

Assessment of bacterial community and characterization of *Pseudomonas aeruginosa* in Diptera flies from livestock kraals and residential dumping sites in Potchefstroom

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DECLARATION

I **Lara de Wet**, declare that the dissertation entitled “ **Assessment of bacterial community and characterization of *Pseudomonas aeruginosa* in Diptera flies from livestock kraals and residential dumping sites in Potchefstroom**”, submitted for the Master of Science in Environmental Sciences degree at the North-West University, has not previously been submitted by me for a degree at this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

Signature:

Date:

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ABSTRACT

The gut microbiota of Diptera flies is poorly understood, especially those that coexist with rural community farms and human waste sites. *Pseudomonas aeruginosa* pose challenges in both clinical and environmental contexts due to its capacity to colonize natural ecosystems and resistance to antibiotics. This study characterized *P. aeruginosa* from illegal residential dumping sites in urban Ikageng and livestock kraals in rural Matlwang village of Potchefstroom, South Africa. Diptera flies were collected in January-February 2022 and were identified using morphological features and by amplification and sequencing of the *Cytochrome Oxidase 1 (CO1)* gene. Meta-taxonomic approach was utilized to investigate the gut bacterial diversity. Furthermore, *P. aeruginosa* was characterized using classical microbiological test, species specific PCR assay targeting the housekeeping *gyraseB* gene, and confirmed by *16S rRNA* gene sequencing. Antimicrobial resistance patterns were investigated on the isolates using disc diffusion assay (DDA) and antibiotic resistant genes (ARGs) by PCR assay. Additionally, six virulence genes (*exoS*, *plcN*, *plcH*, *toxA*, *lasB* and *algD*) were screened among the isolates. The prevalent Diptera flies in Matlwang livestock kraal were identified as *Hemipyrellia* spp. (n = 435/1422, 31%), followed by *Tabanus* spp. (n = 341/1422; 24%) and *Chrysomya* spp. (n = 176/1422; 12%). Ikageng illegal residential dumping site was dominated by *Hemipyrellia* spp. (n = 2461/2793; 88%), followed by *Sarcophagidae* spp. (n = 200/2793; 7.2%), *Chrysomya* spp. (n = 53/2793; 1.9 %), and *Synthesiomyia* spp. (n = 46/2793; 1.6 %). *Tabanus* spp. was only found in Matlwang livestock kraal sites. Meta-taxonomic approach showed that the gut-fly consisted of 92.13% and 90.7% Proteobacteria phyla in Matlwang livestock kraals and Ikageng residential dumping, respectively. *Pseudomonas* species were more prevalent at the Ikageng residential dumping site (98.97%) than in the Matlwang livestock kraal (62.41%). *Rickettsia* spp. were prevalent in Matlwang livestock kraal with a relative abundance of 79%. Culture-based and molecular assays showed that *P. aeruginosa* is prevalent in Matlwang (n = 36/48; 75%) and Ikageng (n = 23/48; 48%). The most commonly detected virulent gene (96.6%) among the isolates was *exoS*, followed by *plcN* and *algD* genes (83.1%), *lasB* (81.4%), *toxA* (76.3%), and *plcH* (47.5%). All isolates were resistant to Metronidazole, Sulphamethoxazole, Cefazolin and Amoxicillin and susceptible to Neomycin (n = 26/59; 44.07%) and Gentamicin (n = 29/59; 49.1%) based on DDA. The sulphamethoxazole, *sulI* gene (n = 52/59; 88.1%) was the most detected ARG found in the isolates, followed by *acc(3)-IV* (n = 36/59; 80.6%) and *rdxA* (n = 21/59; 35.6%) genes that confirms resistance to gentamicin and metronidazole, respectively. The absence of the genes

for Amoxicillin (Pbp1A) and Neomycin (aph (2'')-Ib) in all isolates suggests that they can be utilised to treat *P. aeruginosa* infections. The *P. aeruginosa* was present in both areas; however, it was more common in residential dumping sites, while *Rickettsia* spp. was prominent in livestock kraals. This study has highlighted the significance of the bacteria carried by Diptera flies that are of public health and veterinary importance. The results of this study will assist in informing management decisions on the prevention of bacterial pathogen transmission.

Keywords: Diptera flies, bacterial community, *Pseudomonas aeruginosa*, Residential dumping site, Livestock kraals, Antibiotic resistance.

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LIST OF ABBREVIATIONS AND ACRONYMS

AAT:	African animal trypanosomiasis
<i>acc(3)-IV</i> :	Gentamicin
<i>algD</i> :	Alginate D
AML:	Amoxicillin
<i>aph (2'')-Ib</i> :	Neomycin
ARG's:	Antibiotic resistant genes
ASV's:	Amplicon Sequence Variants
BB:	Binding Buffer
β -lactamases:	Beta-lactamases
BLAST:	Basic Local Alignment Search Tool
BPW:	Buffered peptone water
CLSI:	Clinical and Laboratory Standards Institute
CN:	Gentamicin
<i>CO1</i> :	Cytochrome oxidase 1
DDA:	Disk diffusion assay
DNA:	Deoxyribonucleic acid
EB:	Elution Buffer
EIA:	Equine infectious anaemia
ESBL:	Extended-spectrum β -lactamases
ESKAPE:	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumonia</i> , <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> and <i>Enterobacter</i> spp.
EtBr:	Ethidium Bromide
<i>exoS</i> :	Exoenzyme S
GPS:	Global positioning system
HMM:	Hidden Markov Model
IPM:	Integrated pest management
ITS:	Internal transcribed spacer
IVM:	Integrated vector management
KZ:	Cefazolin

<i>lasB</i> :	Elastas B
LPS:	Lipopolysaccharide
MDR:	Multi-drug resistance
MEGAX:	Molecular Evolutionary Genetics Analysis
MHA:	Mueller-Hinton agar
MTP:	Microbiome Taxonomic Profiling
MTZ:	Metronidazole
N:	Neomycin
NADPH:	Nicotinamide adenine dinucleotide phosphate
NGS:	Next Generation Sequencing
NWU:	North-West University
OMP's:	Outer membrane proteins
ONT:	Oxford Nanopore Technology
OTU:	Operational taxonomic unit
<i>P. aeruginosa</i> :	<i>Pseudomonas aeruginosa</i>
<i>pbp1A</i> :	Amoxicillin
PBS:	Phosphate Buffered Saline
PCR:	Polymerase chain reaction
<i>plcH</i> :	Phospholipase H
<i>plcN</i> :	Phospholipase N
PMI:	Postmortem interval
Pro-K:	Proteinase K
QIIME2:	Quantitative Insight Into Microbial Ecology
<i>rdxA</i> :	Metronidazole
RL:	Sulphamethoxazole
RNA:	Ribonucleic acid
rRNA:	Ribosomal ribonucleic acid
SBA:	Sheep blood agar
SDS:	Sodium dodecyl sulphate
SMA:	Sorbitol McConkey agar

<i>sulI</i> :	Sulphamethoxazole
<i>toxA</i> :	Exotoxin A
UV:	Ultraviolet
WB:	Wash Buffer
WGS:	Whole genome sequencing
WHO:	World health organization

CHAPTER 1

INTRODUCTION

1.1 Background

Agents needed to spread a disease are called vectors, vectors can be classified as ticks, fleas, lice and so forth Müller *et al.* (2019). Vector-borne diseases are illnesses that are caused by viruses, parasites and bacteria. According to Müller *et al.*, 2019, every year more than 700 000 deaths occur due to zoonotic vector-borne diseases. Seventeen percent of the estimated global burden of communicable diseases are due to zoonotic diseases (Müller *et al.*, 2019). Poorer populations living in environmentally degraded environments and houses are more susceptible to be affected by vector-borne diseases. The distribution of vector-borne diseases is dependent on a vast variety of factors such as, social, environmental, and demographic. Vectors are often capable of transmitting the pathogens for the rest of their lives once they have become infected, this occurs during each subsequent bite or blood meal (WHO, 2014).

Diptera are regarded as mechanical vectors that carry multiple pathogenic bacterial species and are one of the most diverse groups of organisms that consist of 128 families and 124 000 species (Skevington & Dang, 2002). The order Diptera is important in controlling pest arthropods, breaking down vegetation that is rotting and provisioning food for other species, they can also be beneficial in forensic sciences, and some play a vital role in transmission of pathogens (Skevington & Dang, 2002). The order Diptera consists of multiple families that include Tipulidae (crane flies), Culicidae (Mosquitoes), Simuliidae (Black flies), Chironomidae (Midges), Tabanidae (Horse and Deer flies), Muscidae (Muscid flies) and Calliphoridae (Blow flies) (Skevington & Dang, 2002).

Metabarcoding analysis using the 16S *rRNA* barcoding gene has proven to be the go-to standard to understand the microbiome associated with the flies (Vences *et al.*, 2016). This makes it easy to study the gut microbiome of the fly and pathogens that are associated with it. Thus, allowing to study uncultured bacteria present in one or multiple samples. This makes it possible to infer relative abundance of bacteria in flies covering considerably greater diversity (Vences *et al.*, 2016). The use of 16S *rRNA* only targets the universal marker of prokaryotes (Vences *et*

al., 2016), however not much information is presented about the antimicrobial resistance and genotyping of the bacterial species and strains. *Pseudomonas aeruginosa* is seen as an opportunistic pathogen that infrequently affects healthy individuals and is commonly associated with ventilator-associated pneumonia together with nosocomial infections (Pang *et al.*, 2019). Continuous growing of antibiotic resistance has made treatment of *P. aeruginosa* a difficult challenge (Lister *et al.*, 2009). β -lactams, aminoglycosides and quinolones are some of the antibiotics that *P. aeruginosa* shows resistance to. The resistance displayed in *P. aeruginosa* can be segregated into three types of resistance namely, adaptive, acquired, and intrinsic resistance (Pang *et al.*, 2019). Biofilm is formed in the lungs of infected patients the biofilms act as a diffusion barrier thus, limiting antibiotic access to the bacterium cells of *P. aeruginosa*. Within the biofilm there is an opportunity for the biofilm to form multidrug-tolerant persister cells that can overcome an antibiotic invasion that can be the causative agent for recurring infections (Mulcahy *et al.*, 2010).

According to Pandey & Cascella (2019) bacterial infections are widely treated and managed with the antibiotic class named β -lactams. The conventional drug classes that are used for the treatment of bacterial infections is the β -lactam antibiotics, but the recurrent occurrence of antimicrobial resistance remains a global health issue. *Pseudomonas aeruginosa* is one of the individual Gram-negative bacilli that is a major matter in question when it comes to the public health challenge in regard to antimicrobial resistance in terms of β -lactams (Pandey & Cascella, 2019). Understanding the mechanisms of resistance is one way to determine which drugs to appoint. The most common resistance to β -lactams is via the production of β -lactams but other mechanisms is also forth coming, such as, the alteration of penicillin resistance in pneumococci (PBP's) of target sites, decreased penetration of the target sites, β -lactamases production generating inactivation and specific pumping mechanisms causing an efflux from the periplasmic space (Pandey & Cascella, 2019).

Clavulanic acids are able to cause inhibition to third generation cephalosporins and aztreonam that is hydrolysed by an evolving group of β -lactams called extended-spectrum β -lactamases [ESBLs] (Paterson & Bonomo, 2005). ESBLs are usually derived from genes that are for *bla_{TEM-1}*, *bla_{TEM-2}* or *bla_{SHV-1}* due to mutations that are able to convert the amino acid configuration around the active site of the β -lactamases (Paterson & Bonomo, 2005). Furthermore, these actions cause the extension of the β -lactams antibiotics susceptible to hydrolysis via these enzymes. Clinical significance is usually coupled with the presence of ESBLs due to ESBLs frequently being plasmid encoded. In relation to plasmid encodement is

the production of ESBL that bears genes that encode for resistance to other drug classes such as, aminoglycosides. Thus, the treatment of ESBL-producing organisms is very restricted (Paterson & Bonomo, 2005).

Classical microbiological and molecular tests remain to be the golden standard for the identification of pathogenic bacterial species (Laupland & Valiquette, 2013). This study employed these methods to isolate and identify enteric pathogens of the family Enterobacteriaceae such as *P. aeruginosa* from Diptera flies collected in Ikageng township and Matlwang village of Potchefstroom, North West province, South Africa. Both Matlwang livestock kraal farming community area and Ikageng residential area are a niche area to investigate the distribution of Diptera flies as carriers for vector-borne diseases.

1.2. Motivation of the study

There is dearth of information Diptera flies and their associated bacterial communities occurring in urban and rural areas of Potchefstroom. Determining the distribution of Diptera is essential for comprehending ecological patterns and biodiversity, since these flies are essential pollinators, decomposers, and environmental health indicators in a variety of environments. Furthermore, monitoring the distribution of Diptera facilitates the tracking of disease vectors (flies) and yields important information for public health campaigns and disease control tactics. As a result, the current study sought to document Diptera flies in urban Ikageng township and rural Matlwang village of Potchefstroom and further fill in scientific information gap on their associated bacterial communities. Due to several Diptera species acting as carriers of pathogenic bacteria such as *P. aeruginosa*, it is important to thoroughly characterize their microbial communities in order to develop targeted disease surveillance and control strategies. This means that knowing the bacterial composition of Diptera can have implications for public health. The gut microbiome of Diptera flies is not well characterized, especially from flies inhabiting in livestock and human surroundings such as residential dumping sites. Antibiotic resistance in *P. aeruginosa* is one of the major concerns in the world. Given that *P. aeruginosa* has the innate ability to develop resistance mechanisms. Therefore, characterizing antibiotic resistance in this pathogen is critical given that it poses a considerable concern in clinical settings. Comprehending the distinct resistance characteristics of *P. aeruginosa* facilitates the development of effective antibiotic treatment approaches, providing medical professionals with relevant medication selection guidance and enhancing the worldwide endeavor to counteract

antibiotic resistance and enhance patient consequences. Ikageng residential dumping site and Matlwang livestock kraals are niche areas for Diptera flies.

1.3. Aim of the study

To investigate the gut microbiome of Diptera flies and characterize *Pseudomonas aeruginosa* from livestock kraals in Matlwang village and illegal residential dumping sites in Ikageng, Potchefstroom, South Africa.

1.4. Objectives of the study

- Identify Diptera flies using morphological and by analyses of *CO1* gene sequences.
- Determine the bacterial community of Diptera flies using 16S metataxonomic approach.
- Isolate and identify *Pseudomonas aeruginosa* strains using microbiological and molecular tests.
- To determine the virulence genes present in *Pseudomonas aeruginosa* isolates.
- To determine the antibiotics resistance patterns of *Pseudomonas aeruginosa* isolates.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Diptera flies

The order Diptera (true flies) is a member of an extensive grouping of neopterous insects that consist of complete metamorphosis and constitute the cohort Oligoneoptera (Rohdendorf, 1974). It consists of an order of insects that comprise of two wings. Insects such as mosquitoes, midges, flies, and gnats form part of the Order Diptera (Day *et al.*, 2002). It is derived from the words “Di”, meaning two and “ptera”, meaning wings, thus indicating that insects belonging to this order are in possession of a single pair of membranous forewings. Diptera are regarded as mechanical vectors that may carry pathogenic bacterial species and transmits to other hosts (Skevington & Dang, 2002). They are one of the most diverse groups of flies that consist of 128 families and 124 000 species (Skevington & Dang, 2002). Diptera consists of multiple families that include Tipulidae (crane flies), Culicidae (Mosquitoes), Simuliidae (Black flies), Chironomidae (Midges), Tabanidae (Horse and Deer flies), Muscidae (Muscid flies) and Calliphoridae (Blow flies) (Skevington & Dang, 2002).

2.2 Biological importance of Diptera

Diptera is important in controlling arthropods pest by breaking down vegetation that is rotting and provisioning food for other species (Skeington & Dang, 2002). They also possess the utmost importance in forensic investigations. A consistent timeframe provided by their life cycle—from egg to larva to pupa to adult—helps in determining the postmortem interval (PMI). Fly eggs are laid on corpses minutes after death because flies are drawn to organic waste that is deteriorating. As they progress through their life cycles, the resulting maggots act as crucial timekeepers. Their existence, species, and stage of development can reveal important details about the circumstances surrounding a death, assisting forensic entomologists in putting together timelines necessary for homicide investigations and judicial actions (Ren *et al.*, 2018). Their function as pollinators is one of their important ecological advantages (Mlynarek, 2022). Many different types of flies help pollinate plants, especially those with pungent blooms that draw them in as major pollinators (Cook *et al.*, 2020). Flies play an important role in the reproduction of many plant species, increasing genetic variety, and preserving healthy

ecosystems, despite being frequently overshadowed by bees and butterflies (Mlynarek, 2022). Diptera flies are crucial to the health of the environment play an important role in pollination, nutrient cycling, and garbage breakdown due to the presence of larvae (Wilk-da-Silva *et al.*, 2018). Their pollination work promotes plant reproduction and genetic variety, both of which are essential for thriving ecosystems. Additionally, their larvae help recycle nutrients, enhancing soil fertility and plant growth (Gold *et al.*, 2018).

However, it's critical to recognize the potential harm that Diptera may cause to the ecology. Some species, like the housefly (*Musca domestica*), have the ability to spread infections to humans and animals, acting as disease vectors (Gupta *et al.*, 2012). They can present serious health problems due to their proximity to populated areas and poor waste management (Qasim *et al.*, 2020). Additionally, some fly species are a problem in agriculture, harming crops and costing farmers money (Issa, 2019). To preserve a healthy coexistence with human and the environment, it is essential to regulate and limit their negative effects, especially with regard to disease transmission and agricultural damage (Gold *et al.*, 2018; Issa, 2019).

2.3 Epidemiology of Diptera in South Africa

There are wide variety of Diptera species present in South Africa (Issa, 2019). Some of the common flies that are well studied are as follows:

The tsetse fly (*Glossina* spp.), which is responsible for spreading the trypanosomes that cause African animal trypanosomiasis (AAT), popularly known as sleeping sickness, is one of the most infamous disease vectors among Diptera in South Africa (De Beer *et al.*, 2016). Both humans and livestock are impacted by this disease, especially in in north-eastern KwaZulu-Natal (De Beer *et al.*, 2016). Tsetse flies are prevalent in particular ecosystems like woodland and savannah regions, where they have a significant negative impact on the well-being of nearby communities (Stich *et al.*, 2002).

The stable fly (*Stomoxys calcitrans*), also part of the housefly family (Muscidae) which is widespread in South Africa, is notorious for spreading a variety of illnesses, including bacteria and parasites, is another worrying vector (Baldacchino *et al.*, 2013). These flies irritate humans and animals by feeding on their blood, which can lead to disease transmission (Rochon *et al.*, 2021). They contribute to the spread of illnesses including brucellosis and summer mastitis in cattle and are particularly common in areas with livestock and inadequate sanitation standards (Baldacchino *et al.*, 2013).

The housefly (*Musca domestica*), another common species in South Africa, is also a well-known mechanical disease vector. Houseflies can spread infections including *Salmonella* spp., *Escherichia coli*, and *Shigella* and feed on a variety of organic substances, including human and animal waste (Alam & Zurek, 2004). They are substantial contributors to the spread of gastrointestinal ailments and a threat to public health because of their capacity to contaminate food and surfaces (Khamesipour *et al.*, 2018). Houseflies seem to be prevalent in the South Africa (Monyama *et al.*, 2022), and are frequently connected to filthy conditions, especially in areas close to habitation and livestock farms (Junqueira *et al.*, 2017). These flies pose a risk of disease transmission to both humans and animals since they carry a variety of bacteria, protozoa, and helminths (Monyama *et al.*, 2023). Due to their function in the decomposition process and their involvement in forensic entomology, Calliphoridae, or blowflies, have forensic significance (Lutz *et al.*, 2018).

2.4 Control measures on fly-vectors in South Africa

By minimising or preventing human contact with vectors, vector control seeks to reduce the spread of infections (Wilson *et al.*, 2020). A multidisciplinary strategy is used in South Africa to manage and control disease vectors, including integrated vector management (IVM) techniques, community outreach programs, and public health activities (WHO, 2012). These programs seek to decrease vector populations, inform the public about vector-borne illnesses, and lower the number of fly breeding grounds by implementing good sanitation and waste management techniques (WHO, 2012). However, none of rural communities' farmers and communities follows these strategies, particularly in the North West province. The epidemiology of diseases spread by Diptera must also be understood in order to design targeted interventions to safeguard public health and lessen the effects of these vectors on society and the environment. Therefore, this requires continual research and disease surveillance.

2.5 Limitations on restrictions and defences against fly vectors

To restrict the spread of infections and lower the incidence of vector-borne diseases in South Africa, fly vector management is essential. The following are some restrictions and defences against fly-vectors in South Africa:

Insecticide-based vector control, frequently used a monotherapy, replaced pre-1940 vector control methods that mainly relied on environmental management and larval control based on an extensive understanding of pathogen transmission (Wilson *et al.*, 2020). Fly-vector

demographic tactics frequently result in decreased genetic diversity within each vector population, which restricts their ability to adapt to vector control interventions by choosing novel traits like insecticide resistance (Schofield & Kabayo, 2008).

2.6 Diptera in livestock kraals

Livestock or livestock kraals are often the result of house vesting many insects, but Diptera is seen as the most abundant insect where livestock is present (Moon, 2018). Diptera present in livestock kraals can be found on the feed, animals and basically all substrates and surroundings where the animals are present (Figure 2.1). Diptera generates dried droplets of fly vomit and feces that causes contamination when it is in contact with the surface of the skin. Diptera plays a vital role in being a vector contributing to enteric pathogenesis. According to Steelman (1976) through-out the year attacks occur on livestock that are originated from a wide variety of arthropod parasites. Diseases can be spread via these vectors that can cause a lack of performance from livestock (such as, growth restriction and health). In terms of the health and welfare of animals, Diptera's presence and effects in livestock kraals (enclosures) in the North West province of South Africa are a major concern, livestock kraals are especially prone to infestations by different Diptera species, such as Muscidae and Calliphoridae (Khamesipour *et al.*, 2018). Studies carried out in this area have revealed the diversity and abundance of Diptera in cattle kraals, highlighting their potential as disease vectors in animals (Khamesipour *et al.*, 2018).

Due to their propensity to organic debris and animal excrement, members of the Muscidae family, which includes houseflies and stable fly, are frequently seen in livestock kraals (Foil & Hogsette, 1994). Additionally, Calliphoridae, or blowflies, are drawn to organic matter that is decomposing and open wounds on livestock (Van Sitter, 2017). Their presence has an impact on the animals' health as well as forensic ramifications for postmortem times (Lutz *et al.*, 2018). Diptera infestations must be well managed and under control in livestock kraals in order to minimize their negative consequences. The fly population can be reduced and the accompanying health hazards for both animals and human or farmers, by employing tactics such proper waste management, routine cleaning, and insecticide application.



Figure 2.1: Matlwang livestock kraal serving as a niche area for Diptera

2.7 Diptera in dumping sites

Dumping sites otherwise known as landfills is seen as a niche area for Diptera. Landfills often occur close to residential areas where citizens often refer to Diptera as a problematic cause that is associated with diseases (Howard, 2001). Due to the implications for public health, environmental hygiene, and waste management, the study of Diptera, in dumping sites, is crucial. Solid garbage is gathered and managed in dumps, which are often referred to as landfills or waste disposal areas (Figure 2.2). Due to the amount of organic waste and ideal breeding conditions, flies, especially those from the Muscidae family, are frequently seen in these locations (Howard, 2001).

Houseflies and stable fly are drawn to the organic material found in landfills because it provides them with adequate nesting grounds and food sources. These flies can spread a variety of bacteria and parasites, making them not just a nuisance but also potentially mechanical vectors that transmit diseases by transporting the causative agent by means of their mouths or feet to humans when they disseminate the disease. To developing efficient waste management plans and public health interventions, research on Diptera in dump sites is essential. To control fly

infestations and lower the risk of disease transmission, integrated waste management strategies, such as efficient waste segregation, covering garbage with soil or other materials, and routine waste disposal, are crucial (Čičková *et al.*, 2015). In order to reduce the appeal of dump sites to flies, safe waste disposal procedures must be promoted through public education and community involvement as well as developing long-term waste management methods and upholding a clean and safe environment, it is essential to comprehend the dynamics of Diptera in dump sites. To create focused treatments that address the issues presented by fly infestations in dumping sites and contribute to the general wellbeing of the populations living in the North West province of South Africa, ongoing monitoring and study in this area are required.



Figure 2.2: Ikageng residential dumping site exhibiting breeding ground for Diptera flies.

2.8 The families of Diptera and their microbiome

One of the largest insect orders is the family Diptera, also known as the fly family. It is a rich and common group of insects with over 150,000 documented species (Grimaldi & Engel, 2005). Dipterans are remarkably ecologically adaptable, inhabiting a variety of environments and engaging in a variety of feeding strategies, such as herbivory, predation, and parasitism (Adler & Courtney, 2019). Their diverse microbiome, which is crucial to their metabolism, nutrition, immunological defences, and behaviour, is frequently credited with their capacity to adapt to various environmental conditions (Chandler *et al.*, 2011). The gut, body surface, and

reproductive organs of Diptera are home to a wide variety of germs, including bacteria, fungus, viruses, and other microbes (Chandler *et al.*, 2011). Since complex polymers like cellulose and chitin are prevalent in their diets, the gut microbiota in particular plays a crucial role in the digestion and metabolism of food (Engel & Moran, 2013). The effective use of various food resources and the acquisition of energy depend on the symbiotic interaction between flies and their gut bacteria.

Diptera's microbiome can affect how they behave and how they interact with the environment and other living things (Gupta & Nair, 2020). In fruit flies (*Drosophila* spp.), for instance, certain bacteria in the gut have been shown to affect mate choice and reproductive success (Sharon *et al.*, 2010). In addition, the gut microbiome of houseflies (Muscidae) and blowflies (Calliphoridae) might change their vector competence, which in turn affects their capacity to transmit pathogens (Junqueira *et al.*, 2017). For several purposes, such as pest control, illness prevention, and biotechnology, it is essential to comprehend the makeup and function of the Diptera microbiome (Husseneder & Grace, 2005). For instance, new approaches to prevent vector-borne illnesses like malaria and dengue fever depend on studies on the gut microbiome of insect vectors like mosquitoes (Culicidae), by leveraging their capacity to break down organic matter and contaminants (Gao *et al.*, 2020). Understanding their gut microbiomes can also be used for waste management, bioremediation, and sustainable agriculture (Husseneder & Grace, 2005). Therefore, the biology, ecology, and interactions of Diptera with their environment are significantly influenced by their microbiome. Understanding the diversity, make-up, and function of Dipteran microbiomes can help us better understand how these organisms adapt, behave, and potentially use them in a variety of contexts.

2.9 Calliphoridae

The Calliphoridae known as blowflies have more than 1,100 species worldwide, are relevant to forensic entomology, medicine, and veterinary sciences as well as important ecological processes (Sukontason *et al.*, 2007). They are of great scientific interest because of their many habitats and distinctive qualities (Hore *et al.*, 2017). Necrophagous is the term used to describe blowflies as they feed mostly on the tissues of deceased animals (Vasconcelos & Araujo, 2012). They are among the first insects to reach decaying animal carcasses, which makes them important in forensic investigations to determine the PMI (Smith, 1986). They serve as useful indications for establishing the sequence of events in forensic cases due to their quick arrival and predictable life cycle (Goff, 1993). By consuming organic matter and helping with nutrient

recycling, blowfly larvae, often known as maggots, contribute significantly to the decomposition of carcasses (Smith, 1986).

Blowflies have four life stages: the egg, larva, pupa, and adult. On suitable substrates like carrion, dung, or open animal wounds, adult blowflies lay their eggs. The larvae that develop from the eggs gorge themselves on the decaying material (Grassberger & Reiter, 2001). After a series of molts, the larvae move away from the food source to pupate when they are at the proper stage. The cycle is completed when the adult flies emerge from the pupae (Figure 2.3).

In addition to their importance in forensics, blowflies can infest life forms including humans and animals with parasites called myiasis (Francesconi & Lupi, 2012). Certain blowfly species' larvae have the capacity to infiltrate live tissues, resulting in uncomfortable sores and possible health problems for the host (Stevens, 2003). In order to reduce the effects of myiasis, blowfly populations must be controlled because it is a problem in veterinary medicine, agriculture, and public health (Francesconi & Lupi, 2012).

