UNIVERSITY OF CAPE COAST

NATURAL INFECTION OF SELECTED BACTERIA IN THE GUT OF POTENTIAL VECTORS OF *LEISHMANIA (MUNDINIA) ENRIETTII* MEMBER (*LEISHMANIA* (*MUNDINIA*) *CHANCEI* IN CUTANEOUS LEISHMANIASIS COMMUNITIES IN GHANA.

FIDELIS KOJO AWOTWE

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BY

FIDELIS KOJO AWOTWE

Thesis submitted to the Department of Microbiology and Immunology of the School of Medical Sciences, College of Health and Allied Sciences, University of Cape Coast in partial fulfilment of the requirements for the award of Master of Philosophy degree in Infection and Immunity.

JULY, 2023

DECLARATION

Candidate Declaration

I hereby declare that this thesis is the result of my original research and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature …….. Date ……

Name: ……

Supervisor's Declaration

We hereby declare that the preparation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature…… Date……

Name: ……

Co-Supervisor's Signature…… Date……

Name: ……

ABSTRACT

The endosymbionts present in the gut of disease vectors play crucial roles in their physiology and vectorial competence, potentially influencing transmission dynamics. This creates avenues for disease control in vectors by exploring the vector microbiome. Leishmaniasis is a significant neglected tropical disease traditionally known to be transmitted by Phlebotomine sandflies. The cutaneous form of the disease caused by the new species *Leishmania chancei* of the *Leishmania* (*Mundinia*) *enrietii* subgenus is endemic in the Volta Region of Ghana, with the vectors responsible for transmission unknown. This study investigated the presence of selected endosymbionts in the guts of potential vectors and their anticipated potential roles in the development of *Leishmania* parasites which may consequently influence disease transmission and lesion exacerbation. A total of 135 sandflies and 750 biting midges were caught by light traps in leishmaniasis-endemic communities in Ghana. The vectors were subsequently pooled (5 sandflies per group and 10 biting midges per group) and subjected to DNA extraction. Universal and specific 16s bacterial primers were employed to perform PCR screening for *Wolbachia*, *Ochrobactrum*, *Ehrlichia*, and *Tsukamurella*. Among the 27 pools of sandflies (135 individuals), 18.5% tested positive for *Wolbachia*, 100% for *Ehrlichia*, 3.7% for *Ochrobactrum* and 70% for *Tsukamurella*. Out of the 75 biting midges pools (750 individuals), 11% tested positive for *Wolbachia*, 41% for *Ehrlichia*, 47% for *Ochrobactrum* and 64% for *Tsukamurella*. Additionally, we amplified the *Leishmania* parasite in 55.6% of sandfly pools and 52% of the biting midges pools. Co-infections of *Leishmania* and bacteria were amplified in 11%, 56%, and 33% of sandflies for *Wolbachia*, *Ehrlichia*, and *Tsukamurella* respectively. Notably, no sandflies showed coinfection with the *Leishmania* parasite and *Ochrobactrum*. On the other hand, biting midges exhibited a co-infection rate of 5.3%, 23%, 24%, and 28% for *Wolbachia*, *Ehrlichia*, *Ochrobactrum* and *Tsukamurella* respectively with the parasite. The presence of *Wolbachia*, *Ehrlichia*, *Ochrobactrum* and *Tsukamurella* in the potential vectors along with the observed coinfections with the *Leishmania* parasite suggests possible interactions between these microorganisms within the vector's gut. These interactions may significantly influence the establishment of infections in vectors and subsequent disease transmission. To gain further insights into the gut microbial community of the potential vectors, sequencing of amplicons should be carried out to uncover the molecular foundations of the intricate relationships between the vectors and their microbiome. This study opens new discourse for investigating the vector microbiome, understanding its role in disease transmission dynamics and how to exploit the microbiome for disease prevention and control.

KEYWORDS

Microbiome

Endosymbiont

Leishmania (*Mundinia*) *enrietii* complex

Culicoides biting midges

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DEDICATION

In loving memory of my kid brother; Francis Kwesi Awotwe.

