and wings are brown in color and tarsi that darken the rest of the body. The head of this mosquito is light brown and in middle it is lightest brown. Antennas and probes are the same length, but in some cases the antenna is slightly shorter than the proboscis. The scales of the thorax are narrow and curved. The abdomen has a light, narrow, round band at the base of each side. Bands barely touch the crescent-shaped bandwidth spot [32]. The pupal of both male and female is same in length but male larval development is short that's why male pupae and adults are short in length and smaller size in population as

compare with female. After fully development adults are ready to feed mate and reproduce for their life cycle [33].

2.3.3 Mating

Mating is coupling of opposite sex and produce their offspring. Mosquitoes also perform mating for sexual reproduction. The swarming patterns of male and virgin females are very similar except some turning points and speed of flight [34]. Their response against swarm "marker" is same. Male mosquito identify and locate female by sound produced by beating of female wings. Wings beating frequency is different for different mosquitoes. In the case of *Cx.quinquefasciatus* the wings beating frequency of female is 500-600 Hz. Response of male may be in form of altering their flight speed or turning behavior on sound once they located [35]. After swarming male and females comes close in face to face and sperms are moved to spermatheca. Male accessory glands secrete a substance matrone, this make female unacceptable for mating for rest of her life. A female can mate at once in her life but male can mate again and again. Female store sperms for fertilizing batches of eggs in rest of her life so she needs male mosquito only once in her life. After that female only needs blood to develop batch of eggs and use sperms stored in spermatheca of female [36].

2.4 Ecological Importance

Cx.quinquefasciatus plays an important role in nature. In the ecosystem, mosquitoes have several roles that are ignored. Therefore, mosquitoes still have ecological functions. The fundamental food of all adult mosquitoes is plant sugar and its associated nutrients, most often in the form of floral nectar. In the process of looking for nectar, mosquitoes pollinate many of the flowers they visit. This is one of the most commonly overlooked ecological functions of mosquitoes. Mosquitoes, however, play essential roles in many ecosystems, serving as food for many species,

helping to flourish by filtering detritus for plant life, pollinating flowers, and also affecting herding paths. Without mosquitoes, thousands of plant species would lose a group of pollinators [37]. Mosquitoes are present everywhere in large number because they reproduce throughout the year with high reproduction rate and are adaptive in almost all kinds of environment. Mosquitoes will affect the ecosystem both positively and negatively. Mosquito larvae remain in water as part of their useful function to provide food for fish and other animals, including larger larvae of other species, such as dragonflies. Mosquito larvae are aquatic insects and, play an important role in the aquatic food chain. Adult mosquitoes, such as butterflies, bats, adult dragonflies and spiders, make up part of the diet of certain insect-eating animals. When they consume nectar, they also help in pollinating certain flowers. Mosquitoes may also play a damaging role, harming other species; however, by being a vector of diseases such as malaria, yellow fever, encephalitis and dengue. Mosquitoes do not themselves cause infections, but only function as vectors [38]. On the lower rungs of the food chain, mosquitoes appear to represent a significant food biomass for wildlife. Mosquito extinction may have a negative impact on the ecosystem if it were achievable. Many scientists, however, believe that the ecosystem could gradually regenerate and that another species might take its place in the environment. Livestock and humans get affected by the increase production of the arthropods because of increase in animal production.

2.5 Medical Importance

Many pathogens of human and wild/domestic animals are carried by *Cx. quinquefasciatus*. Many viruses such as Saint Louis Encephalitis Virus, West Nile Virus and Western Equine Encephalitis Virus are transferred by this species. In South America, Saint Louis Encephalitis Virus is mostly caused by *Cx.quinquefasciatus*. This species reproduces in the body of bird. When a healthy mosquito feeds a blood meal from diseased bird, it got infected and able to transmit this pathogen in a healthy host. This usually occur in spring the nesting season of bird. This virus is then transfer to human through mosquito, and the aged people are more

susceptible to disease than young ones. The symptoms of disease are same as that of flu, including fever and headache. In severe condition, symptoms may include confusion and stiffness. After onset of these symptoms, extreme stress occurs. The brain starts swelling due to drowsiness, sleepiness, excitement or stress [39] [40]. In human blood, there is no extreme level of virus, and hence human is considered as "dead host" [41]. In Florida,*Cx.quinquefasciatus* is not a potential carrier of WNV but have a role in infecting the birds, so infecting humans too [42]. Filariasis and Rift Valley Fever Virus (RVF) caused by *Cx.quinquefasciatus*. There are different types of filaraisis -causing species, some of which cause lymphatic filaraisis 120 M cases of which are approximately reported. The microfilariae are picked up by mosquito from an infested animal/bird. After this, nematode formation occurs in the body of mosquito, and it is transferred to another mosquito afterward [41]. In Asia and Africa,Rift Valley Fever Virus is becoming main cause of epidemics. In Kenya and Southern Somalia more than 300 persons were infected with Rift Valley Fever Virus in 1997 [44].

There are many mosquito species, which are termed as "medically important", because they cause transfer of disease causing agent such as nematodes, bacteria, viruses and protozoan. These entities cause many diseases in human i.e. West Nile Virus, malaria and filaraisis [45]. The transfer of these agents may occur biologically or mechanically. The physiology of these mosquito is quite complex due to the fact that parasite requires a critical period of reproduction or development in carrier. If there is a suitable environment, the parasites is transmitted from one host to another due to blood sucking behavior and mode of nutrition of mosquitos. Mosquito's larval growth is controlled by microbial control management, in many tropical areas. These management agents give many types of trails such as; they provide actual and operative control, they are safe, healthy and eco-friendly and they are effective for human as well as other species. Mosquito borne diseases can be controlled by successful implement of Mosquito Abatement Coordination and by the use of effective vector management [46]. In fact, the exact transmission requires close contact to the host. It allows pathogen to reproduce and develop to mature stages. Actual transfer occur when blood transmitted many times. If

we talk about vector-borne syndromes or infection and mortality caused by them, mosquitos considered as most disastrous entities for humans. More than 3 billion people in different regions are at risk of being infected. Due to this, safety of mankind is now not just a social issue, but it is economic and political too. Most pandemics and epidemics occur in the world, are undeniably due to insect-borne parasites [47]. Up to 17% of these diseases are human infestation, and due to these, 1,000,000 people deceased every year. Mosquitos are very important factor of vector-borne diseases and transmit parasites, but also some others insects i.e. mites, flies, ticks are involved in spread of parasite. There are hundreds of diseases which are caused by mosquitos, some are concisely discussed. *Cx.quinquefasciatus*, is the mosquito species which cause WNV. Wuchereria bancrofti; a is filarial nematode, is important carrier of WNV. In tropical region, flariasis is fastest transmitting disease [2]. Besides this, *Cx.quinquefasciatus* cause some other problems such as allergy and nocturnal discomfort. It affects the people more than a severe disease, sometimes effects are so high, and level of bedlam is unbearable and agonizing [16].

Samples of *Cx.quinquefasciatus* were found in different cities during latest Zika pandemic in Brazil. According to research and new suggestion, this zika virus is cause of emergence of a new virus, Arbo virus. Rendering to WHO, around 146 million people effected with filariasis every year [50].

2.5.1 West Nile Virus

In many countries, the basic cause of mosquito borne infections is WNV (West Nile Virus). There are many countries which are badly affected by this parasite. These may include Greece, Italy, Serbia, USA, Romania and Hungary. According to research, these areas were affected in past by West Nile Virus [48]. The main cause of its transmission in human is biting of infected mosquito. When a healthy mosquito bites an infected bird or mammal, virus is transmitted into that mosquito. This virus is then transmitted to other animals or humans, when infected mosquito bites them. This is usual case, but in some cases this virus has

exceptional transmission i.e. via blood transfusion, laboratory exposure, organ transplantation, during pregnancy, childbirth or lactation. The important point is that, this is not transferred through touching, sneezing and coughing. West Nile Virus usually emerges in late autumn and starting of summers, the mosquito season. There are no treatments or medicines to cure the virus in human. Usually, the person having West Nile Virus feel no illness. The symptoms like fever, could be seen in 1 out of 5 patients. Out of 150 cases, 1 individual develops fatal and serious-type illness and infection. As discussed, there is no treatment for this disease, only prevention can save life. Precautionary measured could be taken by wearing full sleeve shirts and long trousers and by using insect repellent in order to minimize the chance of mosquito biting [49].

2.5.2 Lymphatic Filariasis

Lymphatic Filariasis-an infectious disease which is caused by small sized thread like worm is universally known as NTD (neglected tropical disease). The only host of mature or adult stage is human lymphatic system. The lymphatic system is a system of our body which provides protect from invaders and cause regulation of fluid balance. From a mosquito, Filaraisis is transmitted through person to person. Several conditions i.e. lymphedema or elephantiasis may occur in person suffering from Filaraisis [50]. When a mosquito bite a diseased person, it is infected by microscopic worm which is present in circulatory system of that person. When this infected mosquito bites the normal person, the worm passes via skin of the human body and travel to lymphatic vessels. Here, this worm got maturation or adult stage. The age of adult worm may be up to 7 years. Mating occur in lymphatic vessels and millions of microfilariae (small worms) are produced and released in blood circulatory system. Infected persons can transfer the infection through mosquitos. In effected persons; the infection destroys the lymphatic system completely, but these may be asymptomatic, having no critical symptoms at all. All the permanent disabilities in the world are usually caused by lymphatic filariasis. Persons having such diseases became defaced, and in a society, they

are unwanted and being rejected. Disable or immobilized patients are not able to function or even act properly, due to this disease. This on the other hand also affects their family and communities. The active infection in the body of human can be diagnosed by microscopic examination of infected blood [51]. There exist three different species which can cause lymphatic filaraisis. This affects up to 120M people in 72 countries of sub-tropics and tropics of Asia, Western Pacific, Africa, South America and Caribbean region. The parasite is spread by multiple variety of mosquitos depending on the geographical area. Generally, the dominated carrier of parasite in Africa and America is *Anopheles* and *Culex* respectively. While talking about Asia and Pacific regions, the carrier is *Mansonia and Ades* [52].

2.6 Microbiota of Insects

Microbiota of insects plays a vital role in many biological processes like development, nutrition, digestion, sexual reproduction and most importantly prevention of pathogens [53]. Microbiota is also involved in insecticides resistance. According to recent researches. Many specific bacterias role specific for different purposes like Wolbachia can shorten lifespan of some mosquitoes [54] [55]and also inhibit spread of some viral mosquitoes [56].

2.6.1 Microbiota Associated with Mosquito

There are not only bacteria in gut microbiota of mosquito but also many viruses, prokaryotes and eukaryotic microbes are part of mosquito gut microbiota. They get their microbiota from their parents but mainly derived from environment where there they live that's why microbiota is highly dynamic and varies in different species according to developmental stages and environment they live [56]. To study independent microbiota structure of mosquito gut microbiota there is need to culture these bacteria and then sequencing of 16s rRNA or 18s rRNA is needed [57]. The juvenile stage of mosquito is aquatic and adult is terrestrial. The

larvae of mosquito feed on organic matter in water, unicellular organisms, small invertebrates and other debris adult male and female take nectar from plants but female needs blood of vertebrates for development of eggs. They not only take blood but also responsible for transfer of many pathogens in host. So, these foods may help them to develop their gut microbiota. Studies before 20 century shows that larval and adult mosquito harbor communities of extracellular microbes from their gut microbiota to digestive tract. But recent studies show that the microbes in gut of mosquito have many important roles in biology of mosquito and a hot topic for research. Results of many recent studies suggest that gut microbiota of adult female mosquito effect both positively and negatively on host. It shows that female adult mosquito have ability to maintain and transmit diseases through pathogens. Gut microbiota of female adult mosquito plays vital role in transmission of many diseases [53].

2.6.1.1 Acquisition of Gut Microbiota by Mosquito Larvae

Mosquitoes derive their gut microbiota genetically from parents or they may get it from the environment where there live and feed. Different evidence show that mosquitoes derive their gut microbiota from environment. According to first research evidence that larvae of mosquito obtain their gut microbiota from environment and according to matter in water they do not have extracellular microbes in their gut [53]. According to another research it is indicated that most of microbes in gut of mosquito have structure of microbial community present in water where they live [54]. According to third line of focus mosquitoes have microbes in their gut which cannot be expected from environment these may derived from parents or any other source. Larvae feed on eggshells and other organic matter in water where they live microbes derived from aquatic media where they live their juvenile stages or life. Mosquitoes also transmit intracellular bacteria like Wolbachia and other genera in their eggs. Some viruses are also present and have been transmit but these do not make part of that microbes which make gut microbiota of mosquito [55]. According to culture-based study larval mosquito expel its gut

microbiota into meconium during metamorphosis and adult develop with gut microbiota. Results show that gut microbiota was developed by using water in larval stage and food resources in aquatic medium which is organic matter and extra floral nectaries. Control group show that gut microbiota of larvae was transmitted to adults. The gut microbiota of adult mosquito can be replaced by water, nectar and other food resources containing microbe's intake by mosquito. Blood they feed also contains microbes, composition of blood permanently or temporarily alters the structure of gut microbiota of mosquito by altering the metabolism [58].

2.6.1.2 Composition of Gut Microbiota of Mosquito

According to many recent researches it is evident that gut microbiota of mosquitoes is largely consist of gram negative anaerobic bacteria mostly belonged to four phyla; Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria. These genera of bacteria found in gut of mosquito are also members of general community of insects which get their gut microbiota from habitat. Many of bacteria's which living in gut of mosquito are those which have been isolated and cultured in laboratory. Identification of many viruses is also found in many survey of gut microbiota of mosquito. Most viruses are members of small families and also with small RNA genome like Flaviviridae, which one is also consider as susceptible of many mosquitoes born pathogens. But the phase of virus is not cleared in published studies this suggests that this virus may infects bacterias present in gut of mosquito or the bacteriophages that effects the bacteria in mosquito gut [59].

2.6.1.3 Gut Microbiota of *Culex.quinuefasciatus*

Cx.quinquefasciatus is the major vector of West Nile Virus and filarial nematoda. Bacteria colonized in gut of mosquito are important in mean of association with host of mosquito and pathogens they transmit. These bacterias are present in midgut of mosquito and also effect the physiology of mosquito. Bacteria species found in *Cx.quiquefasciatus* are following.

