

**BACTERIAL DIVERSITY AND THEIR ASSOCIATION WITH *ONCHOCERCA*
VOLVULUS INFECTION IN *SIMULIUM* FLIES IN NORTHERN UGANDA**

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**A DISSERTATION SUBMITTED TO THE DIRECTORATE OF RESEARCH AND
GRADUATE TRAINING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF A MASTER'S DEGREE OF SCIENCE IN MOLECULAR
BIOLOGY AND BIOTECHNOLOGY OF GULU UNIVERSITY**

DECEMBER 2024

DECLARATION AND APPROVAL

DECLARATION

I, **LAKOT AMERIDA**, declare that this is my original work and has not been presented to any University for any award.

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APPROVAL

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DEDICATION

My heartfelt dedication goes to my grandmother Lamunu Rose, whose enduring love and wisdom have shaped me in countless ways. Your stories, lessons, and support are deeply woven into the fabric of my success. To my Mum, Lacha Jackie, whose unwavering love have been the foundation of my journey. To my Uncle Stephen Lukwiya, Aunties: Ketty Achiro, Judith Ayot, and Caroline Amony, whose wisdom and encouragement have always inspired me to strive for excellence. Your guidance has been invaluable.

To my siblings, Winnie Auma, Sandra Aryemo, Clarence Okipi, Stewart Otiti, Akao Esther, Mildred Angwech and my little boy Jaysen Niye whose camaraderie and understanding have provided me with both strength and motivation and it's my desire that this may encourage you to reach for greater heights. Your presence in my life is a constant source of joy and inspiration. And to my friends; Lawrence Okidi, and Veronica Wokibula whose companionship and encouragement have made this journey not only possible but also incredibly rewarding. Your faith in me has been a powerful driving force.

This dissertation is as much yours as it is mine. Thank you for being my support system, my cheer leaders, and my greatest allies.

ACKNOWLEDGEMENTS

I would like to extend my heartfelt gratitude to those who have supported and guided me throughout the course of this research. Firstly, I am deeply thankful to my supervisors, Dr. Robert Opiro, Dr. Godfrey Wokorach and Professor Elizabeth Auma Opiyo, for their exceptional mentorship, invaluable insights, and constant encouragement. Your expertise and dedication have been instrumental in shaping this research, and I am profoundly grateful for your support. I am also grateful to the research assistants, Doreen Atoo Adera and Allan Francis Oloya, whose hard work and assistance were crucial to the success of this project. Your commitment and diligence made a significant difference, and I sincerely appreciate your contributions.

My thanks go to the Chief, Associate Professor Richard Echodu and all the staff of the Multifunctional Research Laboratory. Your support and resources have been vital to the execution of this research, and I am thankful for the collaborative environment you provided. I would also like to acknowledge the staff of the Faculty of Sciences for their assistance and the academic environment they fostered, which greatly contributed to my development throughout this study.

A special thank you to the funder of this research project, the US Agency for International Development (USAID) in partnership with the African Research Network for Neglected Tropical Diseases (ARNTD) supported by the Coalition for Operational Research on Neglected Tropical Diseases (COR-NTD) through the small Grants Programme (SGP) award to Dr. Robert Opiro (SGPV/0102/085) whose financial support made this work possible. Your investment in research is deeply appreciated. Finally, I am profoundly grateful to Uganda Independence Scholarship Trust Fund (UISTF) for awarding me the scholarship to pursue my master's studies in Molecular Biology and Biotechnology. Your support has been invaluable in allowing me to achieve my academic and research goals. Thank you all for your guidance, support, and encouragement throughout this journey.

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ACRONYMS

ACE	Abundance coverage estimate
APOC	African Programme for Onchocerciasis Control
CBM	Christoffel Blinden Mission
CDC	Centre for Disease Control
DDT	Dichlorodiphenyltrichloroethane
DEC	Diethylcarbamazine citrate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
FB	Flush buffer
FLT	Flush tethe
GTZ	German Technical Cooperation Agency
GUMRL	Gulu University Multifunctional Research Laboratories
GUREC	Gulu University Research Ethics Committee
HLC	Human Landing Collection
MDA	Mass Drug (Ivermectin) Administration
NEB	New England Biolabs
NFW	Nuclease free water
OEPA	Onchocerciasis Elimination Program for the Americas
OTU	Operational taxonomic unit
PARMANOVA	Permutational Multivariate analysis of variance
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
QoL	Quality of Life

RPM	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic acid
SDS	Sodium Dodecyl Sulphate
UNSDG	United Nations Sustainable Development Goals
UOEEAC	Uganda Onchocerciasis Elimination Expert Advisory Committee
UOEP	Uganda Onchocerciasis Control Programme
WHO	World Health Organization

ABSTRACT

Onchocerciasis, also known as river blindness, is a chronic and debilitating neglected tropical disease resulting from recurrent infections with the parasitic filarial worm *Onchocerca volvulus*. This disease is transmitted by blackflies (*Simulium* species) and poses significant health challenges to affected populations. To date, vector control of blackflies has largely relied on chemical insecticides, an approach that has several drawbacks. There is a pressing need for alternative biological strategies for vector control, one potential approach being the use of 'probiotic' bacteria to modify the physiology of the vector's gut, thereby creating an environment that is hostile to pathogens. This study aimed to survey the bacterial communities in the guts of blackflies from onchocerciasis foci in northern Uganda to identify species with the potential to interrupt onchocerciasis transmission. *Simulium* flies (n=534) were collected from five sites in Nwoya, Pader, Amuru and Moyo districts in Northern Uganda. The flies were extracted using the SDS method and tests for *O. volvulus* infection were done using nested PCR. Twenty-five infected flies and forty-seven non-infected *Simulium* flies were then run for 16s metagenomic PCR using the 16S Barcoding Kit. The library was then subjected to nanopore sequencing. The sequencing identified 12 bacterial phyla with Proteobacteria as the predominant with a relative abundance of 89.7%. The most abundant genera were Enterobacter with 21.4% out of the 149 bacterial genera identified. The bacterial diversity across the sampled districts were significantly different basing on the Shannon index ($p=0.26$). Eight bacterial genera were significantly associated with flies infected with *O. volvulus*: *Peptoniphilus* ($p=0.009$), *Vogesella* ($p=0.029$), *Aquabacterium* ($p=0.018$), *Curvibacter* ($P=0.034$), *Paucibacter* ($P=0.004$), *Massilia* ($P=0.011$), *Mannheimia* ($p=0.011$) and *Shimwellia* ($P=0.036$). On the other hand, six bacterial genera were identified to be predominantly associated with uninfected status. These included *Enterococcus* ($p= 0.03$), *Citrobacter* ($p=0.043$), *Haemophilus* ($p=0.037$), *Shewanella* ($p=0.035$), *Halotalea* ($p=0.020$) and *Zymobacter* ($p=0.018$). The study identified eight novel bacterial genera linked to *O. volvulus* infection and six associated with non-infection in *Simulium* flies. Antibacterial drugs could be developed to target the infection-associated genera, while symbiotic bacteria in non-infected flies could be modified to produce molecules against *O. volvulus*. Further research is needed to understand the physiological mechanisms of these bacteria, which could lead to effective biocontrol strategies.

Keywords: *Onchocerca volvulus*, *Simulium* flies, Onchocerciasis, Bacterial diversity

CHAPTER ONE

INTRODUCTION

1.1 Background

Onchocerciasis, commonly known as river blindness, is a neglected tropical filarial disease caused by the parasite *Onchocerca volvulus* which is vector borne. It causes visual impairment and may sometimes result in total blindness as well as skin infections (nodular lesions). It is the second most important cause of blindness following trachoma (CDC, 2023). Onchocerciasis is among the 300 diseases with a great impact on disability-adjusted life years (DALYs) (Hotez et al., 2014). It stands at 1.49 million cases annually, 14.6 million with skin diseases and 1.15 million with vision loss giving an overall burden of 30 million (Brattig et al., 2021; CDC, 2023). It has been reported that onchocerciasis is also linked to dwarfism, epilepsy, nodding syndrome, and hernia. The main burden of the disease (99%) occurs in Africa with most of the countries located where over 100 million people live in onchocerciasis endemic areas including Uganda which initially had 17 foci. This puts the population at risk of contracting the disease, especially those living in rural agricultural areas near streams and rivers (CDC, 2023; Kuesel, 2016). According to the Uganda Onchocerciasis Elimination Expert Advisory Committee (UOEEAC., 2022), it is projected that 1.7 million people are at risk since their habitations are near the river systems where the vector breeds and inhabit.

A concerted large-scale intervention programme (African Programme for Onchocerciasis Control) between 1995 and 2015, involving mass drug (ivermectin) administration (MDA) supplemented with vector control using insecticides, reduced the public health burden of onchocerciasis across the region substantially, such that the WHO proposed 2025 as a target for elimination in 80% of African countries. However, significant number of disease foci remain, particularly in remote areas of extreme poverty and social instability (Colebunders et al., 2015). Northern Uganda is a case in point; the large (1.4 million inhabitants) Madi-Mid North focus is one of only two of Uganda's original 17 focus areas where transmission of river blindness continues (Katabarwa et al., 2018) (Figure 1.1). Northern Uganda was excluded from APOC until 2012 due to civil war and its proximity to Southern Sudan, where onchocerciasis control is hindered by civil war of its own, which presents ongoing challenges to local intervention (Katabarwa et al., 2018). The burden in most of the 17 foci within Uganda are minimal with reported success in elimination of onchocerciasis in 7 of the foci, transmission

interruption in 5 foci, suspected interruption in 3 foci and ongoing transmission in the Mid-madi north focus and Lhubiriha (Katabarwa et al., 2018).

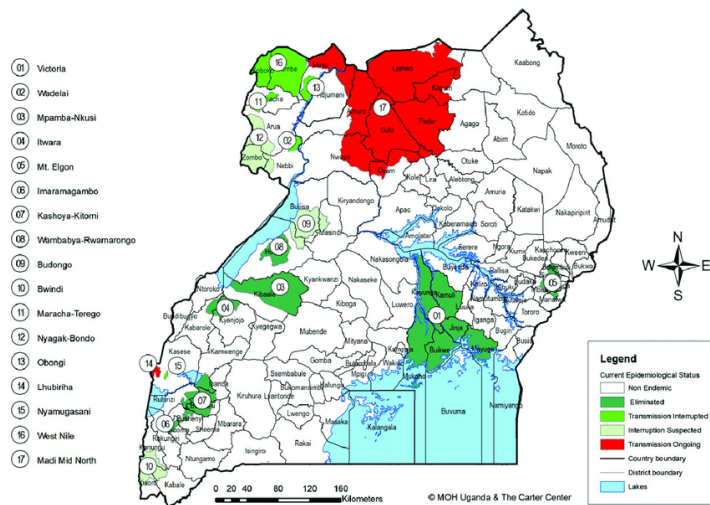


Figure 1. 1:Map of Uganda showing the 17 onchocerciasis foci

To date, vector control of blackflies has relied on chemical insecticides. However, these approaches have numerous pitfalls. Larvicides can be costly to use, may have harmful effects on the environment, and need skilled personnel to determine the correct dosage. (Jacob et al., 2018). Furthermore, the emergence of resistance to temephos and other organophosphates has been encountered in numerous insect populations (Osei-Atweneboana et al., 2011; Valle et al., 2019), compromising their sustainable use. The drawbacks to the use of chemicals have provoked exploration of alternative biological approaches to vector control. A wide variety of approaches have emerged among which are a number that exploit the impact of microbial symbionts on insect physiological homeostasis and thus, indirectly vector competence (Cirimotich, Ramirez, et al., 2011). The gastrointestinal microbiota of insects is being studied more extensively for this purpose, and one innovative approach might involve using 'probiotic' bacteria. These bacteria could change the physiology of the vector's gut, creating an environment that is unfavourable for pathogens. Considerable advancements have been achieved in this area for mosquitoes and tsetse flies (Song et al., 2018; B. L. Weiss et al., 2019) but as yet, exploration of the microbiomes of *Simulium* species has been rudimentary (Tang et al., 2012) and little effort and/or progress has so far been made to quantify microbiomes or identify symbionts of potential value in biological vector control in any of the African *Simulium* species that transmit *O. volvulus*.

1.2 Problem statement

Despite significant efforts to control onchocerciasis, including mass drug administration (MDA) with ivermectin, onchocerciasis remains a significant public health challenge in Uganda, particularly in endemic regions of the Madi-mid north focus being the largest covering 11 districts which continues to affect the UN-SDG3 which focuses on good health and well-being. According to the UOEEAC report (2022), 1.7 million people are at risk due to their proximity to the river systems that harbor *Simulium* flies. Control strategies like mass drug administration using ivermectin are costly requiring 0.78 US\$ per treatment (Ndyomugenyi et al., 2007) causing an economic loss of 28,558.4 US\$ per year to parishes (Kaharuza et al., n.d.). The additional challenge is the large population of 172 million people in need of ivermectin treatment, co-endemicity with Loiasis coupled with its adverse effects (Haselow et al., 2003.) and reported resistance to ivermectin by some *O. volvulus* strains (Awadzi et al., 2004). Doxycycline use on the other hand, has got concerns about its prolonged usage, safety profile and growing threats of antibiotic resistance leading to a reduction in sensitivity of the microbes to the drug from 46% in 2020 to 31% in 2021 (Kuesel, 2016; Sahu et al., 2022).

Meanwhile vector control through bush clearing has not yielded permanent good results due to the seasonal changes that come with re-insurgency of the black flies. Funding has also been insufficient leading to suspension of larviciding for vector control and a rise in the population of *Simulium* flies along major rivers particularly in Northern Uganda (MOH,Uganda., 2023). In addition, use of larvicides and insecticides have been linked with developing resistance against the black flies and toxicity to environment (Jacob et al., 2021; Tirados et al., 2022). It is therefore essential to investigate the role of the blackfly bacteriome in the transmission of *Onchocerca volvulus* and to explore possible strategies for altering the microbiome to decrease parasite development and transmission.

This study aims to explore the bacterial diversity in *Simulium* flies from onchocerciasis-endemic areas in Northern Uganda and its relationship with *Onchocerca volvulus* infection. The interaction between the flies' microbiomes and disease transmission is not well understood, and the role of specific bacteria in influencing infection rates remains unclear. By examining the link between microbial communities and parasitic infections, the research seeks to enhance knowledge of vector biology, which could inform targeted strategies for controlling and eliminating onchocerciasis, ultimately improving public health in the region.

1.3 Objective

1.3.1 General objective

To analyse the bacterial diversity in blackflies from onchocerciasis foci in northern Uganda to identify species with the potential to interrupt onchocerciasis transmission.

1.3.2 Specific objectives

1. To assess the bacterial diversity of blackflies in the various endemic districts of northern Uganda.
2. To determine the relationship of bacterial genera diversity with *O. volvulus* infection and non-infection in *S. damnosum sensu lato*.
3. To determine bacterial genera that can potentially be used as a novel biocontrol against the vector and parasite.