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LIST OF ABBREVIATIONS

CDC –	Centre for Disease Control
CL -	cutaneous leishmaniasis
VL -	visceral leishmaniasis
LCL -	Localized cutaneous leishmaniasis
DMSO -	Dimethyl sulfoxide
GHS -	Ghana Health service
HGA -	human granulocytic anaplasmosis
HME -	Human monocytic ehrlichiosis
LN –	Lymph node
ML -	mucocutaneous leishmaniasis
NTDs -	Neglected Tropical Diseases
PBS –	Phosphate buffered saline
PCR -	Polymerase chain reaction
PKDL -	post-kala-azar dermal leishmaniasis
WHO –	World Health Organisation

CHAPTER ONE

INTRODUCTION

A symbiotic relationship has always existed between animals (including insects) and microorganisms. This could be parasitism, commensalism or mutualism. The gut microbiota of any organism is made up of diverse bacteria, fungi, protozoa and viruses which influence the basic biological process. Leishmaniasis is a vector-borne disease transmitted by an infected Phlebotomine female sandfly. The *Leishmania* parasite should be able to undergo several developmental processes in the gut of its vector to reach the infective form. The gut microbiota of *Leishmania* vectors is suggested to influence the developmental processes of the parasite inside the vector host. Vector(s) responsible for the transmission of cutaneous leishmaniasis in Ghana remains unknown although sandflies and biting midges have been implicated in the transmission. Knowledge of the gut microbiome of potential vectors can provide reasons why either biting midges or sandflies in the endemic communities can or cannot transmit the *Leishmania* parasite as well as propose other vector control methods.

Background of the Study

A disease vector is any agent that has the capacity to harbour and transmit an infectious pathogen to another living organism. Insects that are parasitic, feed on animal or human blood and mainly serve as disease vectors. The diseases caused by these vectors are termed Vector-Borne Diseases (VBDs). Despite the numerous research and efforts to control the spread of these vector-borne diseases, they continue to remain a global and public health threat. VBDs such as malaria, trypanosomiasis and leishmaniasis have an enormous

impact on human and economic health. These diseases pose a global public health issue and impose a significant socioeconomic burden on affected communities and countries. Most of these VBDs are either resurfacing in previously eliminated or low-prevalence regions or emerging in new geographical areas (Beard et al., 2002; WHO, 2014). Arthropod vectors are responsible for over 17% of all infectious diseases and cause a minimum of 700,000 fatalities annually (Marselle et al., 2019; WHO, 2020). Hundreds of millions of vector-borne infections are recorded globally every year, resulting in millions of deaths, and often, the morbidity associated with these vector-borne pathogen infections is inestimable (Beaty, 2000; WHO, 2014). The majority of these diseases are Neglected Tropical Diseases (NTDs) because they affect persons in resource-poor regions in the tropics and subtropics (Hotez et al., 2020; Marselle et al., 2019). The increase in the establishment and spread of VBDs can be attributed to increased international travel, the globalisation of trade and climate change (Hendrickx et al., 2008).

The morphological and physiochemical features of the digestive tract of vectors (mainly insects) are distinct and unique (Engel & Moran, 2013). This provides an environmental requirement that influences gut microbial colonization. This endosymbiotic relationship established in the insect's gut microbial community contributes to the insect's physiology. For instance, the digestion of resistant dietary components (nutrition), development, protection from predators, parasites, and pathogens, contribution to inter and intraspecific communication, reproduction, immune responses, and disease vector efficiency. All these are examples of benefits derived from the symbiotic relationships between insects and microorganisms in their gut (Douglas, 2015;

Engel & Moran, 2013; Fredensborg et al., 2020; Gupta & Nair, 2020; Hassan et al., 2014; Jing et al., 2020; Weiss & Aksoy, 2011). The bacteria found in the guts influence the function of insect vectors in numerous fields of study such as agriculture, medicine, and ecology (Engel & Moran, 2013). In arthropod-vector-parasite interactions, the gut of the vector is the first point of contact after a blood meal (Azambuja et al., 2005). It is in the gut that the parasites begin to undergo various development and transformation stages through attachment and penetration of tissues. Various digestives enzymes, antimicrobial peptides, nitric oxide and naturally existing microorganisms in the gut play an essential role in determining the survival and development of the parasite in the vector (Gillespie et al., 2004; Michel & Kafatos, 2005). The presence of Gram-negative bacteria such as *Serratia marcescens*, *Klebsiella ozaenae*, *Pseudomonas aeruginosa*, *Escherichia coli* and Gram-positive bacteria such as *Enterococcus faecalis* have been detected in the gut microbiota of insects (Demaio et al., 1996; Gonzalez-Ceron et al., 2003). It has further been demonstrated that the midgut bacteria of mosquitoes inhibit the sporogonic development of the parasites (Gonzalez-Ceron et al., 2003; Pumpuni et al., 1993). Microbial infections of the guts of sandflies as well as guts mycoses of sandflies significantly reduce the infection rate of *Leishmania major* in *Phlebotomus papatasi* (Schlein et al., 1985). Earlier studies showed that *Leishmania* promastigotes mostly thrive in an environment uncontaminated with other microorganisms (Schlein et al., 1985). The presence of bacteria and fungi leaves the parasite vulnerable. Prior to sandflies' discovery as *Leishmania* vectors, they were known for their gut sterility (Adler & Theodor, 1929; Killick-Kendrick, 1979). It is postulated that the gut possess a bacterial inhibitor which

is relevant to the biology and ecology of sandflies (Schlein et al., 1985). Another study also showed the dominance of *Acetobacteraceae spp.* as the *Leishmania* parasite underwent metacyclogenesis (Kelly et al., 2017). Interaction between the gut microbiota and the parasite is therefore essential for the transmission of leishmaniasis as well as other VBDs.