TABLE 2.1: Bacterial species in gut microbiota of *Cx. quiquefasciatus*

Sr.No	Bacterial Genera	Species	Accession No.
1	*Acinetobacter*	*A. baumannii*	APD20249
		A. beijerinckii	
		A. junii	
		A. lwofii	
		A. pittii	
		A. soli	
		A. schindleri	
2	*Arthrobacter*	*A. creationlyticus*	ATCC21022
3	*Aerococcus*	*A. uurinaeequi*	ATCC21022
4	*Aeromonas*	*A. enteropelogenes*	AY522923
		A. hydrophila	
		A. ichthiosmia	
		A. veronii	
5	*Bacillus*	*B. anthracis*	AL009126
		B. aryabhattai	
		B. cereus	
		B. circulans	
		B. flexus	
		B. nealsonii	
		B. subtilis	
6	*Citrobacter*	*C. braaki*	MN548424
		C. frei	
7	*Delftia*	*D. lacust*	JOUB01000005
8	*Enterobacter*	*E. asburiae*	ERR1854846
		E. cancergenus	
		E. cloacae	
		E. ludwigii	
		E. ludwi	

Table 2.1 Bacterial species in gut microbiota of *Cx. quiquefasciatus*

Sr.No	Bacterial Genera	Species	Accession No.
9	*Enterococcus*	*E. caccae*	AY754011
		E. faecalis	
		E. hirae	
		E. silesiacus	
10	*Escherichia*	*E. coli*	AE005671
		E. hermannii	
11	*Exiguobacterium*	*E. aurantiacum*	ACB62096
		E.indicum	
		E. profundum	
12	*Janibacter*	*J. melonis*	PRJNA13546
13	*Klebsiella*	*K. oxytoca*	HF536482
		K. pneumoniae	
		K. variicola	
14	*Kocuria*	*K. marina*	CP035504
		K. carniphila	
		K. palustris	
15	*Kytococcus*	*K. schroeteri*	MN911377
16	*Lactococcus*	*L. lactis*	HM219853
17	*Leucobacter*	*L. tardus*	MT335639
18	*Lysinibacillus*	*L. macroides*	MTD16782
19	*Microbacterium*	*M. arborescens*	MG754432
		M. imperiale	
20	*Micrococcus*	*M. lylae*	KM37667
		M.yunnanensis	
		M. maritypicum	
21	*Morganella*	*M. morganii*	KY12032
22	*Pantoea*	*P. anthophila*	CP022427
		P. dispersa	

Table 2.1 Bacterial species in gut microbiota of *Cx. quiquefasciatus*

Sr.No	Bacterial Genera	Species	Accession No.
23	*Proteus*	*P. vulgaris*	AJ250100
24	*Providencia*	*P. alcalifaciens*	KF295828
		P.rettgeri	
25	*Pseudomonas*	*P. cuatrocienegasensis*	MH235964
		P. aeruginosa	
		P. stutzeri	
		P. beteli	
26	*Serratia*	*S. marcescens*	KM492926
27	*Shigella*	*S. flexneri*	AF288197
28	*Sporosarcina*	*S. luteola*	LT601384
29	*Staphylococcus*	*S. agnetis*	CP028841
		S. aureus	
		S. caprae	
		S. epidermidis	
		S. gallinarum	
		S. haemolyticus	
		S. hominis	
		S. saprophyticus	
		S. succinus	
		S. warneri	
		S. xylosus	
30	*Stenotrophomonas*	*S. maltophilia*	MT 335840
31	*Vagococcus*	*V. fluvialis*	SS1994627

2.6.2 Culex Virome

Mosquito act as an exclusive host for a large group of virus which are insect-specific. A metagenomic approach was used to evaluate viral load [60] [61]. 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 [62]. Most resident virome act as commensal microbe due to its inability to infect vertebrate cell lines, prolonged host infection and vertical transmission [63].

2.6.3 Culex 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) [64]. 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 [62]. Different genera of yeast like Candida, Pichia and Wickerhamomyces have been identified in Culex 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 [58].

2.6.4 Role of Microbiota in Mosquito Physiology and Pathogen Transmission

Microbiota of mosquito plays a vital role in many mosquito's biology, as well as nutrition, digestion, mating and sexual reproduction, growth, pathogenesis and immune response functions [65]. 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 [66].

2.6.5 Role of Gut Microbiota of Mosquito in its Nutrition, Reproduction and Development

The gut microbiota of mosquito is developed with its environment and also effect on mosquito's nutrition, reproduction and development. This microbiota is depending on variety of species, development and geography. The microbiota specifically colonized in midgut and rarely in salivery glands and also in reproductive organs. Mosquito microbiota plays a vital role in host nutrition, digestion, coupling, sexual reproduction, growth, immune function and pathogenesis [63].
The scientist compared the transcriptome between infected and uninfected adult female mosquito's feed different diets and found that some of the genes involved in digestion and metabolic processes are stimulated by the presence of microbiota [64].

2.6.6 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. 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. 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.

2.6.7 Applications of Microbiome of Mosquito

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.

2.7 Antibiotic Resistance

Antibiotics are those kinds of compounds that are produced by certain microorganisms that could destroy or inhibit other microorganism's growth. Nowadays many synthetic compounds with similar functions including beta-lactams, cephalosporins and carbapenems are also called antibiotics. Following the initial discovery, the

antibiotics were commonly used as a veterinary medicine in humans, and were important for protecting human and animal health from pathogens. Number of times bacterial infections have been successfully treated with antibiotics, the likelihood of survival and quality of life of humans and other animals worldwide is greatly enhanced. There are lots of uses of antibiotics, seen just after the first application of antibiotics in the 1930s [88].

The rapid development of hospital acquired infections by antibiotic-resistant (ART) pathogens and opportunistic pathogens such as Clostridium di cile, vancomycin-resistant Enterococci (VRE) and Resistant to Fluoroquinolone *Pseudomonas aeruginosa* (FQRP) has become concern of major health problems in recent years [89] [91].

2.7.1 Antibiotics Resistance Mechanisms

The most common survival strategy of antibiotic resistant bacteria survival strategy is by reducing the concentration of the inner cellular antibiotic to the sub-lethal level in the presence of environmental antibiotic. Antibiotic resistant bacteria use three major mechanisms of this strategy i.e. permeability reduction of the cell wall to antibiotics, antibiotics expulsion, and antibiotics destruction by upgrading an antibiotic-inactivating enzymatic pathway [90].

Target-mediated antibiotic resistance is another less frequent strategy, variant target molecule of certain antibiotics with lower binding affinity with the antibiotics were produced by using targeted mediated antibiotic resistance strategy for the normal or near normal metabolic function.

Bacteria follow three routes to develop antibiotic resistance: specific natural cellular property makes it intrinsically resistant (insensitive), target gene under strong selective pressure and transmit the gene vertically to the off spring are the conditions when it accumulate mutations, and acquire resistance through horizontal transfer. Compared to the limited cases of intrinsic resistance and the low frequency of mutation (around 108-109), horizontal transmission of antibiotic resistant determinants plays an important role in the rapid dissemination of antibiotic

resistance [92].

2.8 Insecticides Resistant

Numerous studies have showing that the individual mosquito species are involved in multiple mechanisms of resistance. In particular, two mechanisms increased 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 .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 [71]. 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 [72] Three major metabolic gene families are being involved in the mechanism of the detoxification of insecticides in mosquitoes: Esterases, cytochrome 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 [73]. The largest quantity of the

exogenous aswell as the endogenous compounds in the metabolic detoxification and the excretion are GSTs which are dimeric protein having the property of the solubilization [74] [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] [78]. 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 [79] strongly support the importance of mitochondrial detoxification in insecticide resistance [87]. 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 study 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.quinquefasciatus* the metabolism of permethrin to 4-hydroxypermethrin by microsomal P450 monooxygenases was stated to be significantly greater [80].

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 mosquito 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 [85].

Chapter 3

Materials and Methods

3.1 List of Equipment

1. Autoclave,
2. Magnetic stirrer,
3. Measuring balance,
4. Laminar hood,
5. Incubator,
6. Vortex,
7. Water bath,
8. Microscope,
9. Shaker,
10. Refrigerator,
11. Fishing net,
12. pH balancer.

3.2 List of Apparatus

1. Beakers,
2. Spatula,
3. Conical flask,
4. Eppendorf Tube,
5. Micropipette,
6. Petri dishes,
7. Spirit lamp,
8. Fishing net,
9. Aspirator,
10. Icebox,
11. Inoculation loop,
12. Glass rod,
13. Dissecting needle,
14. Dropper,
15. Parafilm and
16. Graduated cylinders.

3.3 List of Chemicals

1. Nutrient Agar,
2. Blood Agar,
3. MacConkey Agar,

4. Mannitol Salt Agar,
5. Oxidase test,
6. Catalase test,
7. Urease test,
8. Eosin Methylene Blue Agar,
9. Simmon's Citrate Agar,
10. Saline and Distilled Water,
11. Crystal violet and Safranin.

3.4 Methodology Flowchart

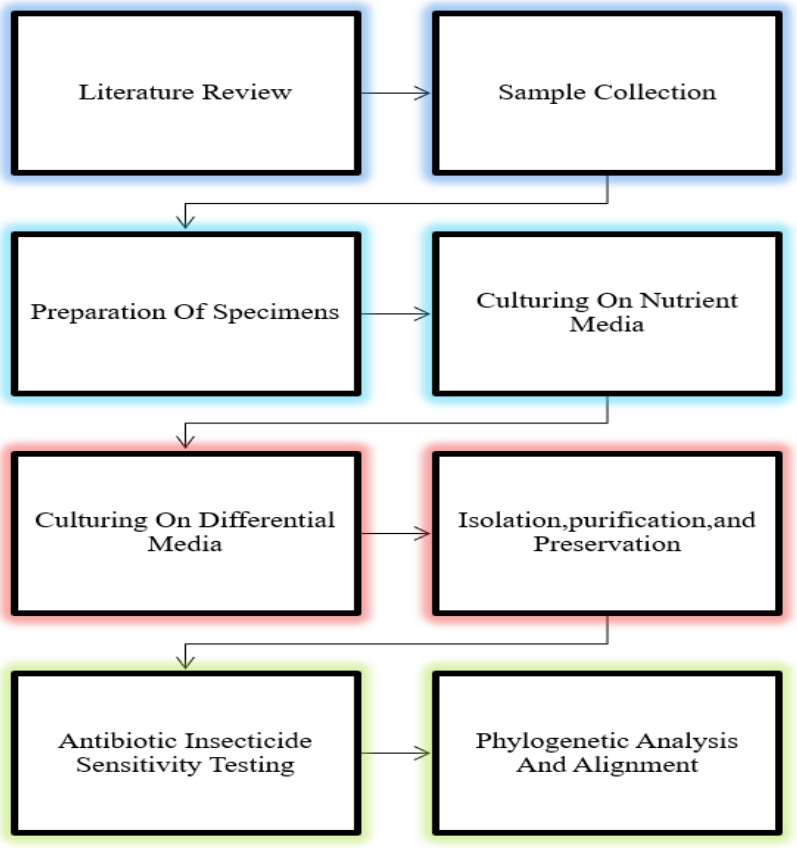


FIGURE 3.1: Methodology of Project

3.5 Sample Collection

Mosquitoes were collected from 2 different categories of Islamabad from houses and the public places.

Randomly mosquitoes were collected from house and public parks. In each location 2 points were selected to capture adult mosquitoes. Mosquitoes were collected in dawn and dusk.

3.6 Sample Collection Method

Sample was collected by mean of Aspirator, Fishing net, Mosquito trapper racket and mosquito landing on hand. Some mosquitoes were collected from walls because they used to rest on walls after feeding. After collection of mosquitoes they were transferred to glass bottles in ice box and transported to the laboratory. They were kept in refrigerator at -4^{o}C.

3.7 Preparation of Sample

A total 300 mosquitoes were separated by mean of their morphology [93]. Prior to the midgut dissection mosquitoes were surface sterilized by washing in 70% ethanol then rinsed four times in 1X PBS.By putting the mosquitoes in a petri dish, they suffer from anesthesia which is kept cold on ice.

FIGURE 3.2: Mosquito before dissection

3.8 Dissection

3.8.1 Protocol

Transfusion of midgut from mosquitoes requires pre-preparation of 1X phosphate buffered saline (1X PBS) solution and anesthesia of mosquito's subject to a temperature of 4^o C, until immobilized. By putting the mosquitoes in a petri dish, they suffer from anesthesia which is kept cold on ice. Other required materials include: 10x objective in light microscope, equipped with pipette, fine tip top forces, glass slide, needle tip probe.

3.8.2 Procedure

- 1X PBS drop was placed on the glass slide with mosquito and glass slides under the light microscope.
- Mosquito was moved to adjust its position on slide under microscope and mosquito's thorax was hold with needle probe.
- Mosquito was held with the probe, by using the straps to grasp the other end of the abdomen and gently pulled the mosquito's abdomen in one motion. The midget was attached to the active thorax.
- Then mid gut was separated from thorax by using force [94].
- Throughout the dissection procedure in laminar flow and observed under microscope dissection needles and forceps were dipped and sprayed in every dissection using 70% of ethanol. Wings, legs and head were removed and gut was separated and confirmed under microscope. As shown in the Figures 3.3,3.4.
- Then the collection of 50 guts were squashed and incubated in water bath and 3ml of 1X PBS was transferred then stirrer on vortex then again incubated in water bath for 4 hours.