1.4 Research Questions

1. What is the bacterial diversity of blackflies in the various endemic districts of northern Uganda?
2. What is the relationship of bacterial diversity with *O. volvulus* infection and non-infection in *S. damnosum sensu lato*?
3. Are there bacterial genera that can potentially be used as a novel biocontrol against vectors and parasite?

1.5 Significance of the study

This study has the potential to advance scientific understanding, contribute to public health improvements, support sustainable environmental practices, and foster economic development, aligning with broader global goals and community needs. By elucidating the relationship between blackfly microbiomes and *Onchocerca volvulus* infection, this study could lead to more targeted and effective interventions for managing and preventing river blindness. Understanding the microbiome's role in infection dynamics may help in developing novel strategies to lower the occurrence of this debilitating disease, thereby enhancing overall community health and quality of life. While the study does not directly target antibiotic resistance, insights gained into the microbiome may inform broader strategies to combat infections more effectively. For instance, identifying microbiome factors that influence

susceptibility or resistance to *Onchocerca volvulus* could indirectly provide clues for addressing issues related to antimicrobial resistance in broader contexts.

By identifying novel bacteria of potential as biocontrol, it may be possible to develop more ecologically sustainable vector management practices that minimize ecological disruption, reduce reliance on harmful chemicals, and ameliorate the adverse outcomes of bush clearing on local ecosystems. The outcomes of this study will address UN-SDGs by potentially improving control strategies for river blindness, the study contributes to reducing disease burden and promoting better health (SDG 3) and promote sustainable land use and protect terrestrial ecosystems. Improved management and prevention of onchocerciasis could have a positive impact on the local economy. Healthier populations are more productive, and reduced disease burden can lower healthcare costs and improve income levels. Additionally, more effective and environmentally friendly pest control methods could enhance agricultural productivity and sustainability, benefiting the economy at both local and national levels.

1.6 Scope of the study

1.6.1 Geographical scope

The research was conducted across four districts within the Madi-Mid North endemic area of Northern Uganda, specifically Pader, Nwoya, Amuru, and Moyo. Sample collection took place at five locations: Angagura near Aruu Waterfalls, Ogelo and Laminatoo along the Ayago River, Atiak along the Unyama River, and Pamulu along the Oluvo River.

1.6.2 Time scope

The samples were collected during the rainy season in the months of April, May and June 2023 for five days from each site.

1.6.3 Content scope

The samples were preserved in Liquid nitrogen and transported to the Gulu University Multifunctional Research laboratories where all the laboratory analysis was carried out. The study included extracting DNA from blackflies using SDS method (Sodium dodecyl sulphate), subjecting them to PCR analysis to test for *O. volvulus* parasite. The infected and uninfected flies were then subjected to 16s metagenomic PCR and then sequenced using the Oxford nanopore technology.

CHAPTER TWO

LITERATURE REVIEW

2.1 Onchocerciasis and burden

Onchocerciasis is a neglected tropical disease caused by a parasite called *Onchocerca volvulus* transmitted by the vector black flies of the *Simulium* genus. The disease is characterized by nodular formation by adult *O. volvulus* worms, skin lesions with severe itching leading to skin discoloration and ocular lesions which sometimes leads to permanent visual impairment (WHO, 2022). The disease has also been closely linked to epilepsy and nodding syndrome in children (Chesnais et al., 2018; Colebunders et al., 2017) and such cases have been observed in Northern Uganda (Deogratius et al., 2016) .

WHO (2021) reports that more than 240 million people live in onchocerciasis endemic areas, which puts them at risk of infection. The global burden of onchocerciasis is at 30 million with 14.6 million cases of skin disease and 1.15 cases of visual impairment (Brattig et al., 2021, WHO, 2018). One hundred and forty thousand (140,000) of the onchocerciasis burden occur in Latin America leaving the major burden of the diseases in Sub-Saharan Africa with 31 endemic countries which account for 99% of the global burden and more than 218 million people at risk of contracting the disease (Frallonardo et al., 2022; WHO, 2019). In the African countries, there is a prevalence of 6.5 million people in Nigeria, 5 million infected and 12 million at risk in Ethiopia meanwhile Cameroon reported a risk burden of 9 million with 5 million infected and 30,000 visual impairment cases due to onchocerciasis (Deribe et al., 2012; Schmidt et al., 2022; WHO, 2021).

In Uganda, 36 districts grouped under 17 foci are endemic putting 3.9 million people at risk (MOH, 2023). Onchocerciasis continues to be a health burden to the country affecting productivity of the population involved since the endemic areas major in agriculture and fishing. Atleast 1.5 million people had been infected by 2009 and according to the Uganda Onchocerciasis Elimination Expert Advisory Committee (UOEAC., 2022), its projected that 1.7 million people are at risk since their habiations are near the river systems where the vector breeds and inhabit. In 2009, as the over all infection rates in Uganda were declining, there was a report of high infection rate of onchocerciasis in Northern Uganda (Madi-mid north focus) were there had been a civil war for over 18 years . The civil war mad it hard to conduct research to know the magnitude of the disease and apply control measures. Nodule prevalence of 30%

and prevalence of microfilaria in skin snips of 29.6% as well as microfilaria in the anterior chamber of the eye as observed from Pader district could indicate ongoing transmission of onchocerciasis in the region (Wakabi, 2009). Additionally are the reported outbreak of nodding syndrome in the endemic districts of Northern Uganda among children aged 5-15 suggesting a link of onchocerciasis to nodding syndrome and epilepsy (Gumisiriza et al., 2021).

Onchocerciasis is a serious public health issue which causes serious disabilities, including blindness, and places a substantial strain on healthcare systems, demanding considerable resources for treatment and prevention initiatives. The lasting health impacts not only harm individual patients but also have wider effects on community health, diminishing the overall productivity and economic output of the affected populations (Ugbomoiko et al., 2022). The quality of life (QoL) for people affected by onchocerciasis is negatively impacted by both its physical symptoms and psychological effects. Research conducted in Nigeria revealed that the disease greatly diminished individuals' QoL, with many experiencing social exclusion and feelings of shame. The sociocultural stigma linked to visible symptoms, like skin lesions, intensifies these challenges, resulting in social isolation. This decline in quality of life can limit access to education and employment, reinforcing ongoing poverty. Onchocerciasis creates significant economic burdens for communities (Ugbomoiko et al., 2022). The disease impairs the ability of affected individuals to work, resulting in lost income and a reduced labour force (Njim et al., 2015). In areas with high infection rates, the cost of treating onchocerciasis can be overwhelming for low-income families, threatening their financial stability (Malatt & Taylor 1992). At the community level, the disease leads to lower agricultural output and reduced economic activity as a result of high illness rates among the workforce. Managing and controlling onchocerciasis requires substantial financial commitment. Initiatives like the African Onchocerciasis Control Programme in West Africa have invested more than US\$257 million between 1995-2010 in disease management, including the widespread distribution of the anti-parasitic drug ivermectin (Coffeng et al., 2013). Despite these investments, the enduring nature of the disease and the need for continuous interventions mean that the socio-economic burden remains significant.

2.2 *Onchocerca volvulus* parasite

O. volvulus is a blood and tissue filarial nematode that causes onchocerciasis and is ranked as the second cause of blindness after trachoma (CDC, 2023). The adult parasites inhabit the subcutaneous tissue where they form fibrous tissue nodules due to their coiling. The male worms are 3-5 cm in length quite smaller than the females that are about 30-80 cm in length and 0.5 cm in diameter (Lindquist & Cross, 2017). *O. volvulus* is projected to have a mean reproductive lifespan of 9-11 years though about 95% reach end of reproduction at 13-14 years however, their average lifespan is 15 years (Plaisier et al., 1991).

Its lifecycle occurs partly in the black fly, the vector that transmits it and the rest of the lifecycle occur in the human host. During a blood meal, the black fly deposits the infective stage 3 larva which penetrates the skin of a host and migrates to the subcutaneous tissue where they form nodules called onchocercomas, they grow further to reach the adult stage. They mate and the fertile females can produce millions of microfilariae of 220– 360 μm with a lifespan of two years. These are disseminated throughout the body causing skin disease characterized by severe itching and lesions, epilepsy and ocular complications.

During a subsequent blood meal, the blackfly takes in microfilaria larvae circulating at the skin surface. The first stage larva penetrates through the stomach wall into the haemocoel then move to the thoracic muscles where they undergo morphogenesis twice to form the larva stage 2 and larva stage 3. The mature stage three larva (L3) then migrates to the head and mouth parts of the black fly where they are transmitted to the new host during a blood meal. Other infective stage three larva move to different organs of the fly like the antenna, brain, ovaries and palps thereby destroying the fly and most flies die as a result leaving only 1% (Boatin & Richards, 2006; Brattig et al., 2021; Ekangou et al., 2022).

2.3 Blackflies and their distribution

Blackflies are a group of small black blood feeding insects ranked third in economic and medical importance. They thrive in fast flowing waters like streams, rivers and waterfalls where they feed and breed. This greatly influences their distribution across the biogeographical realms where about 2200 species of black flies have been described except in Antarctica and a few islands. Of all biogeographic regions, Palearctic has the highest number of blackfly species (700), followed by Oriental with 500, Neotropical has 300 and Nearctic with 256 meanwhile

in Africa, distribution stretches across the subtropical belt from West Africa to East Africa (Adler & McCreddie, 2018; Ajayi & Olusi, 2018).

Simulium (Simuliidae) genus is composed of over 200 species of blackflies but only 1.5% are vectors for human diseases including onchocerciasis (Adler et al., 2010). So, the vector for *Onchocerca volvulus* is the blackfly of the genus *Simulium*. The most common natural vectors for *O. volvulus* are *S. ochraceum*, *S. metallicum* and *S. callidum* while *S. gonzalezi*, *S. haematopotum*, *S. horacioi* and *S. veracruzianum*, are considered as potential vectors in Latin America (Takaoka, 2015).

However, in Africa, *Simulium damnosum* s.l is the most economically important vector species in the transmission of onchocerciasis whereas *Simulium neavei*, *Simulium woodi* and conceivably *Simulium nyalalandicum* account for 10%. Meanwhile in Uganda, *Simulium damnosum sensu lato* accounts for more than 85% of parasite transmission followed by *S. neavei* s.s. which are the two known vectors (Ndyomugenyi et al., 2007). Humans are the host and reservoir of *Onchocerca volvulus* which they acquire after being bitten by a female black fly carrying the parasite (Yameogo et al., 1999).

2.4 Vector control methods

Onchocerciasis control in Uganda began in 1952 focusing on vector control with the use of dichlorodiphenyltrichloroethane (DDT) in River Nile in the Victoria Nile focus in central Uganda which resulted in a successful elimination of *S. damnosum* by 1973. The control strategy was applied to Murchison Nile focus but was met with a re-emergence of *S. damnosum*. DDT larvicide was adopted to eliminate the *Simulium* larvae in Rwenzori and Budongo *S. neavei* focus with partial success (Katabarwa et al., 2018). Vector control using larvicides and insecticides using temephos has been applied by aerial spraying on the river systems such as Aswa, Pager, Agago, Unyama, and Ayago. Larvicides use has been effective in some foci like the Bioko focus in Equatorial Guinea in 2005 eliminating *Simulium yahense* (Traoré et al., 2009) and Itwara foci in western Uganda (1991-2012) following mass drug administration with ivermectin in eradicating *S. neavei* (Lakwo et al., 2013) with support from APOC and in the Victoria Nile focus which achieved the goal in the 1970s (Katabarwa et al., 2018). Despite the achievements met in use of insecticides and larvicides like DDT in the 1970s and organophosphates, showing significant decrease in rate of infection, transmission of river blindness were achieved, there are reported cases of reinvasion of the controlled rivers by the black flies due to seasonal migration and developing resistance to the larvicides and toxicity of

the insecticides like the permethrin and carbosulfan which pose control challenges and limit their usage (Tirados et al., 2022).

Slash and clear has been applied along R.Ayago. Vegetation clearing destroys the habitat and breeding grounds for the blackflies and they have been effective in greatly ameliorating the blackfly density in study areas of South Sudan (>90%) and up to 97% in Amuru district (Jacob et al., 2021; Raimon et al., 2021). Similar reports of reduced blackfly population was observed indicated by a significant reduction in the biting rate of 11% in Nwoya (Jacob et al., 2018). In addition, has been the use of Esperanza window trap (EWT) to capture the flies and all this reduced the vectors biting rate (Lakwo et al., 2020). These were implemented to supplement the MDA using ivermectin which has been used since 1993 in some districts to accelerate the elimination of onchocerciasis. However, there are limitations such as forested areas that may be hard to slash and clear thus continue to provide breeding grounds for the blackflies and the larvae are reported to inhabit the rocks once the bushes are cleared (Jacob et al., 2021).

2.5 Parasitic control methods

The use of chemotherapies has been based on diethylcarbamazine citrate (DEC) and then ivermectin. Initially, Mass drug administration (MDA) with DEC in the Elgon focus and Kigezi area was adopted up to 1992 and a reduction rate of up to 10% was recorded. In 1989, mass treatment with Ivermectin as a broad-spectrum standard treatment for parasitic infections especially onchocerciasis in humans. Ivermectin has an inhibitory effect on the gated chloride ion channels in invertebrate muscle and nerve cells of the microfilaria and impairs normal uterine growth of *O. volvulus* microfilaria (Drug Bank, 2023). This has made ivermectin effective in controlling tissue microfilariae *O. volvulus*. Mass drug administration using Ivermectin as the sole strategy in the countries of the Onchocerciasis Elimination Program for the Americas (OEPA) has been reported to have successfully eliminated onchocerciasis in 11 out of 13 foci in the countries of Colombia in 2013, Ecuador in September 2014, Mexico in July 2015, and Guatemala in July 2016. More than 500 000 people no longer need ivermectin in the Americas (Rodríguez-Pérez et al., 2015; WHO, 2022, 2023).

Ivermectin use in Uganda was initiated by the Uganda Foundation for the blind with funding from Sightsavers. Mass Drug Administration (MDA) commenced in the Budongo foci and was expanded to the Itwara focus with help from the German Technical Cooperation Agency (GTZ). By the midst of 1992, Ivermectin usage was adopted across the other onchocerciasis

foci such as the Maracha-Terego focus in West Nile led by the Ministry of Health and supported by the Christoffel Blinden Mission (CBM). By 1996 the African Programme for Onchocerciasis Control (APOC) was launched and this funded the Uganda Onchocerciasis Control Programme (UOEP) involving mass drug administration (MDA) with ivermectin supplemented with vector control using insecticides for 5 years (Mutabazi, 1998). This reduced the public health burden of onchocerciasis in the Mpamba-Nkusi, Itwara foci and Mt Elgon foci in Eastern Uganda, (Katabarwa et al., 2014), with more than 800,000 people in Uganda and 120,000 people in Sudan not in need of ivermectin by 2015, the year APOC closed (WHO, 2022). However, APOC did not halt the transmission of onchocerciasis and Northern Uganda which was not mapped as part of the onchocerciasis endemic areas in the 1990s was left out. Northern Uganda was excluded from APOC until 2012 due to civil war and its proximity to South Sudan, where onchocerciasis control is hindered by civil war, presenting challenges to local intervention (Katabarwa et al., 2018).