Leishmaniasis as a vector-borne disease is among the topmost important neglected tropical diseases (Alvar, 2012; Hotez et al., 2006; Paranaiba et al., 2018; Torres-Guerrero et al., 2017). Different forms of the diseases; cutaneous, mucocutaneous, and visceral, are widespread in the tropics and sub-tropics (Boakye et al., 2005). Leishmaniasis is reportedly endemic in 102 countries including Ghana (Niño et al., 2023). Since 1999 to date, cutaneous leishmaniasis (CL) – one of the clinical manifestations of leishmaniasis – has been endemic in the Volta region (now Volta and Oti regions) of Ghana(Akuffo et al., 2021a; Akuffo et al., 2021b; Boakye et al., 2005; Kweku et al., 2011). The *Leishmania* species responsible for causing CL in the endemic communities was identified to be novel, belonging to the *Leishmania (Mundinia) enriettii* complex (Kwakye-Nuako et al., 2015; Villinski et al., 2008) and has recently been named *Leishmania* (*Mundina*) *chancei* (Kwakye-Nuako et al., 2023).

In spite of the identification of the disease in these communities and its causative species, the vector(s) responsible for the transmitting parasite is still unknown. Various laboratory investigations have implicated a new vector responsible for transmitting this novel *Leishmania chancei*. In a laboratory infection model using the isolate from Ghana, *Culicoides sonorensis* was noted to support the growth and replication of the *L. chancei* parasites beyond the bloodmeal phase and could transmit the infection to mammalian hosts (Becvar

et al., 2021; Kwakye-Nuako, 2016). A member of the *Mundinia* sub-genus, *Leishmania marcopodum* responsible for leishmaniasis in kangaroos was also identified in the vectors *Forcipomyia* (biting midges) naturally collected from the field in Australia (Dougall et al., 2011)

Problem Statement

Leishmaniasis has been in existence for decades. The disease is endemic in West Africa yet less recognized or under-reported (Boakye et al., 2005; Mann et al., 2021). In Ghana, the first suspected case was identified in 1999 (Boakye et al., 2005). The CL-causing *Leishmania* species in Ghana have been identified as a newly emerging species which is a member of the *Mundinia* (*enriettii*) subgenus (Kwakye-Nuako et al., 2015). To date, research and epidemiological surveillance for CL infections in the endemic communities in Ghana compared to malaria are lacking. Although several studies have been carried out to identify the parasites and study the potential vectors, more ought to be done to understand the disease's transmission dynamics in order to provide appropriate control strategies and treatment methods. Several laboratory infection models have provided evidence that incriminates biting midges (particularly, *Culicoides spp*) as putative vectors for the *Leishmania* species in the *Mundinia enriettii* complex including *L. chancei* from Ghana (Becvar et al., 2021; Dougall et al., 2011; Seblova et al., 2015).

Biting midges are very small hematophagous flies that serve as vectors for viruses (Mellor et al., 2000). They are known for their vicious and nuisance biting in humans, which in extreme cases results in cutaneous pruritic wheal-and-flare reactions and permanent scars (Felippe-Bauer & Sternheim, 2008; Linley et al., 1983; Sherlock, 1965). The biting midges are also known to spread

filarial worms and protozoans to humans, avians, and other animals, as well as cause allergic dermatitis (sweet itch) in horses (Braverman, 1988; Linley, 1985; Meiswinkel et al., 2004; Mellor et al., 2000). Laboratory investigations revealed that the *L. chancei* responsible for CL in Ghana survived, replicated and colonized the gut of *Culicoides sonorensis*, a biting midge but failed to do so in phlebotomine sandflies which are the established vectors of leishmaniasis across the world (Kwakye-Nuako, 2016). Sandflies are arthropod vectors recognised mainly as vectors for the transmission of leishmaniasis. However, they also serve as vectors for other bacteria such as *Bartonella bacilliformis* which causes Carrion's disease as well as several arboviruses, specifically those from the *Phlebovirus* genus (Pons et al., 2016). Not all species of sandflies can even transmit leishmaniasis in humans. About 70 species of sandflies in the genus *Phlebotomus* and *Lutzomyia* are proven as *Leishmania* vectors (Bates, 2007; Young & Duran, 1994). *Sergentomyia* sandflies which are abundant in Ghana are proven vectors for the transmission of *Sauroleshmania* in reptiles and not the human pathogenic species(Ready, 2013). This implies *Sergentomyia* sandflies are unable to support the development of the human *Leishmania* species.