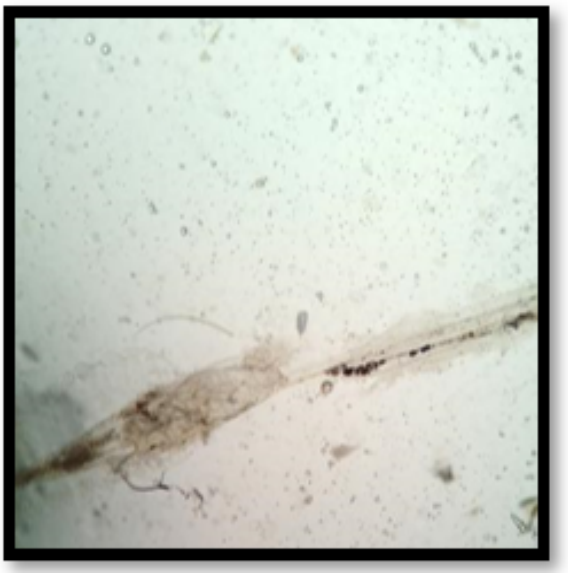


FIGURE 3.3: Gut of *Cx. quinquefasciatus* under microscope 1

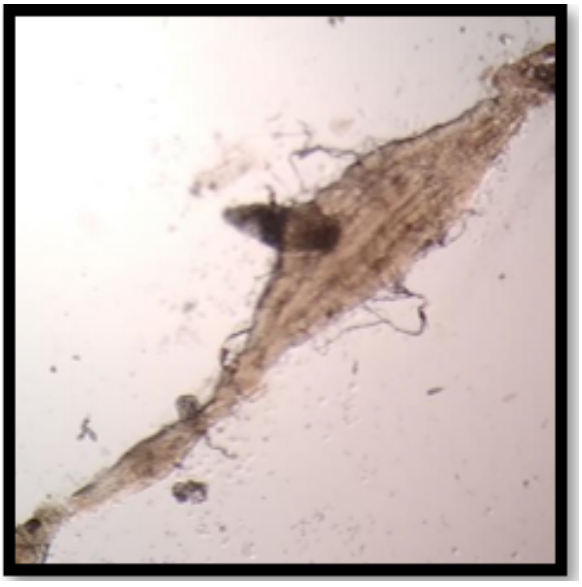


FIGURE 3.4: Gut of *Cx.quinquefasciatus* under microscope 2

3.9 Growth on Nutrient Agar

To verify the presence or association of bacterial pathogens in mosquito's gut, the sample was cultured on the Nutrient agar. Nutrient agar was used for the growth of bacterial pathogens. Nutrient Agar of 5.6g was weighed by measuring balance and added in the 200 ml of distilled water. The mixture was autoclaved at 121^{o}C for 15 to 20 minutes. Autoclaved 20ml of media was poured into sterile petri plates uniformly under laminar flow. The sample was spread uniformly on

the Petri plate through spreader 5ml of prepared sample was poured in plates were spread on 10 plates containing Nutrient Agar. Each location was replicated 5 times. Plates were incubated for 24 hours at 37^{o}C. Plates were incubated in upside down direction to avoid the moisture.

3.10 Growth on Differential Media

Differential media used for identification of aerobic bacteria were MaCconkey Agar (Macc), Mannitol Salt Agar (MSA), Eosin Methylene Blue Agar [EMB], Simon citrate agar [SCA] [95]. Blood Agar (BA) was used for anaerobic flora.

3.10.1 MaCconkey Agar [Macc]

250 ml distilled water was added to 13.75 grams of dry powder of MaCconkey with continuous stirring by magnetic stirrer it was then autoclaved for 15-20 minutes at 121^{o}C. The final media was poured in petri dishes. Under the laminar flow hood total of 10 plates were prepared and were allowed to solidify at room temperature.

3.10.2 Eosin Methylene Blue Agar [EMB]

Volume of 250ml of distilled water was added to 9.375g of Eosin methylene blue agar. Magnetic stirrer was used for stirring and proper mixing of media. Prepared media was autoclaved at 121^{o}C for 15-20 minutes. Media was left for solidification at room temperature after pouring it in petri plates.

3.10.3 Mannitol Salt Agar [MSA]

27.75 grams of powdered MSA was added to conical flask containing 250ml of distilled water. The media was mixed and autoclaved 15 to 20 minutes on 121^{o}C. The media has been left to solidify at room temperature after pouring.

3.10.4 Blood Agar [BA]

7 gram of blood agar powder was added in 250ml of distilled water. Magnetic stirrer was used for stirring and proper mixing of media. Prepared media was autoclaved at 121^{o}C for 15-20 minutes. Media was left for solidification at room temperature after pouring it in petri plates.

3.11 Streaking of Culture Media for Aerobic and Anaerobic Growth

The bacterial colonies grown on Nutrient agar were streaked on the differential media. The criteria for selection of bacteria was color, shape and morphology. Every bacteria taken from nutrient agar plate was streaked on prepared differential media. After streaking plates for aerobic growth were incubated in incubator 37^{o}C for 24 hours and plates of Blood Agar were placed in jar where alka setizer tablet was dissolved in water and jar was air tight for anaerobic bacteria growth. Culturing on different media was replaced for 3 times to get the pure strains. From different media growth two bacterial colonies that were prominent and prevalent were selected on preserved.

3.12 Preservation of Purified Stains

Glycerol stock of 100ml was prepared for the preservation of purified strain. 50% of glycerol was prepared by dissolving 50ml of glycerol and 50ml of distilled water. it was autoclaved at 121^{o}C for 15-20 minutes. 2.5ml eppendorf tubes were taken and autoclaved at 121% for 15 minutes. The eppendorf tubes were numbered in the laminar flow hood. 1ml of glycerol solution was filled in these eppendorf tubes with the help of 1000μL pipette. Suspension was made with loop full of bacteria picked from each differential media and added into eppendorf tubes containing glycerol stock. Eppendorf tubes with bacteria and glycerol were kept at -4^{o}C.

3.13 Gram Staining

3.13.1 Preparation of Crystal Violet Solution

Crystal violet was prepared by dissolving 2g of crystal violet in 10ml of ethanol. Solution was stored in the eppendorf tubes.

3.13.2 Preparation of Gram Iodine Solution

0.03g of iodine pearl, 0.667g of potassium iodide and 0.1g of sodium bicarbonate were dissolved in 10ml of distilled water for preparation of iodine solution.

3.13.3 Preparation of Safranin Solution

0.1g of safranin was dissolved in 4ml of 95 percent concentrated ethanol for the preparation of stock solution. The working solution was obtained by adding one part of stock solution in the five parts of distilled water.

3.13.4 Preparation of Destaining Solution

5ml of 95 percent ethanol was added and mixed with 5ml of acetone for making destaining solution. It was further stored in the eppendorf tube for gram staining purpose.

3.13.5 Gram Staining Procedure

Gram staining procedure was first developed by the Hans Christian Gram in 1844. As a differential staining method, it differentiates gram positive and gram-negative bacteria. A glass slide was cleaned with 75 percent ethyl alcohol then the dilutions was prepared by adding a loop full of purified bacterial culture in the 2ml of

sterilized water in the beaker. A drop of bacterial suspension was poured in middle of slide and slide left air dry. After that heat was provided using spirit lamp for 60 seconds to fix. On the heat fixed bacterial stain drop of crystal violet was added and left for 30 seconds; it was rinsed with sterilized water and blot the water with blotting paper around the bacterial stain. After that, 3-4 drops of Gram iodine were added on the slide and was left for one minute. The slide was again rinsed with sterile water for one minute. Decolorizer was used for washing, which contain 95% ethanol, it was run through the stained area so that it decolorizes the stain and washes out the color, the slide was again rinsed with sterile water. Then 3-4 drops of safranin were added and left for one minute after that rinsed. Cover slip was placed on the slide and blot the moisture from sides and slide was observed under microscope at 40X. The gram negative bacteria shows pink color and gram-positive bacteria shows purple color.

3.14 Biochemical Characterization

Different types of biochemical tests were performed for the biochemical characterization of two prevalent selected strains [96].

3.14.1 Citrate Utilization Test

Bacterial strains with citrate utilization are called citrate positive and those without citrate utilization are called citrate negative. For the execution of this test, 100ml of Simmons citrate solution was prepared. 2.424g of Simmons Citrate was taken and dissolved it in 100ml of distilled water in conical flask. After that, it was autoclaved for 15-20 minutes, at 121^{o}C. Media was poured in the petri plate. Total six plates were prepared for biochemical test. The isolated bacterial strain was inoculated on the Simmons citrate media plates, by taking a loop full of bacteria from each plate. The plates were then incubated in the incubator at 37^{o}C for 48-72 hours after proper wrapping. Green color of media turned blue is called as citrate positive other that don't cause color change are citrate negative.

3.14.2 Urease Test

This test is basically use for the utilization of urea by the bacterial samples. For this test, the Urea Agar Base [UAB] was weighed 2.5g. Then added it in the conical flask with 100ml of distilled water in it. After proper mixing, the conical flask was properly covered and prevented from the contamination, it was autoclaved for 15 to 20 minutes at 121^{o}C. The media was poured into the six plates. The plates were stored in the refrigerator for future use for one day. Streaking of isolated cultures was done on the plates containing Urea Agar Base [UAB]. The plates were incubated in the incubator at 37^{o}C for 48-72 hours. The bacterial strains with pink color are urease positive and other that don't turn the color into pink are urease negative.

3.14.3 Catalase Test

Catalase is an enzyme, enzyme that decomposes hydrogen peroxide into water and oxygen. Hydrogen peroxide forms as one of the byproduct of aerobic carbohydrate metabolism. If this oxidative product remains in the body of bacteria, it becomes lethal for their survival. The reagents that are present in the catalase test contain 3% hydrogen peroxide [97]. A loop full of bacteria from pure culture were taken and placed on the slide. In addition, two drops of 3% H2O2 was added on the slide to check the production of hydrogen peroxide in the bacteria.

3.15 16S rRNA Sequencing

The high throughput is the earliest technique to study the microbial ecology is the use of '16S rRNA sequence that seems to be the most conserved one. It is cost effective approach in a community for the survey of bacteria [98]. In order to determine the microbiota associated with the gut of mosquitoes the preserved strains were send for 16s sequencing, the samples were sequenced from Microgen Korea.

3.16 BLAST and Phylogenetic Analysis

Basic local Alignment Search Tool [BLAST] is the tool available at NCBI that is used for the alignment of sequence with the reference sequence and give the similarity index according to the matches, mismatches, and gaps. The BLAST results for strain S1–785 that gave 99% similarity index with 0% query coverage, MEGAX was used to find out the phylogenetic history of specie. The sequences closest to the strain S1–785 were taken, total of 6 sequences, gave closest similarity to that of S1–785. The sequences were pasted on a separate file. This file was imported in MEGA and then aligned by muscle. After the alignment, the low quality sequences were removed and file was subjected to phylogenetic analysis. The tree was constructed using Maximum likelihood method.

3.17 NCBI Submission

After the removal of low quality sequences, sequences were submitted on the NCBI.

3.18 Antibiotic and Insecticide Sensitivity Test

The most important part of disease management is to determine the antibiotic resistance pattern of bacteria for different antibiotics and insecticides. Kirby and his colleagues A. W. Bauer first developed the disk diffusion method which is alternative of previous broth dilution methods. The test was coined to check the resistance of strains isolated and sequenced that either they are resistant to antibiotics or susceptible. The strains, with less zone of inhibition, show resistant to that specific antibiotic and the strains with more zone of inhibition are susceptible one.

Therefore, in this perspective firstly the nutrient broth was prepared, for the preparation of 100ml TSB, 3g of TSB was taken in the flask having 100ml of distilled

water. After proper shaking the flask was wrapped with aluminum foil, and autoclaved along with six clean wrapped test tubes at 121^{o}C for 15 minutes. It was inoculated with bacteria and kept overnight at 37^{o}C in incubator so that bacteria may grow into the broth.

3.18.1 Kirby Bauer Method Procedures

Muller-Hinton agar media was set having standardized composition. Muller-Hinton agar media was poured into 150 mm petri dishes at a level of 4mm deep. The agar media was maintained at pH range of 7.2 to 7.4 and broth culture was used for inoculation. The culture plates were made inoculated by streaking a sterile swab passed through broth culture of bacteria. The agar media plates inoculated with bacteria was left for about five minutes to dry.
The antibiotics disks were transferred to the inoculated agar plates by using sterilized needles. The discs were gently press by using flame-sterilized forceps to make sure that each disc is in contact with surface of agar media properly. The plates were incubated at incubation temperature of 37^{o}C for the night. The zone of inhibition was measured for each antibiotic disc by using scale or screw gauge which determined the effectiveness status of the antibiotic against bacteria.

3.19 Comparison of Computational and Wet Lab Results

3.19.1 Whole Genome Sequencing

In whole Genome Sequences Extraction 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.

3.19.2 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 [100]. 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.

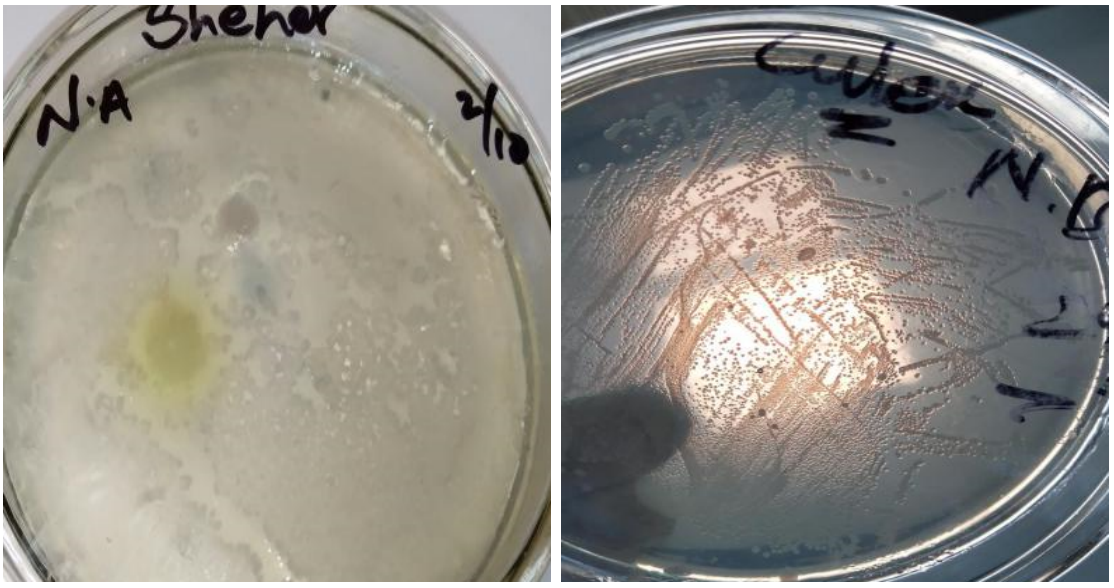
Chapter 4

Results and Discussions

4.1 Nutrient Agar Growth

Bacteria were isolated from gut of mosquito. A general-purpose nutrient agar media was used to culture the isolated bacteria. It is used for the growth of variety of bacteria and fungi [99]. The nutrient agar is chemically composed of peptone, beef extract and agar. This type of simple formula composition provides the sufficient nutrients to bacteria which are favorable for their growth and their genome replication [97].
Nutrient Agar allows the growth of gram-positive as well as gram-negative bacteria. The culture results showed the growth of variety of bacteria.The prepared sample after 24 hours of incubation at 37^oC showed the growth of bacteria.Agar allows the growth of gram-positive as well as gram-negative bacteria which help us to differentited them easily.