Despite registered success in Ivermectin use, challenges have been met in this implementation leading to failure in some countries of sub-Saharan Africa. These challenges include; large coverage area with 172 million people in need of ivermectin treatment, co-endemicity with Loiasis coupled with its adverse effects which discourages the community from participating in ivermectin treatment (Haselow et al., 2003). Reported resistance to ivermectin by some *O. volvulus* strains is also posing a great challenge in its use as a good treatment option as it has been reported of some onchocerciasis patients who failed to respond to treatment with Ivermectin (Awadzi et al., 2004; Katabarwa et al., 2013; Kuesel, 2016). Lakwo et al., (2013) also reports that despite the mass drug administration with ivermectin from 1991 to 1994 the transmission rate has been reduced only from 40 – 22% but remained drastically high which required the adoption of larvicides. Additional is the hard-to-reach areas due to conflicts of which northern Uganda is part following the LRA civil war thus interrupting treatment routines or late start of treatment.

2.6 Use of antibiotics

Doxycycline has been the major antibiotic for treatment of onchocerciasis. Doxycycline which belongs to the tetracycline group of antibiotics is a broad-spectrum antibiotic that inhibits bacterial growth. It is synthetically derived from oxytetracycline, a naturally occurring tetracycline produced by *Streptomyces* bacteria (et al., 2020). Doxycycline works by disrupting bacterial protein synthesis, which is crucial for bacterial growth and reproduction. It does this

by attaching to the 30S ribosomal subunit, a part of the bacterial ribosome. This attachment prevents the aminoacyl-tRNA from binding to the mRNA-ribosome complex, stopping the addition of new amino acids to the growing peptide chain. Consequently, the bacteria cannot produce the proteins required for their survival and multiplication (BiologyInsights Team, 2024). Doxycycline application in onchocerciasis control works by targeting the Wolbachia bacterium leading to its depletion. Wolbachia has been implicated as important in the viability and fertility of *O. volvulus* therefore, targeting the bacteria would render 80-90% of the parasites sterile, unable to reproduce and multiply reducing the microfilaria as they die off at the expiration of their lifespan (Hoerauf et al., 2008). Doxycycline has been effectively used in combination with ivermectin to directly cause the death of microfilariae leading to the death of up to 60% of adult worms. A long-term decrease in the number of microfilariae in the skin and eyes, along with a reduction in the number of adult worms in the body, could help lower the transmission and occurrence of eye disease due to onchocerciasis (Abegunde et al., 2014; Walker et al., 2015).

Despite the achieved milestones in the use of doxycycline, there have been concerns about its prolonged usage, safety profile and growing threats of antibiotic resistance (Kuesel, 2016). With the growing global health threat of antibiotic resistance, doxycycline is not an exception considering its prolonged and multipurpose usage for several conditions as a PEP for sexually transmitted bacterial infections, as an antimalarial drug and in onchocerciasis. Studies indicate that patients with prior doxycycline exposure were more likely to carry a high abundance of ARGs (antibiotic resistance genes) (Carpenter et al., 2024; Kenyon et al., 2023). Following the past pandemic of COVID-19, there was a lot of usage of doxycycline as a therapeutic element against COVID-19 and reports show that repeated usage over the years led to the reduction of its efficacy from 46% in 2019 to 31% in 2021 (Sahu et al., 2022). This therefore calls for research to establish biocontrol against the *O. volvulus* parasite or increase vector refractoriness towards *O. volvulus*. This shall be achieved by exploring the gut microbiome and association to *O. volvulus*.

2.7 Insect microbiome

Insects interact with a variety of pathogenic and non-pathogenic micro-organisms such as viruses, bacteria, nematodes, fungi and protozoa. The relationship between insects and microorganisms is complex and varied. Microbes can be beneficial, neutral, or pathogenic, and these relationships significantly affect insect biology, behavior, and ecology (Mondal et al.,

2023). Acquisition of bacteria by insects marks the onset of microbial symbiosis, which is followed by the adaptation of these bacterial species to the gut environment for prolonged sustenance and successful transmission across generations (Gupta & Nair, 2020). Genetic research has revealed that the relationships between hosts and microbes are often ancient, with both partners likely unable to survive without each other. Over their long co-evolutionary history, the genomes of primary symbionts have significantly shrunk, losing genes and pathways that are no longer needed due to the specialized metabolic environments provided by the hosts. Well-studied primary symbionts include *Buchnera* in aphids, *Carsonella* in sharpshooters, *Blochmannia* in ants, *SOPE* in rice weevils, and *Wigglesworthia* in tsetse flies (Weiss & Aksoy, 2011). Understanding this intricate balance offers important insights for ecological health, and the development of new biotechnological solutions to disease transmission (Gupta & Nair, 2020).

Bacteria have diverse roles they play towards the survival of the insect., for example they help insects digest hard-to-digest substances like animal blood, plant sap and wood providing essential nutrients like amino acids, vitamins, and enzymes, they can also aid the immune system of the insect by outcompeting pathogenic microbes or stimulate immune responses to fight infections (Ferrarini et al., 2022; Mondal et al., 2023). Insects may rely on certain bacteria to improve reproductive success by influencing hormone production, mate attraction, or even protecting eggs and larvae (Wang et al., 2019). Some microorganisms can alter the behaviour of their insect hosts, influencing mating, feeding, and social interactions for example, the parasite *Hymenopimemecis* manipulates its host's behaviour to create the ideal environment for its larvae, *Wigglesworthia* in tsetse flies play a crucial role in the development of the larvae by providing proline which is required for milk yielding, nutrition-wise, they provide vitamins and contribute to the maturation and effective performance of the immune system (Weiss et al., 2022).

On the other hand, these bacteria influence the interaction of the insects and the pathogens affecting also the establishment and transmission of pathogens from one individual to another. The gut microbiome like *Wolbachia* can be regarded as mutualistic for various insects, as it might offer its host protection against viruses, insecticides, or plant defences, while also playing a role in supplying nutritional support. *Wolbachia* has been reported to inhibit replication of dengue virus in the vector mosquito but also beneficial in enhancing survival and fertility of *O. volvulus* (Moreira et al., 2009; Tamarozzi et al., 2011) and this has been the basis for the development of antiwolbachia treatments for onchocerciasis such as doxycycline.

Recent studies in vector control have been focused on identification of bacteria that can be used to prohibit the establishment of the pathogens in the insects and their spread to uninfected humans. For instance the use of naturally occurring bacteria like *Pseudomonas*, *Enterobacter*, *Acinetobacter* and *Bacillus* to block the establishment of *Plasmodium* in *Anopheles* mosquitoes, a discovery of TC1 bacterium from St. Hopkins University as well genetic engineering of symbiotic *P. agglomerans* bacterium to fight malaria (Cirimotich et al., 2011; Wang et al., 2012). Same strategies have been adopted in the control of trypanosomiasis (Medlock et al., 2013; Mfopit et al., 2024; Weiss et al., 2019). For all this to be achieved there is need to study the microbiome of the insect guts and this has been done in tsetse flies, ticks, mosquitoes, and black flies in Mbam river, Cameroon (Ekangouo et al., 2022; Seabournid et al., 2020; Zhang et al., 2019). However, no study has been conducted in the hyperendemic foci of Madi-mid north for which this study will cover.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was conducted in four districts of the Madi-mid north endemic focus of Northern Uganda namely Pader ($2^{\circ}49'59.99''N$ $33^{\circ}04'60.00''E$), Nwoya ($02^{\circ}38'06''N$ $32^{\circ}00'00''E$), Amuru ($3.520846, 32.08355$) and Moyo ($03^{\circ}40.771'N$ $31^{\circ}49.576'E$) (Figure 3.1). Five sites were considered, and these were Angagura near Aruu water falls in Pader district, Ogelo and laminatoo along R. Ayago in Nwoya district, Atiak along R. Unyama in Amuru district and Pamulu along R. Oluvo in Moyo district. The study sites are characterized by wet seasons which, are warm and humid, the dry season is hot, and it is overcast year-round. Over the course of the year, the temperature typically varies from 64°F to 96°F and is rarely below 60°F or above 102°F. These sites were chosen because they have significant population of *Simulium* flies (Hendy et al., 2018).

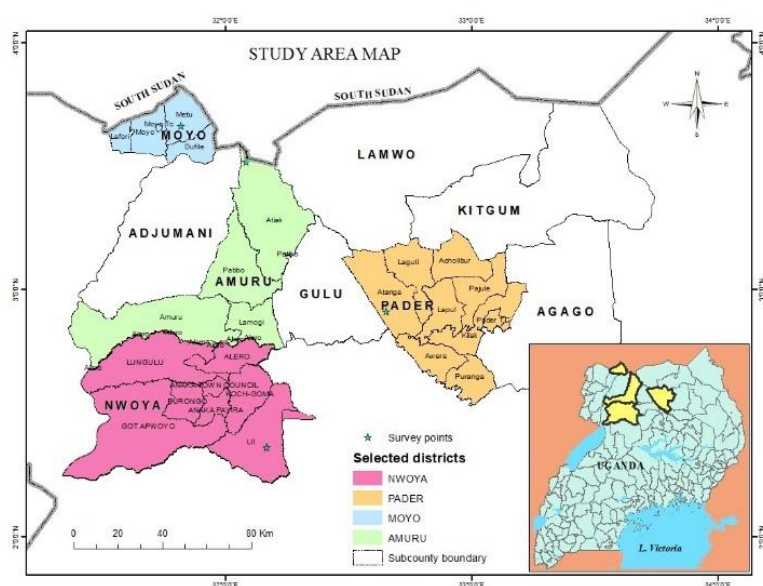


Figure 3. 1: Map of the study area (shaded) showing the districts where sampling was done.

3.2 Study design

The study was cross-sectional in design, with field site selection performed to include the major watersheds in the Madi Mid North focus. Pader collection was performed near the Aruu Falls on River Aswa in Pader district. Nwoya collection was performed along the Ayago river with

the coordinates N 02°25.907' E 032°0.452', Moyo sample collection was performed at Pamulu N03°40.758'E031°49.452' along the White Nile, and Amuru at Atiak along R. Unyama with the coordinates 3.520846, 32.08355.

3.3 Inclusion criteria

Species: *Simulium* species known to be vectors of *O. volvulus* in the study area.

Geographic Origin: Flies collected from endemic regions of Northern Uganda with a high population of *Simulium* flies and ongoing transmission of onchocerciasis.

Development Stage: Adult female *Simulium* flies were included to ensure the presence of potential parasite infections.

Physical Condition: Flies were selected based on their physical condition, excluding damaged or decomposed specimens.

3.4 Exclusion criteria

Misidentified Species: Flies that could not be accurately identified as *Simulium* flies were excluded.

Contamination: Flies contaminated with other insects or environmental microorganisms were excluded to avoid potential bias in microbial community analysis.

Insufficient DNA Yield: Flies that did not yield sufficient DNA for molecular analysis were excluded.

By carefully applying these inclusion and exclusion criteria, the researchers ensured the quality and reliability of the data obtained from the study.

3.5 Sample collection and preservation

Simulium flies totalling 534 samples were obtained from the four districts using human landing collections (HLC) method done by trained community-based participants following standard methods (Baker, 2016). The human landing collection method was used because there was need to determine the biting rate of the *simulium* flies and how many had been captured. The human landing collection method is also simple and cost effective and easily applicable in the remote areas. The collection of flies was done between 07.00 and 18.00hr. The personnel captured blackflies landing on their exposed legs. Flies were collected in individual 15ml falcon tubes and then pooled together in 50ml falcon tubes, which were stored in liquid nitrogen tank and transported to the Gulu University Multifunctional Research Laboratories (GUMRL).

3.6 Sample preparation

DNA extraction was done from individual flies using the sodium dodecyl (SDS) method (Alhassan et al., 2014). Individual black flies were crushed in sterile mortar containing 400µl sodium dodecyl extraction buffer using a pestle. The crushed samples were then placed in individual microcentrifuge tubes and 10µl of proteinase k was added and vortexed. The sample mixture was incubated in a water bath at 65°C for 30 minutes. This was followed by addition of 200µl of 7.5M ammonium acetate and incubation on ice for 10 minutes. The sample mixture was then centrifuged at 13500 RPM (revolutions per minute) for 15 minutes. The supernatant (300 µl) was transferred to a sterile microcentrifuge tube and equal volume of ice-cold isopropanol was added followed by vortexing. The sample mixture was then incubated at -20°C for 1hour. The sample mixture was then centrifuged for 15 minutes at 13500 RPM, and the resultant supernatant was discarded leaving a small cloudy pellet at the bottom of the microcentrifuge tube. Washing was done using 800µl of 70% ethanol and centrifuged at 13500 RPM for 2 minutes. The supernatant was also discarded, and the sample was left to airdry at room temperature for 5 minutes. The sample was the eluted using 50µl of nuclease free water. The quality of extracted DNA was assessed using gel electrophoresis in 1% agarose gel and viewed using the gel imaging system.

3.7 Detection of *O. volvulus* infection in the flies

The DNA extracts were then tested for *O. volvulus* parasite in the *Simulium* flies using the primers Fil1F and Uni1R for the first PCR and Fil2F and Fil2R (Tang et al., 2010) for the second PCR (nested PCR). Primer sequences in table 3.1.

Table 3.1: Table showing primers used for *O. volvulus* detection

Primer name	Primer sequence (5'-3')	Reference
Fil1F	GTGCTGTAACCATTACCGAAAGG	Tang et al., 2010
Uni1R	CGCAGCTAGCTGCGTTCTTCATCG	Tang et al., 2010
Fil2F	GGTGAACCTGCGGAAGGATC	Tang et al., 2010
Fil2R	TGCTTATTAAGTCTACTTAA	Tang et al., 2010

The first PCR master mix consisted of 12.5 µl of Taq 2x master mix (NEB M0270L, thistle scientific), 9.5 µl of nuclease free water, 0.5 µl of forward and reverse primer. This was aliquoted into each well of the 0.2ml PCR strip of 8 tubes (Nolato Treff) and 2 µl of template

DNA was added. Positive control was parasite DNA from the GUMRL cryobank and the negative control was molecular grade water for quality control. The PCR strips were spun in a mini-spin column for the contents to settle. The samples were then loaded in the SimpliAMP thermal cycler. The PCR conditions were as follows; one cycle of initial denaturation at 95°C for 1 minute, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 68°C for 1 minute followed by a final extension at 68°C for 5 minutes.

The second PCR had the same set of reagents, but the primers were Fil2F and Fil2R and the template DNA was the product of the first PCR. The second PCR was also run using the same conditions as the first PCR. The PCR products were then viewed using the gel documentation machine after running on 1% agarose gel at 120 mA and voltage of 400 volts for 1 hour.