2.10 The gut microbiome of Calliphoridae

The ecological and physiological processes of Calliphoridae, depend heavily on their microbiome. These insects have a complex microbiome made up of a wide variety of bacteria, fungus, and other microorganisms that live in their gut (Deguenon *et al.*, 2019; Sontowski & Van Dam, 2020). The Calliphoridae microbiome performs various advantageous tasks, including assisting in digestion and food absorption (Junqueira *et al.*, 2017). In order for the blowfly to effectively utilize nutrients in its diet, bacterial species including Proteobacteria and Firmicutes help break down complex compounds like proteins and carbs. By competing for resources and generating antimicrobial substances, the microbiome can also increase the resilience of the blowflies to diseases, enhancing their general health and likelihood of survival (Sontowski & Van Dam, 2020).

The gut microbiota of the Calliphoridae is not without possible risks, unfortunately as some may create compounds that, when ingested or transmitted through contact, can be toxic to humans and other animals. For instance, some bacteria linked to blowflies can harbour and spread pathogens, endangering public health and food safety such as *P. aeruginosa* (Tomberlin *et al.*, 2017). As a result, even though the Calliphoridae's microbiome is crucial for their physiological processes and ecological interactions, it is crucial to take into account any potential hazards related to their microbial community, especially in situations when they come into contact with humans and environments. The family of flies known as Calliphoridae, or

blowflies, is diverse and significant to the environment due to its necrophagous behaviour and contribution to carcass decomposition. Their importance in numerous scientific fields is highlighted by their forensic importance in determining PMI as well as their propensity to function as disease and myiasis vectors. It is essential to comprehend their biology, life cycle, and behaviour in order to safely use this knowledge to forensic investigations, public health, and animal welfare.

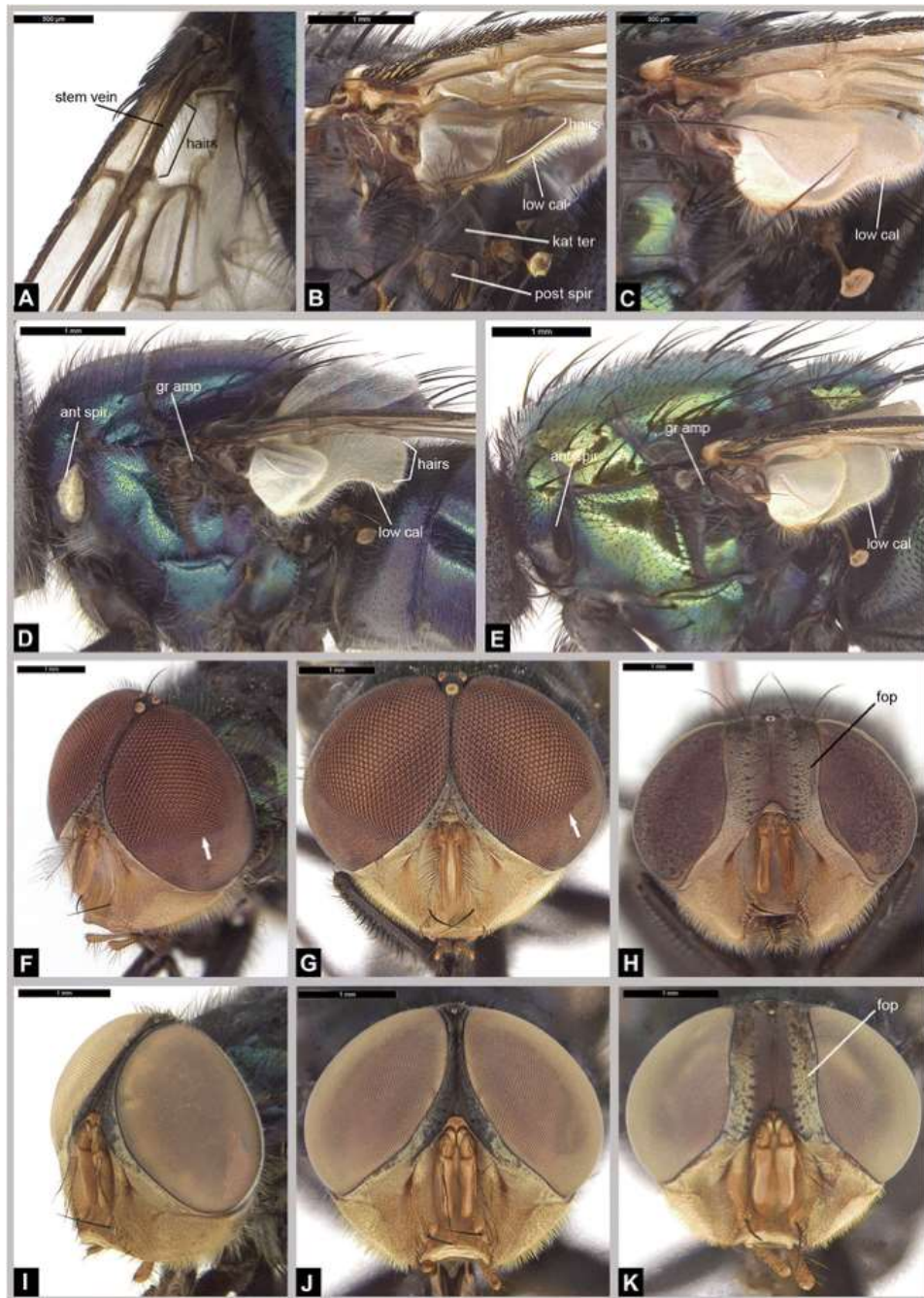


Figure 2.3: Calliphoridae morphology as illustrated in Lutz et al. (2018)

2.11 Muscidae

The order Diptera contains the diversified and widely distributed family of flies known as Muscidae, also known as the housefly. With more than 4,600 documented species, it is one of the most well-known families of flies (Cherix & Pape, 2012). Members Muscidae are significant in terms of their ecological and medicinal significance since they can be found in a variety of habitats, including urban, rural, and natural settings (Geden *et al.*, 2021). One of the most well-known and common members of the Muscidae family are houseflies (*Musca*

domestica). They are widely distributed around the world and have evolved to coexist with humans in close proximity (Peters *et al.*, 2014). In ecosystems, the Muscidae family is crucial as a decomposer and recycler. They consume organic debris, including feces, other detritus, and decaying organic materials. By doing so, they help the environment by breaking down and recycling nutrients (Gerry, 2020). They can also transport and spread a variety of pathogens, including bacteria, viruses, and protozoa that are dangerous to people and animals making them potential disease vectors (Issa, 2019).

Muscidae species biology and life cycles have been extensively researched. For instance, houseflies go through the entire metamorphosis process, going through the egg, larva, pupa, and adult stages (Sanchez-Arroyo & Capinera, 2020). The strong bodies, reddish-brown eyes, and greyish thorax with black stripes of the adult houseflies are their distinguishing features (Figure 2.4). For their larvae, female houseflies prefer to lay their eggs in decomposing organic materials (Kandi *et al.*, 2013). The larvae, also referred to as maggots, are essential for nutrient cycling and waste breakdown. The potential for Muscidae to serve as disease vectors is the source of their medical and economic importance. Particularly houseflies have been linked to the spread of various pathogens that can harm both humans and animals (Khamesipour *et al.*, 2018). The spread of disease that include cholera, typhoid, dysentery, and food poisoning is aided by their ability to take up pathogens from polluted surroundings and transfer them to food, water, and surfaces (De Jesús *et al.*, 2004). To lessen the dangers associated with Muscidae, integrated pest management (IPM) solutions have been developed. IPM uses a comprehensive strategy that combines cultural, biological, and chemical control techniques (WHO, 2012). By reducing breeding grounds and attractants for Muscidae, this strategy seeks to use fewer insecticides and focuses on improving cleanliness and waste management techniques (Geden *et al.*, 2021).



Figure 2.4: Muscidae morphology as illustrated in Couri (2006).

2.12 The gut microbiome of Muscidae

The gut-dwelling microbiome of houseflies is a varied and intricate population of microorganisms. Microorganism facilitates nutrient metabolism and digestion, which helps their diet's efficient breakdown of complex organic molecules. In the gut of houseflies, certain bacterial phyla, such as Firmicutes and Proteobacteria, are common and aid in digestion by dissolving complex compounds to improve nutrient absorption (Park *et al.*, 2019). Additionally, the microbiome can support the decomposition of organic materials, which

supports the health of ecosystems and the crucial ecological role of houseflies as decomposers (De Jonge *et al.*, 2020).

The Muscidae microbiota does, however, have certain potential risks. Houseflies are notorious for their possible role in disease transmission because some microbes in their gastrointestinal tracts may carry and transmit human and animal infections (Park *et al.*, 2019). This feature draws attention to a negative component of their microbiome and emphasizes how crucial it is to manage and reduce house fly populations in locations where they could endanger public health (Hinkle & Hogsette, 2021). To design successful pest management measures that balance their ecological significance with the need to reduce health hazards associated with their presence, it is crucial to understand their biology, behaviour, and their role in the ecosystems.

2.13 Tabanidae

The family of biting insects is known as Tabanidae, and also referred to as horse flies. These insects are notorious for giving extremely painful bites that can irritate and distress in humans and animals (Mullens, 2019). Around 4,500 species of the Tabanidae family can be found all over the world, and they differ remarkably in terms of size, colour, and behaviour (Votýpka *et al.*, 2019). Their wide geographic distribution and presence in a variety of habitats, such as wetlands, grasslands, and woodlands, show how adaptable they are to numerous ecological niches (Mamoudou *et al.*, 2016).

Horse flies and deer flies are recognized for feeding on the blood of their vertebrate hosts (Mullens, 2019). Due to their need for a blood meal to get vital nutrients for egg development, female tabanids are the only blood-feeding species (Lucas *et al.*, 2020). They have mouthparts designed for slicing and piercing their hosts' skin (Figure 2.5), which enables them to easily obtain blood (Lucas *et al.*, 2020). The anticoagulant-rich saliva of tabanids, which prevents the host's blood from clotting during feeding and helps to maintain a continuous flow of blood, can make their bites painful (Gerhardt & Hribar, 2019). They have a history of spreading viruses, bacteria, protozoa, and other pathogens that can lead to illnesses such as equine infectious anaemia (EIA), tularaemia, rocky mountain spotted fever and rickettsialpox (Keita *et al.*, 2020). Additionally, the stress and lower production caused by their aggressive biting behaviour in cattle might negatively impact the animals' general welfare and economic worth (Baldacchino *et al.*, 2014).

To minimize their negative effects on human and animal health, Tabanid control methods are essential. The use of pesticides for chemical control is one method, while habitat management and environmental changes are others (Tudi *et al.*, 2021). For the control of Tabanids, integrated pest management (IPM) strategies that combine a variety of control techniques offer efficient and long-lasting solutions (Baldacchino *et al.*, 2014). Tabanid bite risks can also be decreased in human populations by using insect repellents, protective gear, and good sanitary techniques (Debboun & Strickman, 2013).



Figure 2.5: Tabanidae morphology as illustrated in Mugasa *et al.* (2018)

2.14 The gut microbiome of Tabanidae

The ecological and physiological processes of Tabanidae are significantly influenced by their microbiome. Their microbiome is made up of the wide variety of microorganisms that live in their gut that aid in digestion and nutrition metabolism. Proteobacteria and Firmicutes, are the

two types of gut bacteria that help in breaking down the complex compounds in their diet and for effective nutrient absorption and digestion (Tsakeng *et al.*, 2022).

However, the Tabanidae microbiota may contain bacteria that could create poisons or compounds that are toxic to the fly or other organisms (McKendrick, 1980). Furthermore, some microbes found in the gastrointestinal tract of Tabanidae may serve as pathogen vectors, perhaps spreading diseases to the flies or the animals they feed on (Lefoulon *et al.*, 2021). Because of this, it's vital to take into account the potential hazards connected with specific microbial species, even if the microbiome in Tabanidae provides essential benefits for their digestive systems and general health. In a nutshell the family Tabanidae, which includes horse-flies and deer-flies, is a diversified collection of biting insects renowned for their hematophagous habits and painful bites. Control measures must be put in place to lessen their influence on agricultural and public health because of their capacity to spread disease and discomfort to both human and cattle. To effectively manage tabanid populations and lessen their detrimental effects on society, it is imperative to understand their biology, behaviour, and ecological connections.

2.15 Sarcophagidae

The Sarcophagidae are known as flesh flies that are important contributors to the process of carrion decomposition and waste recycling and are frequently linked to degrading organic debris (Szpila *et al.*, 2015). There are over 2,500 known species in the family Sarcophagidae, with a variety of habitats and ecological roles (El-Bassiony, 2022). They are recognized for their necrophagous feeding habits, which include eating animal carcasses, manure, and other types of rotting waste (Ren *et al.*, 2018). By accelerating the breakdown of organic debris, their function as decomposers is essential in maintaining the balance of ecosystems and nutrient cycling (Mello-Patiu *et al.*, 2014). They aid in the clearance of dead creatures from the environment by depositing their larvae (maggots) on carcasses, which aids in the biodegradation process (Kamal, 1957).

Sarcophagidae undergo a complete metamorphosis during their life cycle, going through egg, larva, pupa, and adult stages (Figure 2.6). In order to provide their larvae with a nutrient-rich environment to grow, female flesh flies frequently lay their eggs in or close to decomposing organic debris (Diaz & Kaufman, 2021). The larvae, also known as maggots, are voracious feeders and are essential to the decomposition process because they consume and decompose the decaying material (Vairo *et al.*, 2017). Since they are often found near decaying carcasses,

flesh flies have forensic significance (Ren *et al.*, 2018). Entomologists can offer vital information regarding the time of death, which is useful in criminal investigations, by examining the phases of development of Sarcophagidae on a corpse (Joseph *et al.*, 2011). In order to calculate the PMI in homicide investigations, forensic entomologists use their life cycle and behaviour (Smith, 1986).

Flesh flies are essential in medical and veterinary entomology in addition to their ecological and forensic value (Ren *et al.*, 2018). Myiasis is a parasite infestation of living vertebrate animals or humans that is known to be caused by a few species of the Sarcophagidae family (Najjari *et al.*, 2020). These flies' larvae can contaminate live tissues, leading to lesions and possibly jeopardizing the host's health (Francesconi & Lupi, 2012).

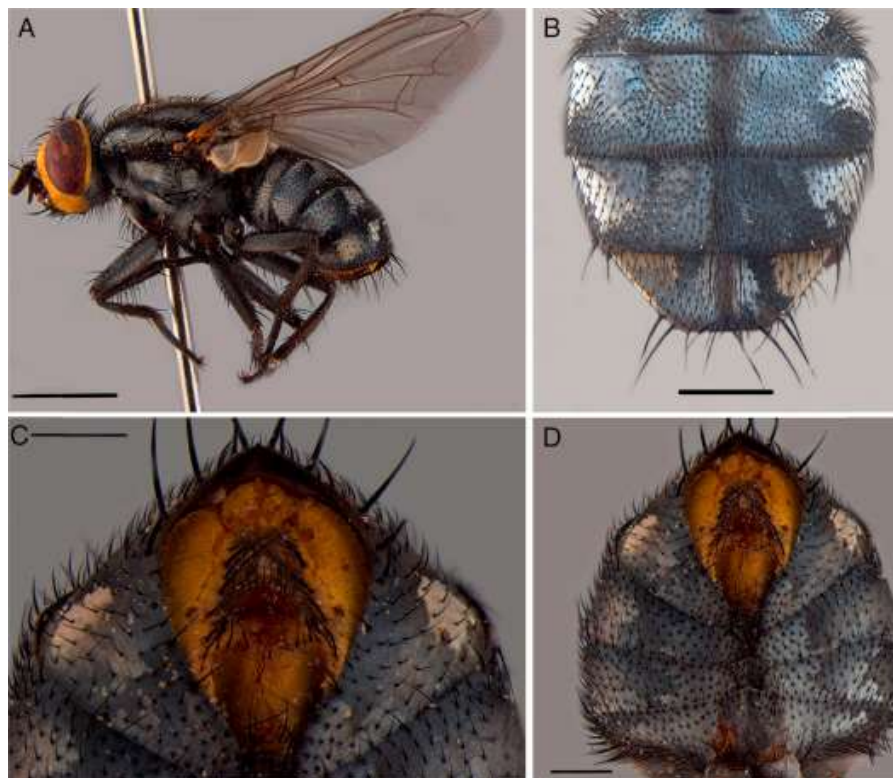


Figure 2.6: *Sarcophagidae* morphology as illustrated by Vairo *et al.* (2015)

2.16 The gut microbiome of Sarcophagidae

Their guts contain a wide variety of microbes that work together to construct a sophisticated microbiome. The digestion and nutrition metabolism of Sarcophagidae are greatly aided by the microbiome. Proteobacteria and Firmicutes, two specific bacteria found in their stomach, help break down complex compounds from their meal to facilitate effective nutrient absorption and digestion (Gupta *et al.*, 2014). Furthermore, the microbiome can support the breakdown of

organic materials, which is a crucial ecological task carried out by flesh flies as detritivores, promoting nutrient cycling and soil health (Ren *et al.*, 2018). Some microbes found in the gut of Sarcophagidae may function as potential infection vectors, endangering both human and animal health (Najjari *et al.*, 2020). Because of this, it's vital to take into account the potential hazards connected with specific microbial species, even if the microbiome in Sarcophagidae provides essential benefits for their digestive processes and ecological responsibilities. Therefore, they are a topic of interest in a number of academic disciplines due to their role in the decomposition process as well as their importance in forensic, medicinal, and veterinary studies (Cherix *et al.*, 2012).

2.17 ESKAPE bacteria and antibiotic resistance

There are six bacteria that possesses antibiotic resistant qualities classified as ESKAPE bacteria. These are known as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. (Figure 2.7). ESKAPE bacteria are associated with multi-drug resistance (MDR) and consist of Gram-negative and Gram-positive bacteria (Mulani *et al.*, 2019). *Pseudomonas aeruginosa* is one of the ESKAPE bacteria that consist of antibiotics resistance genes such as beta lactams found in Diptera's gut. *P. aeruginosa* can develop antimicrobial resistance during anti-pseudomonal chemotherapy and becomes more difficult to treat because of increasing antibiotic resistance (Paprocka, 2022). The classical four-membered Beta-lactamases (β -lactamases) ring amide bond are disrupted via β -lactamases hydrolytic enzymes, which exhibit the antimicrobial ineffective (Poole, 2011). There are four molecular classes of these enzymes, described as A-D, which includes metal dependent (Zn^{2+} - requiring; class B) along with the metal-independent β -lactamases described as (active site serine, classes A, C and D) (Bhalla *et al.*, 2017). *P. aeruginosa* are said to be in possession of consisting of two classes of β -lactamases such as C of cephalosporinase (*AmpC* gene) and class D of oxacillinase *poxB* genes.

Gram-negative bacteria ordinarily possess the common chromosomally encoded enzyme *ampC* which is inducible by a variety of β -lactam antibiotics and cordially contributes to intrinsic resistance (Tamma *et al.*, 2019). The most frequent mechanism of resistance to β -lactams that occur in *P. aeruginosa* is due to the mutation of *AmpC* and goes hand-in-hand with expanded-spectrum cephalosporins and penicillin's (Poole, 2011). Exceptional inducers of *AmpC* are carbapenems, but they possess rapid bactericidal activity together with stability to hydrolysis.

Carbapenems deem effective against *AmpC*⁺ *P. aeruginosa* even though derepressed *AmpC* seems to help carbapenem resistance in addition with other mechanisms of resistance (Poole, 2011). Penicillin's and older, narrow-spectrum cephalosporin were hydrolysed by the initial β -lactamases that were plasmid-encoded restricted-spectrum class A enzymes (Poole, 2011). A wider range of β -lactams such as broad-spectrum cephalosporins, monobactams and carbapenems (Class A, B and D) hydrolyses most β -lactams together with extended-spectrum β -lactamases (ESBL) enzymes (classes A and D). Resistance genes are mobilized and captures by mechanisms of ESBL such as genetic elements and ESBL being encoded by plasmids or transposon-borne genes (integrons). β -lactamase genes are correlated with novel mobile insertion sequences known as ISCR elements in ESBL (Pérez-Etayo *et al.*, 2018). Resistance to broad-spectrum cephalosporins and aztreonam are reported in *P. aeruginosa* due to ESBL hydrolyzation. *P. aeruginosa* isolates often shows resistance to ceftazidime due to the presence of class A ESBLs (Poole, 2011).

The present resistance in *P. aeruginosa* is seen as multifunctional and referable to chromosomal mutations in addition to resistance genes that occur by the means of horizontal gene transfer. Resistance that are impacted by mutational changes incorporate upregulation of multidrug efflux systems that promotes antimicrobial expulsion, the outer membrane permeability being altered to limit the antimicrobial entry and to modify antimicrobial targets and lastly the derepression of *AmpC*, *AmpC* alteration that expand the enzymes substrate specificity (Poole, 2011). Extended-spectrum β -lactamases (ESBL), carbapenems that hydrolyse most β -lactams aminoglycoside-modifying enzymes and 16S rRNA methylases that provide high-level pan-aminoglycoside resistance are mechanisms that contribute to the resistance in *P. aeruginosa* (Poole, 2011). Infections caused by *P. aeruginosa* are usually treated by β -lactams comprising of penicillin's, carbapenems, cephalosporins and monobactams. Antibiotic cleavage by β -lactams enzymes, the reducing drug uptake leading to the loss of outer membrane porin proteins and the antibiotic expulsion by chromosomally encoded efflux mechanisms are a few of the mechanisms that has built to the resistance of agents (Poole, 2011).

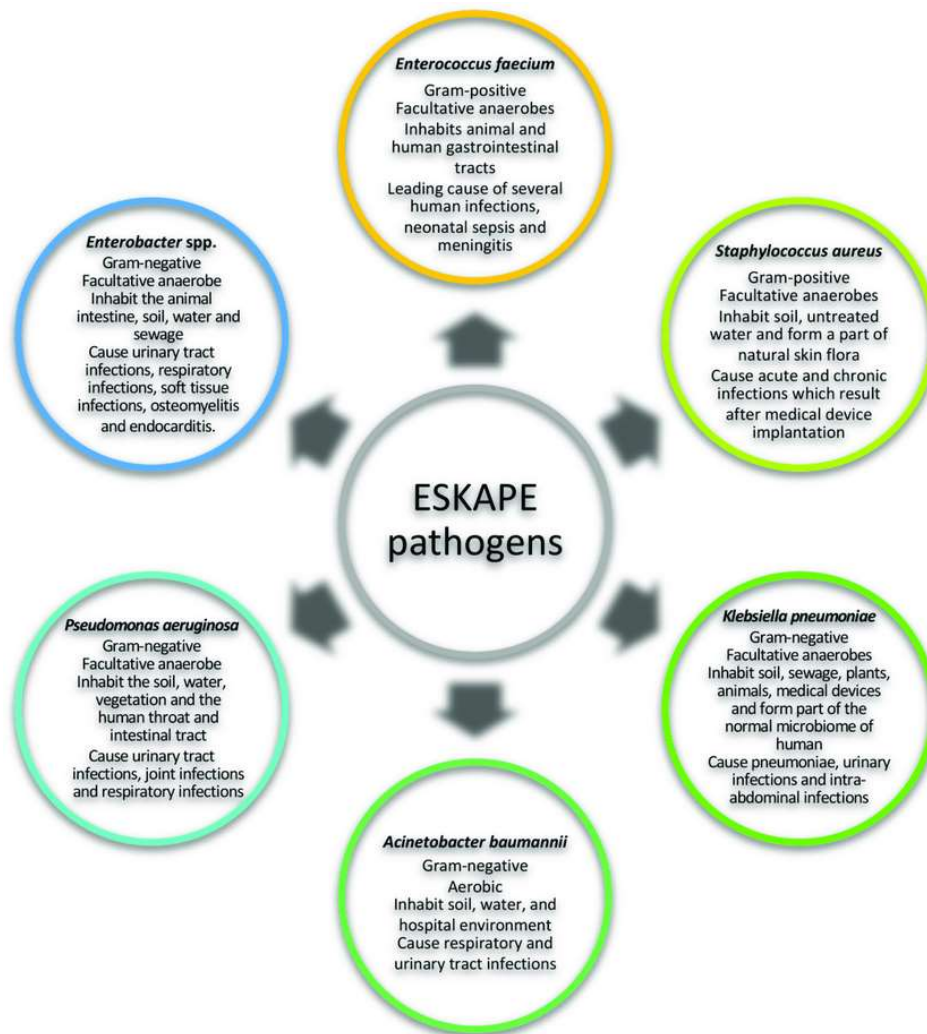


Figure 2.7: Characteristics of ESKAPE pathogens as illustrated in Motiwala *et al.* (2022)

2.18 Aetiology of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic, non-spore forming bacterium that falls within the γ - proteobacteria (Chevalier *et al.*, 2017). It capable of causing a variety of infections in both immunocompetent and immunocompromised hosts, meanwhile a variety of dynamic defences makes it difficult to treat in modern medicine (Mulcahy *et al.*, 2014). It has a potential to colonize a great variety of ecological niches, including and not limited to aquatic ecosystems, animal host (causing severe infection) and lastly rhizosphere (Chevalier *et al.*, 2017). It comprises of a low outer membrane permeability leading to a high inherent resistance to antiseptics along with antibiotics (Pachori *et al.*, 2019). The leading cause for both morbidity and mortality are due to chronic infection with *P. aeruginosa* and frequently occur in patients with cystic fibroses (CF) (Ehsan & Clancy, 2015). This bacterium is highly adaptable to the host environment in addition to the expression of various toxins and virulence factors (Stölting

& Lloyd, 2022). *P. aeruginosa* infections tend to be a challenge to treat, and new effective therapeutic strategies are needed. *P. aeruginosa* can successfully infect and cause disease in individuals due to its ability to adapt to the host environment by means of secreting a variety of virulence factors. These virulence factors include the possession of lipopolysaccharide (LPS). Furthermore, host cells are poisoned via endotoxicity of the lipid A in LPS that sanction attachment, tissue damage and lastly the recognition by host receptors (Qin *et al.*, 2022). Biofilm development and antibiotic tolerance might be directly associated to LPS. The second virulence factor is the outer membrane proteins (OMPs) that contributes to adhesion, antibiotic resistance and nutrient exchange (Qin *et al.*, 2022). The pili, flagellum and other adhesions are correlated to the formation of biofilms that cause multi-drug resistance (MDR). Flagella (T6SS-associated), pili (T4SS) and multi-toxin component type 3 secretion system (T3SS) are only a few of six types of secretion systems that has been identified which function at colonization of the host, swimming, adhesion and finally swarming responding chemotactic signalling. The last factor involves three exopolysaccharides alginate, Psl and Pel that contributes to the facilitation of biofilm formation, while compromising bacterial clearance (Qin *et al.*, 2022). The intracellular environment is affected by the T3SS complex system that critically obstruct the host defence by means of injecting cytotoxins such as *ExoS*, *ExoU*, *ExoT* and *ExoY*. These toxins are known to block phagocytosis and bacterial clearance (Qin *et al.*, 2022).

2.19 Identification of *Pseudomonas aeruginosa*

Gram-negative, rod-shaped *P. aeruginosa* is a widespread bacterium that can be found in a variety of contexts, including soil, water, and hospitals (Habbal *et al.*, 2011). Through a combination of laboratory procedures, it can be quickly identified due to its distinctive properties. The isolation of the organism from a clinical or environmental sample is often the first step in the identification process. Initial identification frequently relies on its distinctive morphology, which manifests as colonies with a bluish-green tint because of the production of pyocyanin pigment (El-Fouly *et al.*, 2015). Additionally, *P. aeruginosa* has the critical enzymatic activity of being oxidase positive, which is utilized to distinguish it quickly from other Gram-negative bacteria (Pachori *et al.*, 2019).