The sample was spread uniformly on the Petri plate through spreader 5ml of prepared sample was poured in plates were spread on 10 plates containing Nutrient Agar. Plates were incubated for 24 hours at 37^o C. Plates were incubated in upside down direction to avoid the moisture. Bacterial colonies with different colours and shape appeared on nutrient agar and these colonies were transferred to differential media.



(a) (b)

FIGURE 4.1: (a) Nutrient agar plate 1 with bacterial growth, (b) Nutrient agar plate 2 with bacterial growth

4.2 MacConkey Agar

The purpose of MacConkey agar used is to isolate the gram negative bacteria extracted from gut of mosquito. MacConkey agar differentiate fermenting gram negative bacteria from lactose non fermenting gram negative bacteria. Chemically it is composed of gelatin and peptones which is an extraction of meat and casein. These different chemicals provide the source for nutrients and vitamins for the growth of microorganisms. The bacterial pathogens which can grow from MacConkey agar i.e. includes *E. coli*, *Enterococcus*, *Klebsiella*, *Aerobacter pseudomonas*.

MacConkey media only allows the growth of gram-negative bacteria hence it inhibits the growth of gram positive bacteria. Bacteria were isolated from gut of *Cx.quinquefasciatus*. MacConkey agar inhibits the growth of gram-positive bacteria. The results showed that all the three locations specimens showed the bacterial growth indicating the presence of gram negative bacteria. MacConkey agar contain Bile salts which prevent most of gram-positive organisms to grow. Neutral red and

crystal violet present in this medium are very lethal to bacteria. Gram-negative bacteria are more resistant to the dyes present in this medium than gram-positive bacteria. Moreover, Bile salts reduces this toxicity for gram-negative bacteria and increase toxicity for gram-positive bacteria. Gram negative bacteria usually shows more significant growth on medium and these bacteria can differentiate due to their lactose fermenting ability.

The lactose fermenting bacterial strains shows red or pink coloured colonies and which may be surrounded by a zone of acid precipitated bile. The pink colored colonies show the presence of *klebsiella* which are rod shaped. The red colored pattern is just due to the releasing of acid from lactose, when pH of medium drops below 6.8 in the result. Absorption of neutral red starts and lateral change in colour of the dye occurs. While lactose non-fermenting bacterial strains like Salmonella and Shigella shows transparent and colourless appearance which normally do not change the medium appearance. The samples were collected from mosquito, some cultured samples showed a shiny pink color colony, some cultured samples showed white appearance and some showed purple.

(a) (b)

FIGURE 4.2: (a) Growth of bacteria on MacConkey Agar plate 1, (b) Growth of bacteria on MacConkey agar plate 2

The results of pathogen isolated showed gram negative bacterial growth. The pink color colonies indicate *klebsiella*. The colorless colonies indicate the probability of

presence of *Staphylococcus* and *Proteus vulgaris* as shown.

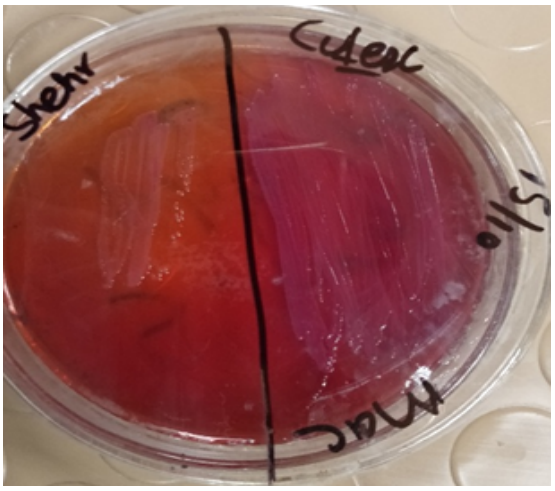


FIGURE 4.3: Growth of bacteria on MacConkey Agar plate 3

FIGURE 4.4: Growth of bacteria MacConkey Agar plate 4

4.3 Mannitol Salt Agar (MSA)

For the isolation of *Staphylococci*, Mannitol salt agar (MSA) is used that is both selective as well as differential medium. This medium consists of 7.5% sodium chloride, that's because it is chosen for those bacteria which can bear high salt concentrations. The only carbohydrate in the MSA is sugar mannitol which is used to distinguish bacteria on the basis of fermentation. Mannitol fermentation is demonstrated by changing of media color, not only by colony color. This process

is predominantly significant as several micrococci are pigmented [98]. All the samples collected from four locations showed growth indicating the probability of presence *Staphylococci* in the isolates.

The absence of any bacterial growth also indicates or confirm the results of MAC and EMB results as MSA inhibits the growth of *E. coli, Klebsiella, Enterobacter and Proteus spp.* The change in color of media depicts the growth of *Staphylococci.* This predict that gut of *Cx.quinquefasciatus* contain the *Staphylococci* that was to be separated on the MSA agar plates.

FIGURE 4.5: Growth of bacteria on Mannitol salt agar plate 1

FIGURE 4.6: Growth of bacteria on Mannitol salt agar plate 2

4.4 Blood Agar

Blood agar is moderate with many nutrients and is prepared with the completion of blood. Blood agar is also a basal medium which can be used as it is so no need to grow on other media first. Nutrient agar or a tropic soy agar can also be used as basal medium for blood agar. Blood agar for the cultivation of fasting bacteria. One of the best ways is to use special nutrients and not add too much nutrients to such media. About 5% of defrosted mammalian blood is automatically incorporated into the basal media to prepare the blood agar medium. It is an enriched medium that supports the growth of certain bacteria. In order for these bacteria to grow, inhibitors in the blood must be inactivated by hot blood agar [99].

Blood agar was used for growth of anaerobic bacteria strains present in gut microbiota of *Cx.quiquefasciatus*. Sample was collected from different areas of Islamabad and blood agar was used as medium for growth of anaerobic bacterial species in gut microbiota of mosquito. Blood agar plates were placed in carbon dioxide chamber which was made by use alka setizer dissolved in 500 ml of water.

Different bacterial strains were collected from this medium. *E. coli, Staphylococcus aureusa* and *Clostridium* were commonly found bacteria on blood agar. Blood agar supports development of fastidious organisms and supply nutrients to support their growth. The blood contained in this base provides maximum nutrition to the medium by providing the additional factors necessary for these bacteria.

Blood agar supports development of fastidious organisms and supply nutrients to support their growth. The blood contained in this base provides maximum nutrition to the medium by providing the additional factors necessary for these bacteria.

For the growth of *Streptococci* and other anaerobic bacteria blood agar is especially used as it provides best results. For pH maintenance and osmatic balance control NaCl is added in blood agar. Water is also used as water make the nutrients easier to absorb by bacteria. Blood agar supports development of fastidious organisms and supply nutrients to support their growth. The blood contained in this base provides maximum nutrition to the medium by providing the additional factors

necessary for these bacteria.

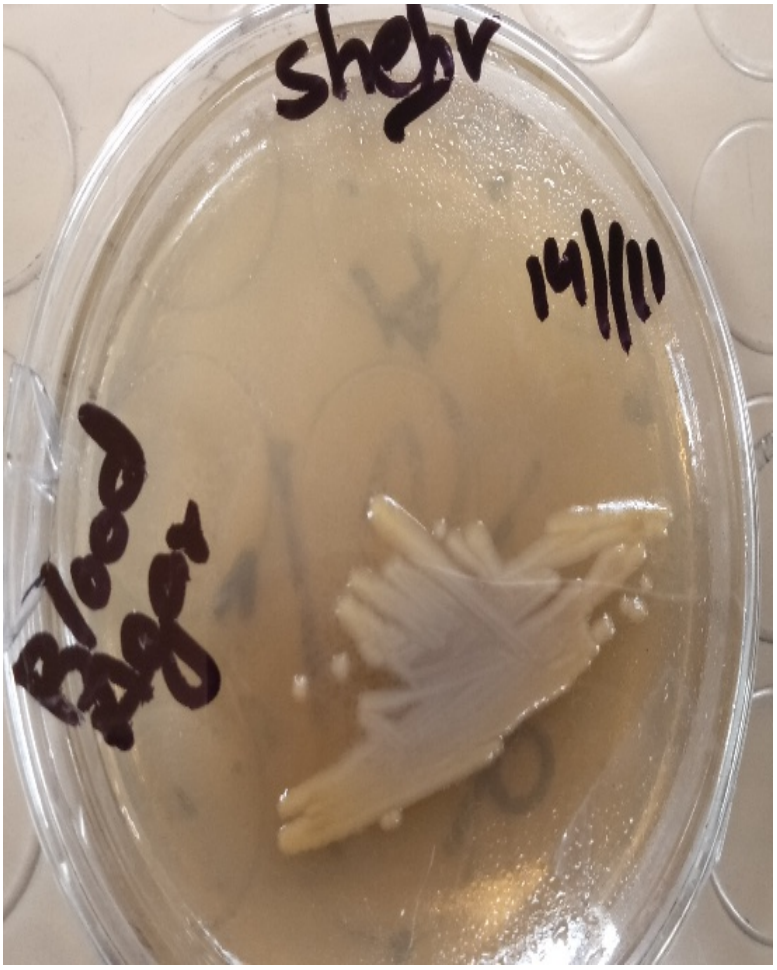
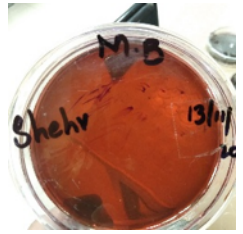


FIGURE 4.7: Bacterial growth on Blood Agar plate 1

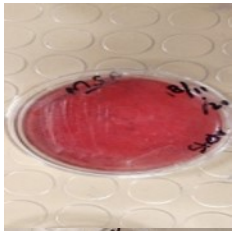
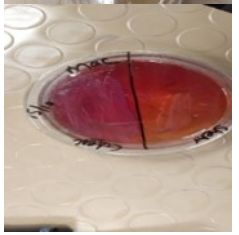
4.5 Bacterial Strains on Differential Media

The culture that was obtained on the differential media was streaked further to isolate the bacteria. Different types of bacteria were obtained with different morphology, different color characteristics, different colony characteristics. Bacterial species or genus were categorized based on the color characteristics and morphology on differential media. Following results including their sample location, colony color, morphology, pigmentation, media, predicted strain name and figure was shown in table 4.1.

Table 4.1: Bacteria isolates on different media

S#	Reference Code	Sample Location	Colony Color	Morphology of Colony	Pigmentation	Media	Predicted name of strain	Figures
1	EMB(P)1	Islamabad	Green sheen	Circular	Green sheen	Eosin methylene blue agar	*E.coli*	
2	MSA(P)1	Islamabad	Yellow	Punctiform	Yellow	Mannitol salt agar	*Staphylococcus*	
3	MACC(P)1	Islamabad	Off white	Circular	Off white	MacConkey agar	*Salmonella*	

4	MSA(P)2	Islamabad	White	Circular	White	Mannitol salt agar	*Staphylococcus aureus*	
5	B.A(P)1	Islamabad	White	Smooth	White	Blood agar	*Lactobacilli*	
6	MACC(P)2	Islamabad	Pink	Circular	Pink	MacConkey agar	*Klebsiella*	
7	MACC(P)3	Islamabad	Purple	Irregular	Purple	MacConkey agar	*Pseudomonas*	

8	MSA(P)3	Islamabad	Crystal White	Spherical	Crystal white	Mannitol salt agar	*Salmonella*	
9	B.A(P)3	Islamabad	White	Puntiform	White	Blood agar	*Staphylococcus*	
10	MACC(P)4	Islamabad	Orange to amber	Circular	Orange to amber	MacConkey agar	*Shigella*	
12	MSA(P)4	Islamabad	Yellow	Circular	Yellow	Mannitol salt agar	*S. aureus*	

4.6 Preservation of Prevalent Strains

The bacterial plate that seems to be more prevalent were further purified by streaking and culturing them repeatedly hence, the purified strains are obtained that was shown below 4.2. These were further stored in the glycerol stock and putin the refrigerator for future use. These pure strains also contain the duplicates, means one strain contain two copies:

TABLE 4.2: Preserved strains from Gut Microbiota of *Culex quinquefasciatus*

S#	Reference Code	Media	Colony Color	Pigmentation	Figures
1	EMB Plate 1	EMB	Purple	Purple	
2	BA Plate 1	BA	White	White	
3	MACC Plate 1	MACC	Pink	Pink	
4	MACC Plate 2	MACC	Pink	Pink	
5	MSA Plate 1	MSA	White	White	
6	MSA Plate 2	MSA	White	No	

4.7 Biochemical Analysis

4.7.1 Staining of Pure Cultures

The staining of pure cultures was performed by Gram staining method. A Danish physician, Hans Christian Gram in 1884 performed staining of pure cultures called as Gram staining method, also called differential stain. This procedure differentiates bacteria into, Gram negative and Gram-positive bacteria. Due to different differences in chemical structure of bacterial cell wall, Gram stain reaction give two different colors. The cell wall of Gram positive bacteria is thicker in peptidoglycan layer as compared to Gram negative and also it is surrounded by outer lipid containing layer.

Lipid is in high contents in Gram negative forming large pores causing the leakage of crystal violet, resulting in the decolonization of the bacterium and take counter stain later. The thick and cross-linked peptides in gram positive cell wall causing its dehydration and closure of pores, retaining the primary stain.

The bacteria which retain the primary stain appear dark blue or violet and not decolorized when stained with Gram's method are called Gram positive, where as those that lose the crystal violet used counter stain, safranin appear red are called as Gram negative [99].