3.8 Library preparation for determination of bacterial communities in *Simulium* flies

3.8.1 16S metagenomic PCR

The DNA extracts were quantified using nanodrop and standardized to a uniform concentration of 10ng using nuclease free water. The standardized samples were then amplified using the 16S Barcoding Kit (SQK-16S024; Oxford Nanopore Technologies, Oxford, UK) having barcoded primers 1-24 at 1µM each. The primer designed targets 16S rRNA gene structured in highly conserved regions interspersed with nine variable regions (V-regions), V1 to V9 of 1,500 bp (Janda & Abbott, 2007). A PCR master mix for a given number of samples plus two to cater for pipetting errors was made under the laminar flow cabinet in a clean room. The reagents constituted 25 µl of LongAmp™ Taq 2× Master Mix (NEB M0533S, New England Biolabs, Ipswich, MA, USA) which is good for long reads, 4.5 µl of nuclease free water and 0.5µl of magnesium chloride for each reaction. After mixing using a vortex mixer and brief spinning, 30 µl of the PCR reaction mixer was aliquoted into each well in a PCR strip of 8 tubes (Nolato Treff). 10 µl of each 16s barcoded primer (1-24) was added to each reaction tube followed by 10 µl of sample. Positive control which was bacterial DNA extract and negative control which was molecular grade water were included in the reactions for quality control.

The capped PCR strips containing reaction mixer were spun in a spin column for 30 seconds then loaded in the simpliAMP thermocycler. The amplification conditions were one cycle of initial denaturation at 95°C for 1 minute, 25 cycles of denaturation at 95°C for 20 seconds, primer annealing at 55°C for 30 seconds and extension at 65°C for 2 minutes, 1 cycle of final extension at 65°C for 5 minutes and final hold at 4°C.

3.8.2 Gel electrophoresis

The PCR products were then viewed on 1% agarose gel made using 0.5X TBE buffer (TBE buffer is a composition of tris base, boric acid, Ethylenediaminetetraacetic acid (EDTA) and sodium hydroxide) and agarose, 1gm of agarose powder. Detailed protocol in Appendix A. The electrophoresis system was then run at 120V, 400 mA for 40 minutes. The gel was placed in a Biorad gel imaging system and the image was captured.

3.8.3 PCR amplicon clean up

The PCR amplicons were then purified using the AMPure XP beads (Beckman Coulter, cat # A63881). Detailed protocol in Appendix B. The eluted samples (1 µl) were then quantified using the Qubit fluorometer.

All the amplicons with different barcodes were pooled making a DNA library and 1 µl of RAP was added. The pooled amplicons with the rapid adapter (RAP) were mixed by flicking the tube and spun down. The solution was left at room temperature for 5 minutes to incubate.

3.9 Sequencing of 16S gene bacterial amplicons

The 16s metagenomic PCR and sequencing was performed following the Oxford nanopore sequencing technology protocol (Branton et al., 2008). The barcoded samples with different barcodes were pooled together and mixed with appropriate volumes of sequencing reagents. The resultant mixture was loaded on the Oxford nanopore minION (minion_mK1c) flow cell (flow_cell_id=FAQ45902) for sequencing. This was done in the sequencing unit of Gulu University Multifunctional Research Laboratories.

3.9.1 Priming

The flow cell priming mix was prepared by adding 30 µl of properly thawed and mixed flush tether (FLT) directly to the tube containing thawed and mixed flush buffer (FB). The solution was mixed by vortexing at room temperature. The priming port was then opened by sliding the priming port cover clockwise. Underneath the cover was checked for any air bubbles. A small volume of buffer was drawn out of the flow cell using a pipette. This was done by setting a 1ml pipette at 200 µl whose tip was inserted into the priming port. The wheel of the pipette was turned until the dial was at 230 µl. A quantity of 800 µl of the priming mix was loaded into the priming port, avoiding introduction of air bubbles. A duration of 5 minutes was given to allow

the priming buffer to move gently through the system. The remaining 200µl of priming buffer was then added to the priming port ensuring that the spotON sample port is also open.

3.9.2 Loading of the spotON minION flow cell

In a new microcentrifuge tube, the sequencing library was prepared using 34µl of sequencing buffer (SQB), 25.5µl of vortexed loading beads (LB), 4.5µl of nuclease free water (NFW) and 11µl of the DNA library making a total volume of 75µl. The sequencing library was gently mixed by pipetting in and out prior to loading. With the priming port open, the 75µl of the library was then added drop by drop to the flow cell via the spotON sample port ensuring that each drop goes in before the next. After loading, the spotON port cover was placed back as well as the priming port cover. The minION (minion_mK1c) device lid was placed back. The sequencing proceeded using the sequencing run parameters (Table 0.2 in Appendix C) for 20hours.

3.10 Data analysis

Data analysis included bioinformatics analysis and statistical analysis (Kai et al., 2019).

3.10.1 Bioinformatics analysis

The sequence reads were processed using the amplicon analysis pipeline of SILVAngs 1.4 (Quast et al., 2013) while individual reads were aligned using the SILVA Incremental Aligner (SINA SINA v1.2.10 for ARB SVN (revision 21008)) (Pruesse et al., 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast et al., 2013) for taxonomic identification and assignment as a project. Shorter reads of less than 50 aligned nucleotides and more than 2% ambiguities were exempted from further processing. Supposed contaminants, artifacts and reads with low alignment quality that is 50 alignment identity, and 40 alignment score were identified and eliminated from downstream analysis. Dereplication and clustering were done on a per sample basis using the VSEARCH (version 2.17.0; <https://github.com/torognes/vsearch>) (Rognes et al., 2016) using the identity criteria of 1.00 and 0.98 respectively. The reference OTUs were classified by BLASTn (2.11.0+; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Camacho et al., 2009) utilizing the standard settings of the distinct version of SILVA SSU Ref dataset as a reference for classification (release 138.1; <http://www.arb-silva.de>). The classification of each OTU reference read was mapped onto all reads that were assigned to the respective OTU yielding quantitative information. Reads without any or weak classifications, where the function “(% sequence identity + %

alignment coverage)/2” did not exceed the value of 93, remain unclassified and were assigned to the meta group “No Relative” in the SILVAngs fingerprint and Krona charts (Ondov et al., 2011).

3.10.2 Statistical analysis

The rest of the statistical analyses were also performed in R programming language using R version: 4.4.1. The relative abundances dataset of bacterial phyla and genera which were in comma delimited format were converted into excel format and transposed then saved in comma delimited format. The datasets were then imported into R, cleaned up, reshaped and filtered using tidyverse (version 2.0.0), reshape (version 0.8.9) and dplyr (version 1.3.1) packages respectively.

Various stacked bar graphs were plotted using the ggplot2 (version 3.5.1) package and other packages like the ggtext (version 0.1.2) and ggthemes (version 5.1.0) were used to enhance the appearance of the plots. Each district had 2 stacked bar graphs plotted for bacterial phyla and bacterial genera. Heatmaps were also plotted to represent the bacterial phyla and genera using the pheatmap (version 1.0.12) package.

The alpha diversity indices (Shannon wiener diversity index, Simpson dominance index, Abundance coverage estimator index (ACE) and Chao1 index) from the genera abundance counts were determined using the vegan package (version 2.6-6.1). The alpha diversity indices dataset were tested for normality by plotting a histogram and carrying out a Shapiro test to determine which statistical test to use. The Kruskal Wallis test was then carried out to determine the statistical differences of bacterial diversity in the four districts at a significant threshold set at $p=0.05$ and a box plot was plotted using the ggplot2 and ggsignif (version 0.6.4) package. The alpha diversity indices were also applied based on the infection status using the Wilcoxon test to check for any significant differences in the bacterial composition in the two groups of infection status. Beta diversity of the different study sites was analyzed based on abundances using Bray Curtis distance matrix on square root transformormed data (Roger & Curtis, 1957.) applying the vegan package in R studio. The permutational multivariate analysis of variance (PERMANOVA) was used to determine the statistical difference for the beta diversity and a pairwise PERMANOVA test was done for the districts. A principle coordinate analysis plot was made to show the distribution of the samples basing on the district and the infection status of the *Simulium* flies.

3.11 Ethical considerations

Ethical clearance for this study was obtained from Gulu University Research Ethics Committee (GUREC) with approval number GUREC-2021-91, and from the Uganda National Council for Science and Technology (UNCST) with approval number HS2291ES. The volunteer fly collectors signed informed consent forms that clearly indicated the purpose, anticipated risks, and benefits of the study. Additionally, volunteers were selected based on their previous experience with the HLC method and exposure to blackfly bites in endemic areas. Moreover, additional refresher training was conducted for volunteers to increase their proficiency in the use of HLC.

CHAPTER FOUR

PRESENTATION, ANALYSES AND INTERPRETATION OF DATA

4.1 General amplification and sequencing results

Of the 534 black fly samples from four districts Nwoya, Amuru, Moyo, and Pader analyzed for *O. volvulus* infection, 39 were positive showing a rate of 7.3% infection. Nwoya had the highest infection rate of 10.8% (18/166) followed by Pader with an infection rate of 6.1% (10/164), Moyo with 7% (7/120), and Amuru with least score of 4 % (4/84) infection rate.

The amplification for 16s gene also showed successful amplification with the positive samples having a band size of 1500bp, while the negative samples had no band showing no amplification (Figure 4.1)

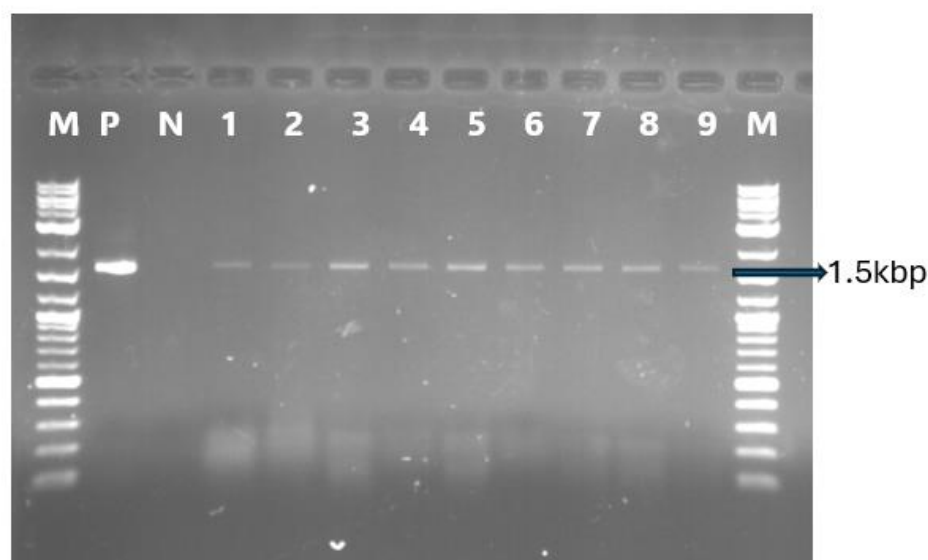


Figure 4. 1: Gel map for 16s PCR for identification of bacteria

Seventy-two black fly samples were analyzed, with 26 which were positive for *O. volvulus*. These were subjected to sequencing using the nanopore sequencing technology. The sequencing generated a total of 826,486 raw sequences of which 1.6% were unsuccessful leaving 812,574 (98.32%) operational taxonomic units, 654 (0.08 %) clustered sequences and 3 replicates. The number of classified sequences was 600,110 (72.15 %) and 213,121 (25.79 %) were of no relative. In the appendix is an interactive Krona graph for all the samples sequenced (Figure 0.4).

Twenty-three OTUs were assigned of which one was of Eukaryotic domain (Obazoa), the remaining 22 were of bacterial domain. After filtering (using dplyr package version 1.3.1) those with low total abundance counts (<10), 12 bacterial phyla remained. The most abundant bacterial phyla were Proteobacteria and Firmicutes with a relative abundance of 89.7% and 9.3% respectively across the seventy-two samples. The stacked bar plot and heatmap show proteobacteria's predominance masking the other phyla of low relative abundances ($<5\%$) (Figure 4.2 and 4.3) and appears across all the samples. However, P01 and P18 from Pader district (Figure 4.9a) have Firmicutes with a relative abundance of $>50\%$ which shows a difference in Pader district as compared to other districts. The remaining ten bacterial phyla were unevenly distributed across all the samples.

A total of 516 bacterial genera were obtained from the OTU assignment. The genera with low total counts <10 and in less than 5% of the samples were filtered out leaving 149 genera. Of the remaining bacterial genera, *Enterobacter* had the highest relative abundance at 21.4%, followed by *Ralstonia* at 13.1%, *Acinetobacter* at 9.8% and *Klebsiella* at 8.7%. None of the genera was found to appear in all the samples. Even the most abundant *Enterobacter* was found missing in one sample (N21 from Nwoya district). The least abundant genera were *Pseudocitrobacter*, *Ottowia*, *Acetobacter*, and *Flavobacterium* with a relative abundance of less than 0.002%. Twenty-four of the genera with relative abundance $\geq 10\%$ are represented in figures 4.4 and 4.5 while those with relative abundance less than 10% are represented as "Others" in only figure 4.4. The dendrogram on the heat map shows two distinct clusters of samples with association to District (location) of origin but no relation to the infection (health) status (Figure 4.5).