2.20 Microbiological test

A key component of laboratory diagnostics is microbiological testing, which enables the identification, characterisation, and analysis of a wide range of microorganisms, including bacteria, viruses, fungi, and parasites. With their ability to provide information on microbial existence, behaviour, and potential dangers, these tests are essential for clinical, scientific, environmental, and industrial uses. Testing for microorganisms uses a variety of methods. On specialized agar media, initial evaluations frequently entail macroscopical observation of growth traits such as colony shape, colour, texture, and growth patterns. This can offer important hints regarding the type of microorganism being studied. A key tool in the identification of microorganisms is biochemical testing. These tests assess metabolic processes, such as the use of particular substrates (such as glucose and amino acids) and enzymatic reactions. The catalase test, the oxidase test, fermentation tests, and the urease test are typical biochemical assays. These tests assist in identifying the metabolic pathways used by *P. aeruginosa* (Table 2.1).

Table 2.1: Characteristic, properties, and enzymatic reaction of Pseudomonas aeruginosa (Wahba & Darrel, 1965; Tang et al., 2017; Aryal, 2022)

Characteristics	Properties of <i>P. aeruginosa</i>
Capsule	Thick polysaccharide capsule
Catalase	Positive
Cetrimide test	Positive
Citrate	Positive
Coagulase	Negative
Flagella	Flagellated (single)
Gas	Negative
Gelatin hydrolysis	Positive
Gram-staining	Negative
Hydrogen sulfide	Negative
Haemolysis	Beta-haemolytic
Indole	Negative
Motility	Motile
Methyl Red	Negative

Nitrate Reduction	Positive
Oxidative-Fermentative	Oxidative
Oxidase	Positive
Pigment	Blue green
Shape	Rod
Spore	Non-sporing
Triple sugar iron agar	Alkali (red)
Urease	Negative
Voges Proskauer	Negative
Fermentation of sugar	
Adonitol	Negative
Arabinose	Negative
Cellobiose	Negative
DNase	Negative
Erythritol	Negative
Fructose	Positive
Galactose	Negative
Glucose	Positive
Hippurate	Negative
Inositol	Negative
Inulin	Negative
Lactose	Negative
Malonate	Positive
Maltose	Negative
Mannitol	Positive
Mannose	Negative
Mucate	Negative
Myoinositol	Negative
Rhamnose	Negative
Ribose	Positive
Sorbitol	Negative
Starch	Negative
Sucrose	Negative
Tartrate	Negative

Trehalose	Negative
Xylose	Negative
Enzymatic reactions	
Acetate utilization	Positive
Arginine dehydrolase	Positive
Esculin hydrolysis	Negative
Lecithinase	Negative
Lipase	Positive
Lysine	Negative
Ornithine decarboxylase	Negative
Phenylalanine deaminase	Negative

2.21 Molecular tests

2.21.1 Salting out DNA extraction method

An excellent and well-researched approach for extracting DNA from Diptera is the salting out method. Using a high-salt solution, like sodium chloride or ammonium acetate, this technique precipitates proteins and extracts DNA from the material (Maurya *et al.*, 2013). The high-salt solution is added after the Diptera tissue has been homogenized, causing protein precipitation and keeping the DNA in solution. The DNA is separated by centrifugation and can then be further refined for use in a variety of molecular biology applications. The salting out technique is well-liked for its ease of use, low cost, and effectiveness in producing high-quality DNA appropriate for genetic research (Maurya *et al.*, 2013).

2.21.2 Polymerase chain reaction (PCR)

A fundamental method in molecular biology, polymerase chain reaction (PCR) allows DNA segments to be amplified. With the use of heat cycling, targeted primers, and DNA polymerase, PCR can produce millions of copies of a target DNA region. DNA is denaturated at a high temperature, primer annealing occurs at a lower temperature, and extension occurs at a middle temperature during the process. PCR's sensitivity, specificity, and speed have transformed a number of sectors, including genetic research, forensics, and diagnostics. It is essential to genetic analysis, mutation detection, and sequencing, leading to a multitude of scientific breakthroughs and uses (Mullis & Faloona, 1987).

2.21.3 16S ribosomal ribonucleic acid (16S rRNA) gene

In microbial taxonomy and phylogenetics, the 16S ribosomal ribonucleic acid (16S rRNA) gene (1550 bp) is a key genetic marker that is widely employed (Woese, 1987). It includes sections with conserved and variable sequences (V1 – V9) and encodes a portion of the small ribosomal subunit in prokaryotes (Figure 2.8). By creating universal primers for PCR amplification, the conserved areas make it easier to examine the diversity of microorganisms and their evolutionary links (Woese, 1987). 16S rRNA gene sequence analysis helps identify and categorize bacteria and archaea, offering important insights into the composition and roles of microbial communities in many settings. The 16S rRNA gene is still a vital tool in microbial ecology and microbiology research since it is preserved in all bacteria (Woese, 1987). In modern day the widely utilized Illumina sequencing platform gives a better throughput, is cheaper in costs, and less work compared with Sanger. Illumina produces short sequences around ≤ 300 bases, due to this a large number of studies only uses a portion of the gene that is necessary for Illumina sequencing.

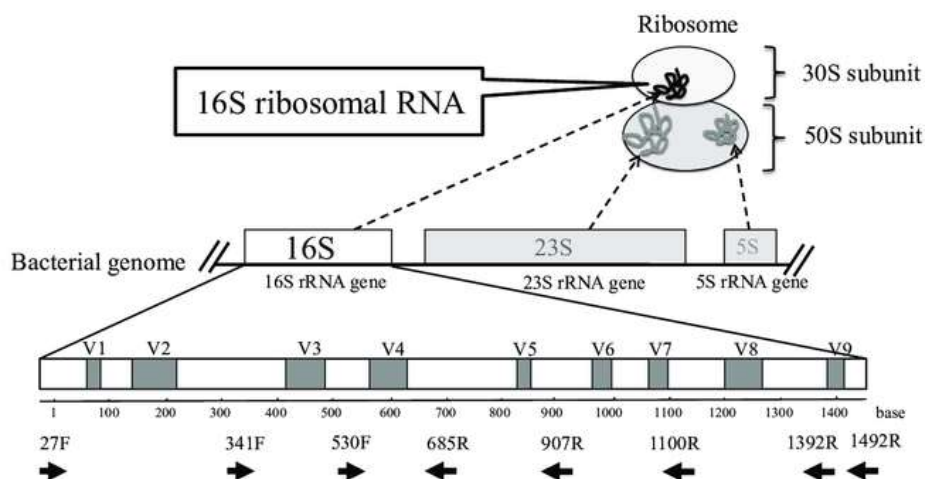


Figure 2.8: Scheme of 16S rRNA gene illustrated in Fukuda *et al.* (2016)

2.21.4 Cytochrome oxidase 1 (COI) gene

A mitochondrial DNA marker called the *cytochrome oxidase 1* (COI) gene has been crucial in the identification and research of Diptera (Hebert *et al.*, 2003). A standardized area of the COI gene, usually 650 base pairs, is amplified and sequenced in DNA barcoding, a technique that makes extensive use of COI. With the use of this DNA barcode, Diptera species can be distinguished from one another according to genetic differences found in the COI sequence. This makes reliable species identification, taxonomic research, and biodiversity assessments

possible. When it comes to identifying cryptic species, clearing up taxonomic ambiguities, and supporting ecological and evolutionary studies pertaining to Diptera populations, *COI* sequencing has been especially useful (Hebert *et al.*, 2003). Furthermore, knowledge of the phylogenetics and evolutionary relationships within the Diptera order has been aided by the *COI* gene. All things considered; the *COI* gene is a useful tool for explaining Diptera diversification. In general, the *COI* gene is a valuable resource for understanding the diversity and biology of Diptera, which is important for many other scientific domains (Hebert *et al.*, 2003).

2.21.5 Sanger sequencing

Frederick Sanger and associates created the fundamental technique for DNA sequencing, known as Sanger sequencing, in 1977 (Figure 2.9). It involves the use of DNA polymerase to replicate DNA, and at certain bases, chain-terminating dideoxynucleotides stop the replication process, resulting in a sequence of fragments that vary in length (Sanger *et al.*, 1977). After size-separating these pieces using gel electrophoresis, the DNA sequence can be ascertained by reading the terminal bases (Sanger *et al.*, 1977). Sanger sequencing is still the gold standard for accuracy and is essential for many molecular biology applications, but Next Generation Sequencing (NGS) methods, which are more affordable and have higher throughput, are replacing it. However, Sanger sequencing is still essential for applications needing high precision and small read lengths, as well as for confirming NGS results.

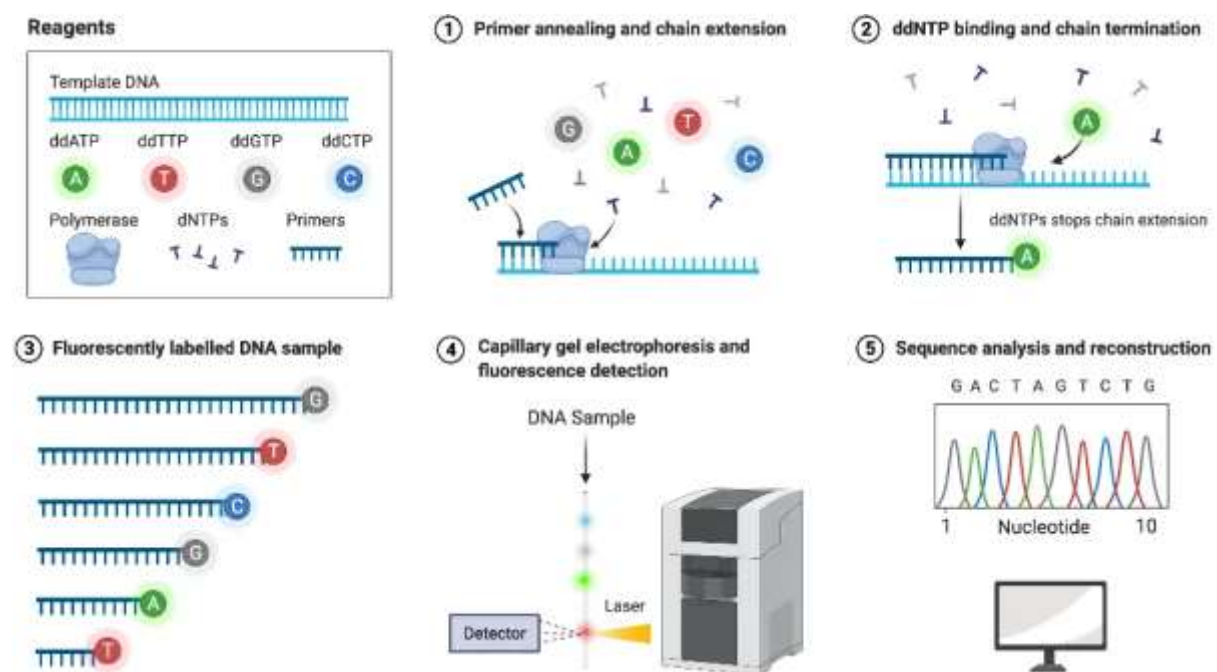


Figure 2.9: Process of Sanger Sequencing as illustrated in Shrestha (2023)

2.21.6 Next generation sequencing

Using next generation sequencing (NGS), genomic research is able to conduct high-throughput, massively parallel sequencing of DNA at a never-before-seen scale and speed (Figure 2.10). In contrast to conventional Sanger sequencing, NGS offers complete insights into genomes, transcriptomes, and epigenomes by processing millions of DNA fragments simultaneously and affordably (Goodwin *et al.*, 2016). Important NGS systems such as PacBio, Oxford Nanopore Technology (ONT), and Illumina use different sequencing chemistry and have different read lengths and throughput capabilities. Because NGS significantly lowers the cost per base, it has revolutionized genomics and made a wide range of applications possible, including transcriptomics, metagenomics, whole genome sequencing, exome sequencing, and more (Goodwin *et al.*, 2016). Research on infectious diseases, cancer genomes, uncommon disease detection, tailored therapy, and comprehension of intricate biological processes have all benefited greatly from this approach (Goodwin *et al.*, 2016). NGS is expected to have a major impact on clinical applications and biomedical research as it develops and interacts with other "omics" technologies.

2.21.7 Illumina

Prominent in Next Generation Sequencing (NGS) technologies, Illumina is a biotechnology leader known for its sequencing platforms and related technologies (Mardis, 2008; Young & Gillung, 2019). By using cutting-edge sequencing-by-synthesis techniques, their sequencing platforms such as the Illumina NexSeq and NovaSeq series achieve high-throughput and high-accuracy sequencing. Millions to billions of DNA fragments may be sequenced simultaneously using Illumina's technology that opened up a wide range of genomic applications such as targeted sequencing, RNA sequencing, whole-genome sequencing, and more (Mardis, 2008). The company's ongoing innovations have made a substantial contribution to the general acceptance and use of NGS in clinical diagnostics, personalized medicine, and genomics research (Mardis, 2008).

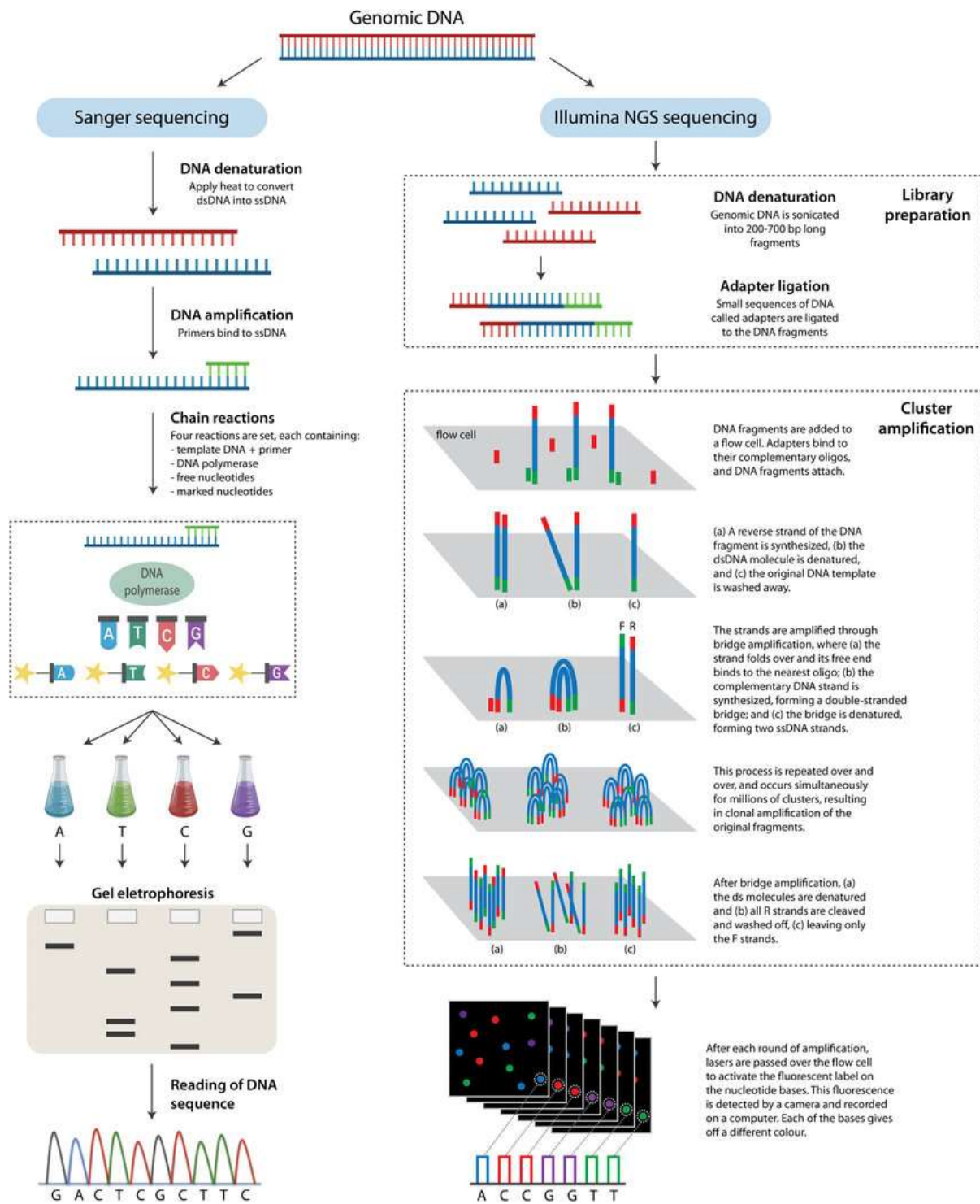


Figure 2.10: Illustration of Sanger Sequencing versus Next Generation Sequencing (Young & Gillung, 2019)

2.22 Metataxonomic approach in flies

The thorough investigation of the microbial communities that live inside Dipteran insects is known as metataxonomic, and it sheds light on the variety of ecological roles and interactions

that these communities play (Wang *et al.*, 2011). Metataxonomics uses high throughput sequencing technologies, including next generation sequencing (NGS), to identify and measure the diversity and composition of microorganisms by focusing on particular genetic markers, such as the internal transcribed spacer (*ITS*) region for fungi and the *16S rRNA* gene for bacteria (Wang *et al.*, 2011). Through the discovery of intricate symbiotic relationships between Diptera and the microorganisms they relate to, this method sheds light on several topics, including behaviour, nutrition, and interactions with pathogens. Understanding the functional relevance of Dipteran microbial communities is made possible by the application of taxonomic analysis, which also advances more general ecological and evolutionary research (Wang *et al.*, 2011). Metataxonomics has been used to study bacterial communities in a variety of Diptera species, such as tsetse flies, houseflies, and fruit flies. These investigations have yielded significant new information about the composition and diversity of bacterial communities in flies and their potential roles in host-microbe interactions. Metataxonomics is a powerful tool for studying bacterial populations in flies.

An investigation into the intestinal bacterial communities of Trypanosome-infected and uninfected *Glossina palpalis* from three foci of human African trypanosomiasis in Cameroon was conducted using *16S rRNA* gene sequencing (Jacob *et al.*, 2017). In a separate study, the bacterial communities of two species of *Colocasiomyia* that consume Araceae were investigated. A meta-analysis was conducted utilizing published data from *Drosophila* bacterial community investigations (Chen *et al.*, 2022). In another study after a metataxonomic examination of four Antarctic sponges, a considerable number of phyla-related Amplicon Sequence Variants (ASVs) were found (Ruocco *et al.*, 2021). Lastly, a study utilizing *16S rRNA* gene sequencing investigated the bacterial communities associated with houseflies (*Musca domestica* L.) residing in South African hospices expressed the need of metataxonomic approach in flies (Monyama *et al.*, 2023).

2.23 Metagenomics approach

In Diptera, a metagenomic method provides an in-depth understanding of the complete microbial community by directly analysing the genetic makeup of every organism found in ambient or intestinal samples (Minard *et al.*, 2013). This method allows the determination of microbial diversity and functional potential by sequencing DNA from the sample using Next Generation Sequencing (NGS) without the need for prior isolation or cultivation (Minard *et al.*,

2013). Diptera's metagenomics provides information about their interactions with pathogens, symbiotic partnerships, and metabolic capacities mediated by their microbiota. It has played a crucial role in revealing the dynamics and complexity of the microbial communities connected to Diptera, advancing our knowledge of their biology and ecological functions (Minard *et al.*, 2013). Metagenomics is a powerful technique for investigating the diversity and functions of microorganisms in flies. Examining the variety and functions of microorganisms in flies can be done effectively with the use of metagenomics (Chellappan & Ranjith, 2022). These investigations have provided significant new insights into the composition and diversity of bacterial communities in flies and their potential roles in host-microbe interactions. Metagenomic techniques have been used to study the bacterial communities associated with a number of fly species, such as *Drosophila melanogaster*, houseflies, and tsetse flies (Chellappan & Ranjith, 2022). Metagenomic sequencing analysis of fly microbiomes has shown that flies can serve as reservoirs for the spread of bacteria (Junqueira *et al.*, 2017).

CHAPTER 3

MATERIALS AND METHODS

3.1 Collection of Diptera flies

Sampling traps were set to collect Diptera flies during the summer season (January - February 2022) from illegal dumping area at Ikageng township of Potchefstroom (Figure 3.1B) 26.7257° S; 27.0500° E as well as at Matlwang livestock kraals (Figure 3.1C) 26.4451°S; 26.5552° E in the North West province. Collection of the fly traps occurred over 2-day intervals in a week (3 times a week). The sites were located and marked using a global positioning system (GPS). Redtop-Trap Disposable Fly Catcher Traps were used to set out for over a week and were further collected in intervals of 2 days. The Redtop-Trap Disposable Fly Catcher traps consisted of dimensions of 22 x 28 x 29.5 cm and weighed 0.015 kg and supplied with bait (Figure 3.2). The traps were set out in Matlwang livestock kraals in open spaces using trees as anchor points to optimize the probability of accumulating different Dipteraflies. Due to Matlwang's significantly greater surface area than Ikageng, a total of 3 fly traps were set out, whereas 2 traps were set at Ikageng. Diptera fly samples were further collected in vials and stored at the North-West University in a refrigerator at 4°C to avoid any further contamination and decrease the rate of growth of any bacteria that may be present.

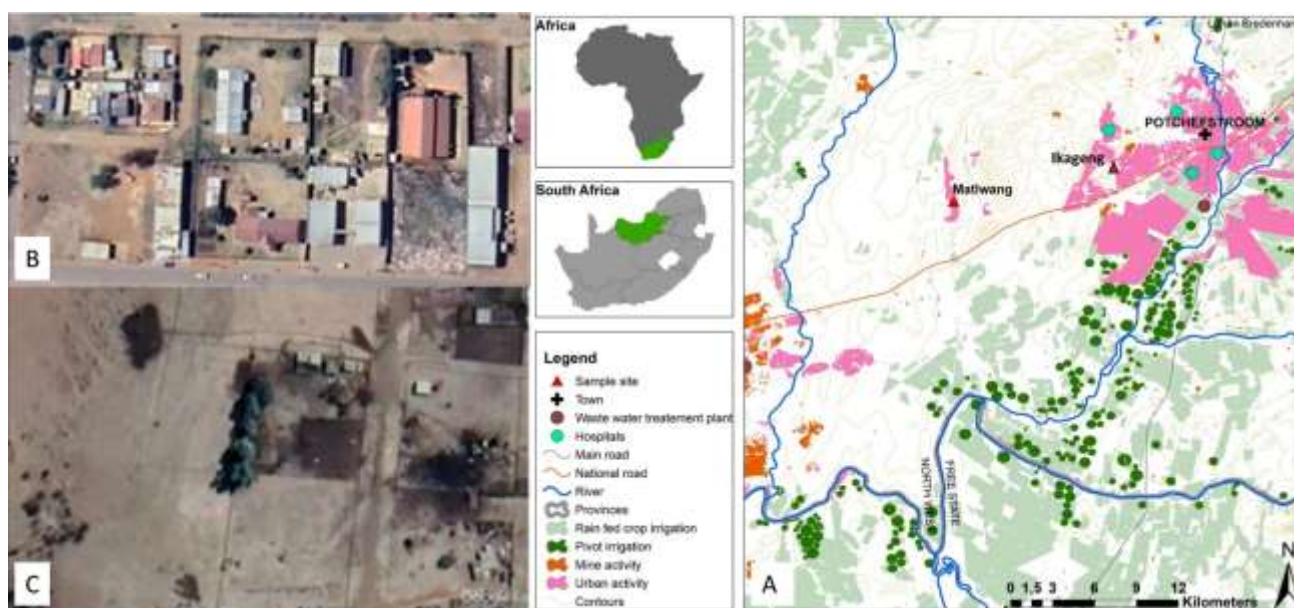


Figure 3.1: A: Image of the North West province in South Africa (Lohan Brendenhann, 2023). B: Ikageng residential dumping site (Google Maps, 2023). C: Matlwang community area (Google Maps, 2023).



Figure 3.2: Image of Redtop-Trap Disposable Fly Catcher trap

3.2 Identification of Diptera flies using microscopy

Diptera flies were sorted based on morpho-species identification and placed in a 90:10 ratio of Phosphate Buffered Saline (PBS) to glycerol to ensure preservation for further downstream processing. Sterile equipment, such as forceps was used aseptically during the identification and sorting of Diptera flies. A stereoscopic zoom microscope, the Nikon SMZ1500, was used to identify the flies based on shape, size, and colour. Based on Ward (2000) descriptions, the phenotypical representation of the Diptera flies was identified. *Hemipyrellia* spp. and *Chrysomya* spp. are grouped under the same family called Calliphoridae and identified by morphological characteristics based on Akbarzadeh *et al.* (2015). The *Tabanus* spp.,s belong to the family of Tabanidae and morphological characteristics of Tabanidae are identified based on Mullens (2019) method furthermore, *Synthesiomyia* spp. belongs to the family Muscidae. Morphological characteristics of the Muscidae family were identified based on previous

method used by Al-Ashba *et al.* (2021). *Sarcophagidae* spp. belong to the family Sarcophagidae, were morphologically identified according to identification keys used by Vairo *et al.* (2015).

3.3 Identification of Diptera flies using metataxonomic approach

3.3.1 DNA extractions of Diptera flies

DNA isolation from Diptera flies was conducted using the salting out method according to Gautam (2022). Briefly, the whole fly was crushed with a metal pistil (± 0.2 mm diameter) in a 1.5 ml Eppendorf tube. About 480 μ L DNA lysis buffer was added (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate (SDS) in the Eppendorf tube. About 10 μ L of Proteinase K (Pro-K) was added to the contents and vortexed for 10 minutes using the tissue lyser and incubated at 56°C for 1 hour. After an hour, an additional 10 μ L of Pro-K was added and incubated again at 56°C overnight. The reaction mixture was then centrifuged for 5 minutes at 12 000 rpm, thereafter, the supernatant was transferred into a new 1.5 mL microcentrifuge tube. About 180 μ L of 5 M NaCl was added to the supernatant, vortex for 30 seconds and centrifuged at 13 500 rpm for 5 minutes. The supernatant was transferred into a new 1.5 mL microcentrifuge along with 420 μ L of ice-cold isopropanol (Propan-2-ol). The reaction mixture was mixed by inverting the tubes 50 times (slowly) followed by centrifugation at 13 500 rpm for 15 minutes at 4°C to precipitate the DNA. After centrifugation, the supernatant was discarded and the DNA pellet containing the DNA was washed with 250 μ L of 75% ethanol. Thereafter, the DNA was vortexed briefly and centrifuge at maximum speed (13 500 rpm) for 5 minutes with this step being repeated. The supernatant was discarded, and the samples were air dried for an hour at room temperature to evaporate the 70% ethanol. Afterwards, DNA was dissolved using 200 μ L of double distilled H₂O. The DNA quality and integrity of the nucleic acid was determined using the Thermo Scientific™ NanoDrop™ One Spectrophotometer Nano-drop and on gel-electrophoresis using 1% agarose stained with 1 μ L of ethidium bromide and visualized under UV light.

3.3.2 PCR using *Cytochrome Oxidase 1 (CO1)*

The *Cytochrome Oxidase 1* gene amplicon was used to amplify the DNA for the fly-species identification, using the forward (LCO1): 5'-GGTCAACAAATCATAAAGATATTGG-3' and reverse (HCO1): 5'-TAAACTTCAGGGTGACCAAAAATCA-3' primers. The PCR conditions consisted of; 95°C for 10 minutes, 95°C for 30 seconds, 47°C for 30 seconds and

72°C for 60 seconds at 35 cycles. The final extension at 72°C for 7 minutes (Mantelatto *et al.*, 2016; Rodrigues *et al.*, 2017).

3.3.3 Sequencing of the *Cytochrome Oxidase 1 (CO1)* and phylogenetic analysis

The PCR products of the *CO1* gene were cleaned up using the PCR Clean-Up System kit in preparation for sequencing. The amplification of the *CO1* gene for sequencing was performed using the universal primers LCO and HCO and sequenced on ProFlex PCR System (ThermoFisher scientific, SA).