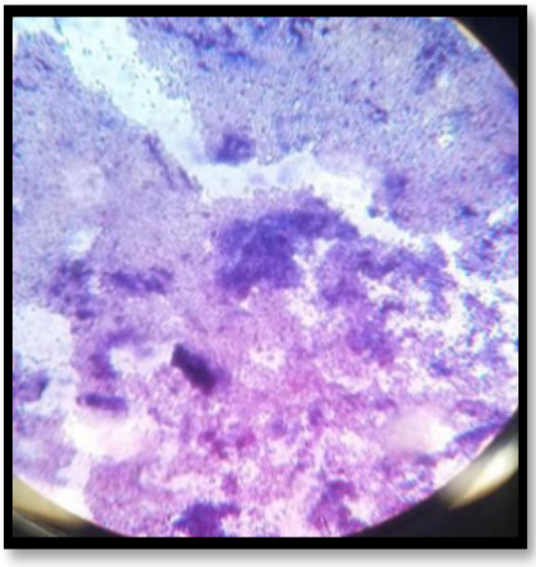


FIGURE 4.8: Staining of Prevalent Strain

The Gram stain uses different reagents in the order as crystal violet, iodine solution, alcohol, and safranin. The results were significant that concluded that the bacterial species obtained on Agar are stained pink which concluded that the species grown on Agar are Gram negative. Moreover, their microscopic examination shows that these are circle.

The strains that are obtained on the Mannitol Salt agar are purple in stain, which indicate that these are Gram Positive. The strains that are obtained on the EMB are stained pink, which means they are Gram negative. The strains that are obtained on the EMB are stained pink, which means they are Gram negative. The strains that are obtained on Blood Agar are stained purple which shows that Gram Positive bacteria are present on Blood Agar.

4.7.2 Urease Test

The urease test that was coined for the analysis that the strains either use the urea or acquire urea after the 2 days examination the strain show positive result with the urease test. The bacterial strains with pink color are urease positive and other that don't turn the color into pink are urease negative.

The result is considered positive if the yellow color of media is turned into pink after the utilization by the strain culturing in that plate. This strain did not change the colour so Urease Test was negative.

FIGURE 4.9: Growth on Urea agar

4.7.3 Citrate Utilization Test

This test involves Simmons Citrate agar which act as only source of carbon. Bromothymol blue act as an indicator turning its color green to blue when pH increases above 7.6. If it uses citrate, then it produces alkaline products [99]. The results shows that strain give positive result in the media and turned into blue after 2 days. That indicate, this specific strain is utilizing the citrate for metabolic activities.

FIGURE 4.10: Simmons Citrate Agar 1

4.7.4 Catalase Test

Catalase test is performed to check the presence of catalase enzyme which catalase oxygen release from hydrogen peroxide (H2O2). For aerobic strains 3% hydrogen peroxide was used and 15% H2O2 was used for detection of catalase in anaerobic strain. This test is used to differentiate bacteria which produce catalase to those bacteria which do not produce catalase.

When catalase test was applied on prevalent strain from Blood Agar medium it did not showed any bubble formation so catalase test was negative for this strain. The lack of bubble production showed that catalase is not present in strain.

FIGURE 4.11: Bubble formation in catalase test

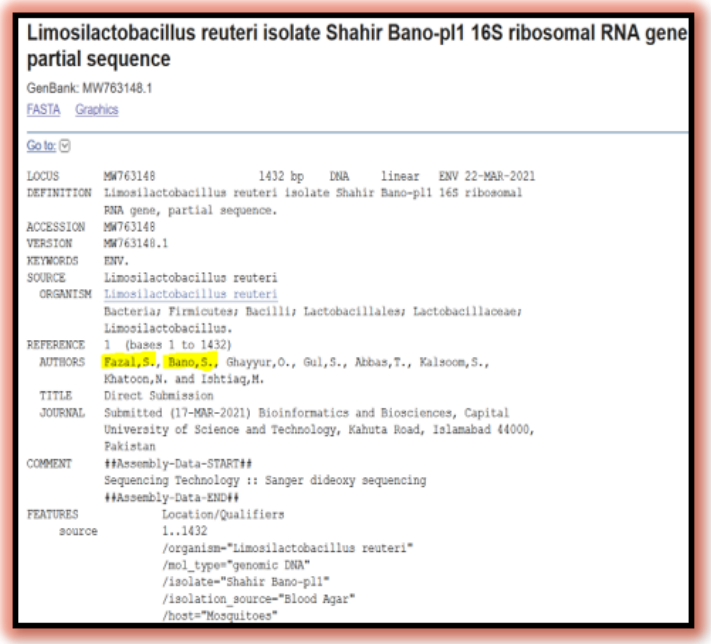
After the biochemical analysis of most primitive strain it is concluded that isolated bacter has rod shape and anaerobic type bacteria. According to results of gram staining it is shown that bacteria is gram positive and urease negative. It changes colour of simmon sitrate media so it is citrate positive as it showed no bubbles in hydrogen peroxide so it is gram negative.

TABLE 4.3: Characteristics of *Lactobacillus reuteri*

Sr.No	**Basic Characteristics**	**Properties**
1	Shape	Rod Shape
2	Type	Anaerobic
3	Gram Staining	Positive
4	Urease	Negative
5	Citrate Test	Positive
6	Catalase Test	Negative

4.8 NCBI Submission

After the removal of low quality sequences, the strain sequence was submitted in NCBI and the accession number given from the GenBank is MW763148.1.



Limosilactobacillus reuteri isolate Shahir Bano-pl1 16S ribosomal RNA gene partial sequence

GenBank: MW763148.1

FASTA Graphics

Go to:

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KEYWORDS    ENV.
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  ORGANISM  Limosilactobacillus reuteri
            Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae;
            Limosilactobacillus.
REFERENCE   1  (bases 1 to 1432)
  AUTHORS   Fazal,S., Bano,S., Ghayyur,O., Gul,S., Abbas,T., Kalsoom,S.,
            Khatoon,N. and Ishtiaq,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-MAR-2021) Bioinformatics and Biosciences, Capital
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//
```

(b)

FIGURE 4.12: (a) Submission on NCBI, (b) Sequence on NCBI

4.9 Multiple Sequence Alignment of Sequence

The multiple sequence alignment was done in ClastralW and the results of multiple sequence alignment showed that there was variation in sequence with name sahar Bano CUST Blood Agar. When this sequence was aligned with other sequences of Lactobacillus (Lactobacillus reuteri strain VB4 16s ribosomal RNA gene and Lactobacillus reuteri strain 149 16s ribosomal gene) the variation of fifteen base.

```
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HM124253.1      ----AGAGTTTG-ATCCTGGCTCAGGATGAACGCCGGCGGTGTGCCTAATACATGCAAGT
MW074915.1      TTTTAGAGTTTGGATTCATGCTCAGGATGAACGCCGGCGGTGTGCCTAATACATGCAAGT
                          ****.  .:*:.  * * ****   ****************

Sahar           CGTACGCATTGGCCCAACTGATTGATGGTGCTTGCACCTGATTGACGATGGATCACCAGT
HM124253.1      CGTACGCACTGGCCCAACTGATTGATGGTGCTTGCACCTGATTGACGATGGATCACCAGT
MW074915.1      CGTACGCACTGGCCCAACTGATTGATGGTGCTTGCACCTGATTGACGATGGATCACCAGT
                ******** **************************************************

Sahar           GAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCCGGAGCGGGGGATAACATTTG
HM124253.1      GAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCCGGAGCGGGGGATAACATTTG
MW074915.1      GAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCCGGAGCGGGGGATAACATTTG
                ************************************************************

Sahar           GAAACAGATGCTAATACCGCATAACAACAAAAGCCGCATGGTCTTTTGTTTGAAAGATGG
HM124253.1      GAAACAGATGCTAATACCGCATAACAACAAAAGCCACATG-GCTTTTGTTTGAAAGATGG
MW074915.1      GAAACAGATGCTAATACCGCATAACAACAAAAGCCGCATG-GCTTTTGTTTGAAAGATGG
                ***********************************.****  ******************

Sahar           CTTTGGCTATCACTCTGGGATGGACCTGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCT
HM124253.1      CTTTGGCTATCACTCTGGGATGGACCTGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCT
MW074915.1      CTTTGGCTATCACTCTGGGATGGACCTGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCT
                ************************************************************

Sahar           TACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGAACTGAGAC
HM124253.1      TACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGAACTGAGAC
MW074915.1      TACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGAACTGAGAC
                ************************************************************

Sahar           ACGGTCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTG
HM124253.1      ACGGTCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTG
MW074915.1      ACGGTCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTG
                ************************************************************

Sahar           ATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTGGAGA
HM124253.1      ATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTGGAGA
MW074915.1      ATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTGGAGA
                ************************************************************

Sahar           AGAACATGTGTGAGAGTAACTGTTCACGCAGTGACGGTATCCAACCAGAAAGTCACGGCT
HM124253.1      AGAACGTGCGTGAGAGTAACTGTTCACGCAGTGACGGTATCCAACCAGAAAGTCACGGCT
MW074915.1      G-AACGTGCGTGAGAGTAACTGTTCACGCAGTGACGGTATCCAACCAGAAAGTCACGGCT
                . ***.** ************************************************

Sahar           AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGG
HM124253.1      AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGG
MW074915.1      AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGG
                ************************************************************