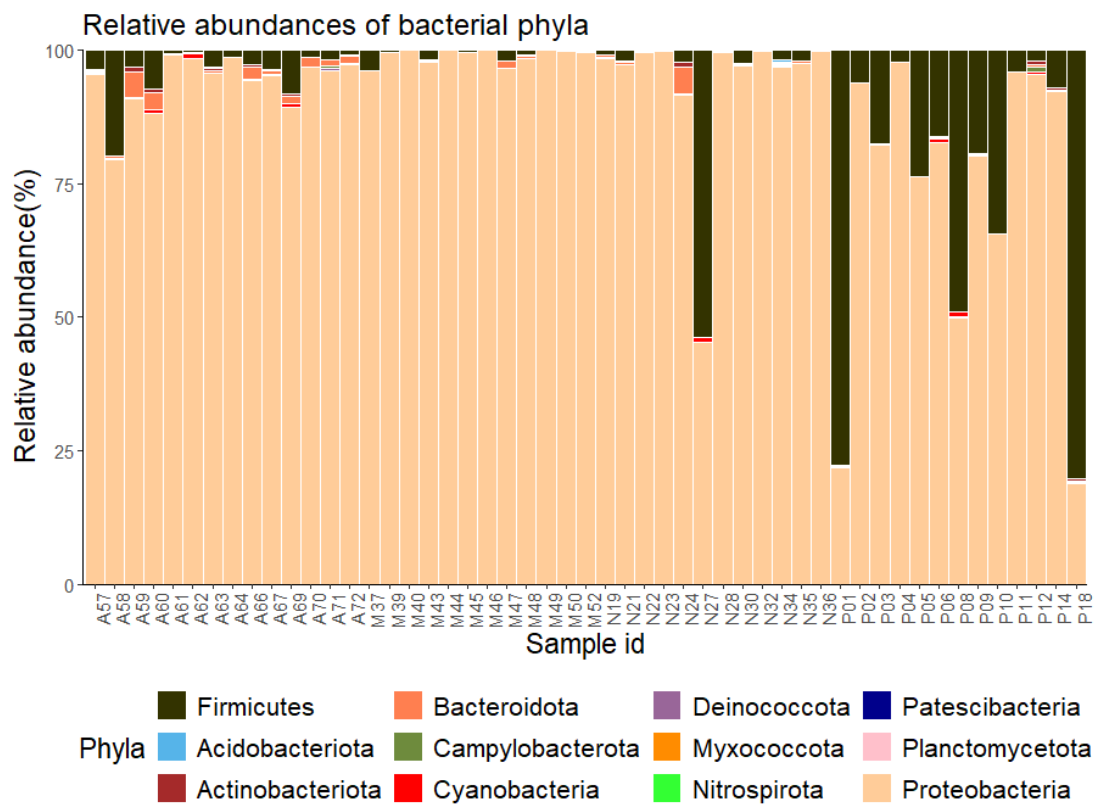


Figure 4. 2: General bacterial phyla for all districts

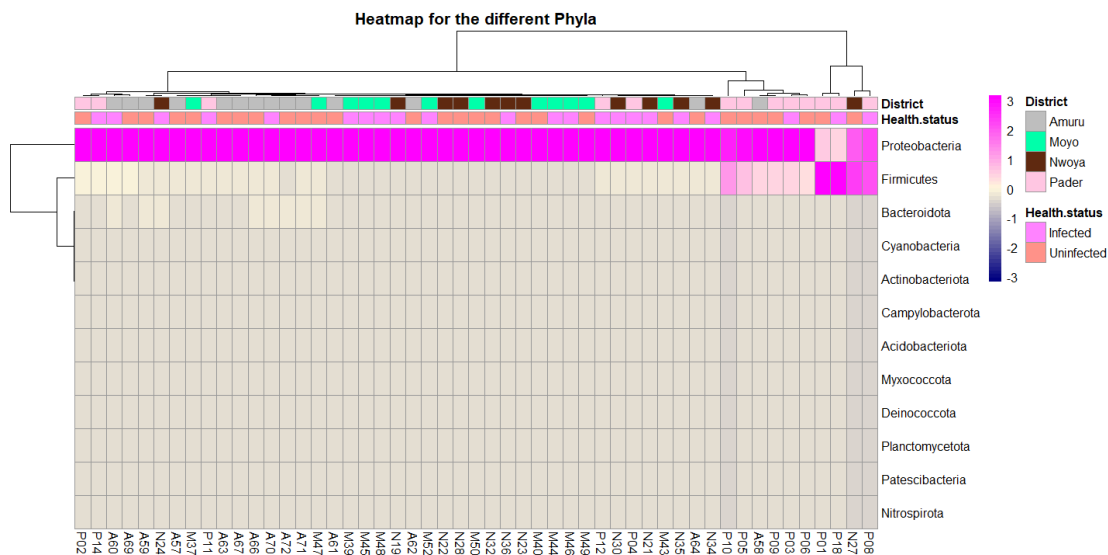


Figure 4. 3: Heatmap for bacterial phylum

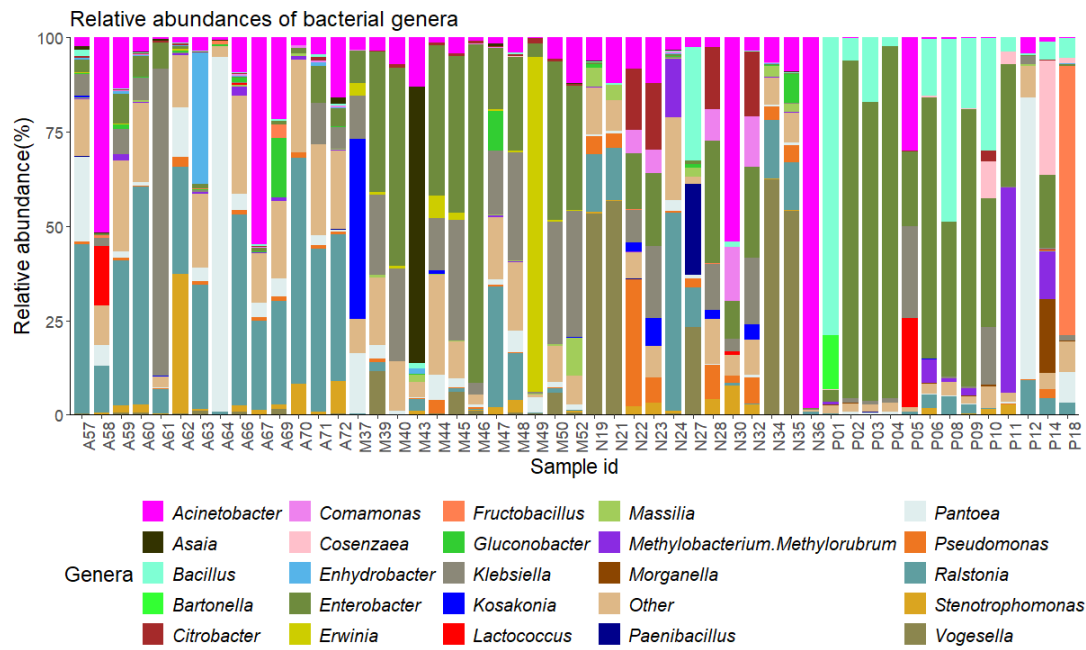


Figure 4. 4: General bacterial genera for all districts

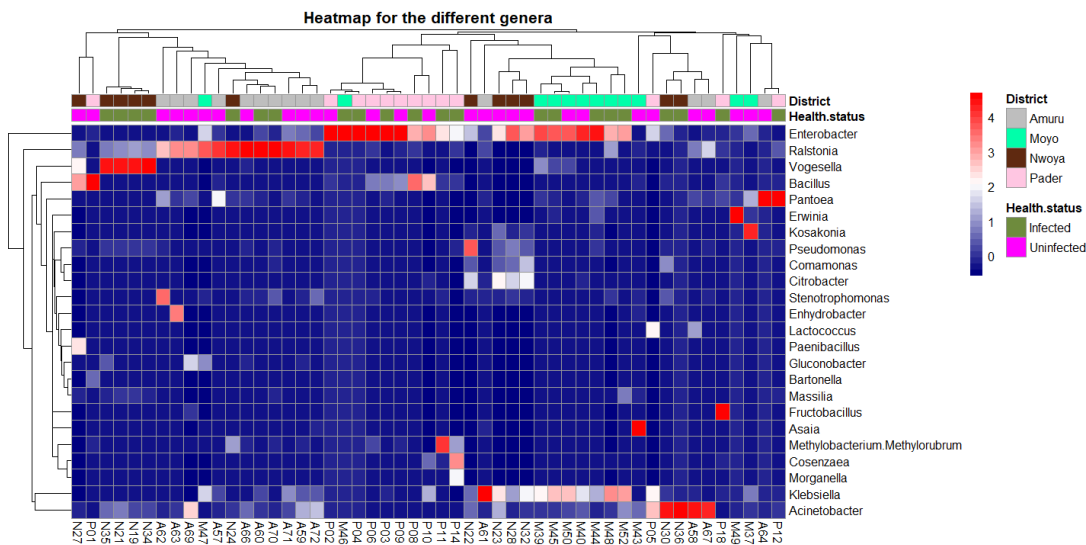


Figure 4. 5: Heatmap for bacterial genera and association with location and infection (health) status of *Simulium flies*

4.1.1 Bacterial diversity in Amuru district

The bacterial community in blackflies from Amuru district were composed of 12 phyla which is marked by a high abundance of Proteobacteria across all the samples with relative abundance ranging from 79% to 99% across the samples (Figure 4.6a). Firmicutes was also fairly represented across all the samples with the highest relative abundance at 19.8% and the lowest

at 0.9%, respectively. The other ten bacterial phyla were marginally represented with Nitrospirota, Campylobacter, and Patescibacteria appearing in only one sample each at 0.02, 0.04 and 0.05 relative abundance (%) respectively.

Amuru portrays a high diversity of bacterial genera with 127 different genera. The most abundant bacterial genera are *Ralstonia*, *Klebsiella*, *Pantoea*, and *Acinetobacter* with relative abundances at 33.3%, 8.5%, 11.3% and 13.3%, respectively. The eight mostly represented genera with fair (>10%) relative abundance and those with less than 10% represented as others are shown in figure 4.6b. In appendix is figure 0.2, a sample Krona graph for sample A69 showing bacterial diversity from bacterial domain to genera level.

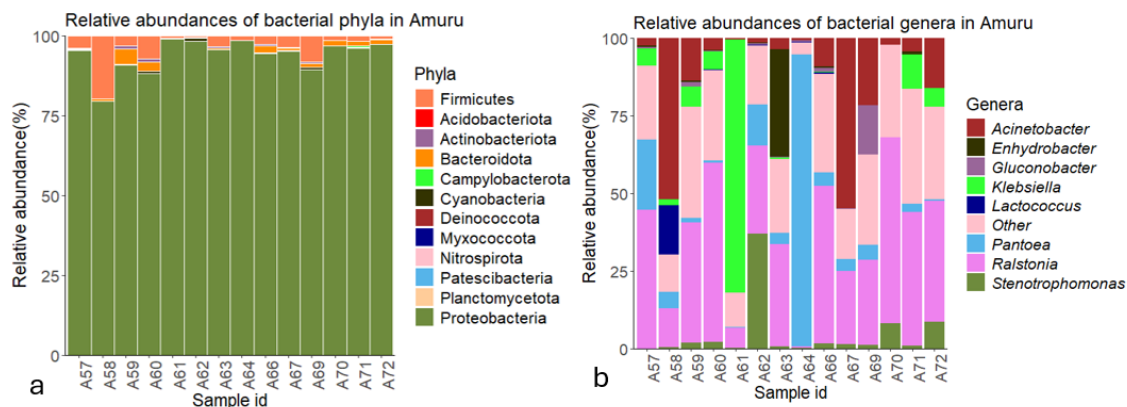


Figure 4. 6: Bacterial community in Amuru district, (a); Bacterial Phyla, (b); Bacterial genera and those with relative abundance <10% as others

4.1.2 Bacterial diversity in Moyo district

The black fly community in Moyo is composed of 10 phyla. Proteobacteria is the most abundant phyla distributed across the 12 samples with relative abundances of 96%-99%. The remaining 9 bacterial phyla are of low relative abundance (Figure 4.7a). The least represented phyla are Campylobacterota appearing in only one sample.

In Moyo district, there is a high bacterial diversity with 134 genera. The most abundant bacterial genera were *Enterobacter* (32.4%), *Klebsiella* (18.1%), *Erwinia* (8.5%) and *Asaia* (6.2%). *Citrobacter*, *Enterobacter*, *Klebsiella*, *Erwinia*, *Pantoea* and *Acinetobacter* was distributed across all the samples. The other genera show uneven distribution across the samples. A genus with at least 10% relative abundance in a sample is represented in figure 4.7b. while those with low relative abundance are represented as others.

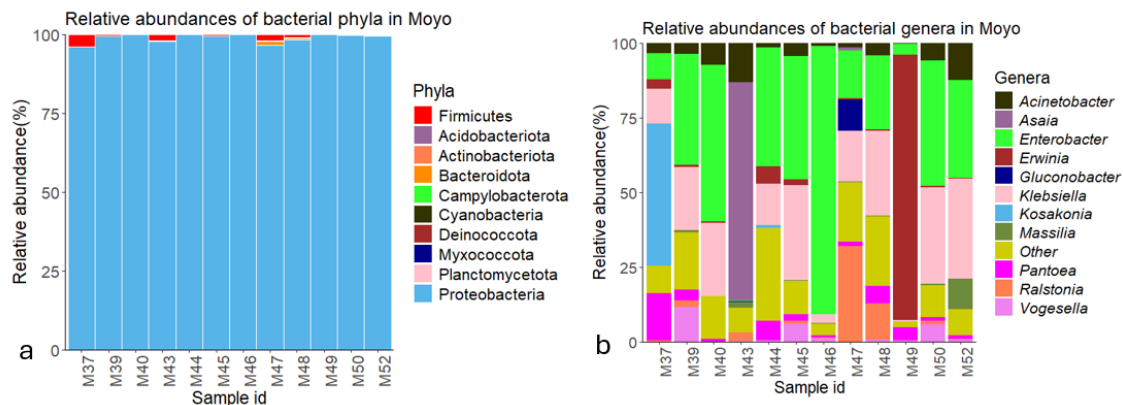


Figure 4. 7: Bacterial diversity in Moyo district, (a); Bacterial Phyla, (b); bacterial genera with those <10% represented as others

4.1.3 Bacterial diversity in Nwoya district

Like the previous districts, Nwoya is characterised by a high relative abundance of proteobacteria across 11 samples of 91-99% making it the most abundant phyla of all the 12 phyla represented (Figure 4.8a). However, sample N27 shows a different pattern, it has the least relative abundance of proteobacteria 45.3% and the highest relative abundance of firmicutes (53.8%). The least represented bacterial phyla were Campylobacterota, Patescibacteria, and Nitrospirota which are in one sample each with a relative abundance of 0.07%, 0.06% and 0.17%, respectively.

Nwoya district has got also a high diversity of 129 bacterial genera distributed across the blackfly community. The most represented and abundant genera were *Vogesella*, *Acinetobacter*, *Ralstonia*, and *Enterobacter* with a relative abundance of 20.8%, 18.3%, 10.1% and 8.6%, respectively. *Acinetobacter* is the only genus that is well represented in all the samples (Figure 4.8b).

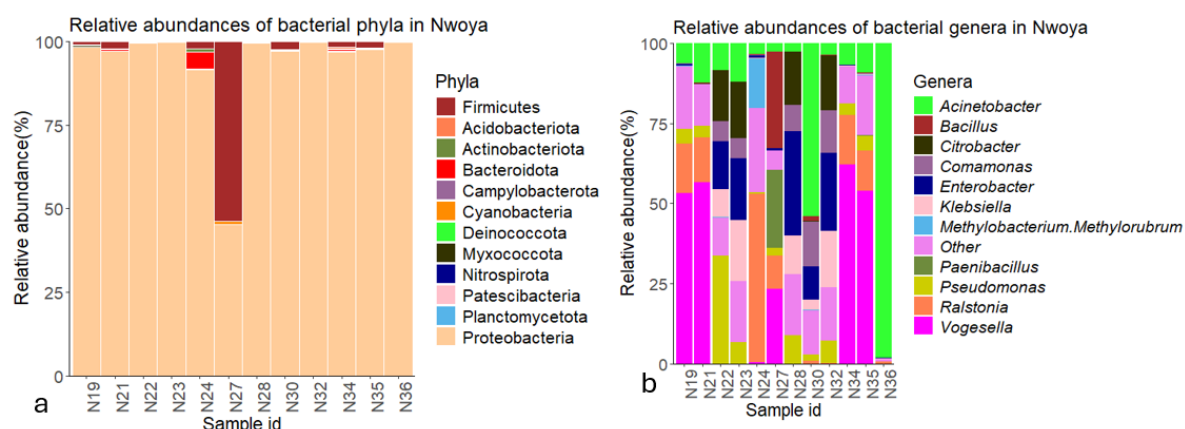


Figure 4. 8: Bacteria diversity in Nwoya district, (a); Bacterial phyla, (b); Bacterial genera with those of relative abundance <10% represented as others

4.1.4 Bacterial diversity in Pader

Pader district paints a different picture of bacterial phyla diversity from the other districts of Amuru, Moyo and Nwoya. Though proteobacteria is still the most abundant across 76.9% (10/13) of the total samples with a relative abundance 65-97%, there is a characteristic high abundance of firmicutes in samples P01 and P18 (Figure 0.3) with a relative abundance of 77.7% and 80.1% respectively (Figure 4.9a). A non-significant difference was observed in sample P08 which has 49.0% of proteobacteria and 49.8% of firmicutes. Firmicutes are well represented with an overall percentage of 26.1% to the 73.3% of proteobacteria as compared to their distribution in samples from the former three districts. Campylobacterota and Patescibacteria are poorly represented.