3.3.4 Bioinformatics analysis on Diptera fly *CO1* gene.

FinchTV (Treves, 2010) was used to edit the base calls of the sequenced gene displayed on a chromatogram. Ambiguous bases were trimmed from the 5' and 3' end using the FinchTV (Treves, 2010). BioEdit (Tippmann, 2004) was used to join the forward and reverse strands. The nucleotide Basic Local Alignment Search Tool (BLASTn) (Chen *et al.*, 2015) was used to identify the query sequence of the fly species. Reference sequences were retrieved from GenBank of National Centre for Biotechnology Information (NCBI) to infer phylogenetic relationship of the fly species. Multiple sequence alignment was conducted using BioEdit. Molecular Evolutionary Genetics Analysis (MEGAX) (Kumar *et al.*, 2018) was used to generate the phylogenetic trees using maximum likelihood (Kumar *et al.*, 2018). A bootstrap value of 1000 was used to ensure confidence of the tree.

3.3.5 Library preparation and 16S *rRNA* gene sequencing using metataxonomic approach

The 16S *rRNA* gene sequencing of the bacteria on the Diptera flies was executed using the 16S *rRNA* gene primers performed according to Illumina DNA library preparation protocol (Illumina, USA). Amplicon was administered by a reaction set up as described in the 16S Metagenomic Sequencing Library Preparation manual using the V3-V4 sequencing primers (Illumina, USA). The PCR reactions were performed on a thermalcycler using the following conditions, 95°C for 3 minutes, 25 cycles of: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 72°C for 5 minutes and an infinite hold at 4°C (Lorenz, 2012). Samples were confirmed by running them on a 1.5% agarose gel. Sequencing was performed at the North-West University (NWU) in Potchefstroom, North West, South Africa on a MiSeq sequencer using the manufacturer's guideline (Illumina, 2013).

3.3.6. Bioinformatics analysis on the 16S rRNA gene sequencing

Processing of the metabarcoding data was performed using Quantitative Insight Into Microbial Ecology (QIIME2) pipeline using the default settings, using the SILVA 138 reference database. The Microbiome Taxonomic Profiling Pipeline (MTP) workflow used in EzBioCloud is as follows (Yoon *et al.*, (2017): Two sequences (forward and reverse) of the same PCR amplicon were merged via overlapping of sequence information. Primer sequences were removed for PCR using the 16S rRNA gene due to primers used for PCR not being considered as “sequenced”. The processing was conducted using an in-house code. Low quality sequences were filtered out using these parameters: sequence lengths of <100 bp, average Q value <25 and sequence not predicted as 16S according to Hidden Markov Model (HMM) based search. Identical sequences as de-replicated were extracted to decrease computational time. The de-replicated sequences were used for taxonomic assignment using the VSEARCH program as suggested by Rognes *et al* (2016). VSEARCH calculated and searched the sequence similarities of the NGS reads against the EzBioCloud 16S rRNA database at a 97% similarity for cut-off percentage for species-level identification. Chimeras were inspected on the remaining reads using the UCHIME program. An “open reference” method or algorithm was used for OTU’s assignment using 4 steps namely. A 97% cut-off for species-level identification. UCLUST tool was used to cluster the sequences not matched by the 97% similarity boundary. The final set of OTU’s comprised of the species and was used for calculating alpha diversity indices. Singletons that remain were ignored in the OTU’s assigning. This is crucial for Illumina short reads due to a possibility of over-estimating the number of OTU’s. The OTU information was used for various alpha-diversity indices such as Shannon and Simpson diversity and specie richness and later secondary analysis. The output files were formatted to a compatible format for MicrobiomeAnalyst for downstream processing and visualization.

3.3.6 Culture isolation of *Pseudomonas aeruginosa* in Diptera flies

Culture isolation of *P. aeruginosa* was conducted in fly species collected from Matlwang livestock kraals and Ikageng residential dumping sites in Potchefstroom, North West, South Africa. The exterior surface of the flies was washed using 70% ethanol by leaving the flies in ethanol for 1 hour. The tubes were filled with a 1:1 ratio, consisting of one fly and 1 mL of phosphate-buffered saline (PBS) at pH:7. The flies were then crushed in PBS followed by a 20 s vortex. 1 mL of the PBS was placed in 9 mL buffered peptone water (BPW) and incubated

for 24 hours with agitation at 37°C. A spread plate method was utilized using a 10-fold dilution (10^{-1}), 100 µL of the BPW were transferred onto 90 mm agar plates of Sorbitol McConkey agar (SMA). The spread plate method was carried out in duplicate to ensure that the results were harmonized. All the inoculated plates were incubated at 37°C for 24 hours for culture isolation. All presumptive *P. aeruginosa* colonies were identified and sub-cultured on SMA. Gram-staining was conducted for identification and confirmation of cells, observed using light microscope.

3.4 Identification of *Pseudomonas aeruginosa* using molecular tools

3.4.1 DNA extraction of *Pseudomonas aeruginosa*

Culture isolation was conducted on samples from two sampling areas (Matlwang and Ikageng) using Sorbitol MacConkey agar. The overnight incubated cultures of *P. aeruginosa* were used for DNA extraction using the Chemagic DNA Cyto Pure Kit protocol. In brief, 200 µL lysis buffer and 25 µL proteinase-K were added to microcentrifuge tubes containing microbial cells. Incubation occurred at 600 rpm at 56°C until the lysis was complete (3 hours) using a shaking incubator. The material was then spun down and the supernatant was then used. About 75 µL resuspended magnetic beads were added with 400 µL Binding Buffer (BB) to the lysate by pipetting 10X to mix. The mixture was then incubated for 10 minutes at room temperature. The tubes were then placed in the chemagic stand and the beads were then drawn to the sides. The tubes were left for 2 minutes, and the supernatant was discarded, and the tubes were removed from the magnet position. A total of 500 µL Wash Buffer 1 were added to the tube and resuspended. The magnetic bead/DNA complexes were separated for 1 minute and then the supernatant was discarded. The tubes were then removed from the stand and the washing procedure was conducted 3 more times using 500 µL of Wash Buffer 2 (WB2) and then Wash Buffer 3 (WB3). When all the wash buffers have been removed, the tubes were left in the magnet position. A 1.5 mL wash buffer 4 was added and left for 90 seconds without resuspension, the supernatant was then discarded. 200 µL of Elution Buffer (EB) were added to the tube and resuspended. Incubation occurred for 5 – 10 minutes at room temperature. The tubes were placed in the chemagic stand for 2 minutes and the elute that contained the DNA was then transferred to a clean tube (chemagic DNA Cyto Pure Kit, Protocol). DNA quantification was conducted using the NanoDrop (ThermoFisher™) spectrophotometer and 1.5% agarose gel.

3.4.2 Identification of *Pseudomonas aeruginosa* using *gyraseB* gene

Conventional PCR was conducted to identify *Pseudomonas* spp. from Diptera flies targeting the *P. aeruginosa* housekeeping gene *gyraseB* (Forward: CCT GAC CAT CCG TCG CCA CAA; Reverse: CGC AGC AGG ATG CCG ACG CC) (Hosu *et al.*, 2021). Each PCR reaction consisted of a total reaction of 25 µL consisting of 12.5 µL of a 2X DreamTag Green Master mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, 4 mM MgCl₂, and loading buffer) (ThermoFisher Scientific, South Africa), 8.5 µL of nuclease-free water, 2 µL of the template DNA, and 1 µL of each oligonucleotide primer. The following conditions were used: 94°C for 5 minutes, 94°C for 1 minute, 58°C for 1 minute, 72°C for 1.5-minutes, 72°C for 7 minutes with an infinite hold at 4°C. The amplified PCR mixture were then confirmed by running the mixture on 1.5% agarose gel, stained with 10 µL Ethidium Bromide (EtBr).

3.4.3 The 16S rRNA gene sequencing of the *Pseudomonas aeruginosa*

The amplification of the 16S rRNA was conducted using PCR and a universal primer pair namely, 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3') (Frank *et al.*, 2008). Each PCR reaction consisted of a total reaction of 25 µL consisting of 12.5 µL of a 2X DreamTag Green Master mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, 4 mM MgCl₂, and loading buffer) (ThermoFisher Scientific, South Africa), 8.5 µL of nuclease-free water, 2 µL of the template DNA, and 1 µL of each oligonucleotide primer. The following PCR conditions were used ; 95°C for 5 minutes, 95°C for 5 minutes (x30), 55°C for 30 seconds, 72°C for 1 minute and 72°C for 5 minutes followed by an infinite hold at 4°C. The PCR products were cleaned up using a PCR Clean-Up System kit, in preparation for sequencing using the ABI 1500 machine.

3.4.4 Detection of virulence genes on *Pseudomonas aeruginosa*

Six *P. aeruginosa* virulence genes exoenzyme S (*exoS*), phospholipase N, (*plcN*), phospholipase H (*plcH*), exotoxin A (*toxA*), elastase B (*lasB*) and alginate D (*algD*) were screened for the detection and identification of *P. aeruginosa* virulence factors (Faraji *et al.*, 2016). A 25 µL total reaction consisted of 12.5 µL of a 2X DreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, and 0.4 mM dTTP, 4 mM MgCl₂, and loading buffer), 8.5 µL of nuclease-free water, 2.0 µL of template DNA, and 1.0 µL of each

oligonucleotide primer. PCR conditions are listed in (Table 3.1). The amplified PCR product was then verified by using 1.5% agarose gel stained with 10 μ L Ethidium Bromide (EtBr).

Table 3.1: *Pseudomonas aeruginosa* virulence genes, primers, and PCR conditions used in this study.

Target gene	Primer name	Sequences	PCR conditions	Base pairs	References
<i>algD</i>	algD-F:	CGTCTGCCGCGAGATCGGCT	94°C - 1 min (30x)	313 bp	Faraji <i>et al.</i> , 2016
	algD-R:	GACCTCGACGGTCTTGCGGA	60°C - 1.5 min 72°C - 1 min		
<i>lasB</i>	lasB-F:	GGAATGAACGAAGCGTTCTCCGAC	94°C - 1 min (30x)	284 bp	Faraji <i>et al.</i> , 2016
	lasB-R:	TTGGCGTCGACGAACACCTCG	60°C - 1.5 min 72°C - 1 min		
<i>toxA</i>	toxA-F:	CTGCGCGGGTCTATGTGCC	94°C - 1min (30x)	270 bp	Faraji <i>et al.</i> , 2016
	toxA-R:	GATGCTGGACGGGTCGAG	63°C - 1.5 min 72°C - 1 min		
<i>plcH</i>	plc H-F:	GCACGTGGTCATCCTGATGC	94°C - 1 min (30x)	608 bp	Faraji <i>et al.</i> , 2016
	plc H-R:	TCCGTAGGCGTCGACGTAC	60°C - 1.5 min 72°C - 1 min		
<i>plcN</i>	plc N-F:	TCCGTTATCGCAACCAGCCCTACG	94°C - 1 min (30x)	481 bp	Faraji <i>et al.</i> , 2016
	plc N-R:	TCGCTGTCGAGCAGGTCGAAC	60°C - 1.5 min		

			72°C - 1 min		
<i>exoS</i>	exo S-F:	CGTCGTGTTCAAGCAGATGGTGCTG	94°C - 1 min (30x)	444 bp	Faraji <i>et al.</i> , 2016
	exo S-R:	CCGAACCGCTTCACCAGGC	60°C - 1.5 min 72°C - 1 min		

3.5 Antimicrobial resistance profile (AMR) and hemolytic test on *Pseudomonas aeruginosa* isolates

Antimicrobial susceptibility screening to Neomycin (N), Gentamicin (CN), Metronidazole (MTZ), Sulphamethoxazole (RL), Cefazolin (KZ) and Amoxicillin (AML) was conducted on the *P. aeruginosa* isolates. The disk diffusion assay (DDA) also known as Kirby-Bauer method was used to determine the antimicrobial profiles of the isolated *P. aeruginosa* isolates (Table 3.2). A standardized inoculum (100uL) was spread over the surface of Mueller-Hinton agar (MHA). Reproducibility depends on the log growth phase of organisms thus fresh subcultures was used for this method. The zone inhibition of the antibiotics was measured in millimetre (mm) according to the Clinical and Laboratory Standards Institute (CLSI) (Igbinosa *et al.*, 2017). All isolates were tested for haemolytic activity on sheep blood agar (SBA) using spot inoculation. Haemolytic activities were observed for the isolates and recorded according to α , β or γ patterns.

Table 3.2: Antibiotics reference guide used for antibiotic susceptibility testing in *Pseudomonas aeruginosa* (Hudzicki, 2009).

Diameter of zones inhibition (mm)				
Antibiotics	Disk symbol	R	I	S
Neomycin	N	≤ 12	13-14	≥ 15
Gentamicin	CN	≤ 12	13-14	≥ 15
Metronidazole	MTZ	≤ 10	11-15	≥ 16
Sulphamethoxazole	RL	≤ 10	11-15	≥ 16
Cefazolin	KZ	≤ 14	15-17	≥ 18
Amoxicillin	AML	≤ 13	14-16	≥ 17

3.6 Phenotypic characterisation of extended beta-lactamases-producing *Pseudomonas aeruginosa*

Phenotypic detection of extended beta-lactamases (ESBL)-producing *P. aeruginosa* was conducted in fly species collected from Matlwang and Ikageng in Potchefstroom, North West, South Africa. Spot inoculation of the *P. aeruginosa* isolates was initiated, and plates were incubated aerobically at 37°C for 24 hours on CHROMagar™ ESBL.

3.7 Detection of ESBL- encoding genes in *Pseudomonas aeruginosa* isolates

Detection of antibiotics resistance genes was conducted on the *P. aeruginosa* isolates using conventional PCR. The presence or absence of β -lactams (*TEM*, *CTX-M*, *OXA* and *SHV*) resistance genes were screened from *P. aeruginosa* isolates. All β -lactam primers were acquired from Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa. Each PCR reaction consisted of a total reaction of 25 μ L consisting of 12.5 μ L of a 2X DreamTag Green Master mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, 4 mM MgCl₂, and loading buffer) (ThermoFisher Scientific, South Africa), 8.5 μ L of nuclease-free water, 2 μ L of the template DNA, and 1 μ L of each oligonucleotide primer. Amplified PCR mixture was then confirmed using a 1.5% agarose gel, stained with Ethidium Bromide (EtBr) and observed with ultraviolet (UV) light using the ENDURO GDS Gel Documentation System (Labnet International Inc., US). PCR condition and primer sequences are shown in Table 3.3.

3.8 Detection of antibiotic resistance genes on *Pseudomonas aeruginosa* isolates

Detection of antibiotics resistance genes was conducted on the *P. aeruginosa* isolates using conventional PCR. The presence or absence of five different antibiotics were tested. All β -lactam primers were acquired from Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa. Each PCR reaction consisted of a total reaction of 25 μ L consisting of 12.5 μ L of a 2X DreamTag Green Master mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, 4 mM MgCl₂, and loading buffer) (ThermoFisher Scientific, South Africa), 8.5 μ L of nuclease-free water, 2 μ L of the template DNA, and 1 μ L of each oligonucleotide primer. Amplified PCR mixture was then confirmed using a 1.5% agarose gel, stained with Ethidium Bromide (EtBr) and observed with ultraviolet (UV) light using the ENDURO GDS Gel Documentation System (Labnet International Inc., US). PCR condition and primer sequences are shown in Table 3.4.

Table 3.3: Extended beta-lactamase genes that were tested against *P. aeruginosa* isolates that includes PCR conditions and sequences

Target gene	Primer name	PCR conditions	Sequence	References
<i>OXA</i>	OXA – F OXA – R	94°C 5min 94°C 45s x35 55°C 30sec 72°C 1min 72°C 10min	ACACAATACATATCAACTTCGC AGTGTGTTTAGAATGGTGATC	Liu <i>et al.</i> , 2018
<i>SHV</i>	SHV – F SHV – R	94°C 5min 94°C 45s x35 55°C 30sec 72°C 1min 72°C 10min	CACTCAAGGATGTATTGT G TTAGCGTTGCCAGTGCTCG	Liu <i>et al.</i> , 2018
<i>TEM</i>	TEM-F TEM-R	94°C 5min 94°C 45s x35 55°C 30sec 72°C 1min 72°C 10min	TTC TTG AAG ACG AAA GGG C ACGCTCAGTGGAACGAAAAC	Liu <i>et al.</i> , 2018
<i>CTX-M</i>	CTX-M-F CTX-M-R	94°C 5min 94°C 45s x35 55°C 30sec 72°C 1min 72°C 10min	GTTACAATGTGTGAGAAGCAG CCGTTTCCGCTATTACAAAC	Liu <i>et al.</i> , 2018

Table 3.4: Antimicrobial resistant confirmation genes in *Pseudomonas aeruginosa* isolates

Antibiotic name	Primer name	PCR conditions	Sequence	References
Gentamicin	AME 14ACC(3) IV R AME 13ACC(3) IV F	95°C 3min 95°C 1min x 35 54°C 1min 72°C 5min 72°C 10min	F: AGTTGACCCAGGGCTGTCGC R: GTG TGC TGC TGG TCC ACA GC	Bräu <i>et al.</i> , 1984
Metronidazole	rdxA F rdxA R	95°C 5min 94°C 30s x 40 55°C 30s 72°C 1min 72°C 10min	F: AAT TTG AGC ATG GGG CAG A R: GAA ACG CTT GAA AAC ACC CCT	Diab <i>et al.</i> , 2018
Amoxicillin	Pbp1A F Pbp1A R	95°C 3min 95°C 1min x 35 54°C 1min 72°C 5min 72°C 10min	F: GCG ACA ATA AGA GTG GCA R: TGC GAA CAC CCT TTT AAA T	Diab <i>et al.</i> , 2018
Neomycin	aph(2'')-Ib F aph(2'')-Ib R	94°C 4min 94°C 1min x 30 55°C 2min 72°C 1min 72°C 10min	F: CTTGGACGCTGAGATATATGAGCAC R: GTTTGTAGCAATTCAGAAACACCCTT	Chow <i>et al.</i> , 2001

Sulphamethoxazole	sulI F	95°C 1min 30sec	F: CGCACCGGAAACATCGCTGCAC R: TGAAGTTCCGCCGCAAGGCTCG	Rahmani <i>et al.</i> , 2013
	sulI R	95°C 30sec x 40 65°C 30sec 72°C 30sec 72°C 7min		

CHAPTER 4

RESULTS

4.1 Morpho-species identification of the Diptera flies

The Diptera flies collected from two distinct areas, namely Matlwang and Ikageng in Potchefstroom, North West, South Africa, were identified and sorted through microscopic analysis by considering phenotypic characteristics such as color, shape, and size of the body. A total of 1422 and 2793 flies were collected, respectively, in the illegal residential dumping sites of Ikageng and the livestock kraals of Matlwang (Figure 4.1). Matlwang livestock kraal fly samples contained *Hemipyrellia* spp. (n = 435/1422, 31%), *Tabanus* spp. (n = 341/1422; 24%) and *Chrysomya* spp. (n = 176/1422; 12%). Ikageng illegal residential dumping site samples contained *Hemipyrellia* spp. (n = 2461/2793; 88%), *Synthesiomyia* spp. (n = 46/2793; 2%), *Sarcophagidae* spp. (n = 200/293; 7%) and *Chrysomya* spp. (n = 53/2793; 2%). In both regions, Diptera flies that were not identified were assigned as “unknown” and were excluded in this study.

4.1.1. Description of the identified Diptera flies

Hemipyrellia spp. and *Chrysomya* spp. are grouped under the same family called Calliphoridae which is identified according to some of the following characteristics (Akbarzadeh *et al.*, 2015): A stem-vein with or without row hairs above are observed, a lower calypter with or without hairs on dorsal surface and the thorax seen as bright green metallic, cuprous, or blueish. The greater ampulla with stiff erect hairs or short hairs and the dorsal surface of lower calypter with dense hairs. The colour of the anterior spiracle appears dark, brownish, or bright white yellowish. Identification occurred by the morphology of the basal part of the upper calypter as well as the lower calypter. The darkened anterior wing margin exhibit a presence of hairs on the katepisternal setae 0+1; conspicuous dusting of dorsal part of thorax and a third antennal segment colour furthermore the upper and lower calypters expressed colours as one pair of acrostichal setae on post-sutural surface of the thorax are present. Calliphoridae possess mouth-edges and facial ridges.

Tabanus spp., belongs to the family Tabanidae that had the following morphological characteristics: a prominent and anteriorly extended antennae together with enlarged

flagellomeres usually consisting of 4 – 8 on the flagellum and large ommatidial facets with smaller facets ventrally that makes up the eyes. Median or basal callus and an absence of ocelli are visible together with two-segmented and enlarged maxillary palps at the base of the apical palpomere, stout and toothed proboscis with blade like mandibles and maxillary laciniae. A stout thorax are present with strong flight muscles and a prominent notopleural lobe. Fairly strong legs with notable tibial spurs are visible. Consistency occurs within Tabanidae. The thorax and abdomen are the same width but dorsoventrally slightly compressed and slightly colored or patterned.

Synthesiomyia spp. belong to the family Muscidae with the following morphological characteristics: an undeveloped postscutellum appear with a hypopleuron that is without strong bristles and a front wing margin along with an anal vein evanescent occurs. Antennae and palpi depicting in an orange-yellowish color as well as the final terminal segment of the abdomen portrays in a yellow color. The length of body is ± 7 -10 mm and grey to black in color. Eyes are compound without any hairs and the head consist of hard short bristles together with a pedicle with longitudinal fissure. The maxillary palpi is yellow and the flagellum occurs yellow with arista bear. Four longitudinal vittae occur on the mesothorax in thorax region along with four long bristles present on the sternopleuron. The legs are black in color and the inner posterior margin contains hairs. The abdomen is gray with a checkered pattern.

Sarcophagidae spp. belong to the family Sarcophagidae with the following morphological characteristics: one pair of proclinate orbital setae are shown along with an inner vertical setae differentiated from the postocellar setae and tergite including a dorsolateral golden light spot. Tergite is undivided as well as a spiracle in membrane and a spiracle sclerite appears and a strong marginal setae. The absence of tergite 7. Two lateral sclerotized bare plates, two times the size of cercus occurs. The epiproct is absent and sternites have dark-brown colour and the posterior corners are rounded. A strong setae in the posterior margin is present.

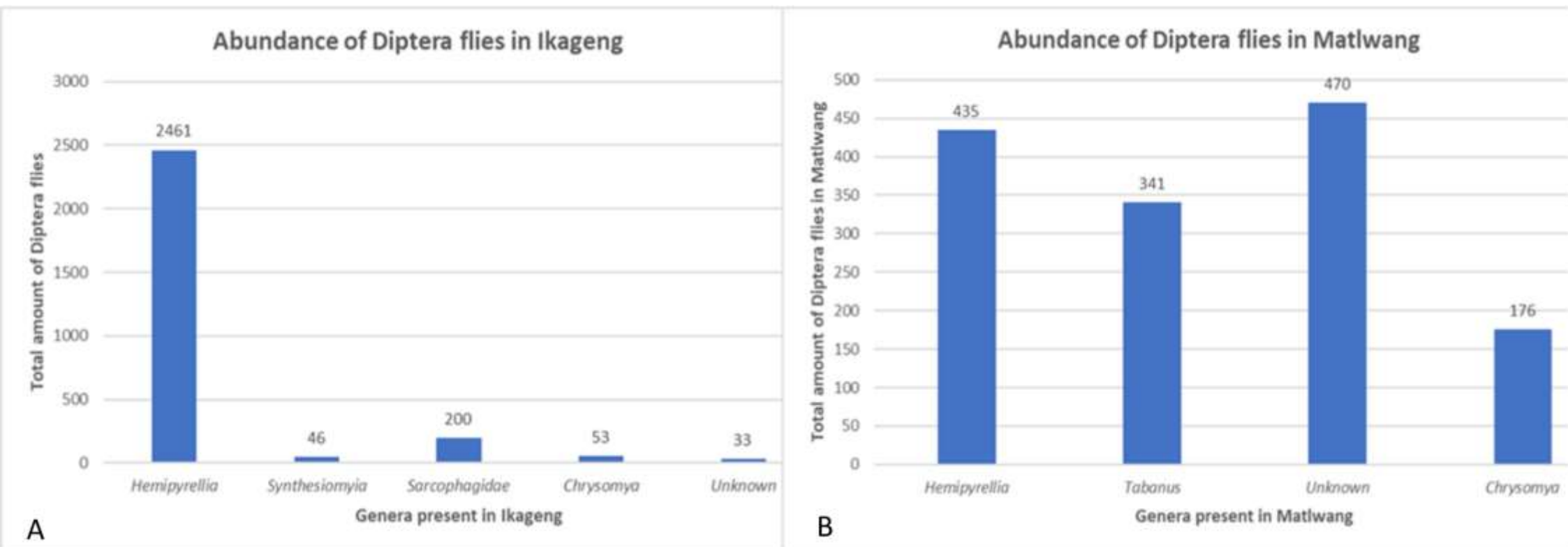


Figure 4.1: A chart plot showing the prevalence of genera Diptera flies in A: Ikageng residential dumping site and B: Matlwang livestock kraals.

4.2 Molecular identification of the Diptera flies

An 1888 bp region of the *CO1* gene was used for genetic identification of the Diptera flies collected from Matlwang and Ikageng in Potchefstroom. The use of BLAST nucleotide analysis identified the sequenced flies belonging to different genera species, supported by percentage identities between query cover to percentage identity with an E-value of 0.0 (Appendix T7.1). A paraphyletic phylogenetic tree of the Diptera flies in Ikageng formed a clear distinction of 4 genera species grouping them as *Hemipyrellia* spp., *Chrysomya* spp., *Synthesiomyia* spp. and *Sarcophagidae* spp. (Figure 4.2). Within the genera *Hemipyrellia*, there are two species that were identified in Ikageng dumping site. This included the *H. fernandica* and *H. pulchra* that grouped as a sub-clade supported by a bootstrap value of >99% at the node. Ikageng dumping site also consisted of the *Chrysomya megacephala* that clustered with the GenBank sequences (MK075818.1 and MK075815.1). *Chrysomya* and *Hemipyrellia* are closely related and can be classified under the same family as Calliphoridae. The other genera found in this dumping site was *Synthesiomyia nudiseta* and showed a high bootstrap confidence of 100% with the reference GenBank sequence (MK038739.1 and MF511766.1). *Sarcophagidae* found in another clade consisted of *Sarcophagidae* spp. and *S. tibialis* with a bootstrap value of 63% (Figure 4.2).

Meanwhile, 3 genera species were classified as *Hemipyrellia* spp., *Chrysomya* spp. and *Tabanus* spp. in Matlwang livestock kraals (Figure 4.3). The *Hemipyrellia* spp. (*H. fernandica* and *H. ligurriens*) was identified in Matlwang grouping with the *Chrysomya* (*Chrysomya marginals*) in the same clade. Two *Tabanus* species were classified belonging to the family of Tabanidae, three *Tabanus* species that formed their own sub-clade were identified in this study from the livestock kraals. These were classified as *T. trivittatus* voucher, *T. birmanicus* and *T. par* that grouped in their relative minor sub-clades of the species.