Sahar           CGTAAAGCGAGCGCAGGCGGTTGCTTAGGTCTGATGTGAAAGCCTTCGGCTTAACCGAAG
HM124253.1      CGTAAAGCGAGCGCAGGCGGTTGCTTAGGTCTGATGTGAAAGCCTTCGGCTTAACCGAAG
MW074915.1      CGTAAAGCGAGCGCAGGCGGTTGCTTAGGTCTGATGTGAAAGCCTTCGGCTTAACCGAAG
                ************************************************************
```

FIGURE 4.13: Multiple sequence alignment of sequences Sahar Bano Blood agar, Uncltured *Latobacillus* and *Lactobacillus reuteri*

4.10 Phylogenetic Analysis

The phylogenetic tree consists of the 4 major clades i.e. Clade A, Clade B, Clade C and Clade D. The sequences of 25 bacterial strains were retrieved from the NCBI genbank and the new strain's sequence is also included in the tree. Phylogenetic analysis using Maximum Parsimony indicates that the sequenced strain seems to independently evolved in Clade C and shows ancestral similarity with *Uncultured lactobacillus sp*, and *Lactobacillus reuteri*.

Multiple sequence alignment also revealed fifteen base pairs variation in sequence when aligned with these two bacterial species. This bacterial strain is reported to be found in the *Aedes albopictus* but from this research it is clear that it is also found in the gut of *Culex quiquefasciatus*. From the phylogenetic tree it can be seen that the sequence with the name of Sahar Bano CUST Blood Agar is diverging as the separate sub group from the Clade A sub group 3.

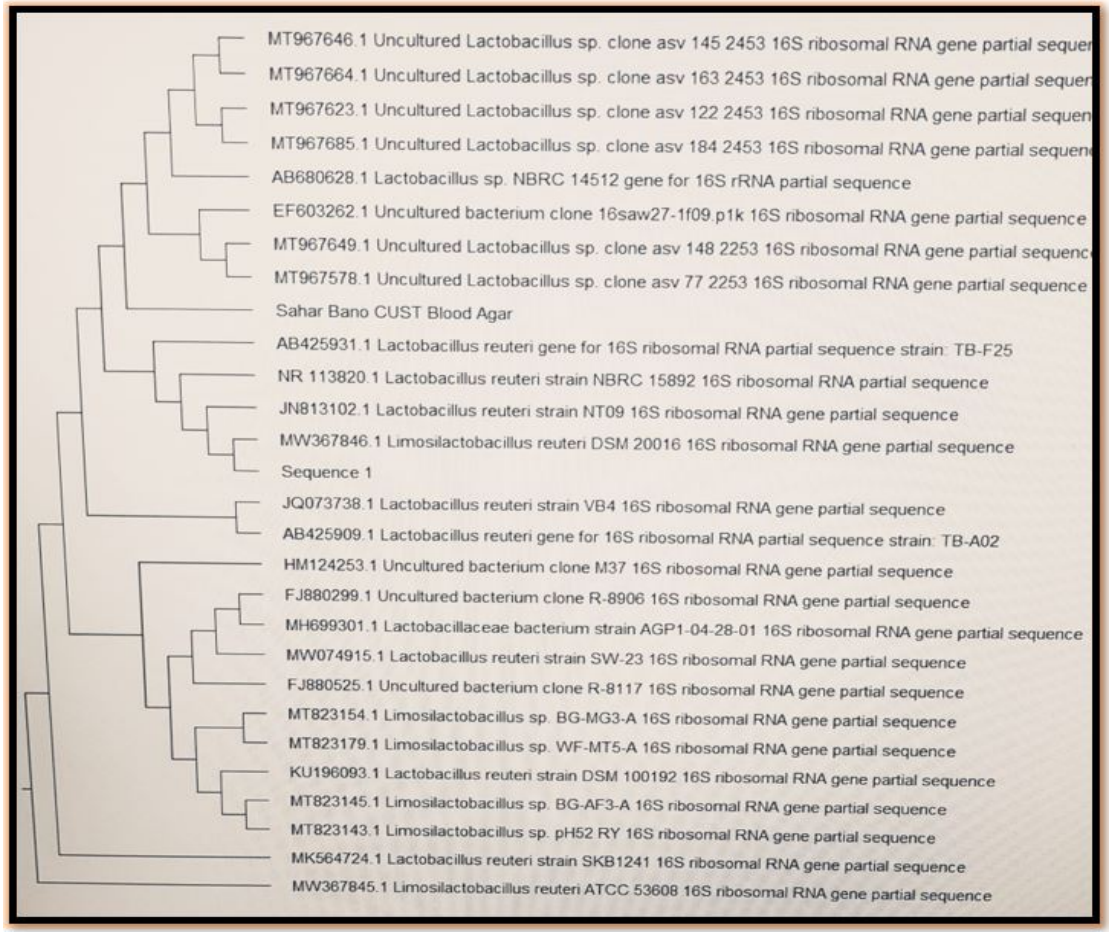

FIGURE 4.14: Phylogenetic Tree of *Culex quiquefasciatus*

4.11 Antibiotic and Insecticide Sensitivity Test

The most prevalent strain of bacteria was isolated and undergone for antibiotic and insecticide sensitivity test. Total 3 antibiotics and 3 insecticides were used for this purpose. The Disk diffusion method was used to check the antibiotic and insecticide sensitivity. Ceftriaxone, Imipenem and Nalidixic acid were used for antibiotics and Allethrin, Resmethrin and Thiacloprid were used for insecticides. Zone of inhibition for the three antibiotics and three insecticides are mentioned in appendix 1 and the percentage of resistance is mentioned in 4.4 and 4.5. Highest percentage of resistance i.e, 93.30% was recorded in Imipenem with a least resistance for Nalidixic acid 15.38%. Imipenem showed 1% sensitivity for *Lactobacillus reuteri*. Insecticide resistant sensitivity tests were also performed and they showed no resistance to this bacteria.

TABLE 4.4: Percentage of resistance of antibiotics for *Lactobacillus reuteri*

Sr.No	**Antibiotics Names**	Imipenem	Ceftriaxone	Nalidixic acid
1.	**Resistant**	14	12	2
2.	**Intermediate**	0	3	1
3.	**Sensitivity**	1	0	10
4.	**R%**	93.3%	80%	15.38%
5.	**I%**	0%	20%	7.69%
6.	**S%**	6.6%	0%	76.92%

TABLE 4.5: Percentage of resistance of insecticides for *Lactobacillus reuteri*

Sr. No	**Insecticides Names**	Allethrin	Resmethrin	Thiacloprid
1.	**Resistant**	0	0	0
2.	**Intermediate**	0	0	0
3.	**Sensitivity**	0	0	0
4.	**R%**	0%	0%	0%
5.	**I%**	0%	0%	0%
6.	**S%**	100%	100%	100%

4.12 Comparison of Computational and Wet Lab Results

When we compare the computational results of the functional genomic of the whole genome obtained by the NCBI Genome and RAST server, it was observed that *Lactobacillus species* were not involved in any of the aromatic compound metabolic pathways but 15 subsystems were identified to be involved in the antibiotic resistance pathways. *Lactobacillus specie* was involved in the antibiotic resistance and showed no relation in insecticide resistance as shown in 4.15

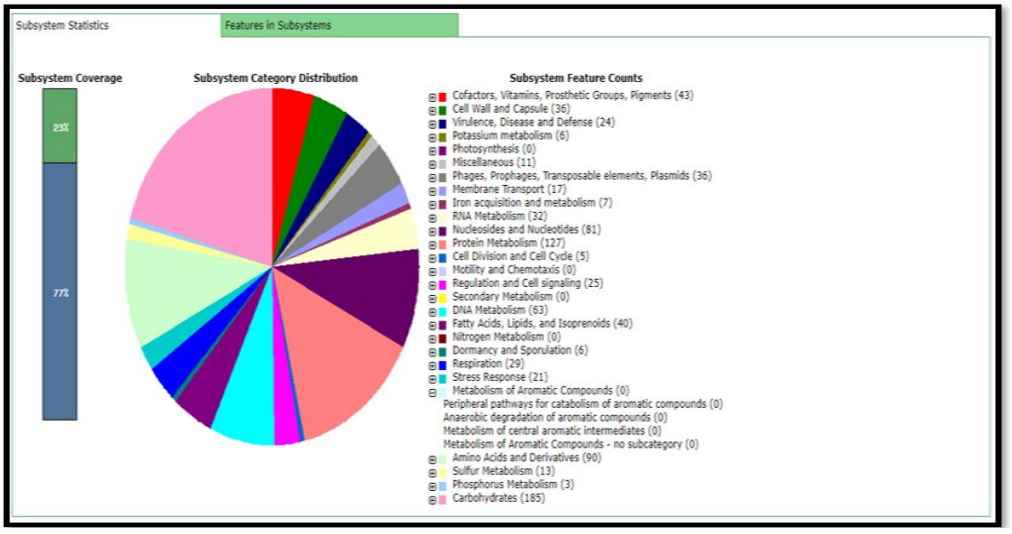

FIGURE 4.15: Shows the subsystems involved in antibiotic and insecticide resistance

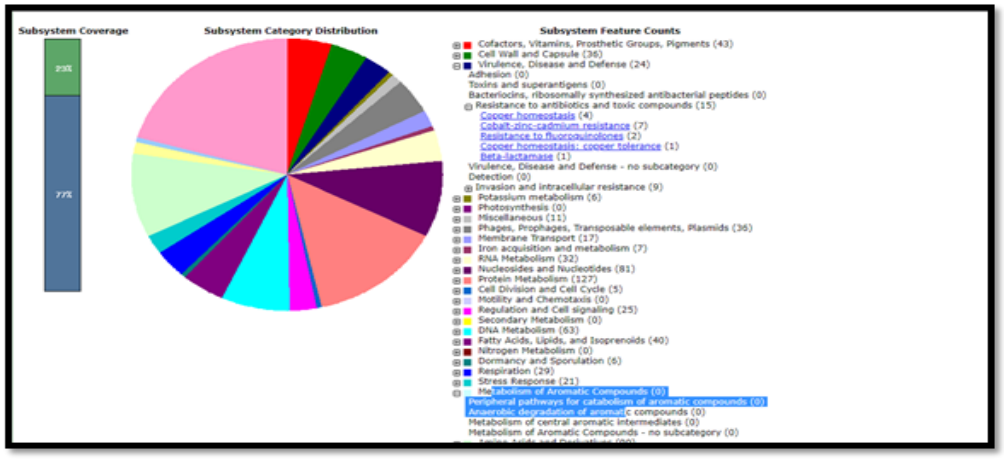

FIGURE 4.16: Shows the subsystems involved in antibiotic and insecticide resistance

4.13 Discussion

The bacteria inhabiting mosquito midgut has drawn special attention in recent past due to their interactions with both mosquito host as well as disease causing parasites. *Cx. quinquefasciatus* adapted to survive in domestic and peridomestic habitats in urban and semi urban environmental conditions and are most dominant species in these environments [17]. They prefer paddies that grow in large numbers and cover floating vegetation, and plants with cold dirty water. Canals with deeper climates with less sulfur water, less emerging vegetation and more floating vegetation. *Cx.quinquefasciatus* plays an important role in nature. The fundamental food of all adult mosquitoes is plant sugar and its associated nutrients, most often in the form of floral nectar. In the process of looking for nectar, mosquitoes pollinate many of the flowers they visit. Many pathogens of human and wild/domestic animals are carried by *Cx. quinquefasciatus*. Many viruses such *Saint Louis Encephalitis Virus, West Nile Virus and Western Equine Encephalitis* are transmitted by this vector. Besides this, *Cx. quinquefasciatus* cause some other problems such as allergy and nocturnal discomfort. There are not only bacteria in gut microbiota of mosquito but also many viruses, prokaryotes and eukaryotic microbes are part of mosquito gut microbiota.

They get their microbiota from their parents but mainly derived from environment where there they live that's why microbiota is highly dynamic and varies in different species according to developmental stages and environment they live [56]. To study independent microbiota structure of mosquito gut microbiota there is need to culture these bacteria and then sequencing of 16s rRNA or 18s rRNA is needed [57]. The microbiome of adult *Cx. quinquefasciatus* midgut were isolated on differential culture media showed the presence significant number of bacteria microbiome of larvae of *Cx. quinquefasciatus* vary according to instar and contamination in water where instar live [108]. The results of 16s rRNA sequencing indicates its close association with *Lactobacillus reuteri* specie with 99% similarity. As *Lactobacillus reuteri* is reported in mid gut of mebers of Culicidae family such as *Anopheles gambiae*, *Anopheles stephensi* and *Aedes albopactis* [101] [102] [103].

Lactobacillus reuteri is found in gut of human and other animals. It is also present in gut of mosquito family Culicidae and work as syngeneic manner also in antimicrobial metabolisms and modulation of immune responses [105]. It is not reported in *Cx. quinquefasciatus* yet. The juvenile stage of mosquito is aquatic and adult is terrestrial. 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. Blood they feed also contains microbes, composition of blood permanently or temporarily alter the structure of gut microbiota of mosquito by altering the metabolism [58]. Soil and aquatic environment also plays significant role in acquisition of gut microbiota of mosquito and role in colonization of bacteria in midgut from environment where they breed or get nectar or feed blood [107].

Mosquitoes have microbes in their gut which cannot be expected from environment these may derived from parents or any other source [53]. 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 [68]. All these factors are interrelated and can determine the pathogenicity and non pathogenicity in mosquitoes. Many of bacteria present in the midgut of mosquito are capable to kill the parasites. Many of bacteria present in gut of mosquito like *Enterococcus*, *Escherichia coli*, *Serratia marcescens* and *Enterobacter clacae* produce haemolysin exert activity against pathogens. Protease and prodigiosin are also produced by *Serratia marcescens* which show the potent activity against *Trypensoma curzi* and *Plasmodium falciparum* [109]. Numerous studies have showing that the individual mosquito species are involved in multiple mechanisms of resistance. In particular, two mechanisms increased 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 [106]. The bacterial strain tested for insecticide showed 0% insecticide resistance against Allethrin , Resmethrin and Thiacloprid.

Many of gram positive species like *Streptococcus pyrogenes* (ATCC 19615) and gram negative bacterial species like *Escherichia coli* (ATCC 25922) have role in degradation of deltamethrin or malathion [110]. Microbiota of mosquito is also involved in pharmacokinetic responses like absorption and transformation of xenobiotic [111]. *L. plantarum* is also responsible for conversion of chlorpyrifos into more potent insecticidal metabolite [112].

Controversy in our result might be due to the limited number of insecticides with narrow range of chemical classification tested. The results have been further analyised using RAST server, it was observed that Lactobacillus species were not involved in any of the aromatic compound metabolic pathways but 15 subsystems were identified to be involved in the antibiotic resistance pathways. The strain antibiotic sensitivity was checked against three antibiotics. The strain showed highly resistance against Imipenem with 93.3% and least with Nalidixic acid with 15.38% [13]. *Lactobacillus* strains were studied to check antibiotic resistance in 15 antibiotics of different type and nature and showed resistance against lincomycin, tetracycline and clindamycin [113].

Many of researches focused on investigation of bacteria in gut of mosquito which have important role in antibiotic resistant [114] [115] [116].

Antibiotic exposure dot not always indicate that there is presence of antibiotic resistance. As mosquito are known as public health threat for many viral and bacterial diseases, findings of research shows that mosquito are also carry antibiotic resistant bacteria. Virus pathogens are also transmitted by mosquitoes [117]. Mosquitoes travel a long distance in air so they are also vactors for spreading of antibiotic resistant bacteria between environments [117].

Chapter 5

Conclusions and Recommendations

The mosquitoes plays a significant role in public health. These are primary vector for transmission and spread of many economic importance diseases like systematic reaction, nuisance and local skin reaction and also responsible for many acute and death causing diseases. The larva of *Cx.quiquefasciatus* , live mainly in highly contaminated habitats such as cesspools and drains. The immature, larvae of mosquitoes has been challenged by a number of insecticides. The aquatic stage is also where larvae acquire microbes that colonize them and eventually become gut microbiota. This microbial environment, mediated by gut microbiota, is critical for subsequent survival and success, especially during the adult stage. Bacteria in gut microbiota of mosquito plays significant role in growth, nutrition, digestion, reproduction, prevention against pathogens and also cause resistance against insecticides.

In present research one of our aim was to isolate and identify most prevalent bacterial specie from gut of mosquito species *Cx.quinquefasciatus* and to evaluate insecticide resistantance in most prevalent bacterial species in gut of mosquito.

The microbiome of adult *Cx.quinquefasciatus* midgut were isolated on differential culture media and the results indicate the presence of *E. coli, Staphylococus,*

Klebsiella spp and *Lactobacillus spp.* Biochemical characterization of prevalent bacterial specie revealed it a gram positive, urease negative, citrate positive and catalase negative.

The results of 16s rRNA sequencing indicates its close association with *Lactobacillus reuteri* specie with 99% similarity. Phylogenetic analysis showed that the sequenced strain seems to independently evolved and revealed ancestral similarity with *Uncultured Lactobacillus* sp, and *Lactobacillus reuteri.* The strain antibiotic sensitivity was checked against three antibiotics. The strain showed highly resistance against Imipenem with 93.3% and least with Nalidixic acid with 15.38% and showed 0% insecticide resistance against Allethrin , Resmethrin and Thiacloprid. The computational results of the functional genomic of the whole genome obtained by the NCBI Genome and RAST server, it was observed that *Lactobacillus* species were not involved in any of the aromatic compound metabolic pathways but 15 subsystems were identified to be involved in the antibiotic resistance pathways. The study must be expand in other areas such as:

a In current study only one prevalent microbial specie has been focused these is a need to further explore the midgut microbiome of *Cx.quinquefasciatus* in Pakistan.

b The role of these microbiome must be explore in mosquito life history and their survival as a vector in the environment.

c Larval stages must also be studied to reveal microbiome present in midgut of mosquito.