Pader district presented 128 bacteria genera. The bacterial genera were well presented across the samples with at least over 5 bacterial genera (*Acinetobacter*, *Bacillus*, *Enterobacter*, *Klebsiella* and *Ralstonia*) in all samples from Pader as shown in figure 4.9b, *Enterobacter* was the most abundant genera (42.7%), followed by *Bacillus* (17.7%), *Pantoea* (6.6%), *Fructobacillus* (5.5%) and *Methylobacterium* (5.9%).

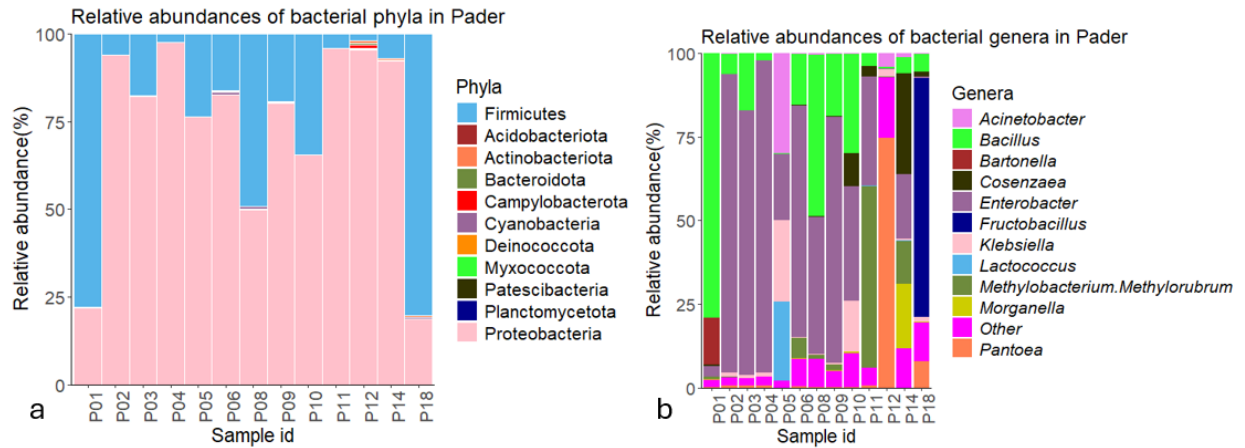
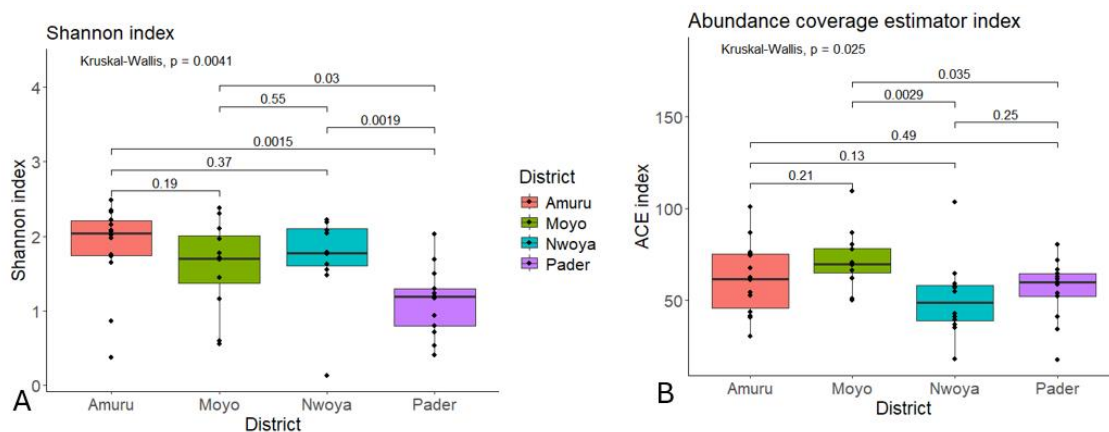


Figure 4. 9: Bacterial diversity in Pader district, (a); Bacterial phyla, (b); bacterial genera with those of relative abundance <10% represented as others

4.2 Relationship between bacterial composition of *Simulium* flies and district of location and infection status

The relationship between the bacterial diversity of blackflies and the district of origin was determined based on the alpha diversity indices. From the results of the Kruskal Wallis test on Shannon index, ACE and Chao index, the P-value showed that the bacterial diversity across the districts is significantly different (Figure 4.10). However, Simpson dominance index showed that the bacterial diversity is not significantly different across the districts (Figure 4.10C).



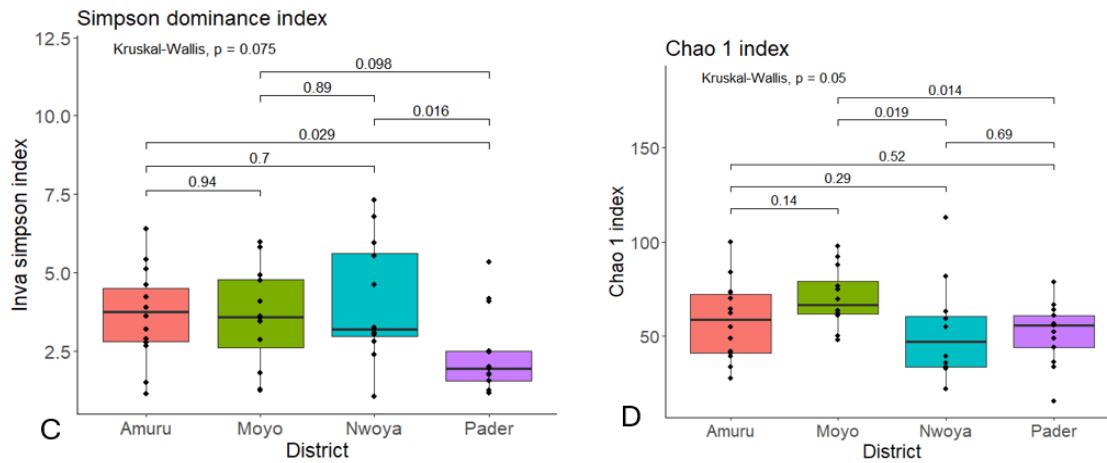
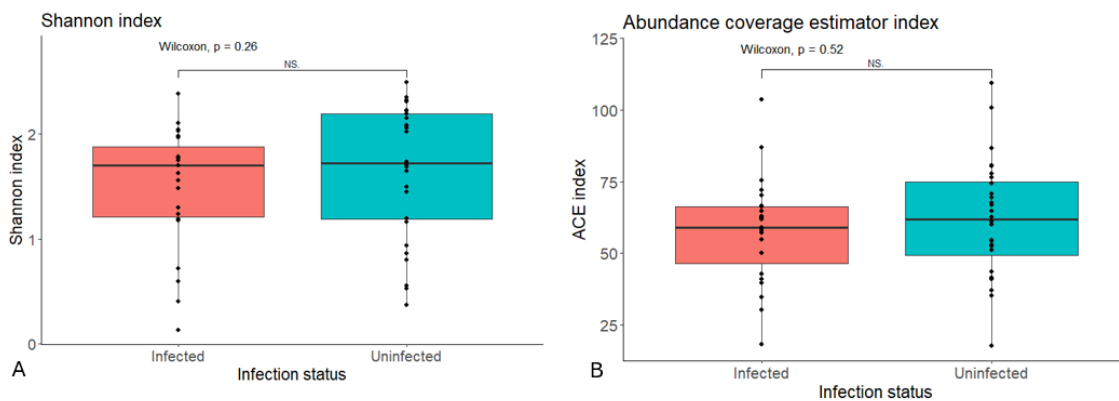


Figure 4. 10: Alpha diversity indices in association with location, (A) Shannon index ($p = 0.0041$), (B) ACE index ($p = 0.025$), (C) Inva Simpson index ($p = 0.075$), (D) Chao1 index ($p = 0.05$)

4.3 Relationship between bacterial composition of *Simulium* flies and infection status

All the alpha diversity indices demonstrate that the relationship between bacterial diversity in the *Simulium* flies and their infection status do not differ significantly; Shannon index ($p = 0.26$), Simpson dominance index ($p = 0.14$), Abundance coverage index ($p = 0.52$) and Chao1 index ($p = 0.65$) (Figure 4.11).



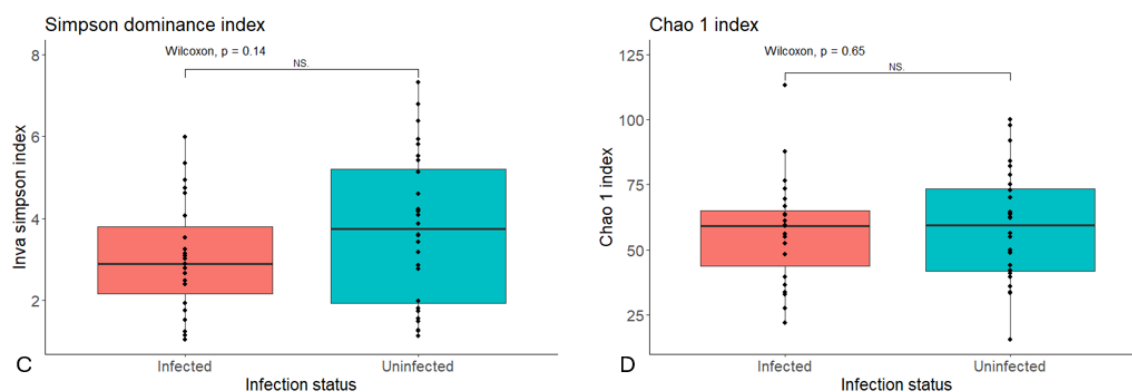


Figure 4. 11: Alpha diversity indices in association with infection status, (A) Shannon index ($p = 0.26$), (B) ACE index ($p = 0.52$), (C) Inva Simpson index ($p = 0.14$), (D) Chao1 index ($p = 0.65$)

4.4 Beta-diversity

I used Bray Curtis distance (Bray and Curtis., 1957) for beta diversity analysis. A distance matrix was determined on the square root transformed data based on Bray-Curtis distances (Bray and Curtis., 1957). The ordination plot results demonstrated by the permutational multivariate analysis of variance (PERMANOVA) test resulted in a significant difference as seen by the p-value of 0.0001 (Table 4.1). A pairwise PERMANOVA on the distance matrix showed significant differences between the districts (Table 4.2).

Table 4.1: PARMANOVA table

	Df	Sum Of Sqs	R2	F	Pr(>F)
Location	3	3.3039	0.28877	6.3609	1e-04 ***
Residual	47	8.1374	0.71123		
Total	50	11.4413	1.00000		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 4.2: Table showing the pairwise statistical differences between districts

	Amuru	Moyo	Nwoya
Moyo	0.0015**	-	-
Nwoya	0.0024**	0.0030**	-
Pader	0.0015**	0.0015**	0.0015**

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

The Principal Coordinate analysis (PCoA) plot (Figure 4.12) demonstrated how samples clustered differently across the space due to their geographical location. Two clusters depicted in the PCoA plot which are similar in the dendrogram on the heatmap (Figure 4.3). Samples from Pader district on the bottom right hand were quite different in their bacterial composition as compared to the samples from the other three districts of Amuru, Nwoya and Moyo which clustered slightly below and above the horizontal axis. *Simulium* flies from Amuru district also showed a different pattern of bacterial composition in comparison with those from Nwoya and Moyo. The Community in Amuru clustered on the upper left part of the plot. *Simulium* flies from Moyo clustered to the upper right part of the plot. Nwoya community, however, showed an overlay slightly above the Moyo community and slightly below and within the Amuru community. The clustering of the samples was not different in terms of the infection status.

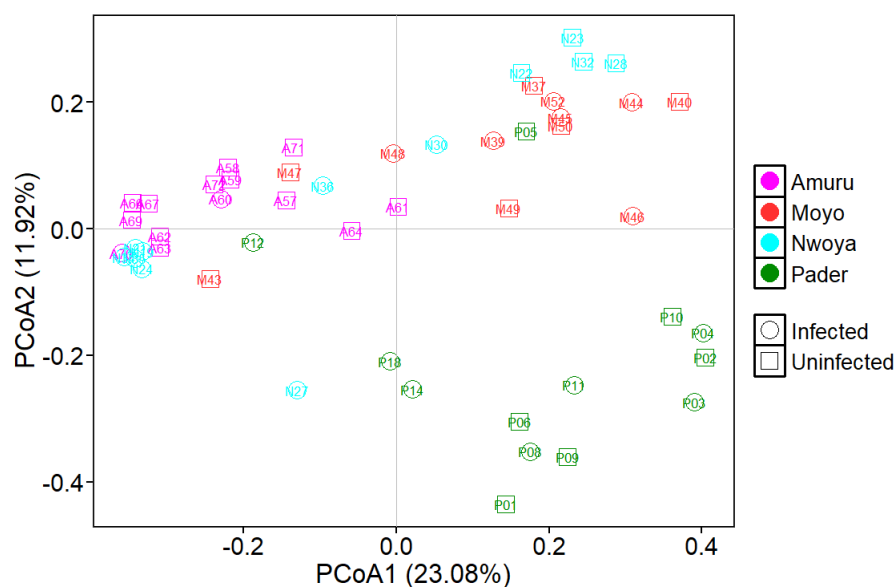


Figure 4. 12: PCoA plot for beta diversity basing on the Bray Curtis dissimilarity index in association with the location and infection status of *Simulium* flies

4.5 Potential biomarkers of blackfly microbial communities

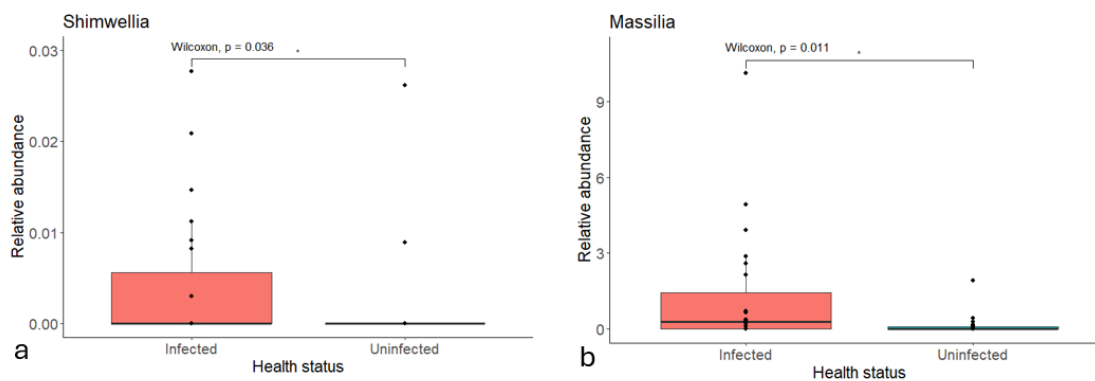
A Wilcoxon test was conducted comparing all the 149 individual genera in the infected and non-infected samples. Those with a significant difference ($p\text{-value} < 0.05$) were filtered to identify which infection status they were more associated with (Table 4.3) and box plots were plotted in R.