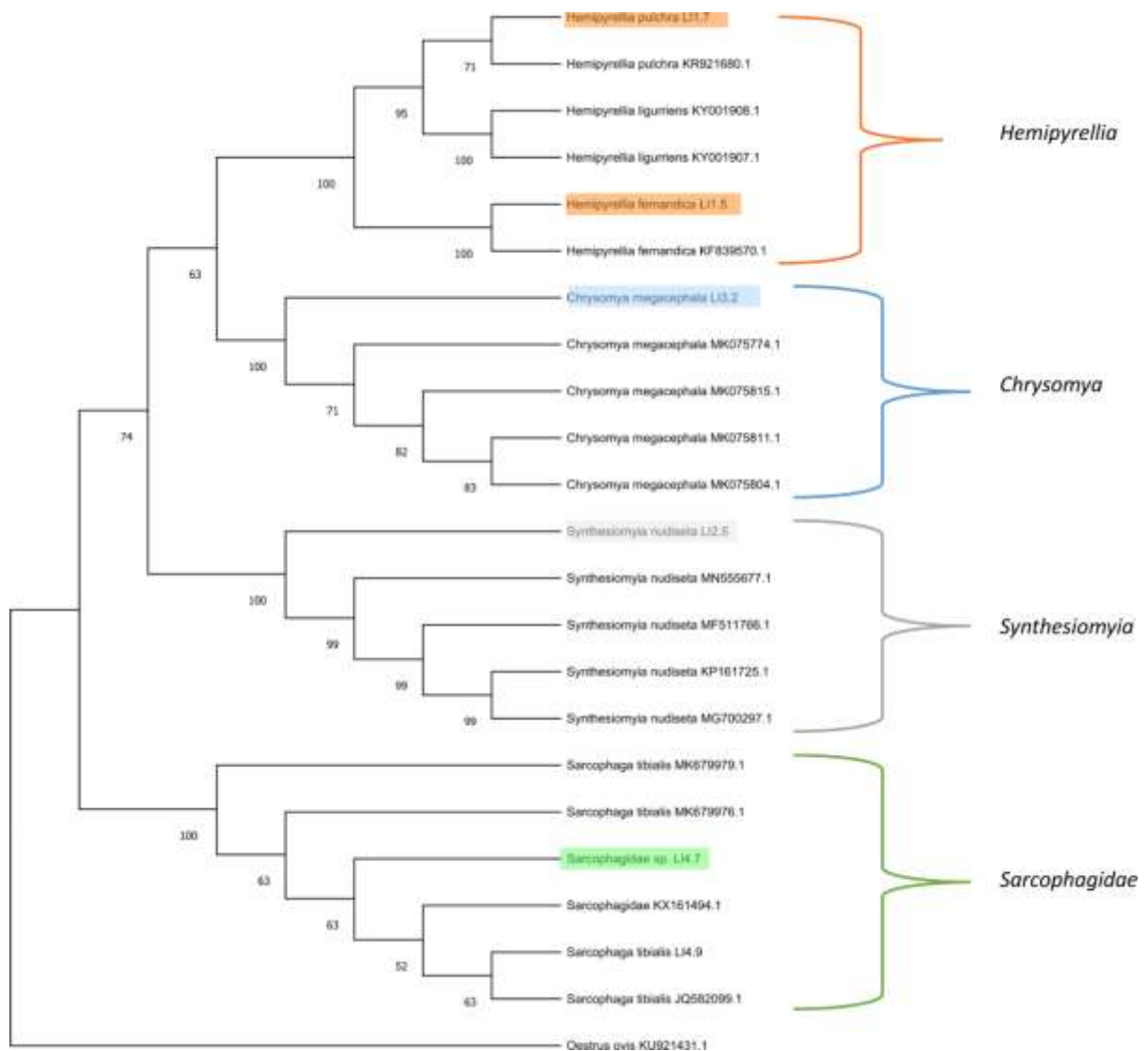


Figure 4.2: Neighbour joining phylogenetic tree of the fly genus species collected in Ikageng residential dumping site sequenced using the cytochrome oxidase 1 (CO1) gene. Bootstrap support values (>63%) are indicated at the internodes. Samples sequenced in this study are highlighted. The *Oestrus* KU921431.1 was used as an outgroup to root the tree.

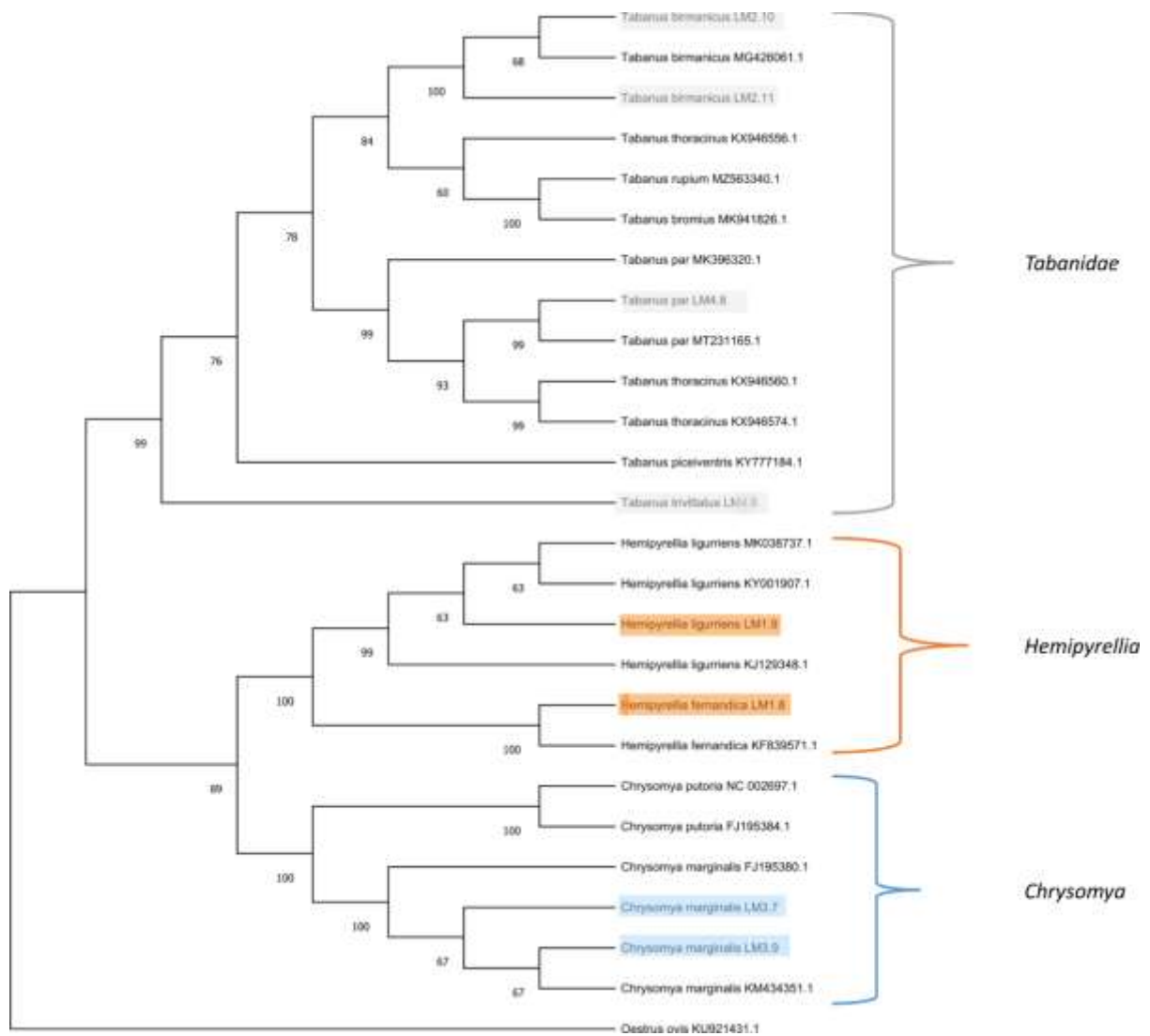


Figure 4.3: Neighbour joining phylogenetic tree of the fly genus species collected in Matlwang livestock kraals sequenced using the cytochrome oxidase 1 (CO1) gene. Bootstrap support values (>49%) are indicated at the internodes. Samples sequenced in this study are highlighted. The *Oestrus ovis* KU921431.1 was used as an outgroup to root the tree.

4.3 Metataxonomic analysis: quality control

Sequence reads for each fly sample from the Ikageng residential dumping site and Matlwang livestock kraals were processed for quality control (Appendix T7.2). The sequence reads were processed and filtered based on removing chimeras, non-target amplicons, and low-quality amplicons. After quality processing, the sequenced reads ranged from 970 to 23 812. The highest number of total valid reads was 23 812 obtained from the genus *Tabanus* spp. found in Matlwang. The lowest number of reads was 970 obtained from Ikageng in the genus *Synthesiomyia*, whereby 18 low quality amplicons were removed, with no non-target amplicons.

4.4 The relative abundances of the bacteria in Diptera fly guts

Diptera flies (Ikageng n = 20) and (Matlwang n = 21) were assessed for the abundance of gut bacterial communities (Figure 4.4 and Figure 4.5), respectively. The fly bacterial composition from Matlwang livestock kraals was mostly made up of Proteobacteria phyla (92.13%). Alphaproteobacteria were the most relative abundant class presented in the Matlwang livestock kraals at 80.51%. The family Rickettsiaceae presented in copious amounts, with the genus *Rickettsia* being the most abundant (Figure 4.5). This was represented by the *Rickettsia rickettsii* group with a 79% relative abundance. A total of 21 Diptera flies from Matlwang were assessed of different *Rickettsia* species. A total of 31 *Rickettsia* species were identified within the *R. rickettsia* group. Matlwang Diptera flies consisted of all the 31 *Rickettsia* species. However, the abundance of each *Rickettsia* species varies in each fly host (Appendix F7.2).

The most abundant phylum presented within the Diptera flies of the Ikageng dumping site was Proteobacteria (98.97%), and the second most prevailing phylum is Firmicutes (78.52%). The Gammaproteobacteria class and the Pseudomonadaceae family were most dominant in the different classes and families found in Ikageng dumping site with both prevailing at 98.97%. The genera *Pseudomonas* and *Lactobacillus* showed to be more prevalent in Ikageng (Figure 4.6). The *P. synxantha* group at species level (Figure 4.7), showed a relative abundance of 98.97%.

The family Calliphoridae of the fly consists of both fly genera *Hemipyrellia* spp. and *Chrysomya* spp. that is present in both Ikageng residential dumping site and Matlwang livestock kraals (Appendix F7.1). *Hemipyrellia* spp. fly species in Ikageng presented with the

following phylum: Fusobacteria (2.17%), Actinobacteria (2.55%), Firmicutes (56.67%), Bacteroidetes (8.93%) and Proteobacteria (75.7%). *Hemipyrellia* spp. in Matlwang livestock kraals presented with the following Phylum: Fusobacteria (5.13%), Actinobacteria (3.22%), Firmicutes (25.87%), Bacteroidetes (4.19%) and Proteobacteria (80.78%). *Hemipyrellia* spp. in Matlwang presented with an abundance of 69.72% of the *R. rickettsii* group in contrast to the *Hemipyrellia* spp. in Ikageng with an abundance of 40.18% of the *R. rickettsii* group. *Pseudomonas synxantha* group present with an abundance of 48.1% in Ikageng and 14.93% in Matlwang.

Chrysomya spp. in Ikageng residential dumping site presented with the following phylum: Fusobacteria (1.02%), Actinobacteria (1.13%), Firmicutes (76.72%), Bacteroidetes (2.54%) and Proteobacteria (90.7%). *Chrysomya* spp. in Matlwang livestock kraal presented with the following phylum: Fusobacteria (13.7%), Actinobacteria (3.77%), Firmicutes (5.7%), Bacteroidetes (5.14%) and Proteobacteria (85.37%). *Chrysomya* in Matlwang was presented with an abundance of 79.00% of the *R. rickettsii* group in contrast to the *Chrysomya* in Ikageng with an abundance of 0.49% of the *R. rickettsia* group. *Pseudomonas synxantha* group is present with an abundance of 89.57% in Ikageng dumping site and 1.41% in Matlwang livestock kraal. The fly *Chrysomya* spp. in Ikageng has a relative abundance of 44.52% of *Lactobacillus sakei* group and 24.26% of the *Paraclostridium benzoelyticum* group.



Figure 4.4: Stacked bar graph of assessment of bacterial communities of Diptera flies at genus level, collected from Matlwang livestock kraals in Potchefstroom, South Africa



Figure 4.5: Stacked bar graph of assessment of bacterial communities at species level of Diptera flies in Matlwang, North West, South Africa

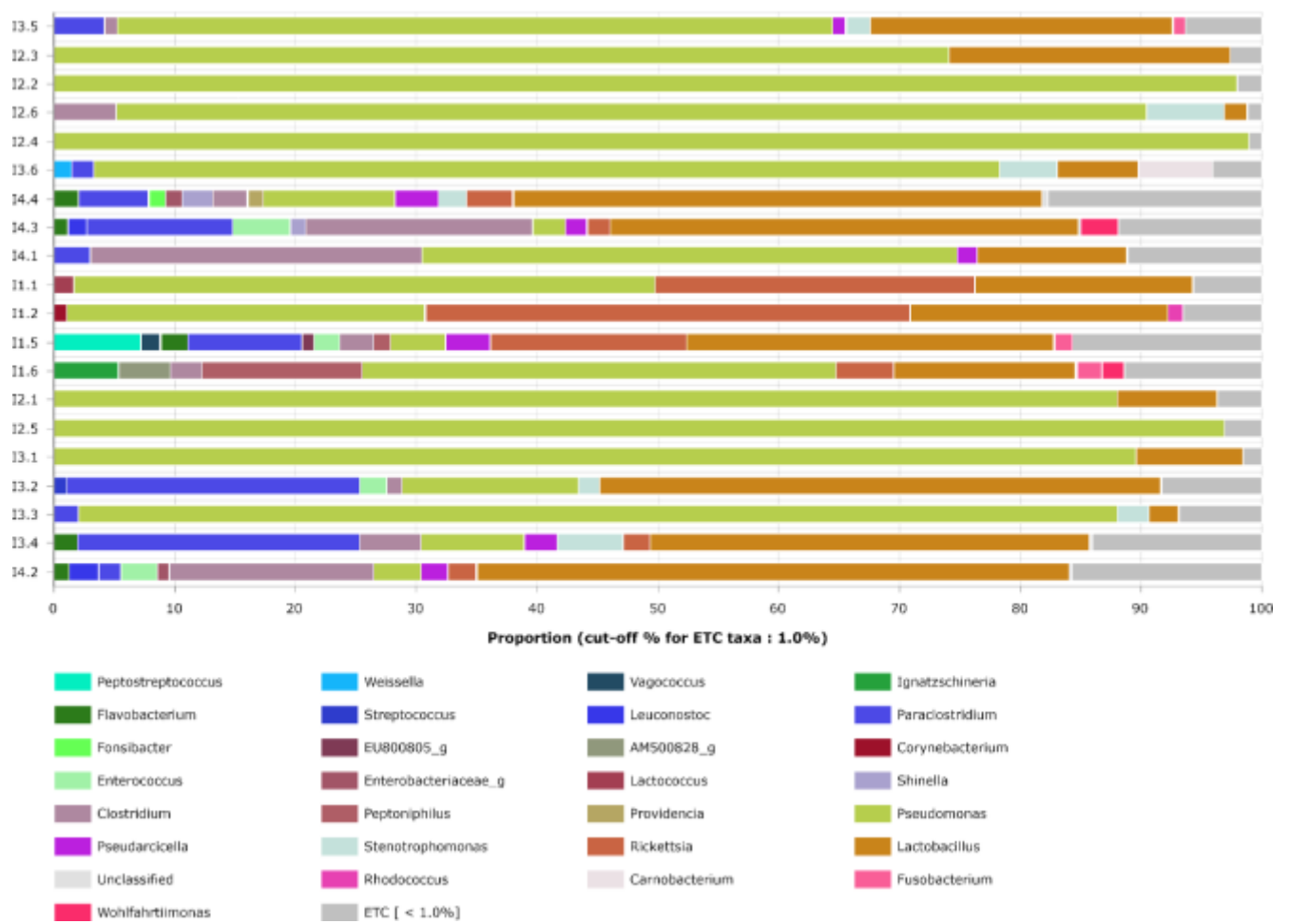


Figure 4.6: Stacked bar graph of assessment of bacterial communities of Diptera flies at genus level in Ikageng, North West, South Africa



Figure 4.7: Stacked bar graph of assessment of bacterial communities present in Diptera flies at species level in Ikageng, North West, South Africa.

4.5 α -Diversity of gut microbiome of Diptera flies

Diversity rarefaction curves were generated using OTU's to interpret the sequence depth of the microbial diversity within the gut microbiome of the Diptera flies based on the *16S rRNA* amplicon. This reflected the diversity of bacteria in each fly species collected from different sites i.e., Matlwang livestock kraal (Figure 4.8) and Ikageng residential dumping site (Figure 4.9). The maximum reads per OTU for each fly was determined, that presented depth of coverage that ranged from a minimum of 1 to a maximum of 23812 reads in Matlwang. Ikageng residential dumping site depicted more diversity with higher number of sequenced reads per OTUs compared to flies of the Matlwang livestock kraals with a minimum read of 1 and a maximum of 15956 reads.

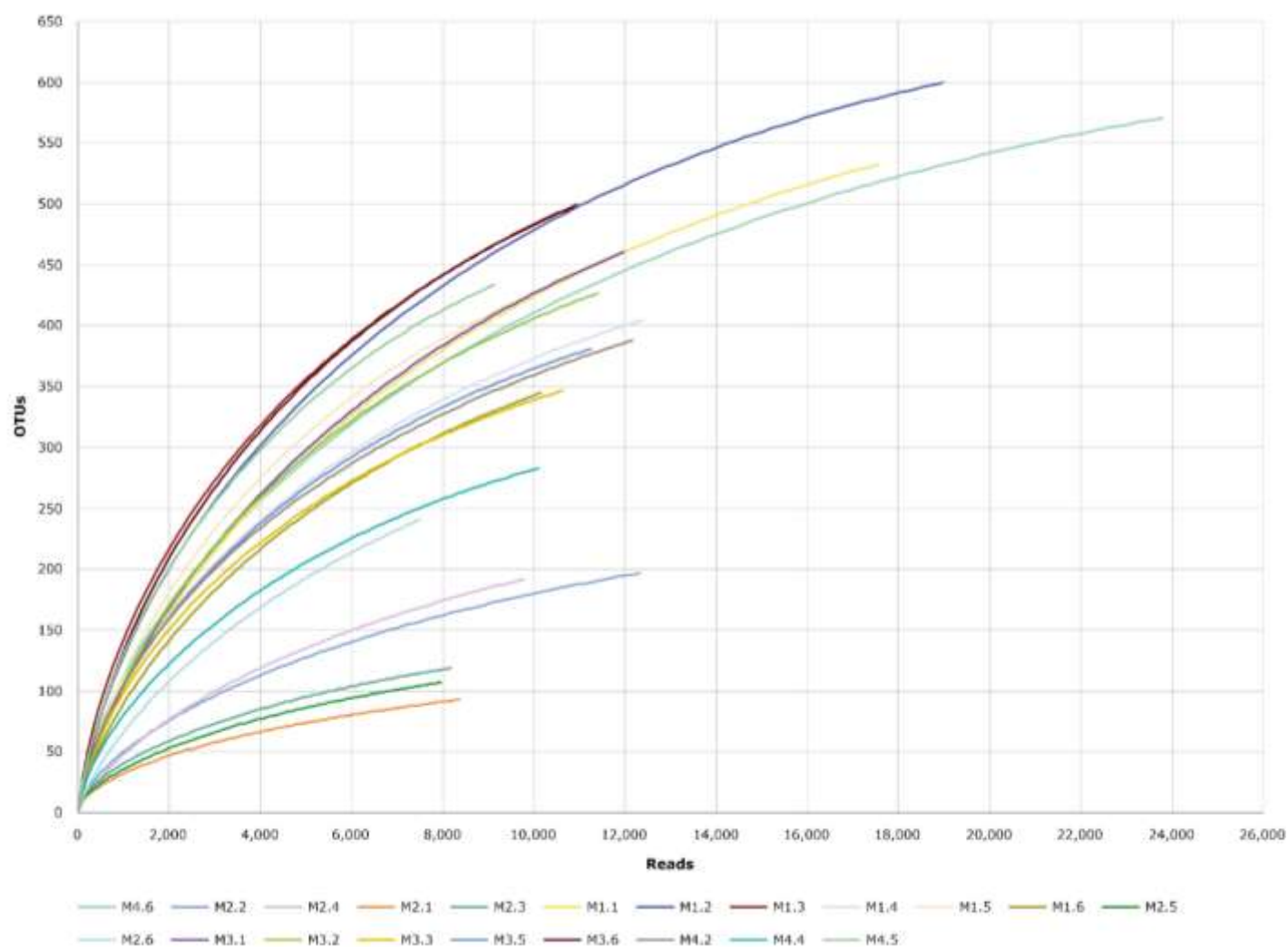


Figure 4.8: Alpha-diversity rarefaction curves of diversity within the gut microbiome of the Diptera flies in Mathwang based on the 16S rRNA amplicon.

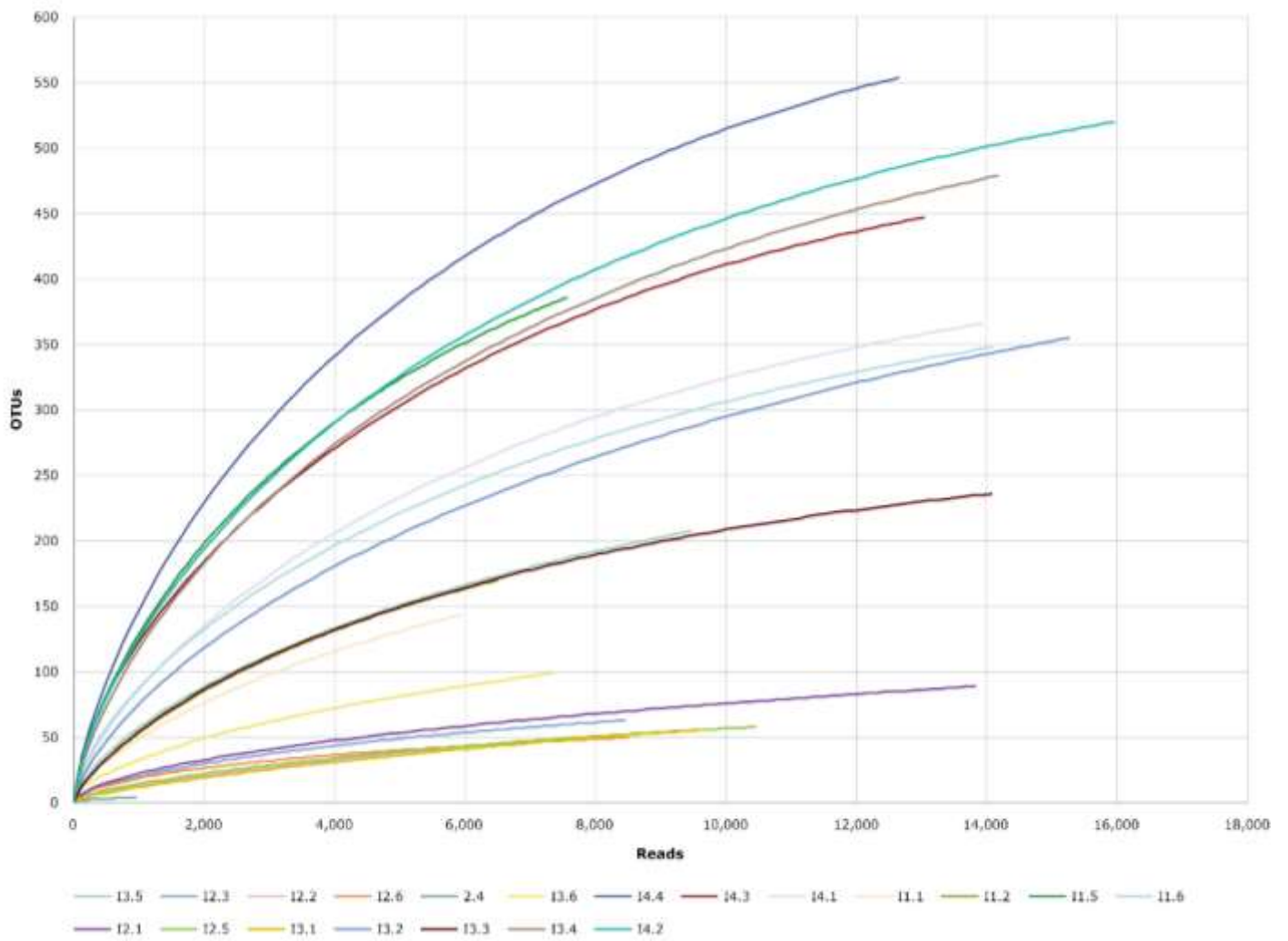


Figure 4.9: Alpha-diversity rarefaction curve to interpret the sequence depth of the microbial diversity within the gut microbiome of the Diptera flies in Ikageng based on the 16S rRNA amplicon.

4.6 α -Diversity of gut microbiota of Diptera flies Chao1 plot

The microbial diversity present within the Diptera flies from Matlwang and Ikageng was assessed using alpha matrices such as Chao1, Observed (Richness), Shannon and Simpson (evenness). Box plots (Figure 4.10) were used to illustrate the diversity within the different family of the flies and locations. The Diptera found in Ikageng proved to be more diverse than in Matlwang as illustrated in all the diversity metrics. Tabanidae demonstrated more diversity than any other genera. A nonparametric test named the Kruskal-Wallis test was used to determine the distribution of the Diptera flies (Table 4.1). P-values for each diversity metrics were determined (Observed OTU's p-value = 0.0010475; Shannon index p-value = 0.054594; Simpson index p-value = 0.054594 and Chao1 p-value = 0.0010475). The p-values that are equal/below 0.05 are deemed significant, meaning that the null hypothesis can be discarded whereas the p-values greater than 0.05 suggest that no effect was observed or not significant, indicating that there was insufficient data to rule out the null hypothesis and draw the conclusion that the groups are not the same. The Kruskal-Wallis test compares two or more independent samples.

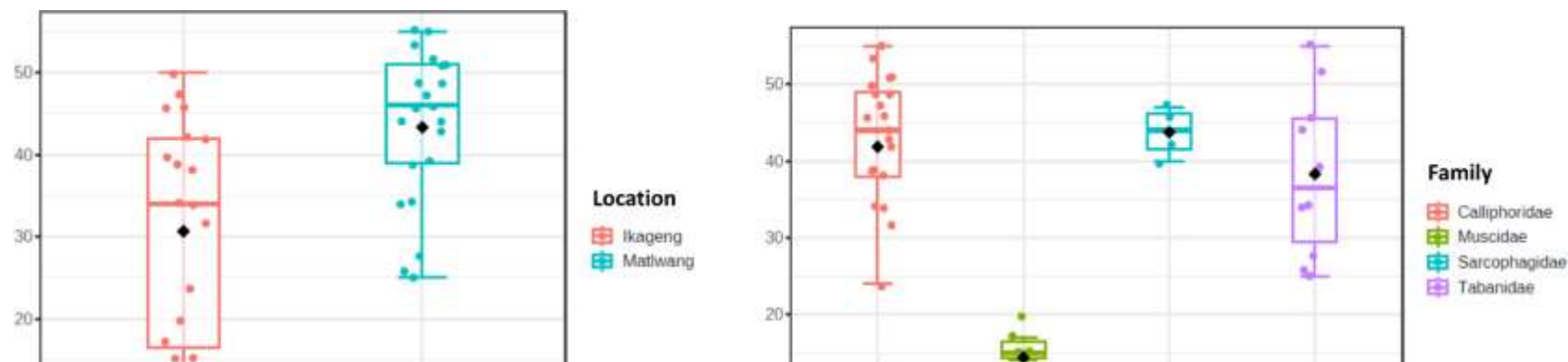


Figure 4.10: Alpha diversity Chao1 box plot in A: location and B: family. Circles are preventative of individual samples, grouped by location (A) and family (B). First and third quartiles of the distribution are represented with the median represented as the line within the box plot.

Table 4.1: Overview of alpha diversity metrics using Observed OTUs, Shannon, Simpson and Chao1 between locations and Diptera fly genera.

Diversity metrics	Ikageng Diptera microbiota	Matlwang Diptera microbiota	Matlwang vs Ikageng Diptera microbiota
	Average; STD	Average; STD	P-value
Observed OTU's	41,85±17,50	50±39.5	0,0010475
Shannon index	1,78±0,55	2,50±1,85	0,054594
Simpson index	0,758±0,230	0,760±0,625	0,058066
Chao1	41,85±17,50	50±39.5	0,0010475

STD indicates Standard deviation.

4.7 β -Diversity

Beta diversity was investigated in this study to determine the similarities of the Diptera fly microbiome between the two regions (Matlwang and Ikageng) using the Bray-Curtis Index. Figure 4.11 demonstrates the two distinct clusters that formed coinciding within each region. Distinct separation between the two clusters is visible within Matlwang and Ikageng. However, a small overlap of the two clusters was observed among these two regions, also indicated on the phylogeny in the middle cluster. The overlapping occurs within the similarities in bacterial species composition of Proteobacteria, particularly *P. aeruginosa*. A clear difference between Matlwang (blue clade) and Ikageng (red clade) can be observed due to the low level of similarity. Owing to the distinct bacterial species that are present in these two areas. In this regard, *Pseudomonas* spp. was detected in high abundance in the Ikageng dumping side, whereas *Rickettsia* spp. was more prevalent in Matlwang.