d More insecticides and antibiotics must be tested to identified *Lactobacillus spp.*

e The role of these microbiome must also be explored in designing vector control strategies.

f The role of these microbiome must also be explored in degradation of xenobiotic compounds.

Bibliography

[1] K. Chandel et al., "Midgut microbial community of *Culex.quinquefasciatus* mosquito populations from India," *PLoS One*, vol. 8, no. 11, 2013, doi: 10.1371/journal.pone.0080453.

[2] E. Nchoutpouen et al., "*Culex* species diversity, susceptibility to insecticides and role as potential vector of Lymphatic filariasis in the city of Yaoundé, Cameroon," *PLoS Negl. Trop. Dis.*, vol. 13, no. 4, pp. 1–16, 2019, doi: 10.1371/journal.pntd.0007229.

[3] S. Bhattacharya, P. Basu, and C. Sajal Bhattacharya, "The Southern House Mosquito, *Culex quinquefasciatus*: profile of a smart vector," *J. Entomol. Zool. Stud. JEZS*, vol. 73, no. 42, pp. 73–81, 2016.

[4] R. P. Lopes, J. B. P. Lima, and A. J. Martins, "Insecticide resistance in *Culex quinquefasciatus* Say, 1823 in Brazil: A review," *Parasites and Vectors*, vol. 12, no. 1, pp. 1–12, 2019, doi: 10.1186/s13071-019-3850-8.

[5] M. Ashfaq and U. Ashfaq, "Pakistan entomologist evaluation of Mosquitocidal activity of water extract moringa olifera seeds against *Culex quinquefasciatus(Diptera: Culicidae)* in Pakistan." *Pakistan Entomol.*, vol. 34, no. 1, pp. 21–26, 2012.

[6] H. Weissenböck, Z. Hubálek, T. Bakonyi, and N. Nowotny, "Zoonotic mosquito-borne flaviviruses: Worldwide presence of agents with proven pathogenicity and potential candidates of future emerging diseases," *Vet. Microbiol.*, vol. 140, no. 3–4, pp. 271–280, 2010, doi: 10.1016/j.vetmic.2009.08.025.

[7] E. C. Uttah, G. N. Wokem, and C. Okonofua, " The Abundance and Biting Patterns of *Culex quinquefasciatus* Say (Culicidae) in the Coastal Region of Nigeria ," *ISRN Zool.*, vol. 2013, pp. 1–7, 2013, doi: 10.1155/2013/640691.

[8] V. J. Pidiyar, K. Jangid, M. S. Patole, and Y. S. Shouche, "Studies on cultured and uncultured microbiota of wild Culex quinquefasciatus mosquito midgut based on 16S ribosomal RNA gene analysis," *Am. J. Trop. Med.Hyg.*, vol. 70, no. 6, pp. 597–603, 2004, doi: 10.4269/ajtmh.2004.70.597.

[9] WHO 2020, Pictorial identification key of important disease vectors in the WHO South-East Asia Region 2020. 2020.

[10] H. M. Tahir, T. Ishaq, M. K. Mukhtar, S. Y. Khan, and K. Ahmed, "Potential use of Calotropis procera (milkweed) to control *Culex.quinquefasciatus* (Diptera: Culicidae)," *Pak. J. Zool.*, vol. 45, no. 3, pp. 615–621, 2013.

[11] A. M. Samy, A. H. Elaagip, M. A. Kenawy, C. F. J. Ayres, A. T. Peterson, and D. E. Soliman, "Climate change influences on the global potential distribution of the mosquito *Culex quinquefasciatus,* vector of West Nile virus and lymphatic filariasis," *PLoS One*, vol. 11, no. 10, 2016, doi: 10.1371/journal.pone.0163863.

[12] S. A. de Morais, C. Moratore, L. Suesdek, and M. T. Marrelli, "Genetic-morphometric variation in Culex quinquefasciatus from Brazil and La Plata, Argentina," *Mem. Inst. Oswaldo Cruz,* vol. 105, no. 5, pp. 672–676, 2010, doi: 10.1590/s0074-02762010000500012.

[13] W. Akram, H. A. A. Khan, F. Hafeez, H. Bilal, Y. K. Kim, and J. J. Lee, "Potential of citrus seed extracts against dengue fever mosquito, Aedes albopic tus (Skuse) (Culicidae: Diptera)," *Pakistan J. Bot.*, vol. 42, no. 5, pp. 3343–3348, 2010.

[14] O. O. Lewis, "Toxic Stress of Lead on Clarias Gariepinus (African Catfish) Fingerlings," vol. 6, pp. 101–104, 2003.

[15] V. Control, "Microbial Flora in Gut of *Culex Quinquefasciatus*," vol. 23, no. 2, 1992.

[16] K. Barnard, A. C. S. N. Jeanrenaud, B. D. Brooke, and S. V. Oliver, "The contribution of gut bacteria to insecticide resistance and the life histories of the major malaria vector Anopheles arabiensis (Diptera: Culicidae)," *Sci. Rep.*, vol. 9, no. 1, pp. 1–11, 2019, doi: 10.1038/s41598-019-45499-z.

[17] M. A. Sattler et al., "Habitat characterization and spatial distribution of Anopheles sp. mosquito larvae in Dar es Salaam (Tanzania) during an extended dry period," *Malar. J.*, vol. 4, pp. 1–15, 2005, doi: 10.1186/1475-2875-4-4.

[18] E. J. Muturi et al., "Environmental factors associated with the distribution of Anopheles arabiensis and Culex quinquefasciatus in a rice agro-ecosystem in Mwea, Kenya," emphJ. Vector Ecol., vol. 33, no. 1, pp. 56–63, 2008, doi: 10.3376/1081-1710(2008)33[56:efawtd]2.0.co;2.

[19] S. Hill and C. R. Connelly, "Southern House Mosquito *Culex quinquefasciatus* Say," *Univ. Florida*, pp. 1–5, 2013.

[20] E. Willott and F. Ramberg, "Identification and Geographical Distribution of the Mosquitoes of North America, North of Mexico," *J. Wildl. Dis.*, vol. 43, no. 4, pp. 806–807, 2007, doi: 10.7589/0090-3558-43.4.806.

[21] D. S. Suman, "Evaluation of enhanced oviposition attractant formulations against Aedes and Culex vector mosquitoes in urban and semi-urban areas," *Parasitol. Res.*, vol. 118, no. 3, pp. 743–750, 2019, doi: 10.1007/s00436-019-06228-7.

[22] D. A. LAPOINTE1,"Dispersal of *Culex quinquefasciatus (Diptera: Culicidae)* in a Hawaiian Rain Forest" *J. Med. Entomol.*vol.45(4):pp.600-609, 2008.

[23] A.Ralph Barr,."The Distribution of *Culex p.pipiens and C.P.quinquefasciatus* in North America".*Department of Entomology, University of Kansas*,vol.6,no.1,pp.1-13,1957.

[24] Gerberg EJ, Barnard DR, Ward RA. "Manual for Mosquito Rearing and Experimental Techniques." *American Mosquito Control Association Bulletin* vol. 5: pp.61–62, 1994.

[25] Subra R, . Biology and control of *Culex pipiens quinquefasciatus* Say, 1823 (Diptera, Culicidae) with special reference to Africa." *Insect Science and its Application*, vol.1(4):pp.319-338, 1981.

[26] Barr, A. Ralph, and Abdulla Al-Azawi. "Notes on oviposition and the hatching of eggs of Aedes and Psorophora mosquitoes (Diptera: Culicidae)". *University of Kansas*, 1953.

[27] Mohrig, Werner. "Die Culiciden Deutschlands." *Anzeiger für Schädlingskunde 42*,vol. 8,pp.126-127, 1969.

[28] Gillett, J. D. "Abdominal pulses in newly emerged mosquitoes, Aedes aegypti." *Mosquito news 43*,vol. 3,pp.359-361, 1983.

[29] Sutcliffe, D. W. "The chemical composition of haemolymph in insects and some other arthropods, in relation to their phylogeny." *Comparative Biochemistry and Physiology* vol.9, no. 2 (1963): 121-135.

[30] Mclver, Susan B. "Sensilla of mosquitoes (diptera: Culicidae)." *Journal of Medical Entomology* vol. 19, no. 5 (1982): 489-535.

[31] Clements, Alan Neville. "The biology of mosquitoes: development, nutrition and reproduction". *London: Chapman and Hall*, Vol. 1. 1992.

[32] Kettle, D. S. "Medical and veterinary entomology. CAB International." *Wallingford, UK*, 1995.

[33] Woodring, J., and E. W. Davidson. "Biological control of mosquitoes." *The biology of disease vectors*. Colorado: University Press of Colorado, pp.530-48, 1996.

[34] Lehane, M. J. "Managing the Blood Meal". *Biology of blood-sucking insects*." 1991.

[35] Russell, Richard C., Domenico Otranto, and Richard L. Wall. "The encyclopedia of medical and veterinary entomology". CABI, 2013.

[36] Bruce-Chwatt, Leonard Jan, and Julian De Zulueta."The rise and fall of malaria in Europe: a historico-epidemiological study". *Published for the Regional Office for Europe of the World Health Organization by Oxford University Press, Walton Street*, Oxford OX2 6DP, 1980.

[37] Relative densities of freely breeding populations of *Aedes (S.) polynesiensis Marks and A. (S.) albopictus Skuse*. A large cage experiment. Rozeboom LE Am J Trop Med Hyg. ; 20(2):pp.356-62,1971 Mar.

[38] Essa, Z. I., Essack, S. Y.,and Connolly, "*C.. Staphylococcus aureus* from public hospitals in KwaZulu Natal, South Africa—Infection detection and strain—Typing". Southern African *Journal of Epidemiology and Infection*,24(1), 4-7, 2009.

[39] CDC. "CDC answers your questions about *St. Louis encephalitis*". Center for Disease Control.2019

[40] CDC. "Saint Louis Encephalitis. Center for Disease Control". June 2019

[41] Foster WA, Walker ED."Mosquitoes (Culicidae)",. *In Mullen G, Durden L. (editors). Medical and Veterinary Entomology*. Academic Press. New York, pp. 245-249,2002.

[42] Day JF, Stark LM."Frequency of Saint Louis encephalitis virus in humans from Florida", *Journal of Medical Entomology*, vol 37: pp.626-633, 2000, USA: 1990-1999.

[43] WHO,"Lymphatic filariasis". World Health Organization. June 2019.

[44] DHPE.Rift Valley Fever. Directors of Health Promotion and Education.(2005)

[45] Aregawi, Maru, Richard E. Cibulskis, Mac Otten, and Ryan Williams." World malaria report". World Health Organization, 2009.

[46] Kerwin, J.L., 2007."Oomycetes: Lagenidium giganteum". *In: Floore, T.G. (Ed.), Biorational Control of Mosquitoes. Am. Mosq. Control Assoc. Bull.*vol. 7,pp: 50–57.

[47] World Health Organization."Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces": report of the WHO informal consultation, Geneva, 28-30 September 1998. No. WHO/CDS/CPC/MAL/98.12. Geneva: World Health Organization, 1998.

[48] Zeller HG Schuffenecker I. "West Nile virus: an overview of its spread in Europe and the Mediterranean basin in contrast to its spread in the Americas".*Eur J Clin Microbiol Infect Dis.* ;pp.23:147–56,2004.

[49] Gubler DJ, Campbell GL, Nasci R, Komar N, Petersen L, Roehrig JT. "West Nile virus in the United States: guidelines for detection, prevention, and control". emphViral Immunol. vol.13(4):pp.469-75. doi: 10.1089/vim.2000.13.469. PMID: 11192293.

[50] Lammie PJ, Cuenco KT, Punkosdy GA. "The pathogenesis of filarial lymphedema: is it the worm or is it the host?" *Ann NY Acad Sci.*vol.979:pp.131-42; discussion 188-96,2000.

[51] Debrah AY, Mand S, Marfo-Debrekyei Y, et al. "Macrofilaricidal effect of 4 weeks of treatment with doxycycline on Wuchereria bancrofti". *Trop Med Int Health.*vol.12(12):pp. 1433-41,2007.

[52] Mak JW."Epidemiology of lymphatic filariasis". *Ciba Found Symp.*pp.127:5-14. doi: 10.1002/9780470513446.ch2. PMID: 2885159.

[53] McMeniman, Conor J., Roxanna V. Lane, Bodil N. Cass, Amy WC Fong, Manpreet Sidhu, Yu-Feng Wang, and Scott L. O'Neill. "Stable introduction of a life-shortening Wolbachia infection into the mosquito Aedes aegypti." *Science.*vol. 323, no. 5910:pp.141-144,2009.

[54] Dutra, Heverton Leandro Carneiro, Marcele Neves Rocha, Fernando Braga Stehling Dias, Simone Brutman Mansur, Eric Pearce Caragata, and Luciano Andrade Moreira. "Wolbachia blocks currently circulating Zika virus isolates in Brazilian Aedes aegypti mosquitoes." *Cell host and microbe.*vol.19, no. 6:pp.771-774,2016.

[55] Van den Hurk, Andrew F., Sonja Hall-Mendelin, Alyssa T. Pyke, Francesca D. Frentiu, Kate McElroy, Andrew Day, Stephen Higgs, and Scott L. O'Neill. "Impact of Wolbachia on infection with chikungunya and yellow fever viruses in the mosquito vector Aedes aegypti." *PLoS neglected tropical diseases.*vol 6, no. 11,2012.

[56] Wang, Sibao, André LA Dos-Santos, Wei Huang, Kun Connie Liu, Mohammad Ali Oshaghi, Ge Wei, Peter Agre, and Marcelo Jacobs-Lorena. "Driving mosquito refractoriness to Plasmodium falciparum with engineered symbiotic bacteria." *Science.*vol.357, no.6358:pp. 1399-1402,2017.

[57] Strand, Michael R. "Composition and functional roles of the gut microbiota in mosquitoes." *Current opinion in insect science.*vol 28:pp.59-65,2018.

[58] Wilke ABB, Vidal PO, Suesdek L Marrelli."Population genetics of Neotropical *Culex quinquefasciatus* (Diptera: Culicidae)". *Parasites and Vectors*; pp.7:468,2014.

[59] Karlekar SR, Deshpande MM, Andrew RJ."Present susceptibility status Of *Culex quinquefasciatus*, Say to three insecticides in Nagpur district of India.*J Sci Res and Tech.* vol.1(2),pp.12-14.

[60] Demaio J, Pumpuni CB, Kent M, Beier JC Am J."The midgut bacterial flora of wild *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae mosquitoes. Trop Med Hyg.*vol.54(2):pp.219-23,1996.

[61] M. Shi, P. Neville, J. Nicholson, J.-S. Eden, A. Imrie, and E. C. Holmes, "High-resolution metatranscriptomics reveals the ecological dynamics of mosquito-associated rna viruses in western australia," *Journal of Virology*, vol. 91, no. 17, 2017

[62] B. G. Bolling, S. C. Weaver, R. B. Tesh, and N. Vasilakis, "Insect-specific virus discovery: significance for the arbovirus community," *Viruses, vol*, vol.7, no. 9, pp. 4911–4928, 2015.

[63] N. Vasilakis and R. B. Tesh, "Insect-specific viruses and their potential impact on arbovirus transmission," *Current opinion in virology*, vol. 15, pp.69–74, 2015.