Table 4.3: Table showing the potential biomarkers for *O.volvulus* infection and non-infection *Simulium* flies

Genus	Mean(sd)		p-value
	Infected	Uninfected	
<i>Enterococcus</i>	0.034(0.091)	0.272 (0.754)	0.030*
<i>Peptoniphilus</i>	0.029(0.052)	0.006(0.025)	0.009**
<i>Vogesella</i>	11.766(21.688)	0.404(1.100)	0.029*
<i>Aquabacterium</i>	0.014(0.030)	0.0002(0.0008)	0.018*
<i>Curvibacter</i>	0.023(0.055)	0.006(0.028)	0.034*
<i>Paucibacter</i>	0.035(0.070)	0.0004(0.002)	0.004**
<i>Massilia</i>	1.288(2.380)	0.134(0.370)	0.011*
<i>Citrobacter</i>	0.192(0.251)	2.743(5.896)	0.043*
<i>Shimwellia</i>	0.004(0.008)	0.001(0.005)	0.036*
<i>Haemophilus</i>	0(0)	0.011(0.028)	0.037*
<i>Mannheimia</i>	0.047(0.18)	0(0)	0.011*
<i>Shewanella</i>	0.160(0.325)	0.401(0.592)	0.035*
<i>Halotalea</i>	0(0)	0.022(0.052)	0.020*
<i>Zymobacter</i>	0.026(0.097)	0.191(0.488)	0.018*
<i>Serratia</i>	1.462(2.368)	2.017(2.277)	0.11

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

The results showed that eight genera were significantly associated with the infected status and these included *Peptoniphilus* (p=0.009), *Vogesella* (p=0.029), *Aquabacterium* (p=0.018), *Curvibacter* (P=0.034), *Paucibacter* (P=0.004), *Massilia* (P=0.011), *Mannheimia* (p=0.011) and *Shimwellia* (P=0.036) (Figure 4.13), and of these *Mannheimia* was found only in the infected flies.



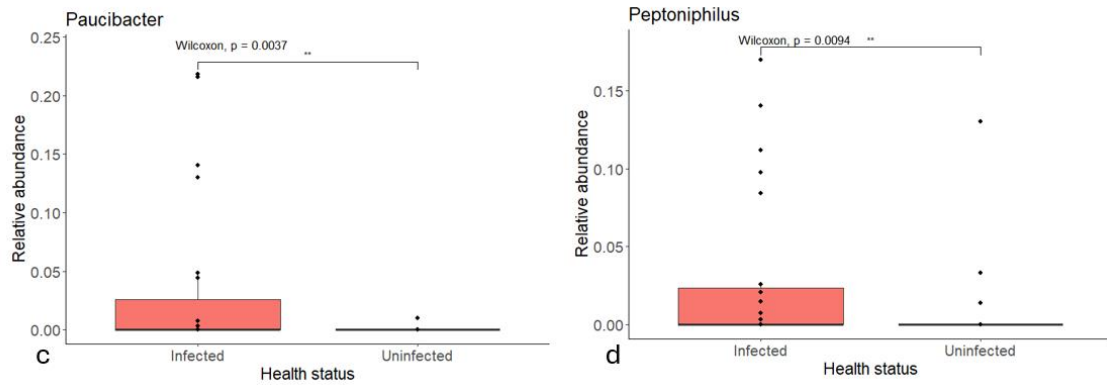


Figure 4. 13: Bacterial genera significantly associated with Infected *Simulium* flies, (a) *Shimwellia* ($p=0.036$), (b) *Massilia* ($p=0.011$), (c) *Paucibacter* ($p=0.0037$), (d) *Peptoniphilus* ($p=0.0094$)

On the other hand, six bacterial genera were identified to be significantly associated with uninfected status. These included *Enterococcus* ($p= 0.03$), *Citrobacter* ($p=0.043$), *Haemophilus* ($p=0.037$), *Shewanella* ($p=0.035$), *Halotalea* ($p=0.020$) and *Zymobacter* ($p=0.018$) (Figure 4.14). Two (*Haemophilus* and *Halotalea*) of the six bacterial genera were found only in the uninfected *Simulium* flies.

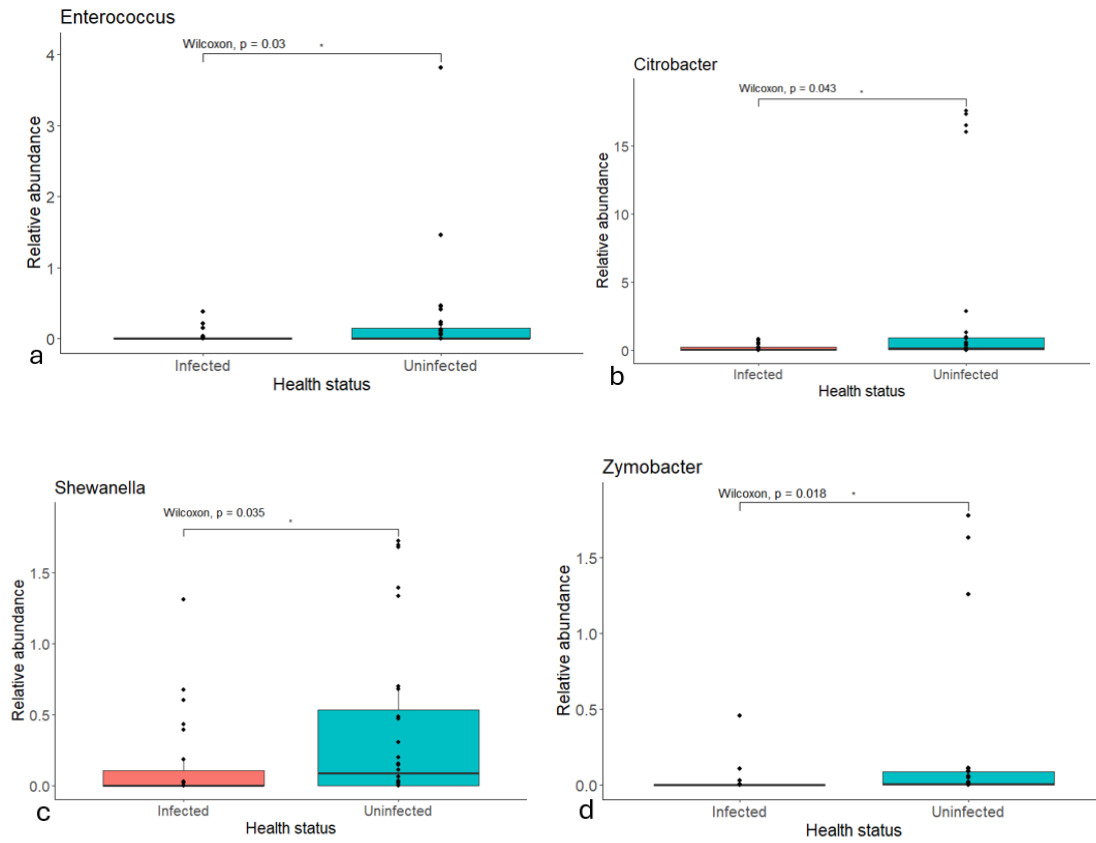


Figure 4. 14: Bacterial genera significantly associated with uninfected *Simulium* flies, (a) *Enterococcus* ($p=0.03$), (b) *Citrobacter* ($p=0.043$), (c) *Shewanella* ($p=0.035$), (d) *Zymobacter* ($p=0.018$)

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

This study assessed the bacterial community in the *Simulium* flies to determine the bacterial diversity in the different districts of Northern Uganda and how they are associated with infection and non-infection with *O. volvulus*. This could result in identification of potential biocontrol against onchocerciasis transmission. Sequencing using the Oxford nanopore technology enabled identification of 12 bacterial phyla and 149 bacterial genera that met the filtering criteria of >10 total counts and appearance in at least 5% of the samples. Earlier studies on the microbiome of insect vectors have shown identification of six phyla and nine phyla basing on the 16S rRNA and 18S rRNA respectively in mosquito pool (*Aedes aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus*) using the pyrosequencing technology (Thongsripong et al., 2018). *Glossina spp* microbiome study identified eighty-three bacterial genera using the Illumina sequencing technology (Jacob et al., 2017). In addition, a study on the microbiome in ticks yielded 30 phyla and 654 genera using the Illumina (Jiang et al., 2024). Likewise, in the black fly community, the recent study in Cameroon basing on the 16S rRNA allowed the identification of 19 phyla and 210 genera using the same technology of Illumina (Ekangouo et al., 2022). The differences can be attributed to the sequencing technology and the geographical location (Jiang et al., 2024; Yun et al., 2014).

The most abundant bacterial phyla were Proteobacteria with a mean relative abundance of 89.7%. This agrees with a previous study on blackflies (Ekangouo et al., 2022) and other insects (mosquitoes, weevil, and tsetse flies) on bacterial diversity where the same patterns were observed (Tchioffo et al., 2016; Thongsripong et al., 2018). Proteobacteria had a relative abundance of 62.1% of the total reads in a study which sampled 218 insect species belonging to 21 different orders (Yun et al., 2014). Proteobacteria has a symbiotic relationship with insects offering help in nutrient acquisition, essential amino acids and vitamin biosynthesis, lipid, protein and carbohydrate digestion, energy metabolism, essential in the fertility of insects as well as improving the viability of insects and in the detoxification (Jing et al., 2020; Mondal et al., 2023).

Firmicutes were the second most abundant though with low relative abundance at 9.3%. However, in Pader district, there was a fair abundance of Firmicutes with certain samples

having over 70% relative abundance. This has been noted in blood feeding ticks with higher relative abundance of Firmicutes at 69.23% and another study indicated Firmicutes at 7.4% to 98.6% as compared to the relative abundance of Proteobacteria at 0.5% to 60.6% (Rousseau et al., 2021). Higher abundances of Firmicutes have been associated with the larvae stage (Fu et al., 2023; Sajnaga et al., 2021) as compared to adults normally dominated by Proteobacteria. This study together with different studies in ticks indicate how differences in species and environmental factors affect the composition of bacteria in insects.

The predominant bacterial genera in this study were *Enterobacter* with a relative abundance of 21.4%. Other studies reveal comparable results in insects such as tsetse flies (Geiger et al., 2009; Gómez-Govea et al., 2024). Studies have shown that certain bacterial species of *Enterobacter* do confer benefits to the host insects in terms of nutrient acquisition, improving the fitness of the host in fertility and fighting pathogens as well as detoxification (Augustinos et al., 2015; Kyritsis et al., 2019).

The alpha diversity indices showed a significant difference in the bacterial community in the different districts. The results were not any different from the PERMANOVA test which also revealed significant differences (p-value of 0.0001) in the community composition of the bacteria across the different districts. A pairwise PERMANOVA showed significant differences between the districts. It should still be noted that bacterial community composition in Pader district varied from the community composition of the three districts of Amuru, Nwoya and Moyo viewed by two significant clusters. This can be explained by the differences in their geographical location due to several factors like the climate, topography, and edaphic factors. Moyo district is in west Nile part of Northern Uganda 97kilometers from Amuru, 118km from Nwoya and 179km from Pader (Around the world, 2024). Pader is in the heart of Northern Uganda while Moyo, Amuru and Nwoya are in the western part of Northern Uganda (Figure 1.1). With this it can be noted how these districts are spatially distributed within Northern Uganda.

River Oluvo in Moyo is located within a mountainous area which is inhabited by wild animals. This is a similar phenomenon with Ogelo village in Nwoya which is neighbouring Murchison Falls National Park, a home for the wild animals. This shows a link in the blood meal source of the wild animals which might have influenced some similarity in the bacterial composition of the *Simulium* flies, differences can be traced to their spatial distribution geographically. Similarities in bacterial composition of flies from Amuru and Nwoya can be attributed to their

proximity being neighbouring districts. River Unyama in Amuru however is surrounded by bushy areas known habitats for the *Simulium* flies but not a wildlife habitat with no nearby game reserve or park. It should also be noted that the *Simulium* flies from Pader were collected from a domestic grazing area for cattle 1km away from Aruu falls. The differences in bacterial diversity due to geographical location has been observed before in tsetse fly community in different districts of Uganda (Aksoy et al., 2014). This was also observed in cricket population from the wild and laboratory reared (Ng et al., 2018) and *Spodoptera frugiperda* community (Fu et al., 2023). In addition to geographical location, diet having influence was presented before by Yun et al. (2014) in a study that involved 218 species of insects from fifteen different sites in South Korea as well as in dung beetles (Franzini et al., 2016). It is expected that samples from similar geographical location are likely to have the similar bacterial composition as observed in blackflies from three communities within Bafia health district (Ekangou et al., 2022b), tsetse flies in southern Cameroon (Tsagmo Ngoune et al., 2019). The bacterial diversity in either infected or uninfected flies was not significantly different. The hierarchical cluster dendrogram showed two significant clusters which were not associated to infection status but poorly defined by the locations. This can be attributed to the similar locations of sampling so no difference would be expected in bacterial diversity associated with infection statuses. Ekangou et al. (2022) also showed that there were no significant differences in the bacterial diversity of infected and uninfected black flies, this was also observed in tsetse flies (Jacob et al., 2017). Comparatively Tong et al. (2020) showed that significant differences could be observed in gut microbiome of diarrhoeic and healthy frogs.

Notwithstanding, this study identified bacterial genera that were strongly associated with the infected and uninfected groups, and some were even found in either of the groups exclusively. Statistical tests showed the following to be significantly associated with infection status, *Peptoniphilus*, *Vogesella*, *Aquabacterium*, *Curvibacter*, *Paucibacter*, *Massilia*, *Mannheimia* and *Shimwellia*. *Peptoniphilus*, *Vogesella*, *Aquabacterium*, *Curvibacter*, *Paucibacter*, *massilia*, *Mannheimia* and *Shimwellia* strong association with the infection status suggest their importance in alleviating the vulnerability of the *Simulium* flies to the parasite *Onchocerca volvulus*. *Peptoniphilus* is known as a commensal found in the skin, vagina, and gut of animals. However, 17 case reports identified species of *Peptoniphilus* in blood stream infections likely to be associated with polymicrobial infection and connected to skin infections (Brown et al., 2014; Müller-Schulte et al., 2019). This genus has also been linked to eye infections following a case report that identified the species *Peptoniphilus indolicus* from the orbital of a patient

(Shires et al., 2020). This suggests the association of *Peptoniphilus* with other pathogens like the parasites in causing diseases such as the blood stream infections.