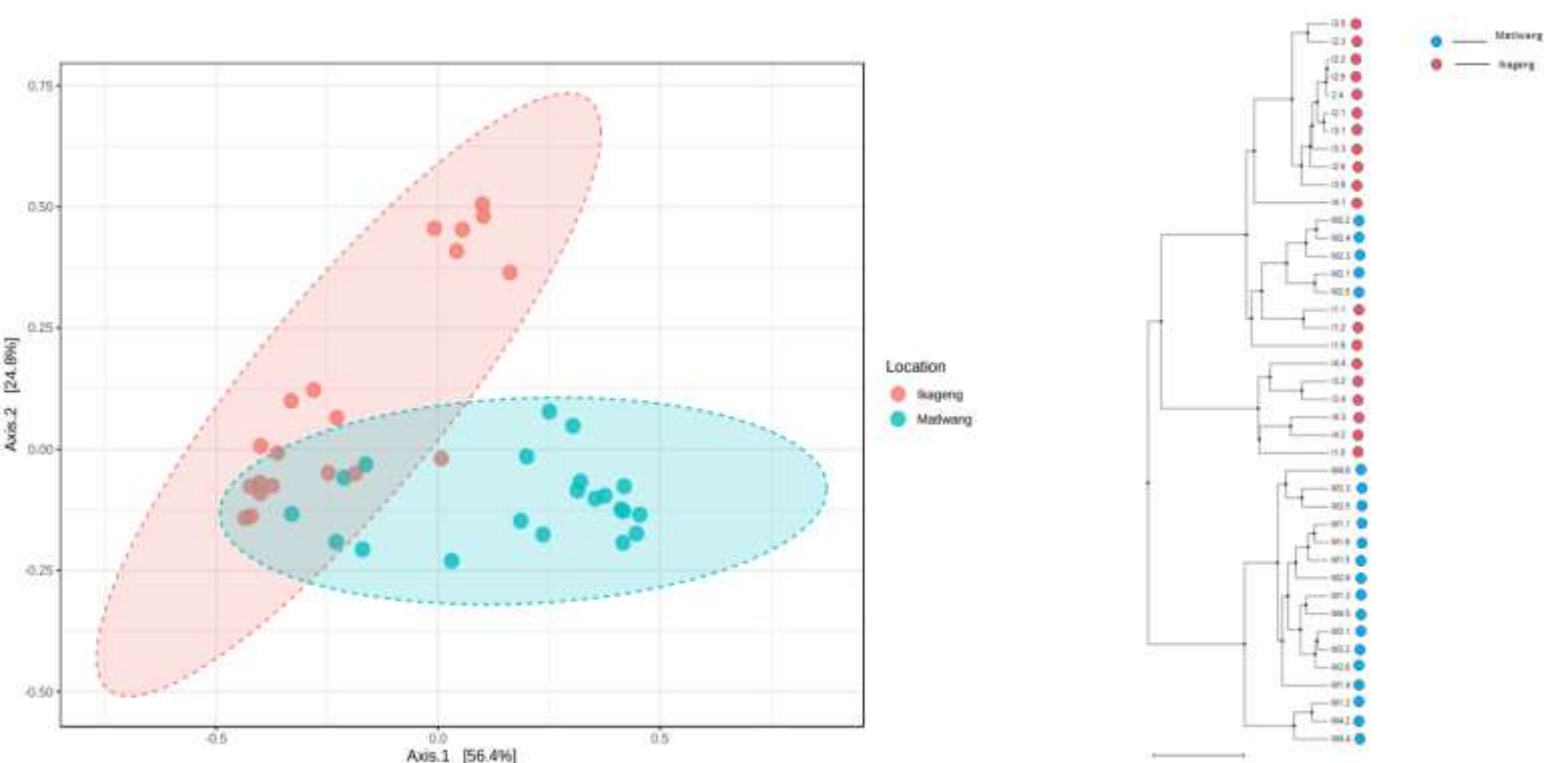


Figure 4.11: Beta-diversity analysis (Bray-Curtis Index) showing the comparison between the two regions (Matlwang & Ikageng), Potchefstroom

4.8 Culture isolation and identification of *Pseudomonas aeruginosa*

The following results indicate the presumptive number of colony forming units (CFU) per milliliter for *Pseudomonas aeruginosa* at each site Matlwang (Appendix F7.2B) and Ikageng (Appendix F7.2A) after a 24 hour incubation period at 37°C on Sorbitol MacConkey agar. Out of the 48 fly samples collected from Ikageng an isolate growth ratio of (n=7/12 = 58.33%) for *Hemipyrellia* spp., (n=7/12 = 58.33%) for *Synthesiomya* spp., (n=4/12 = 33.33%) for *Chrysomya* spp. and (n=5/12 = 41.67%) for *Sarchophagidae* spp was shown. Out of the 48 fly samples collected from Matlwang showed (n=10/12 = 83.33%) for the genera of *Hemipyrellia* spp., (n=11/12 = 91.67%) for *Tabanus* spp., (n=8/12 = 66.67%) for *Chrysomya* spp. and (n=7/12 = 58.33%) for *Tabanus* spp.

4.9 Molecular identification of *Pseudomonas* species

A total of 48 fly samples were tested for the identification of *Pseudomonas* species from Ikageng dumping site and Matlwang livestock kraal. About 23 and 36 presumptive cultures were isolated from these different fly species in Ikageng and Matlwang, respectively. The

isolates were subjected to species-specific PCR using the *gyrase B* gene to identify *P. aeruginosa*. The presumptive isolates n = 23/23 (100%) and n = 36/36 (100%) tested positive for the presence of *P. aeruginosa* in Ikageng and Matlwang, respectively. The use of BLASTn analysis confirmed these isolates as *P. aeruginosa*. The GenBank accession numbers were assigned to some of the represented strains (n = 5). The accession number of the *16S rRNA* gene sequences were assigned to the *P. aeruginosa* isolates (sequence 1: OR122642; sequence 2: OR122643; sequence 3: OR122644; sequence 4: OR122645; sequence 5: OR122646).

4.10 Detection of virulence genes in *Pseudomonas aeruginosa* isolates

Pseudomonas aeruginosa strains (n = 58) obtained from several fly genera and identified at both sites were investigated for their virulence genes amplified using PCR. Six different virulence genes were used to confirm the virulence factors of the *P. aeruginosa* isolates. The six virulence genes used included *exoenzyme S* (*exoS*), *non-hemolytic phospholipase C* (*plcN*), *hemolytic phospholipase C* (*plcH*), *exotoxin A* (*toxA*), *elastase* (*lasB*) and *GDP-mannose 6-dehydrogenase* (*algD*). In Ikageng, about 23 isolates from different genera *Hemipyrellia* spp. (n = 7), *Synthesiomyia* spp. (n = 7), *Chrysomya* spp. (n = 4) and *Sarcophagidae* spp. (n = 5) were tested for the presence and absence of these virulence genes (Figure 4.12A). *ToxA* and *plcN* genes were detected in all isolates (n = 23) from the four fly genera in Ikageng. The *lasB* and *plcH* genes were detected in the isolates from *Hemipyrellia* spp., *Synthesiomyia* spp. and *Sarcophagidae* spp., while only 3 isolates from *Chrysomya* spp. tested positive. The *exoS* gene was detected in all the isolates from fly-genera *Synthesiomyia* spp. and *Chrysomya* spp., (n = 6/7; 85.71%) in *Hemipyrellia* spp. and (n = 4/5; 80%) in *Sarcophagidae* spp. In Matlwang, 36 isolates isolated from different fly-genera *Hemipyrellia* spp. (n = 9), *Tabanus* spp. (n = 17) and *Chrysomya* spp. (n=8) were profiled for the virulence genes (Figure 4.12B). The *plcH* and *ExoS* genes were present in all the 36 isolates. The *lasB* gene was detected in *Hemipyrellia* spp. (n = 6/9; 66.67%), *Tabanus* spp. (n = 13/17; 76.47%), and *Chrysomya* spp. (n = 5/8; 62.5%), respectively. The *algD* gene was present in *Hemipyrellia* spp. (n = 6/9; 66.67%), *Tabanus* spp. (n = 16/19; 84.21%), and *Chrysomya* spp. (n = 5/8; 62.5%) , respectively. The *toxA* gene was detected in (n = 3/9; 33.33%) isolates from *Hemipyrellia* spp., (n = 12/17; 70.59%) in *Tabanus* spp. and (n = 5/8; 62.5%) in *Chrysomya* spp.

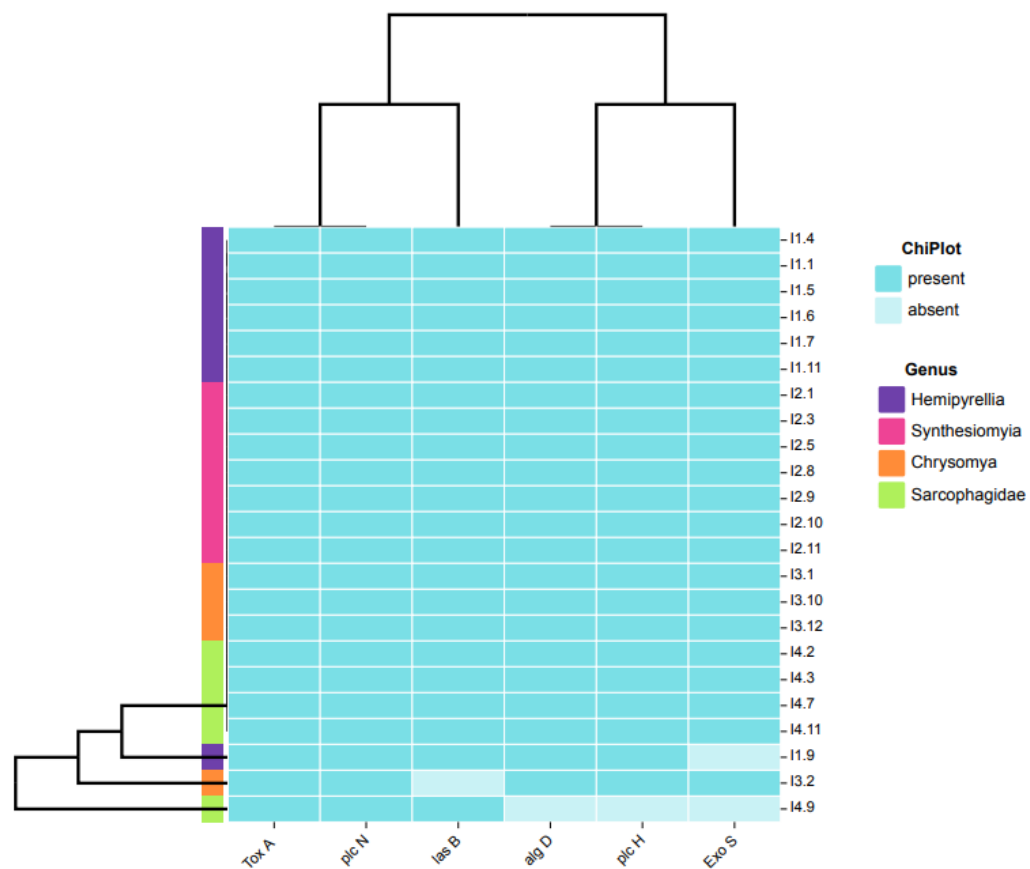


Figure 4.12A: Phylogenetic clustering of the virulence genes that are present or absence in *Pseudomonas aeruginosa* isolates in relation to fly-genus species collected from Ikageng, Potchefstroom.

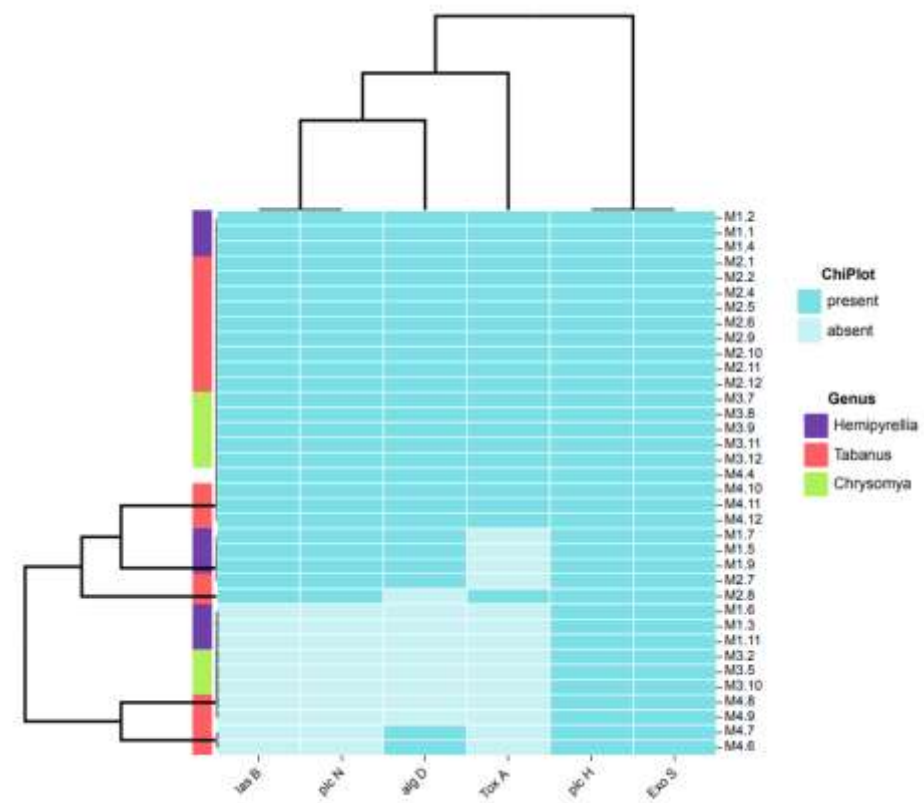


Figure 4.12B: Phylogenetic clustering of the virulence genes that are present or absence in *Pseudomonas aeruginosa* isolates in relation to fly-genus species from Matlwang, Potchefstroom.

4.11 Antimicrobial susceptibility testing of *Pseudomonas aeruginosa* and determination of ESBL-producing isolates

Antimicrobial susceptibility test was performed to profile *P. aeruginosa* isolates isolated from Ikageng (Figure 4.13A) and Matlwang (Figure 4.13B). Six different antibiotics were used and chosen based on selection criteria for Enterobacteriaceae. All the *P. aeruginosa* isolates in these two regions were resistant to metronidazole, sulphamethoxazole, cefazolin and amoxicillin. The isolates from both sites were resistant to metronidazole, sulphamethoxazole, cefazolin and amoxicillin. In both sites, the *P. aeruginosa* isolates showed different antibiotic resistance patterns on gentamicin and neomycin. In Ikageng a total of (n = 3/23; 13%) of the isolates were resistant to gentamicin, while (n = 20/23; 86.96%) were susceptible to gentamicin. In Ikageng, (n = 9/23; 38.89%) of the isolates were resistant to neomycin, meanwhile (n = 7/23; 30.43%) and (n = 12/23; 52.17%) were classified as susceptible and intermediate to neomycin, respectively. In Ikageng, (n = 9/23; 38.89%) of the isolates were resistant to gentamicin, while (n = 6/23; 26%) were susceptible to gentamicin. Neomycin was the most effective antibiotic in Ikageng as (n = 12/23; 52.17)% and (n = 7/23; 30.43%) of the isolates showed to be intermediate and susceptible to neomycin, respectively. This trend was also seen on the Matlwang isolates as (n= 13/36; 36.11%) and (n = 19/36; 52.77%) were intermediate and susceptible to neomycin, respectively. However, it is imperative to note that some of the isolates were resistance to neomycin in these two sites. The extended-spectrum beta-lactamases (ESBL) was determined among the confirmed *P. aeruginosa* isolates (n = 59). All the investigated *P. aeruginosa* isolates were considered as ESBL producing. The ESBL activity was noted as metallic blue colour on the colony in all the isolates (Appendix T7.3A). The majority of isolates in Matlwang (n = 24/36; 66.67%) and Ikageng (n = 18/23; 78.26%) showed β -haemolysis growth on sheep blood- (Appendix T7.3A).

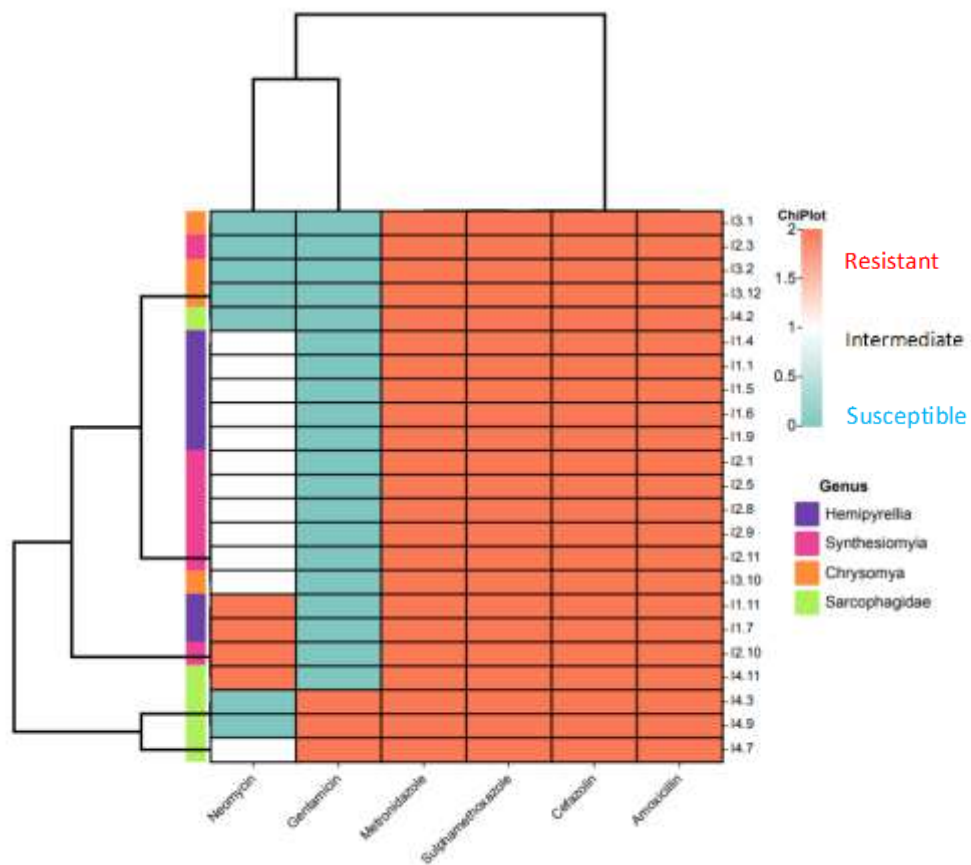


Figure 4.13A: Heatmap analysis of the antibiotic susceptibility for *Pseudomonas aeruginosa* isolates in Ikageng using the Kirby-Bauer method or disk diffusion assay.

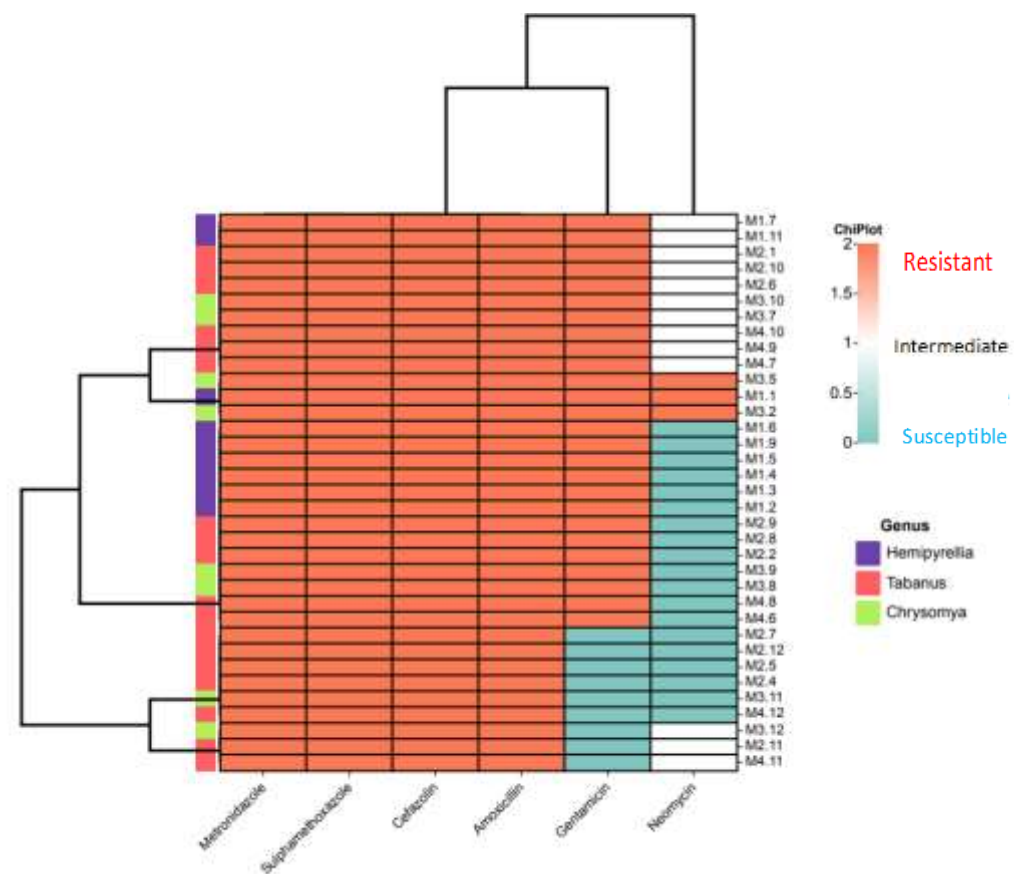


Figure 4.13B: Heatmap analysis antibiotic susceptibility for *Pseudomonas aeruginosa* isolates in Matlwang using the Kirby-Bauer method or disk diffusion assay.

4.12 Antibiotic resistance genes in *Pseudomonas aeruginosa* isolates

Five different antibiotics resistance genes (ARGs) were determined in *P. aeruginosa* isolates from Ikageng (n = 23) and Matlwang (n = 36). The ARGs determined in this study included neomycin (*aph* (2'')-Ib), gentamicin (*acc*(3)-IV), metronidazole (*rdxA*), sulphamethoxazole (*sulI*) and amoxicillin (*pbp1A*). All Ikageng *P. aeruginosa* isolates tested positive for *acc*(3)-IV gene that confers resistance to gentamicin (Figure 4.15A). This differs from the disc diffusion assay (DDA) where in Ikageng, 38.89% of the isolates were resistant to gentamicin. The disc diffusion assay's resistance to metronidazole and sulphamethoxazole coincides with the results of the PCR assay, which also revealed the presence of the *rdxA* and *sulI* genes, respectively. About 43.5% and 86.9% of the isolates consisted of the *rdxA* and *sulI* genes, respectively. The absence of the *aph*(2'')-Ib gene in *P. aeruginosa* isolates indicates their susceptibility to neomycin. The lack of the *pbp1A* gene is inconsistent with the DDA, whereby most of the isolates showed to be resistant to amoxicillin.

Three ARGs were detected in *P. aeruginosa* isolates from Matlwang livestock kraal (Figure: 4.15B). This included *acc*(3)-IV (n = 29/36; 80.55%), *sulI* (n = 32/36; 88.89%), and *rdxA* (n = 11/36; 30.56%) genes that confers resistance to gentamicin, sulphamethoxazole, and metronidazole, respectively. The existence of these genes provides significant support for this, as DDA displayed the similar pattern. The *pbp1A* and *aph* (2'')-Ib genes that confers resistance to amoxicillin and neomycin, respectively were absent in all the *P. aeruginosa* isolates. The absence of *pbp1A* gene is inconsistent with the disc diffusion assay, whereby all the isolates were resistant to amoxicillin. The absence of neomycin (*aph* (2'')-Ib) is consistent with the DDA.

4.13 Detection of ESBL- encoding genes in *Pseudomonas aeruginosa* isolates

Four different β -lactam genes were examined among *P. aeruginosa* isolates from Ikageng (n = 23) (Appendix F7.4) and Matlwang (n = 36) (Appendix F7.5). The 4 β -lactam genes included *bla_{OXA}*, *bla_{SHV}*, *bla_{TEM}* and *bla_{CTX-M}*. Only one isolate (n = 1/23 = 4.35%) from Ikageng showed the presence of the *bla_{CTX-M}* gene. The presence of *bla_{CTX-M}* gene was noted in the *P. aeruginosa* isolated from the genus *Hemipyrrellia* spp. in Ikageng. Only one isolate from Matlwang consisted of the *bla_{CTX-M}* gene from the *Hemipyrrellia* spp.

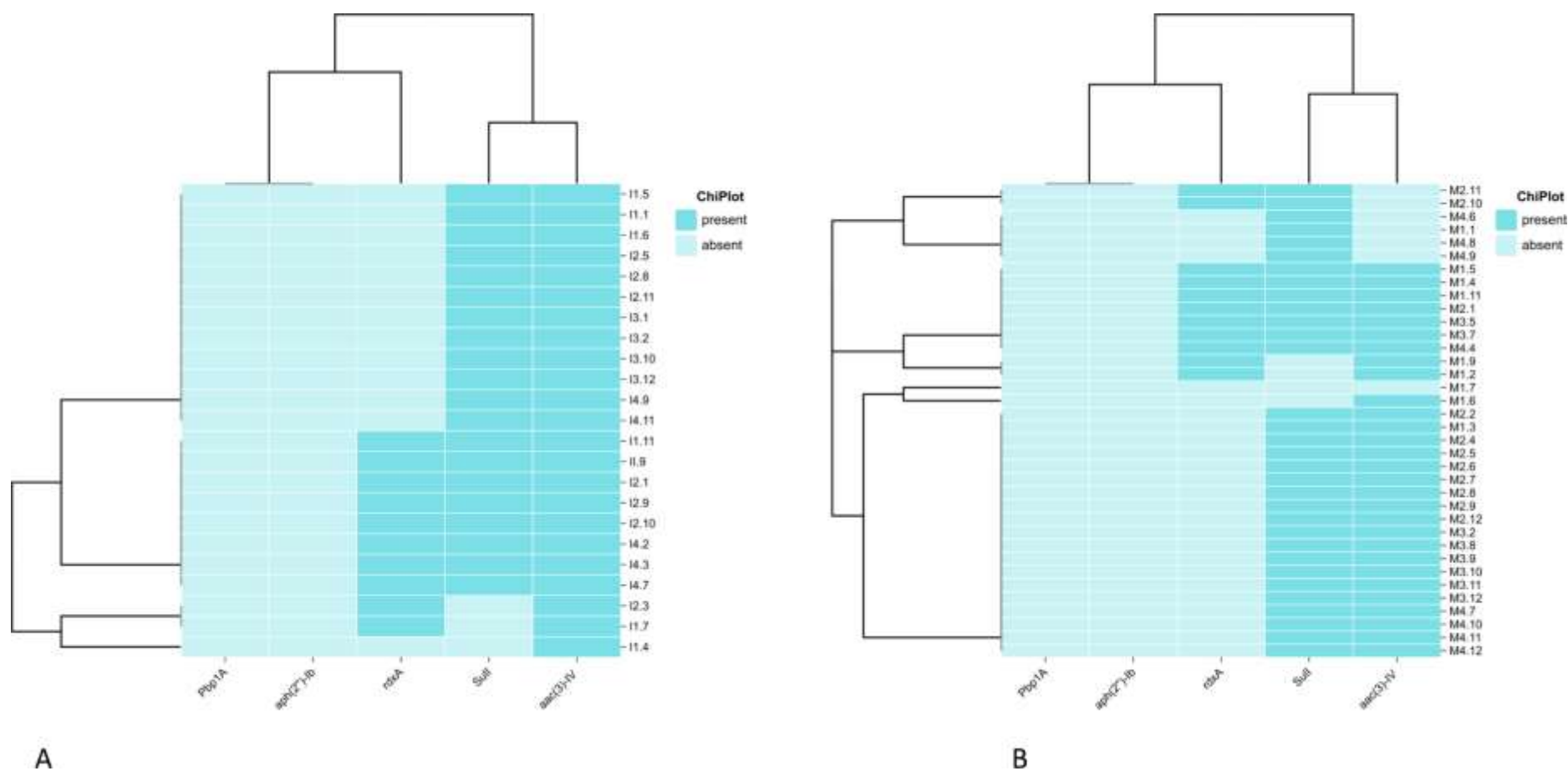


Figure 4.14: Distribution of antibiotic resistance genes detected in *Pseudomonas aeruginosa* isolates from A: Ikageng residential dumping site and B: Mathwang livestock kraals.