[64] P. A. Lynch, U. Grimm, M. B. Thomas, and A. F. Read, "Prospective malaria control using entomopathogenic fungi: comparative evaluation of impact on transmission and selection for resistance," *Malaria journal*, vol. 11, no. 1, p. 383, 2012

[65] Dong, Yuemei, Fabio Manfredini, and George Dimopoulos. "Implication of the mosquito midgut microbiota in the defense against malaria parasites." *PLoS pathogens*,vol. 5, no. 5,2009.

[66] A. de O Gaio, D. S. Gusm˜ao, A. V. Santos, M. A. Berbert-Molina, P. F.Pimenta, and F. J. Lemos, "Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti (diptera: Culicidae)*(l.)," *Parasites and vectors*, vol. 4, no. 1, pp. 1–10, 2011.

[67] de O Gaio, Analiz, Desiely S. Gusmão, Adão V. Santos, Marília A. Berbert-Molina, Paulo FP Pimenta, and Francisco JA Lemos. "Contribution of midgut bacteria to blood digestion and egg production in Aedes aegypti (Diptera: Culicidae)(L.)." *Parasites and vectors*,vol. 4, no. 1:pp. 1052011.

[68] B. T. Beerntsen, A. A. James, and B. M. Christensen, "Genetics of mosquitovector competence," *Microbiology and molecular biology reviews*,vol. 64,no. 1, pp. 115–137, 2000.

[69] M. B. Kingsolver, Z. Huang, and R. W. Hardy, "Insect antiviral innate immunity: pathways, effectors, and connections,"emphJournal of molecular biology, vol. 425, no. 24, pp. 4921–4936, 2013.

[70] M. Krishnan, C. Bharathiraja, J. Pandiarajan, V. A. Prasanna, J. Rajendhran, and P. Gunasekaran, "Insect gut microbiome–an unexploited reserve for biotechnological application," *Asian Pacific journal of tropical biomedicine*, vol. 4, pp. S16–S21, 2014

[71] A. de O Gaio, D. S. Gusm˜ao, A. V. Santos, M. A. Berbert-Molina, P. F. Pimenta, and F. J. Lemos, "Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti (diptera: Culicidae)(l.)*," *Parasites and vectors*, vol. 4, no. 1, pp. 1–10, 2011.

[72] N. Liu, "Insecticide resistance in mosquitoes: impact, mechanisms, and research directions," *Annual review of entomology*, vol. 60, pp. 537–559, 2015.

[73] A. J. Ketterman, C. Saisawang, and J. Wongsantichon, "Insect glutathione transferases," *Drug metabolism reviews*, vol. 43, no. 2, pp. 253–265, 2011.

[74] H. Ranson and J. Hemingway, "Mosquito glutathione transferases," *Methods in enzymology*, vol. 401, pp. 226–241, 2005.

[75] H. RANSON, L. ROSSITER, F. ORTELLI, B. JENSEN, X. WANG, C. W. ROTH, F. H. COLLINS, and J. HEMINGWAY, "Identification of a novel class of insect glutathione s-transferases involved in resistance to ddt in the malaria vector anopheles gambiae," *Biochemical Journal*, vol. 359, no. 2, pp. 295–304, 2001.

[76] J. G. Scott, "Cytochromes p450 and insecticide resistance," *Insect biochemistry and molecular biology*, vol. 29, no. 9, pp. 757–777,

[77] G. A. Rosenthal and M. R. Berenbaum, "Herbivores: their interactions with secondary plant metabolites: ecological and evolutionary processes".*Academic Press*, 2012, vol. 2.

[78] X. Li, M. A. Schuler, and M. R. Berenbaum, "Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics," *Annual review of entomology*, vol. 52, 2007.

[79] M. A. Schuler, "The role of cytochrome p450 monooxygenases in plant-insect interactions." *Plant physiology*, vol. 112, no. 4, p. 1411, 1996.

[80] S. Kasai, I. S. Weerashinghe, and T. Shono, "P450 monooxygenases are an important mechanism of permethrin resistance in *Culex quinquefasciatus* say larvae," *Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America*, vol. 37, no. 1, pp.47–56, 1998.

[81] J. M. Riveron, H. Irving, M. Ndula, K. G. Barnes, S. S. Ibrahim, M. J. Paine, and C. S. Wondji, "Directionally selected cytochrome p450 alleles are driving the spread of pyrethroid resistance in the major malaria vector anopheles funestus," *Proceedings of the National Academy of Sciences*, vol. 110, no. 1, pp. 252–257, 2013.

[82] C. S. Wondji, H. Irving, J. Morgan, N. F. Lobo, F. H. Collins, R. H. Hunt, M. Coetzee, J. Hemingway, and H. Ranson, "Two duplicated p450 genes are associated with pyrethroid resistance in anopheles funestus, a major malariavector," *Genome research*, vol. 19, no. 3, pp. 452–459, 2009.

[83] N. Pasteur and M. Raymond, "Insecticide resistance genes in mosquitoes: their mutations, migration, and selection in field populations," *Journal of Heredity*, vol. 87, no. 6, pp. 444–449, 1996.

[84] M. Raymond, C. Chevillon, T. Guillemaud, T. Lenormand, and N. Pasteur, "An overview of the evolution of overproduced esterases in the mosquito culex pipiens," *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, vol. 353, no. 1376, pp. 1707–1711, 1998.

[85] A. Vaughan and J. Hemingway, "Mosquito carboxylesterase esta 21 (a2). cloning and sequence of the full-length cdna for a major insecticide resistance gene worldwide in the mosquito *Culex quinquefasciatus*," *Journal of Biological Chemistry*, vol. 270, no. 28, pp. 17 044–17 049, 1995

[86] S. Kasai, T. Shono, and M. Yamakawa, "Molecular cloning and nucleotide sequenceof a cytochrome p450 cdna from a pyrethroid-resistant mosquito,

Culex quinquefasciatus say,"*Insect molecular biology*, vol. 7, no. 2, pp. 185–191, 1998.

[87] H. Liu, E. W. Cupp, K. M. Micher, A. Guo, and N. Liu, "Insecticide resistance and cross-resistance in alabama and florida strains of *Culex quinquefaciatus*," *Journal of medical entomology*, vol. 41, no. 3, pp. 408–413, 2004.

[88] Shah, P. J.,and Ryzner, K. L."Evaluating the appropriate use of piperacillin/tazobactam in a community health system: A retrospective chart review".*Pharmacy and Therapeutics*, vol.38(8),pp.462,2013.

[89] Sheehan, J. C. "The enchanted ring: The untold story of penicillin". *AMCA. Mosquito Information: Control. American Mosquito Control Association.*June 2019.

[90] WHO." Pictorial identification key of important disease vectors in the WHO South-East Asia Region", 2020.

[91] Coleman, J., Juhn, J., and James, A. A. A. "Dissection of midgut and salivary glands from *Ae. aegypti mosquitoes*".*Journal of Visualized Experiments*,vol. 5, 2007.

[92] Chandel, K., Mendki, M. J., Parikh, R. Y., Kulkarni, G., Tikar, S. N., Sukumaran, D., Prakash, S., Parashar, B. D., Shouche, Y. S., and Veer, V."Midgut microbial community of *Culex quinquefasciatus* mosquito populations from India".*PLoS ONE*,vol.8(11),2013.

[93] Fouda MA, Hassan MI, Al-Daly AG, Hammad KM (2001) Effect of midgut bacteria of *Culex pipiens L.* on digestion and reproduction.*Journal of the Egyptian Society of Parasitology*,vol.31:pp. 767–780.

[94] Lindh JM, Terenius O, Faye I."16S rRNA Gene-Based identification of midgut bacteria from field-cought *Anopheles gambiaeSensu Lato* and *A. Funestus mosquitoes* reveals new species related to known insect symbionts. *Appl Environ Microbiol*, vol.71: pp.7217–7223,2005.

[95] Chun J, Lee JH, Jung Y, Kim M, Kim S, et al."EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences".*Int J Syst Evol Microbiol.*vol. 57: pp.2259–2261,2007.

[96] Gusamao DS, Santos AV, Marini DC, Bacci M, Berbert-Molina MA, et al. "Culture-dependent and culture-independent characterization of microorganisms associated with *Aedes aegypti(Diptera: Culicidae)(L.*) and dynamics of bacterial colonization in the midgut". *Acta Trop*,vol.115: 7,2010.

[97] Demaio J, Pumpuni CB, Kent M, Beier JC."The midgut bacterial flora of wild *Aedes triseriatus, Culex pipiens, and Psorophora columbiae mosquitoes*". *Am J Trop Med Hyg.*vol. 54:pp. 219–223,1996.

[98] Ferguson MJ, Micks DW." Micro organisms associated with mosquitoes, Bacteria isolate from adult *Culex fatigans*,Wiedmann. *Journal of Insect Pathology.*vol3:pp.112–119,1961.

[99] Riehle MA, Lorena MJ."Using bacteria to express and display anti parasite molecules in mosquitoes, Current and future strategies".*Insect Biochemistry and Molecular Biology*,vol.35:pp.699–707, 2005.

[100] R. K. Aziz, D. Bartels, A. A. Best, M. DeJongh, T. Disz, R. A. Edwards, K. Formsma, S. Gerdes, E. M. Glass, M. Kubal et al., "The rast server: rapid annotations using subsystems technology," *BMC genomics*, vol. 9, no. 1, pp. 1–15, 2008.

[101] Z. Kambris, P. E. Cook, H. K. Phuc, and S. P. Sinkins, "Immune activation by life-shortening wolbachia and reduced filarial competence in mosquitoes," *Science*, vol. 326, no. 5949, pp. 134–136, 2009.

[102] M. K. Kajla, O. Andreeva, T. M. Gilbreath III, and S. M. Paskewitz, "Characterization of expression, activity and role in antibacterial immunity of anopheles gambiae lysozyme c-1," *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, vol. 155, no. 2, pp. 201–209, 2010.

[103] Dieng, H., Talukder, P., Satho, T., Nakashima, Y., Nwachukwu, I. N., Frederick, A., Saifur, R. G. M., Salmah, C., Ahmad, A. H.,and Miake, F. (2010). *Lactobacillus* infection related to midgut protein synthesis in the dengue vector *Aedes albopictus*: Platform of non-symbiont bacteria for the control of Aedes vectors. 2(March), 14–21.

[104] A. V. Gra¸ca-Souza, C. Maya-Monteiro, G. O. Paiva-Silva, G. R. Braz, M. C. Paes, M. H. Sorgine, M. F. Oliveira, and P. L. Oliveira, "Adaptations against heme toxicity in blood-feeding arthropods," *Insect biochemistry and molecular biology*, vol. 36, no. 4, pp. 322–335, 2006.

[105] Arichehreh, S. P., Ahmasbi, G. T., and Arafrazi, A. S. (2018). Isolation and identification of Lactobacillus bacteria found in the gastrointestinal tract of the dwarf honey bee , *Apis florea Fabricius* ,(Hymenoptera : Apidae). 430–438, 1973.

[106] A. Vaughan and J. Hemingway, "Mosquito carboxylesterase esta 21 (a2). cloning and sequence of the full-length cdna for a major insecticide resistance gene worldwide in the mosquito *culex.quinquefasciatus*," *Journal of Biological Chemistry*, vol. 270, no. 28, pp. 17 044–17 049, 1995.

[107] Chandel, K., Mendki, M. J., Parikh, R. Y., Kulkarni, G., Tikar, S. N., Sukumaran, D., Prakash, S., Parashar, B. D., Shouche, Y. S., & Veer, V. (2013). Midgut microbial community of culex quinquefasciatus mosquito populations from India. PLoS ONE, 8(11).

[108] Marcus J. Pennington1,2, Sean M. Prager1, William E. Walton1 & John T. Trumble1,2

[109] Id, J. H., Gorham, C., Brackney, D. E., & Id, B. S. (2019). Antibiotic resistant bacteria and commensal fungi are common and conserved in the mosquito microbiome. 1–16.

[110] Kirsten Barnard1, Alexander C. s. N. Jeanrenaud 2,3,. Basil D. the contribution of gut bacteria to insecticide resistance and the life histories of the

major malaria vector Anopheles arabiensis(Diptera: Culicidae) Brooke2,3 & shüné V. oliver).

[111] Lee W-J, Brey PT. 2013. How microbiomes influence metazoan development: insights from history and Drosophila modeling of gut-microbe interactions. Annu Rev Cell Dev Bio 29:571–592. doi:10.1146/annurev-cellbio-101512-122333).

[112] Microbiota-Mediated Modulation of Organophosphate Insecticide Toxicity by Species-Dependent Interactions with Lactobacilli in a Drosophila melanogaster Insect ModelBrendan A. Daisley, a,b Mark Trinder, a,b Tim W. McDowell,c Stephanie L. Collins,a,b Mark W. Sumarah,c and Gregor Reidcorresponding authora.

[113] (Antibiotic resistance of potential probiotic bacteria of the genus Lactobacillus from human gastrointestinal microbiome S. G. Botina, E. U. Poluektova, A. A. Glazova, N. V. Zakharevich, N. V. Koroban, V. V. Zinchenko, M. M. Babykin, O. G. Zhilenkova, A. M. Amerkhanova & V. N) (Danilenko Danielsen M., Wind A., Susceptibility ofLactobacillus spp. to antimicrobial agents,Int. J. Food Microbiol. 82 (2003) 1–11.).

[114] Mohammed AN, Abdel-Latef GK, Abdel-Azeem NM, El-Dakhly KM. Ecological study on antimicrobial-resistant zoonotic bacteria transmitted by flies in cattle farms. Parasitol Res. 2016 Oct;115(10):3889-96. doi: 10.1007/s00436-016-5154-7. Epub 2016 May 31. PMID: 27245073.

[115] Alves TDS, Lara GHB, Maluta RP, Ribeiro MG, Leite DDS. Carrier flies of multidrug-resistant Escherichia coli as potential dissemination agent in dairy farm environment. Sci Total Environ. 2018 Aug 15;633:1345-1351. doi: 10.1016/j.scitotenv.2018.03.304. Epub 2018 Apr 2. PMID: 29758886.

[116] Onwugamba FC, Fitzgerald JR, Rochon K, Guardabassi L, Alabi A, Kühne S, Grobusch MP, Schaumburg F. The role of 'filth flies' in the spread of antimicrobial resistance. Travel Med Infect Dis. 2018 Mar-Apr;22:8-17. doi: 10.1016/j.tmaid.2018.02.007. Epub 2018 Mar 1. PMID: 29482014.

[117] Zurek L, Ghosh A. Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. Appl Environ Microbiol. 2014 Jun;80(12):3562-7. doi: 10.1128/AEM.00600-14. PMID: 24705326; PMCID: PMC4054130.

An Appendix

Table 5.1

Imipenem	Ceftriaxone	Nalidixic acid
43	19	15
40	13	13
37	15	19
19	12	10
12	9	7
12	18	6
18	7	18
20	25	20
23	33	8

Table 5.2

Allethrin	Resmethrin	Thiacloprid
0	1	0
0	0	0
1	0	0
0	2	0
0	0	1
0	0	2
2	1	0
0	0	0