Species of *Massilia* genus have been noted to be associated with eye infections causing cornea abscess (Ballesterro-Téllez et al., 2016). A study by Mwamundela et al. (2023) identified *Massilia* in bedbugs that had fed on human and Guinea pigs, there is therefore a possibility that the infected blackflies might have obtained the pathogenic bacteria during their blood meal from animals and humans. *Vogesella* is not known to be pathogenic though there is a report of its isolation from human urine (Lan et al., 2020). *Aquabacterium*, *Curvibacter* and *Paucibacter* were initially isolated from water. Less is documented regarding their association to parasites, other insects, and animals though a study showed prominent level of *Aquabacterium* in blood of a patient who was suffering from fibromyalgia (Jensen, 2019) which may not be associated with *Onchocerca volvulus* infection. These can potentially be indicators of *O. volvulus* infection (Swanson et al., 2023).

This study also identified six bacterial genera to be strongly significant in the non-infection status of *Simulium* flies. Those that were significantly associated with the non-infection status were *Enterococcus*, *Citrobacter*, *Haemophilus*, *Shewanella*, *Halotalea* and *Zymobacter*. The commensal *Enterococcus* which inhabits the gut of insects, animals and humans has been shown to reduce infection in other insects. A study to demonstrate the interactions between microsporidia and gut microbiota in *Bombyx mori* showed that *Enterococcus* reduced *Nosema bombycis* infection. This occurred by modifying the host's immunity and biochemical reactions as well as lowering the germination of the parasite by producing lactic acid which reduces the PH of the gut creating an unfavourable environment for parasite growth (Yuen & Ausubel, 2014; X. Zhang et al., 2022).

Citobacter has also been demonstrated to unveil microbicidal properties against *Photorhabdus* spp and *Xenorhabdus* spp of insect killing nematodes (Mandal et al., 2013; Shanks et al., 2012). These antibacterial elements discovered in myriad studies (Mandal et al., 2013; Shanks et al., 2012) could show an interaction between the microbiome of *O. volvulus* and the microbiome of the *Simulium* flies. *Haemophilus* genus of bacteria are exclusively obligate parasites found in warm blooded animals, birds, and humans (Britannica, 2022) which makes it unusual to find it in insects. However, *Simulium* flies being blood feeders could explain their presence in the gut. It should also be noted that *Haemophilus* was identified in *Musca domestica* (houseflies) (Stabile et al., 2024) which is found in close associations with humans.

It remains unclear of *Haemophilus* association with the non-infection status of *Simulium* flies which may need further investigations to unravel this.

The significant association of *Shewanella* with the non-infection status was also demonstrated in a study where the colonization of the *C. elegans* by *Bacillus nematocida* B16 infection showed a significant higher number of *Bacillus* but *Shewanella* numbers were low (Niu et al., 2016). Lastly, *Zymobacter* and *Halotalea* belonging to the same family Halomonadaceae are free-living bacteria that have been identified in various insects but none of their associations has been related to protection against pathogens or parasites (Duguma et al., 2019; Maccaro et al., 2024; Milenovic et al., 2022; Ntougias et al., 2015; Suenami et al., 2019; Tarabai et al., 2023).

5.2 Conclusions

This study aimed to assess the bacterial composition of blackflies in the various endemic districts of northern Uganda, with a particular focus on understanding the microbial communities present in these vectors of onchocerciasis. By identifying and characterizing the bacterial genera associated with blackflies from different regions, the study has provided valuable insights into the microbial diversity of these insects and their potential role in disease transmission. The results indicate that blackflies in the endemic districts harbor a wide variety of bacterial species, some of which may have implications for the ecology of disease transmission. The microbial communities differed between districts, suggesting that local environmental factors and vector populations may play significant roles in shaping these communities. Moreover, certain bacteria identified in this study may be involved in the complex interactions between blackflies and the pathogen they transmit, *Onchocerca volvulus*.

These findings underscore the need for further research into the potential relationship between the bacterial microbiota of blackflies and their capacity to carry or transmit diseases, which could ultimately inform new control strategies for onchocerciasis and other vector-borne diseases in northern Uganda.

5.3 Recommendations

This study has revealed novel bacterial genera associated with *Onchocerca volvulus* infectivity and non-infectivity in *Simulium* flies. It is recommended to conduct a deeper investigation into the presence of pathogenic bacteria in blackflies, particularly those that may interact with

Onchocerca volvulus. Identifying specific bacteria that might enhance or inhibit the transmission of these diseases could provide novel targets for vector control. Given the variation in bacterial communities across different endemic districts, further studies should focus on a more detailed, comparative analysis of environmental and ecological factors that influence microbial composition in blackflies. This may include factors like water sources, vegetation, and human activity that could shape the microbiome of blackflies.

Based on the findings of this study, the development of integrated vector control strategies should consider not only the biological aspects of the blackfly as a vector but also the role of its microbiome. Potential interventions might target the disruption of harmful bacterial communities, thereby reducing the capacity of blackflies to transmit diseases. It is recommended to establish long-term monitoring and surveillance programs to track changes in bacterial composition in blackfly populations over time, especially in response to interventions such as vector control or changes in local environmental conditions. This could help in understanding the dynamics of vector-borne disease transmission and bacterial resistance patterns.

5.4 Limitation of the Study

The sample size for this study was relatively small which may not adequately represent the broader population of *Simulium* flies and the diverse bacterial communities from the different geographical locations. In addition, the study was confined to northern Uganda region with findings which may not be applicable to other areas with different ecological and environmental conditions. There is also a possibility of PCR bias since PCR amplification was used during library preparation potentially skewing the representation of certain bacterial phylum and genera in the final sequencing results. Lastly, nanopore has a high error rate compared to other sequencing technologies which can lead to incorrect base calling and affect the accuracy of diversity estimates.

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APPENDICES

Appendix A: Preparation of agarose gel, loading and running the electrophoresis system

1. The 1% agarose gel was prepared by weighing one gram of agarose powder (CSL CSL-AG100)
2. The agarose powder was dissolved in 100mls of 0.5X TBE buffer
3. The mixture was then heated in a microwave for two minutes to dissolve the agarose powder
4. This was followed by cooling for a few minutes in cold water
5. 5ul of ethidium bromide was added to the solution.
6. The solution was then poured into a well-prepared cast tray with an appropriate comb and left to stand for 30 minutes to solidify.
7. The comb was then picked out leaving wells in which was loaded a mixture of 5µl PCR amplicon and 2µl of blue gel loading dye (NEB B7021S).
8. A quick load purple ladder (NEB N0468S) was put in the first well as a marker. The electrophoresis system was then run at 120V,400 mAH for 40minutes.

Appendix B: Sample cleaning protocol

1. Re-suspend the AMPure XP beads by vortexing.
2. Add 30ul of re-suspend AMPure XP beads to the reaction sample and mix by pipetting.
3. Mix gently manually for 5minutes.
4. Insert the sample tubes into a magnetic rack.
5. While maintained on the magnetic rack, pipette off the supernatant.
6. Wash the beads with 200ul of freshly prepared 70% ethanol without disturbing the pellet.
7. Remove the ethanol.
8. Repeat steps 6 and 7.
9. Pipette off any residual ethanol and leave to air dry for 30seconds.
10. Remove the sample tube from the magnetic rack and re-suspend the pellet in 10ul of nuclease free water.
11. Incubate at room temperature for 2minutes.
12. Insert the samples back into the magnetic rack to pellet the beads until eluent is clear and colourless.

- Transfer the 10ul of the sample into a new 1.5ml microcentrifuge tube and dispose of the pelleted beads. Sample ready for use in other downstream processes.

Appendix C: Quantification using Qubit fluorometer

- Prepare a quantification buffer for all the samples and two standards as follows in the table.

Table 0.1: Table showing the sample volumes for preparing the Qubit reagents

Qubit reagent	Vol/sample	Vol/74 samples
Qubit dsDNA BR buffer	200ul	14800ul
Qubit dsDNA BR reagent 200x	1ul	74ul

- Aliquot 190ul of buffer into two tubes for standard then add 10ul of each standard 1 and 2.
- Aliquot 199ul of buffer into qubit tubes for all the samples
- Add 1ul of sample into the buffer in the qubit tube.
- Vortex and spun briefly then leave to stand for minutes.
- Set the program in the qubit machine to dsDNA broad range.
- Quantify the two standards then proceed to quantify the samples.
- Record the qubit concentrations and original concentration in ng/ml.

Table 0.2: Table showing Sequence run parameters

Flow Cell Type	FLO-MIN106
Kit	SQK-16S024
Initial bias voltage	-180 mV
FAST5 output	Enabled
FASTQ output	Enabled
BAM output	Disabled
Bulk file output	Disabled
Active channel selection	Enabled

Basecalling	Enabled
Specified run length	20 hours
FAST5 reads per file	4000
FAST5 output options	vbz_compress, fastq,raw
FASTQ reads per file	4000
FASTQ output options	compress
Mux scan period	1 hour 30 minutes
Reserved pores	0 %
Basecall model	Fast basecalling

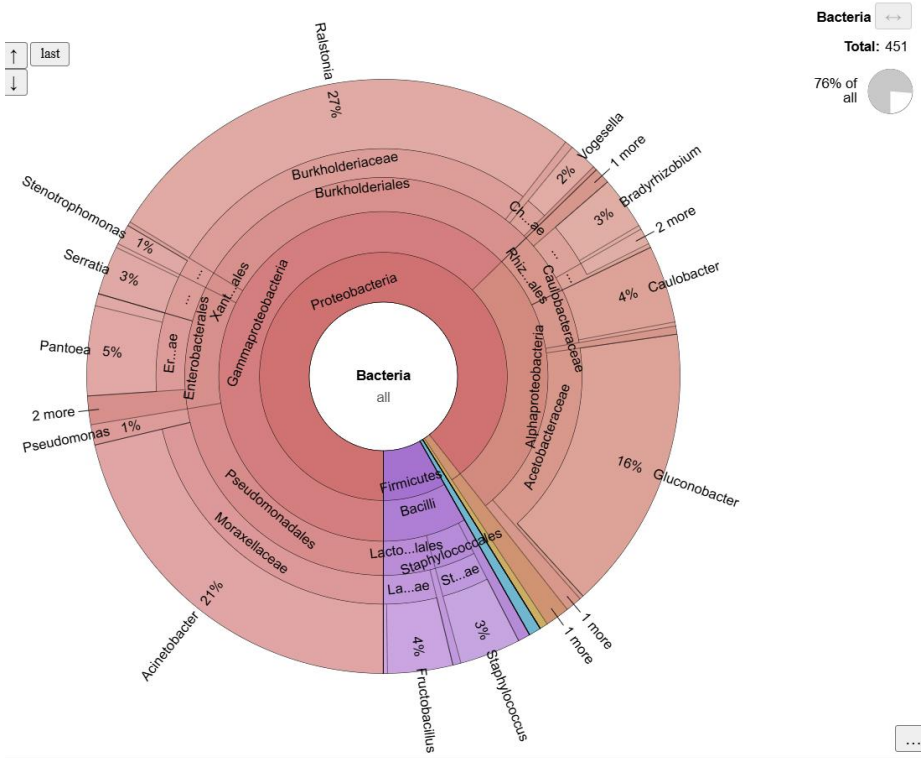


Figure 0. 1:Krona graph showing bacterial composition in sample A69

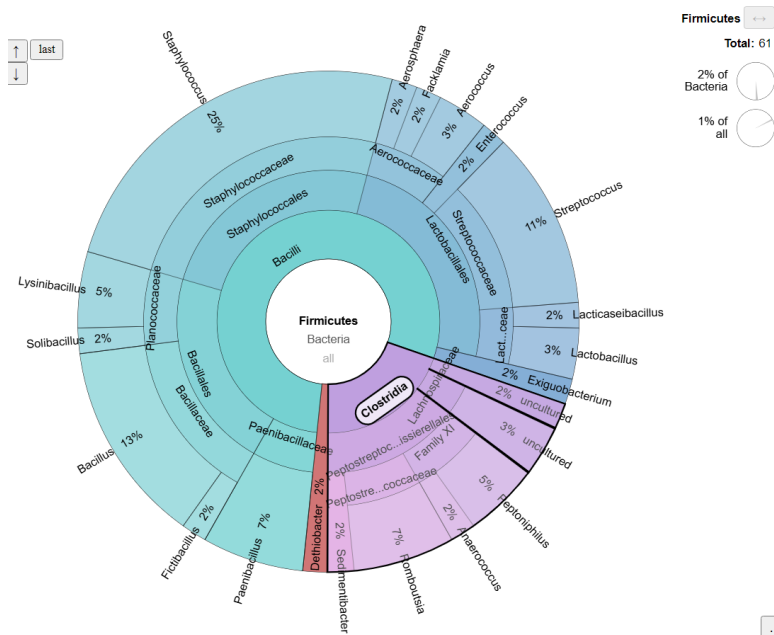


Figure 0. 2: A zoom of the krona graph showing the Firmicutes in sample N35

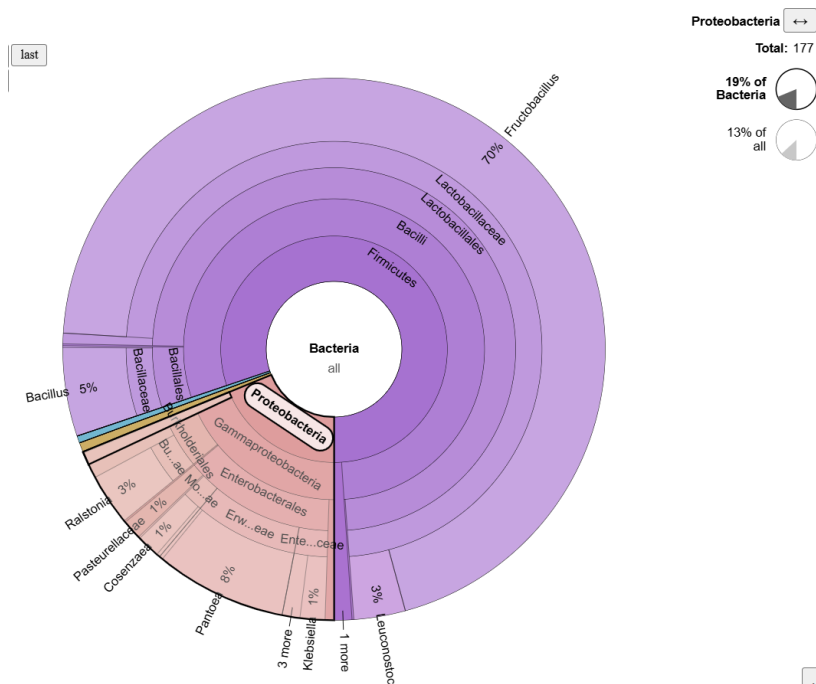


Figure 0. 3: Krona graph for sample P18 with Firmicutes highlighted as the most abundant bacterial phylum

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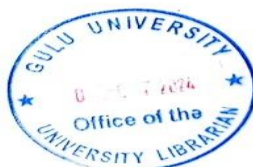


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