CHAPTER 5

DISCUSSION

This study examined the gut bacterial diversity in Diptera flies collected from Ikageng residential dumping site and Matlwang livestock kraals, Potchefstroom North West province, South Africa. Furthermore, Diptera flies in these two regions were characterized by the presence of *P. aeruginosa* to determine the virulence and antibiotics resistance genes profiles.

5.1 Morphological and genetical identification of Diptera flies

This study showed that Ikageng residential dumping site had a higher number of Diptera flies (n = 2793) compared to Matlwang (n = 1422 flies) livestock kraals (Figure 4.1). The high number of Diptera flies collected from Ikageng residential dumping site in this study could be due to the unhygienic environment from the dumping site. This site tend to have lot of decaying material such as food that produce odor serving as attractant for the flies. Within the dumping site, the nutrient availability and temperature serve as a source for feeding and breeding for the Diptera flies (Goulson *et al.*, 1999). A humid, warm environment with ample food is present in the dumping sites and is seen as a keen breeding ground for Diptera (Goulson *et al.*, 1999). Matlwang livestock kraals presented with a lower amount of Diptera flies likely due to only a few species under the order of Diptera are associated with livestock such as cattle (Shety *et al.*, 2022). Correlation to environmental factors such as temperature, humidity, and rainfall can have an impact on the amount of Diptera present (Shety *et al.*, 2022). Livestock kraals are a harsher environment with limited food and breeding space for Diptera flies that are suitably adapted for such environments can thrive there (Shety *et al.*, 2022), particularly blood feeding and those breeding on faecal material.

Both Matlwang livestock kraal and Ikageng illegal residential dumping site presented with high number of *Hemipyrellia* spp. at 31% and 88%, respectively. *Hemipyrellia* spp. are likely to breed in environmental conditions such as refuse, dead animals, as well as both human and animal faeces (Hore *et al.*, 2017). Thus, *Hemipyrellia* spp. are predominant in both Ikageng and Matlwang, due to Ikageng dumping site consisting of the perfect breeding and feeding ground with refuse, dead animals, and faeces. Matlwang livestock kraals consist of cattle that soil the ground and attract *Hemipyrellia* spp. for feeding and breeding.

Twenty four percent of the *Tabanus* spp. were only present in Matlwang livestock kraal classified as biting horseflies that forms part of the family Tabanidae (Mullens, 2019). Females usually require a bloodmeal for reproduction proposes which indicates that the females are the vectors of disease-causing agents. This makes Matlwang the perfect breeding and feeding ground of *Tabanus* spp. due to the presence of cattle and other livestock in Matlwang (Snyman *et al.*, 2020). In South Africa, *Tabanus* spp. are prevalent in warm wet areas such as tropical climates in KwaZulu-Natal (Esterhuizen, 2006).

In this study, 7% of the *Sarcophagidae* spp. was only found in Ikageng. This might be due to these Diptera flies being a part of the family known as flesh flies which differs from the Tabanid that is known as biting horse flies (Rafinejad *et al.*, 2014). *Sarcophagidae* spp. are ovoviviparous, meaning they are opportunistically depositing hatched or hatching maggots and not eggs (Ren *et al.*, 2018). They usually deposit on carrion, decaying material and dung which makes the dumping site the optimum area to breed as there are ample decaying material and unhygienic materials to give the maggots the best chances for survival (Ren *et al.*, 2018). *Sarcophagidae* spp. have also been reported in residential areas in Cusco, Peru (Ly *et al.*, 2018).

Cytochrome oxidase 1 (*CO1*) analysis of the sequenced Diptera flies allowed the genera and species to be identified, which had the BLAST nucleotide identities with the public available GenBank sequences. On a paraphyletic phylogenetic tree, the Diptera flies sequenced from Ikageng clustered into four clades representing genera, which were identified as *Hemipyrellia* spp., *Chrysomya* spp., *Synthesiomyia* spp., and *Sarcophagidae* spp. Whereas, three clades of genera were identified in the livestock kraals of Matlwang: *Hemipyrellia* spp., *Chrysomya* spp., and *Tabanus* spp. Through the removal of challenges associated with traditional taxonomy and morphological characterization such as the deplorable condition that Diptera flies are in when arriving for identification (Williams *et al.*, 2022), the use of *CO1* in this study offered a quick and precise way to identify Diptera species (Smith *et al.*, 2006).

5.2 Gut microbiome in Diptera flies from Matlwang livestock kraals and Ikageng residential dumping site

In Matlwang livestock kraals, the majority (92.13%) of the composition of the gut' fly microbiome was made up of Proteobacteria phyla. Proteobacteria might be seen as a possible microbial trademark of diseases and are presumptively the reason why the abundance of this phyla is so general (Rizzatti *et al.*, 2017). Proteobacteria are also the largest phylum to date that consist of six classes and seen as

a preparation agent of the gut for colonization of the strict anaerobes that help to sustain a healthy gut function (Shin *et al.*, 2015). In Matlwang, the family Rickettsiaceae was presented in copious amounts, whereby the *Rickettsia rickettsii* group was more prominent with 79% relative abundance. *R. rickettsii* group are most prominently found in ticks and form part of tick-borne diseases (Ribeiro *et al.*, 2021). Thus, the bacterium is often carried by ticks that are present around cattle may be the cause of the abundance of *R. rickettsii* group identified in the Matlwang livestock kraal area. The intracellular pathogen *R. rickettsii* is considered as the cause of spotted fever and zoonotic illnesses that can result in haemorrhage and organic dysfunctions (Ribeiro *et al.*, 2021; Hornok *et al.*, 2014). The presence of *R. rickettsii* group in flies is not well documented as potential vectors for transmission of this pathogen. This study highlights the first report to detect this pathogen bacterium in Diptera flies found in Matlwang, Potchefstroom, North West province, South Africa. The presence of the *R. rickettsii* group in Diptera flies found in livestock kraals is alarming as it has only been thought *R. rickettsii* group is mostly transmitted by ticks (Hornok *et al.*, 2014). Therefore, more research should be conducted to investigate the transmission of *R. rickettsii* and the transmission of tick-borne pathogens to other vertebrates that are exposed to livestock and farmers that are at risk for the potential to being infected by these pathogens. Other veterinary important pathogens noted in Matlwang livestock kraals was *Clostridium* spp. such as *Clostridium senegalense* found in *Hemipyrellia* spp. and *Tabanus* spp. It is a bacterium usually present in soils and the intestinal tract of humans and animals. *Clostridium* spp. include several significant human pathogens that can be the causative agent for gas gangrene, food poisoning, botulism, tetanus and pseudomembranous colitis (Khiav & Zahmatkesh, 2021).

The most abundant phylum presented within the Diptera flies of Ikageng dumping site was Proteobacteria (98.97%). The Pseudomonadaceae family were most dominant in Ikageng dumping site prevailing at 98.97%. Ikageng showed more prevalence in the genus *Pseudomonas* and *Lactobacillus*. *Pseudomonas* spp. is known to naturally colonize Muscidae intestines and are an important agent in protecting the larvae from entomopathogenic fungal infections (Zhang *et al.*, 2021). The presence of the genus *Pseudomonas* in Ikageng residential dumping site might be the reason why a large abundance of *Pseudomonas* spp. was detected and might be a causative agent for diseases such as meningitis, pneumonia, and septicemia in humans that is relatively close and living close to dumping sites (Body *et al.*, 1983). *Pseudomonas* spp. is one of the ESKAPE bacteria that consist of antibiotic resistance genes (Denissen *et al.*, 2022), and can survive in harsh weather conditions ranging from 4°C to 42°C (LaBauve and Wargo 2012). It can grow and sustain itself under hypoxia as well as high cell densities (Moradali *et al.*, 2017).

Lactobacillus spp. in the gut of Diptera are a promoting factor for growth of the larvae when there are nutrient-limiting conditions (Storelli *et al.*, 2011). *Lactobacillus* are also beneficial for the gut-epithelial homeostasis, protection against enteric infection, nutrient allocation and metabolism in Diptera (Matos & Leulier, 2014). Other prevailing phyla that were found in the Diptera flies included Firmicutes, Fusobacteria, Actinobacteria and Bacteroidetes found in Ikageng. The presence of these multiple phyla is in consensus with the finding in other studies on Muscidae (*Synthesiomyia*) (Gupta *et al.*, 2011), and Calliphoridae (*Hemipyrellia* & *Chrysomya*) (Wang *et al.*, 2018). The genera *Ignatzschineria*, *Vagoccus* and *Wohlfahrtiimonas* were present in the microbiome even though the abundance differs, but these genera have been isolated from numerous species and are often detected together in insects that are linked with environments of unhygienic nature (Deguenon *et al.*, 2019). *Ignatzschineria* and *Wohlfahrtiimonas* has both been previously isolated in Diptera belonging to the family Sarcophagidae, Calliphoridae and Muscidae (Deguenon *et al.*, 2019). Both *Ignatzschineria* and *Wohlfahrtiimonas* exhibit strong chitinase activity, but other known roles are not yet clearly investigated.

In this study, the high relative abundance of the bacterial species found in the gut of the family Calliphoridae that consist of *Hemipyrellia* spp. and *Chrysomya* spp. in both Matlwang and Ikageng was the phyla of Proteobacteria, and the second highest phyla present were Firmicutes. Each site (Matlwang & Ikageng) showed a homogenous of commonly detected bacterial phylas among the different fly-genera species. However, the bacterial composition or abundance was different in each fly genera even-tough *Pseudomonas* spp. were present in both areas. *Hemipyrellia* spp. and *Chrysomya* spp. was detected in both Ikageng and Matlwang meaning that the gut microbiome of both these genera share similar composition that could be because of the diet being similar. The relative high abundance of Proteobacteria and Firmicutes found in Calliphoridae was also reported in Calliphoridae flies from the suburban farm in North Carolina, USA (Deguenon *et al.*, 2019). The least abundant phylum found in both regions within the Calliphoridae family was Actinobacteria. This also coincides with the results found in Calliphoridae found in a suburban farm in North Carolina, US (Deguenon *et al.*, 2019).

Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria and Bacteroidetes were the predominant phyla in the Diptera sequenced from Matlwang livestock kraal. This result is in accordance with the results found in other studies done on the gut microbiome of Calliphoridae Junqueira *et al* (2017) and Tabanidae (Lefoulon *et al.*, 2021). The bacterial composition of Diptera flies found in Ikageng are more diverse than in Matlwang. This is due to that Diptera flies tends to feed on human food and

other decaying waste that are in copious amounts as residents tend to add more waste in a continuous manner and have optimum living conditions for breeding and feeding (Geden *et al.*, 2021). Despite the high abundance observed in Ikageng, a large pool of bacterial community composition consisting of pathogenic bacteria that includes *Pseudomonas* spp., *Rickettsia* spp., *Clostridium* spp. was conserved in Matlwang flies. Nevertheless, all the p-values presented are below 0.05 and deemed statistically significant, meaning that the null hypothesis can be discarded. These results are not unexpected as previous studies has proclaimed that the bacterial composition in dumping sites is expected to be diverse and in copious amounts (Selvarajan *et al.*, 2022).

5.3 Abundance and virulence factors of *Pseudomonas* species

Pseudomonas spp. isolates from Diptera flies in Ikageng residential dumping site and Matlwang livestock kraals were characterized in this study. *Pseudomonas* spp. is one of the ESKAPE pathogens that is associated with having antibiotic resistance (Pang *et al.*, 2019). In Ikageng, the high prevalence of presumptive *Pseudomonas* spp. isolates was found in the *Hemipyrellia* spp. (n = 7/12; 58.33%) followed by *Synthesiomya* spp. (n = 7/12; 58.33%) followed by *Sarcophagidae* spp. (n = 5/12; 41.67%) and lastly *Chrysomya* spp. with n = 4/12 (33.33%). The use of metatransomic approach showed that the genus with the most isolated *Pseudomonas* spp. was *Chrysomya* spp. (98.97%), *Synthesiomya* (89.58%), *Hemipyrellia* (48%) and *Sarcophagidae* (44%), which contradicts the composition using culture-dependent method. It is known that culture-dependent rely on growing microorganisms, which may not be able to grow all the microorganism due to selective growth conditions leading to incomplete representation of the bacteria community (Hiergeist *et al.*, 2015). This trend was also noted in Matlwang whereby the most isolated *Pseudomonas* spp. was *Tabanus* spp. (73.27%), *Hemipyrellia* spp. (15%) and *Chrysomya* spp. (1.53%) using metatransomic approach. In this study, a higher abundance (n = 59/96; 61.46%) of *P. aeruginosa* in Diptera flies in both regions was still recovered using culture-dependent method that shows a public health concern. The findings of this investigation do not support the findings of Odetoyin *et al.* (2020), as few *P. aeruginosa* isolates (n = 3/67 = 4.5%) was isolated from Diptera flies.

The isolates in this study were classified as β -hemolysis, respectively. *P. aeruginosa* β -hemolysis isolates have also been reported from a nosocomial infection that were 100% positive for β -hemolysis (Al-Daraghi & Al-Badrwi, 2020). The presence of β -hemolysis as a virulence factor implies that the isolates have a potential to produce toxic by-products such as streptolysin O in blood and can destroy red blood cells (Buxton, 2005). *P. aeruginosa* has several virulence factors such as *exotoxin A*, *alginate*, *exoenzyme S*, *elastase* and *phospholipase* which are regulated via signaling systems (Faraji

et al., 2016). Six virulence genes were screened among the identified 59 isolates in these two regions that included *exoenzyme S* (*exoS*), *phospholipase N*, (*plcN*), *phospholipase H* (*plcH*), *exotoxin A* (*toxA*), *elastase B* (*lasB*) and (*alginate D*) *algD*. The *lasB* gene is responsible for promoting IL-1 β activation, neutrophilic inflammation, and destruction of lung architecture when patients are infected by *P. aeruginosa* (Sun *et al.*, 2020). The *toxA* and *plcN* were detected in all the isolates. These results are not in correspondence with a presence rate of *toxA* (63.1%) and *plcN* (60%) found in Faraji (2016), where these two genes were present in *P. aeruginosa* isolates in children with Cystic Fibrosis and burn wounds in Iran. Thus, it is not clear whether all *P. aeruginosa* strains have the *toxA* gene. The *toxA* gene is responsible for the regulation of the exotoxin A synthesis and known to effectively cause tissue damage and decreases phagocytic activity of leukocytes in infected patients (Dong *et al.*, 2015; Pollack, 1983). The *plcN* gene is responsible for suppressing neutrophil respiratory burst activity (Terada *et al.*, 1999), whereas the *plcH* gene are responsible for maintaining the virulence during infections of *P. aeruginosa*. The *plcH* gene was detected in all the isolates investigated in this study, except in one isolate (I4.9) from *Sarcophagidae* spp. in Ikageng residential dumping site. This strain can be classified as an avirulent as other virulent genes were also not detected in *algD* and *exoS*. *P. aeruginosa* is protected from the host defense mechanism due to the *algD* gene. This is achieved by the reduction of phagocytosis as well as the inhibition of activation of complement proteins (Edward *et al.*, 2023). While *exoS* impairs phagocytosis in the lungs which exacerbates infection (Faraji *et al.*, 2016).

In Ikageng, the *lasB* gene was detected in all the isolates, except in one strain from *Chrysomya* spp. The virulence factor of *lasB* in *P. aeruginosa* is responsible for proteolytic actions that can range from tissue damage to compromising the host immune system (Cathcart *et al.*, 2011). Moreover, the same gene was absent in 6/36 isolates from *Hemipyrellia* spp., 13/36 from *Tabanus* spp. and 5/36 from *Chrysomya* spp. in Matlwang. The latter mentioned strains lack other virulence genes such as *toxA*, *lasB*, and *plcN*. These strains can be classified as avirulent strains of *P. aeruginosa*. Avirulent *P. aeruginosa* have been identified in clinical host (Luzar & Montie, 1985). The number of virulent *P. aeruginosa* isolates are significantly higher in all four genera in Ikageng residential dumping site, meanwhile 10 isolates could be classified as avirulent from Matlwang livestock kraal. Overall, the research findings indicate that *P. aeruginosa* has virulent and avirulent strains, which can be further distinguished from each other by their virulent genes they possess. Whole genome sequencing could further be employed to significantly differentiate their metabolic pathways and other genetic make up

5.4 Antibiotic resistance of *Pseudomonas aeruginosa*

In the current study, an antimicrobial susceptibility test was conducted in the *P. aeruginosa* isolates. The use of phenotypic antibiotic resistance patterns showed that the isolates in this study were mostly intermediate or susceptibility to neomycin (n = 52/59; 88.14%). This was augmented on PCR assay as all isolates tested negative to *aph(2'')-Ib* gene on PCR. The susceptibility rate is much higher than the susceptibility tested on *P. aeruginosa* isolated from different animal species such as dog, cat, horse, penguin, and brown bear that present with otitis externa, where a susceptibility of 56.25% was found (Leigue *et al.*, 2016). Susceptibility to neomycin found in *P. aeruginosa* isolates from Diptera has not been well investigated. Based on disc diffusion assay (DDA), all isolates were resistance to amoxicillin. However, amoxicillin (*Pbp1A*) gene was absent based on PCR in *P. aeruginosa* isolated from Ikageng and Matlwang, this agrees with the study of (Gad *et al.*, 2008) where 100% of the *P. aeruginosa* isolates were found to be resistant to amoxicillin. There is a discrepancy between these two assays, suggesting that the *Pbp1A* gene used in this study does not confine the *P. aeruginosa*. According to an alternate study on *Helicobacter pylori* (Gram-negative bacteria) in Mexico, antimicrobial resistance to amoxicillin does not correlate with the DDA and PCR *Pbp1A* confirmation (Matta *et al.*, 2023). This is due to the three mutations in the DNA that code for the PBP1A-1 protein, which led to two alterations in amino acids. These mutations relationship may be explained by their proximity to the PBP protein's C-terminal region's enzymatic activity sites (SKN368-371, SNN433-435, and KTG555-557) (Matta *et al.*, 2023). This is the point at which mutations alter the PBPs' structure, lowering their affinity for binding amoxicillin and producing resistance phenotypes, which could explain the resistance of amoxicillin was found in all isolates in this study using DDA.

All isolates were resistance to sulphamethoxazole, based on DDA. The *sulI* gene was detected in 87.72% of the *P. aeruginosa* isolates indicating resistance against sulphamethoxazole. The resistance of the aminoglycosides is in correspondence with Poole (2005) stating that resistance to aminoglycosides persists even though they are still effective medications for treating severe *P. aeruginosa* infections. About 88.1% of the isolates tested positive for the *aac(3)-IV* gene that confers resistance to gentamicin. The observation was also noted on disc diffusion assay whereby (n = 29/59; 49.15%) of the isolates showed resistance to gentamycin. These results agree with Hemmatinezhad *et al.* (2015) where *P. aeruginosa* isolated from *M. domestica* in Iran expressed an 49.1% resistance rate in the isolates. In another study *P. aeruginosa* isolates (n = 49/137; 35.77%) were resistant to one aminoglycosides such as gentamicin and that (n = 39/49; 79.6%) of those isolates were in possession of AME genes (Atassi *et al.*, 2023). All isolates in this study were resistant to cefazolin and this agrees with a study conducted in Iran on patients admitted to a hospital and *P. aeruginosa* isolated from the

patients, with a 97% prevalence of resistance, cefazolin showed the highest level of antibiotic resistance (Miladi *et al.*, 2020). In this study, all the isolates were resistance to metronidazole based on DDA, these results do not coincide with Marais *et al.* (2003) where the PCR assay screening of *rdxA* gene that confers to Metronidazole where (n = 11/36; 30.56%) were present based on the PCR confirmation, the *rdxA* gene is mostly tested on *Helicobacter pylori* isolates. It has been discovered that mutations in *rdxA*, a gene encoding an oxygen-insensitive NADPH nitroreductase, and *frxA*, a gene encoding a NAD(P)H-flavin oxidoreductase, are linked to and promote metronidazole resistance in *Helicobacter pylori*. *H. pylori* may develop a metronidazole resistance phenotype with mutations alone in *frxA* or without mutations in either *rdxA* or *frxA* (Marais *et al.*, 2003). This could suggest that phenotypical resistance might be present in the *P. aeruginosa* isolates even without the mutations of the *rdxA* gene being present.

Only one *bla_{CTX-M}* gene was present in both Ikageng and Matlwang *P. aeruginosa* isolates, this could imply that the primer is not specific to the isolates of *P. aeruginosa* that are the subject of this investigation. The presence of *bla_{CTX-M}*, which encode extended-spectrum beta-lactamases (ESBLs), can vary among strains of *P. aeruginosa*. The lower prevalence of *bla_{CTX-M}* gene (4.35%) differs from isolates that were isolated from patients in Mthatha, Eastern Cape province, South Africa, whereby 31.7% of the isolates tested positive (Hosu *et al.*, 2021). The resistance present to antibiotics poses a major threat in the treatment of *P. aeruginosa* and the rehabilitation of infected patients. Antimicrobial resistance is a growing concern in the world. In this study the *P. aeruginosa* isolated from Diptera flies indicated a high resistance to improper usage of antibiotics by farmers, lack of the healthcare system with providing correct treatment of residence nearby that are infected and might spread to the Diptera flies living and breeding at the dumping sites and thus contributing to antibiotic resistance, old or expired medicine being used or picked up at the dumping sites.

CONCLUSION

In conclusion, Diptera flies were successfully identified and sequenced using the *CO1* gene. Four genera of Diptera flies were identified in Ikageng; *Chrysomya* spp., *Hemipyrellia* spp., *Synthesiomyia* spp., and *Sarcophagidae* spp. Diptera in Matlwang were identified under the genera *Chrysomya* spp., *Hemipyrellia* spp., and *Tabanus* spp. The gut microbiome from Diptera flies were successfully investigated and analyzed. The gut microbiome was mainly composed of the family *Calliphoridae* from Ikageng and Matlwang. Phylum Proteobacteria was the most abundant whilst the least abundant phylum was Actinobacteria. The study demonstrated that Diptera are carriers of multi-drug resistant *P. aeruginosa*. This is the first report of *R. rickettsii* group from flies found in Matlwang village of Potchefstroom.

RECOMMENDATIONS

- The Ikageng illegal residential dumping site and the Matlwang livestock kraals of Potchefstroom, North West province, South Africa were examined in this study. Further research on other residential dumping sites or livestock kraals outside of the North West province may be conducted in the future to determine the presence of Diptera. This will allow researchers to better understand the abundance of genera in other provinces and consider environmental factors such as temperature and nutrient sources.
- Additional studies can be implemented for seasonal sampling for Diptera flies as it offers valuable year-round insights into the species makeup and dynamics of their populations. Researchers may accurately create ecological models by capturing the temporal fluctuations in diversity and abundance. The information gathered from seasonal sampling can reveal notable variations in the frequency, habits, and geographic range of fly species, providing important details for efficient pest control or conservation plans.
- Further studies can be conducted for assessing the microbiome from other Diptera families. In the current study, a small region of the *16S rRNA* gene (V3-V4 sequencing primers) was used for identification of the isolates. Future studies might suggest sequencing of the isolates using a full *16S rRNA* gene that can help with a rapid and accurate identification of the isolates using long read chemistry.
- Additional β -lactamases genes, including carbapenems and other significant ARGs, can be utilized to enhance comprehension and provide novel therapeutic approaches for severe infections resulting from pathogens. In the current study the gut microbiome of Diptera flies were investigated but a broad and more in-depth investigation can be achieved using whole genome sequencing (WGS) that will allow a comprehensive identification of microbial species and their role, which provides previously unattainable insights into the microbiome of Diptera flies. Understanding host-microbe interactions, evolution, and possible uses in disease control are aided by this.
- Further studies can be implemented on the investigation of β -lactamases genes (*bla_{CTX-M}*) that can be more primer specific by means of optimizing primer design and guarantee selective amplification of the target sequence and reduce non-specific binding, utilize stringent PCR settings in addition, such as modifying annealing temperatures.

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APPENDIX

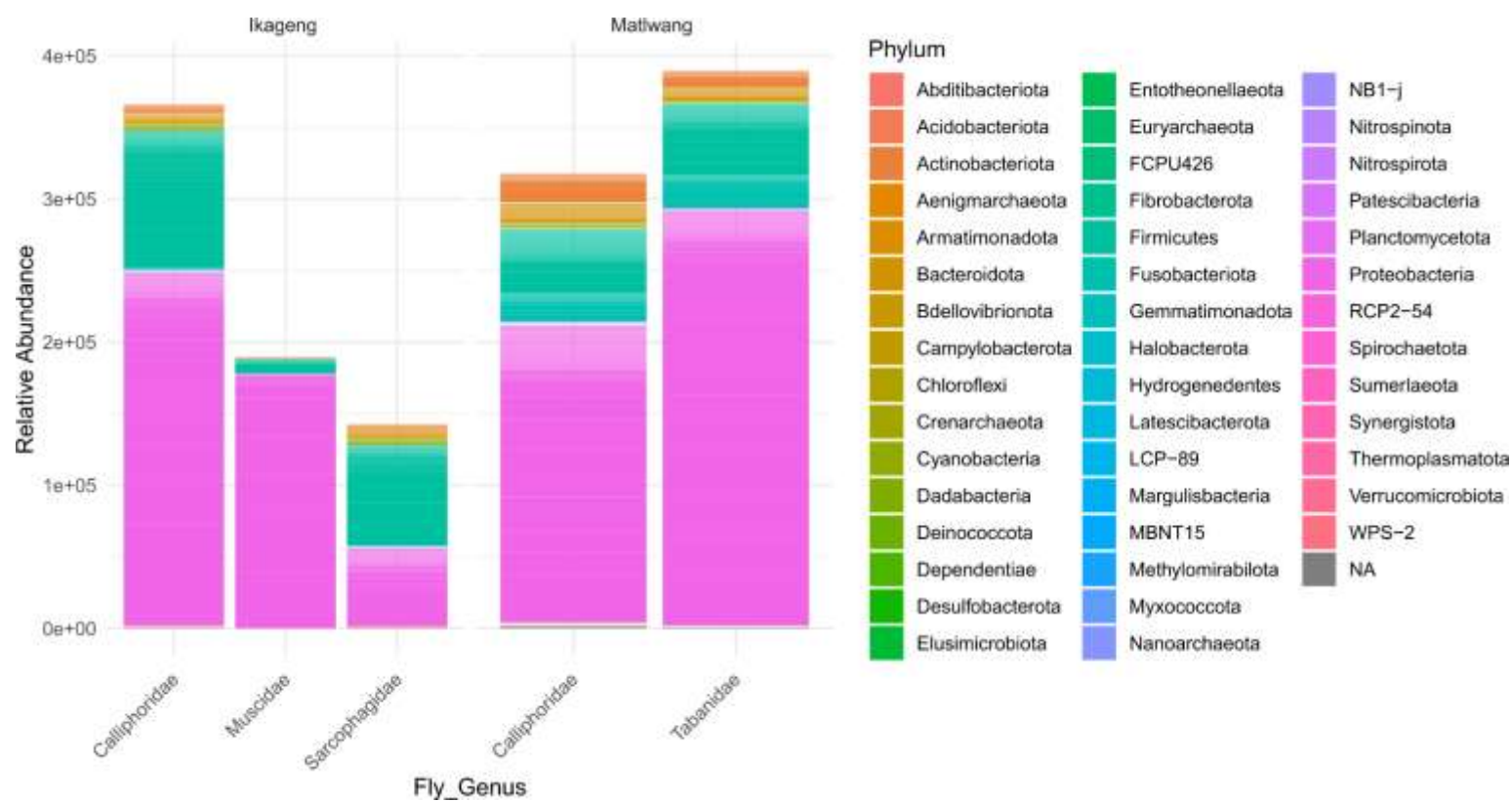
Appendix T7.1: BLASTn results of the COI gene used for identification of the Diptera flies collected from Matlwang and Ikageng in Potchefstroom.

Sample ID	Scientific name	Query cover (%)	E-value	Percentage identity (%)	Closest accession number
LI1.5	<i>Hemipyrellia fernandica</i>	100	0.0	100	FR719160.1
LI1.7	<i>Hemipyrellia pulchra</i>	100	0.0	100	KR921678.1
LI2.5	<i>Synthesiomyia nudiseta</i>	100	0.0	100	MK038739.1
LI3.2	<i>Chrysomya megacephala</i>	100	0.0	100	MK075818.1
LI4.7	<i>Sarcophagidae sp. sc_01765</i>	100	0.0	100	KX054678.1
LI4.9	<i>Sarcophaga tibialis</i>	100	0.0	100	JQ582061.1
LM1.8	<i>Hemipyrellia fernandica</i>	100	0.0	100	FR719160.1
LM1.9	<i>Hemipyrellia ligurriens</i>	100	0.0	100	KY001904.1
LM2.10	<i>Tabanus birmanicus</i>	100	0.0	100	MG426062.1
LM2.11	<i>Tabanus birmanicus</i>	100	0.0	100	MG426063.1
LM3.7	<i>Chrysomya marginalis</i>	100	0.0	100	MH765375.1
LM3.9	<i>Chrysomya marginalis</i>	100	0.0	100	MH765375.1
LM4.8	<i>Tabanus par</i>	100	0.0	100	MT231166.1
LM4.9	<i>Tabanus trivittatus</i>	100	0.0	100	KY777158.1

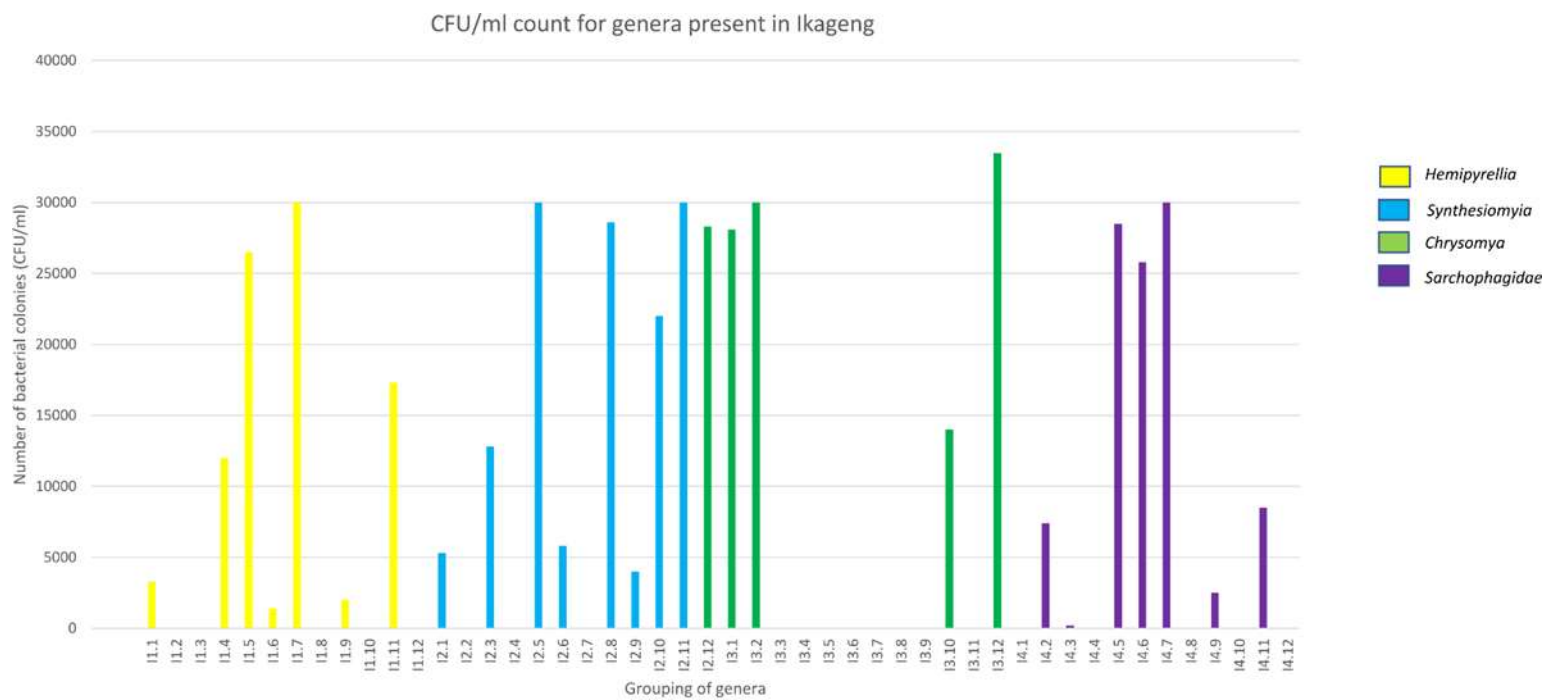
Appendix T7.2: Overview of the total valid reads, low quality amplicons, non-target amplicons, chimeric amplicons, and genera of the sequenced Diptera samples

Sample ID	Total reads after pre-filter	Total valid reads	Low quality amplicons removed	Non-target amplicons removed	Chimeric amplicons	Genera
I1.1	6628	5962	146	41	479	<i>Hemipyrellia</i> spp.
I1.2	7306	6505	195	85	521	<i>Hemipyrellia</i> spp.
I1.5	9457	7570	301	710	876	<i>Hemipyrellia</i> spp.
I1.6	16588	14091	538	513	1446	<i>Hemipyrellia</i> spp.
I2.1	14472	13836	283	21	332	<i>Synthesiomyia</i> spp.
I2.2	5777	5621	100	20	36	<i>Synthesiomyia</i> spp.
I2.3	8953	8476	150	14	322	<i>Synthesiomyia</i> spp.
I2.4	992	970	18	0	4	<i>Synthesiomyia</i> spp.
I2.5	10861	10462	224	43	132	<i>Synthesiomyia</i> spp.
I2.6	9079	8505	204	23	347	<i>Synthesiomyia</i> spp.
I3.1	10010	9654	179	17	160	<i>Chrysomya</i> spp.
I3.4	17364	14181	702	927	1554	<i>Chrysomya</i> spp.
I3.5	10715	9479	260	298	678	<i>Chrysomya</i> spp.
I3.6	7946	7369	151	72	354	<i>Chrysomya</i> spp.
I4.1	16645	13932	745	697	1271	<i>Sarcophagidae</i> spp.
I4.2	20368	15956	1077	1262	2073	<i>Sarcophagidae</i> spp.
I4.3	16153	13045	917	707	1484	<i>Sarcophagidae</i> spp.
I4.4	16118	12656	658	1087	1717	<i>Sarcophagidae</i> spp.
M1.1	20219	17590	809	311	1509	<i>Hemipyrellia</i> spp.
M1.2	21826	18988	942	195	1701	<i>Hemipyrellia</i> spp.
M1.3	12949	10960	645	305	1039	<i>Hemipyrellia</i> spp.
M1.4	14001	12421	474	194	912	<i>Hemipyrellia</i> spp.
M1.5	12068	10405	562	250	851	<i>Hemipyrellia</i> spp.
M1.6	11776	10168	629	179	800	<i>Hemipyrellia</i> spp.
M2.1	9271	8392	205	33	641	<i>Tabanus</i> spp.
M2.2	13435	12339	334	72	690	<i>Tabanus</i> spp.
M2.3	8709	8194	178	21	316	<i>Tabanus</i> spp.
M2.4	10567	9798	234	68	467	<i>Tabanus</i> spp.
M2.5	8695	7980	228	23	464	<i>Tabanus</i> spp.

M2.6	8287	7515	204	96	472	<i>Tabanus</i> spp.
M3.1	13498	11982	484	317	715	<i>Chrysomya</i> spp.
M3.2	12946	11418	596	221	711	<i>Chrysomya</i> spp.
M3.3	11765	10649	405	102	609	<i>Chrysomya</i> spp.
M3.5	12411	11259	421	160	571	<i>Chrysomya</i> spp.
M3.6	12883	10885	646	396	956	<i>Chrysomya</i> spp.
M4.2	13731	12178	593	125	835	<i>Tabanus</i> spp.
M4.4	11640	10121	545	122	8524	<i>Tabanus</i> spp.
M4.5	10518	9153	448	204	713	<i>Tabanus</i> spp.
M4.6	26149	23812	799	271	1267	<i>Tabanus</i> spp.

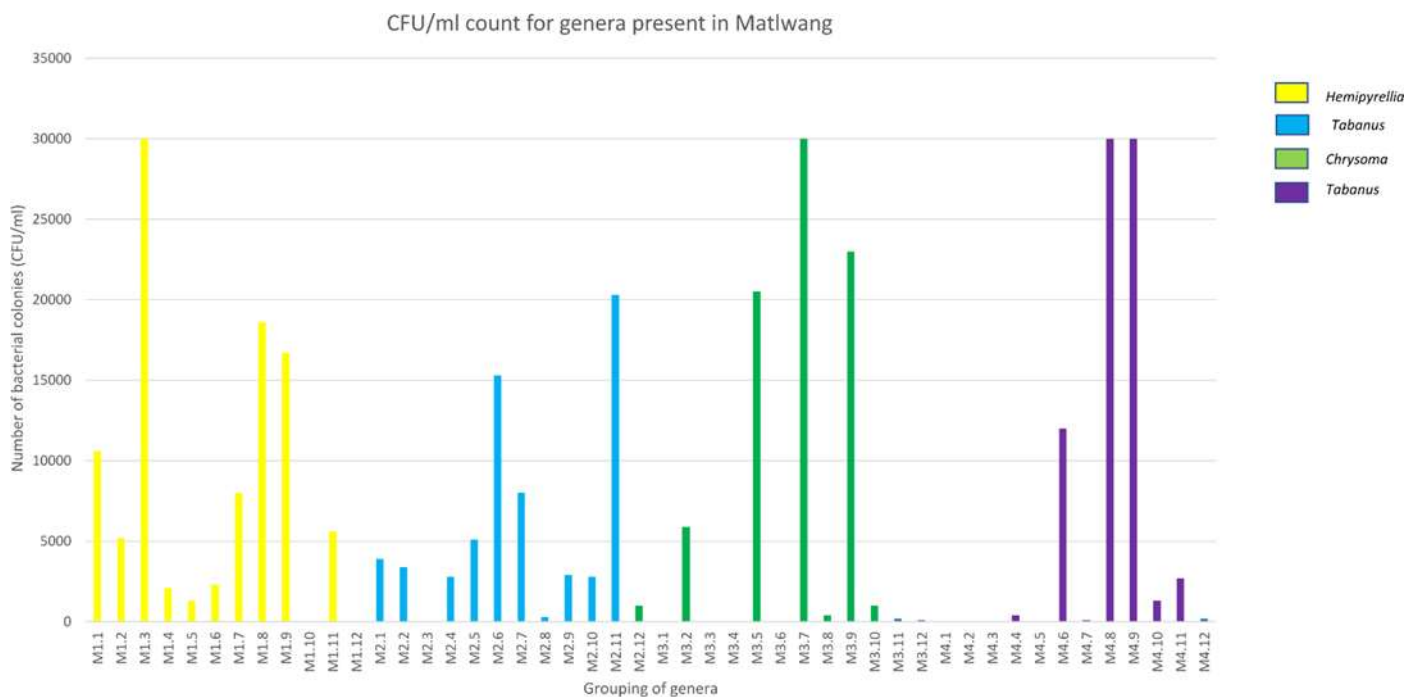


Appendix F7.1: Phylum present from Diptera in the different families based on location from Matlwang and Ikageng



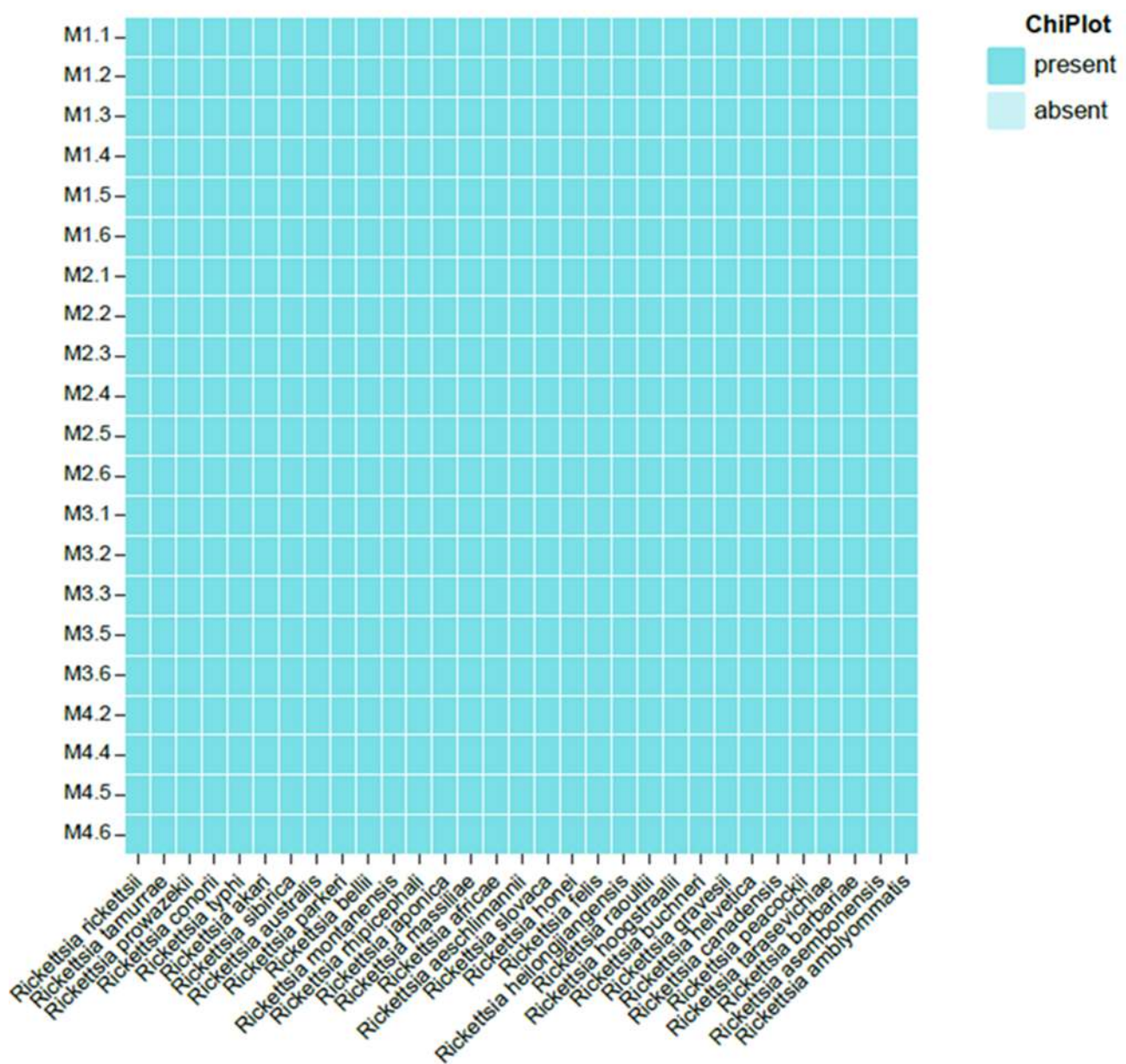
A

Appendix F7.2.A: Bar graph illustrating the prevalence of *Pseudomonas* species isolated from flies in Ikageng, Potchefstroom



B

Appendix F7.2B: Bar graph illustrating the prevalence of *Pseudomonas* species isolated from flies in Ikageng, Potchefstroom



Appendix F7.3: Heatmap illustrating present, and absence of species found in the *Rickettsia rickettsia* group in livestock kraals from Matlwang, Potchefstroom.

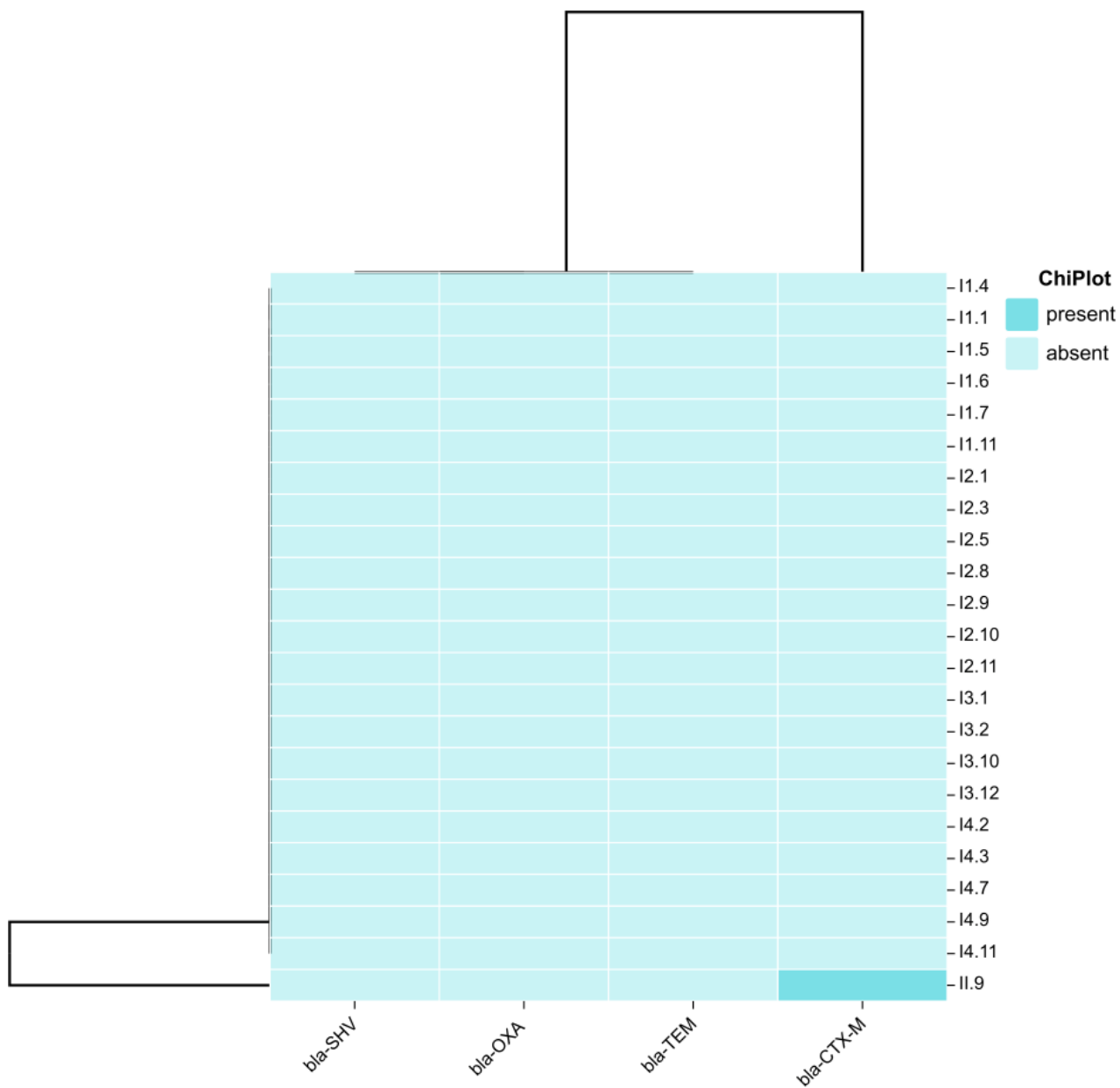
Appendix T7.3A: Hemolytic and ESBL activity in different genera present in Mathwang.

Sample ID	Hemolysis ($\alpha/\beta/\gamma$)	Fly host
M1.1	β	<i>Hemipyrellia</i> spp.
M1.2	β	<i>Hemipyrellia</i> spp.
M1.3	γ	<i>Hemipyrellia</i> spp.
M1.4	β	<i>Hemipyrellia</i> spp.
M1.5	β	<i>Hemipyrellia</i> spp.
M1.6	α	<i>Hemipyrellia</i> spp.
M1.7	β	<i>Hemipyrellia</i> spp.
M1.9	β	<i>Hemipyrellia</i> spp.
M1.11	γ	<i>Hemipyrellia</i> spp.
M2.1	β	<i>Tabanus</i> spp.
M2.2	β	<i>Tabanus</i> spp.
M2.4	β	<i>Tabanus</i> spp.
M2.5	β	<i>Tabanus</i> spp.
M2.6	β	<i>Tabanus</i> spp.
M2.7	β	<i>Tabanus</i> spp.
M2.8	β	<i>Tabanus</i> spp.
M2.9	β	<i>Tabanus</i> spp.
M2.10	β	<i>Tabanus</i> spp.
M2.11	β	<i>Tabanus</i> spp.
M2.12	β	<i>Tabanus</i> spp.
M3.2	α	<i>Chrysomya</i> spp.
M3.5	γ	<i>Chrysomya</i> spp.
M3.7	β	<i>Chrysomya</i> spp.
M3.8	β	<i>Chrysomya</i> spp.
M3.9	β	<i>Chrysomya</i> spp.
M3.10	α	<i>Chrysomya</i> spp.
M3.11	β	<i>Chrysomya</i> spp.
M3.12	β	<i>Chrysomya</i> spp.
M4.4	γ	<i>Tabanus</i> spp.
M4.6	γ	<i>Tabanus</i> spp.
M4.7	α	<i>Tabanus</i> spp.
M4.8	α	<i>Tabanus</i> spp.

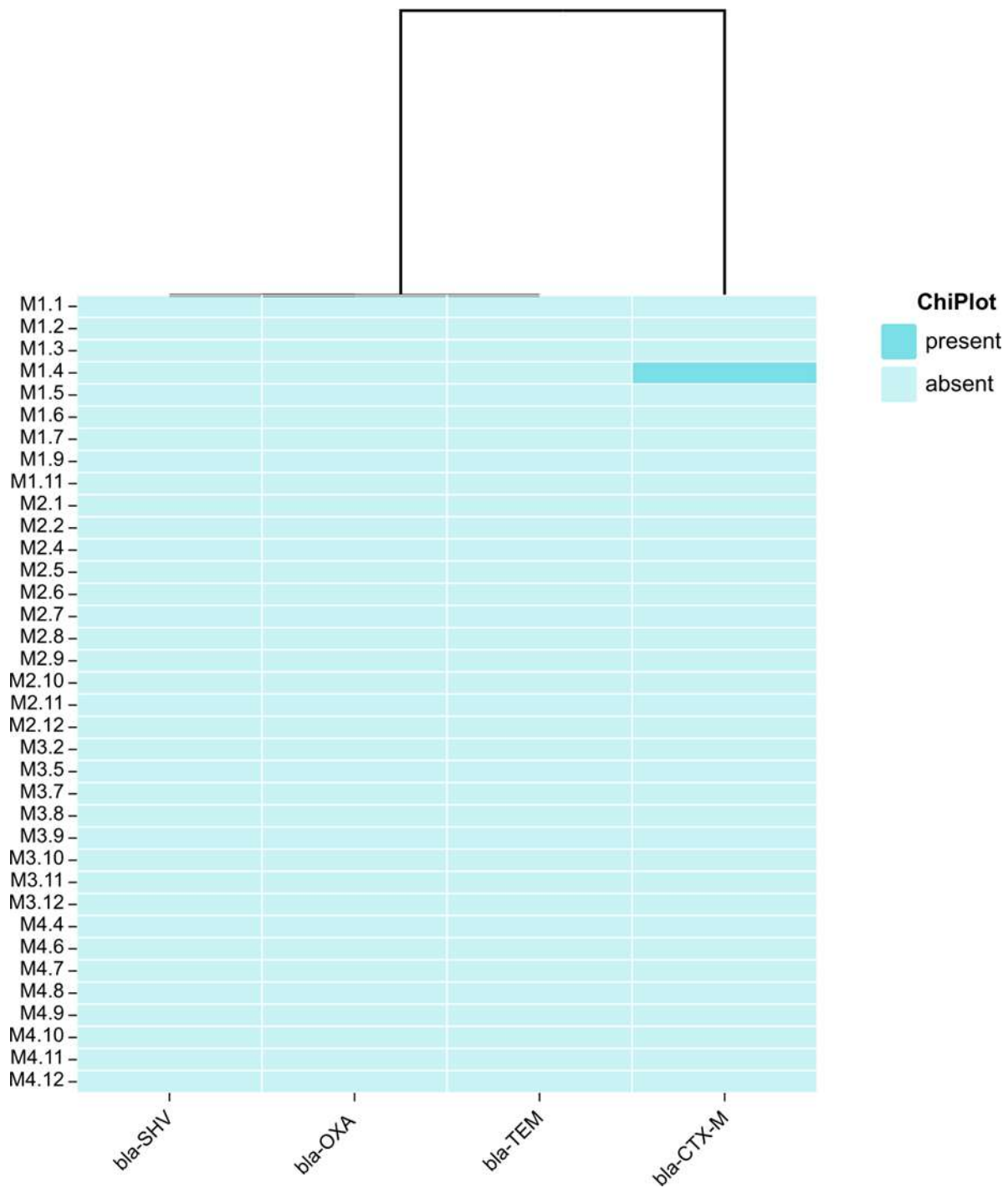
M4.9	Y	<i>Tabanus</i> spp.
M4.10	β	<i>Tabanus</i> spp.
M4.11	Y	<i>Tabanus</i> spp.
M4.12	β	<i>Tabanus</i> spp.

Appendix T7.3B: Haemolytic and ESBL activity in different genera present in Ikageng.

Sample ID	Hemolysis (α/β/Y)	Fly host
I1.1	β	<i>Hemipyrellia</i> spp.
I1.4	β	<i>Hemipyrellia</i> spp.
I1.5	Y	<i>Hemipyrellia</i> spp.
I1.6	Y	<i>Hemipyrellia</i> spp.
I1.7	β	<i>Hemipyrellia</i> spp.
I1.9	α	<i>Hemipyrellia</i> spp.
I1.11	β	<i>Hemipyrellia</i> spp.
I2.1	β	<i>Synthesiomyia</i> spp.
I2.3	β	<i>Synthesiomyia</i> spp.
I2.5	β	<i>Synthesiomyia</i> spp.
I2.6	β	<i>Synthesiomyia</i> spp.
I2.8	β	<i>Synthesiomyia</i> spp.
I2.9	β	<i>Synthesiomyia</i> spp.
I2.10	β	<i>Synthesiomyia</i> spp.
I2.11	β	<i>Synthesiomyia</i> spp.
I2.12	β	<i>Synthesiomyia</i> spp.
I3.1	β	<i>Chrysomya</i> spp.
I3.2	β	<i>Chrysomya</i> spp.
I3.10	β	<i>Chrysomya</i> spp.
I3.12	β	<i>Chrysomya</i> spp.
I4.2	β	<i>Sarcophagidae</i> spp.
I4.5	β	<i>Sarcophagidae</i> spp.
I4.6	β	<i>Sarcophagidae</i> spp.
I4.7	α	<i>Sarcophagidae</i> spp.
I4.9	β	<i>Sarcophagidae</i> spp.
I4.11	α	<i>Sarcophagidae</i> spp.



Appendix F7.4: Clustered heatmap illustrating beta-lactams confirmation in *Pseudomonas aeruginosa* isolates from Ikageng residential dumping site.



Appendix F7.5: Clustered heatmap illustrating beta-lactams confirmation in *Pseudomonas aeruginosa* isolates from Matlwang livestock kraals./