After a blood meal, the vector should be able to support the development of the *Leishmania* parasite. In the gut, the parasite attaches to the gut to survive expulsion via defecation with other components of the blood meal (Ashford, 2000). During the various developmental stages, various chemicals such as trypsin and promastigote secretory gel are produced which are essential for its development (Bates & Rogers, 2004). Similarly, the microbial ecology of the gut of the vector also plays key roles in the establishment of an infection inside

the gut of the vector (Dennison et al., 2014; Louradour et al., 2017). Thus, the absence or presence of certain bacteria can either support or impede the development of the *Leishmania* parasite. In *Culicoides sonorensis,* the replication of bluetongue virus (BTV) and epizootic hemorrhagic fever virus (EHDV) was inhibited when it was infected with *Wolbachia* (Matthews et al., 2022).This study seeks to investigate the gut microbial community of the two potential vectors of *L. chancei*, biting midges and sandflies collected from endemic communities in the Volta region.

Other studies have shown that the commensal bacteria residing in the gut of vectors of other parasitic diseases like mosquitoes and tsetse flies contribute to the vectorial competence of the vectors (Dennison et al., 2014; Ferreira et al., 2023). The presence of *Serratia odorifera* in the midgut of Aedes aegypti mosquitoes makes them more susceptible to the Dengue-2 virus (Apte-Deshpande et al., 2012). Tsetse flies harbour the three primary endosymbiotic bacteria *Wigglesworthia glossinidia, Sodalis glossinidius* and *Wolbachia pipientis* (O'Neill et al., 1993). Tsetse flies become sterile and more susceptible to *Trypanosome* infection when *Wigglesworthia* is eliminated (Wang et al., 2009). When *Sodalis* is eliminated, the lifespan of the tsetse fly decreases substantially (Wang et al., 2009). *Wolbachia* has been shown to induce cytoplasmic incompatibility in tsetse flies (Alam et al., 2011). Some commensals also prevent the establishment of infection in the vector. For example; *Kosakonia cowanii* Zambiae (*Kco_Z*) confers resistance to *Plasmodium* in *Anopheles gambiae*. *P. falciparum* infection in *Anopheles stephensi* is reduced by the Gram-negative bacteria *Xanthomonas maltophilia, Pseudomonas aeruginosa, Escherichia coli, Cedecea lapagei*, and *Wingella*

americana (Pumpuni et al., 1993; Pumpuni et al., 1996). In *Ae. aegyti*, *Wolbachia* -wMel confers resistance to the dengue and chikungunya viruses (Rancès et al., 2012; Van Den Hurk et al., 2012; Walker et al., 2011) and *P. gallinaceum* (Moreira et al., 2009). In *A. stephensi*, *Wolbachia* causes a reduction of its susceptibility to *P. falciparum* (Bian et al., 2013). As such, characterizing the vector-associated microbiota of the potential vectors of *L. chancei* (i.e. sandflies and biting midges) would provide important information about microbes that render the vectors highly susceptible to *Leishmania* infections and provide cues as to the members of the microbiota that could be targeted for disease and vector control. This study, therefore, contributes to the much-needed understanding of *Leishmania* transmission within the endemic communities in Ghana. In the absence of approved commercial vaccines, vector control techniques will remain one of the most effective preventative methods for leishmaniasis (Tabbabi et al., 2022) and the vector's microbiome is one of the promising fields to explore.

Aim

To examine the gut microbiome and the microbial interaction with *Leishmania* parasites in biting midges and sandflies caught in the CL-endemic communities in Ghana.

Specific Objectives

i. To determine the bacteria present in the guts of the biting midges and sandflies using PCR

ii. To confirm the presence of the *Leishmania* parasites and the bacteria co-existing in the gut of biting midges and sandflies using PCR

Hypothesis

i. The co-existence of bacteria with *Leishmania* will impact the vectorial capacity of biting midges and sandflies in the transmission of the parasite.

ii. The presence/absence of *Ehrlichia* and *Tsukamurella* in midges or sandflies can result in co-infection or secondary infection during the transmission of cutaneous leishmaniasis.

Significance of Study

Insect vectors have developed a wide array of mechanisms to maintain their vectorial capacity. This includes their ability to inactivate toxic substances or develop resistance to drugs and pesticides against the parasites and vectors respectively (Beaty, 2000; Council, 1986). Alteration in the behavioural patterns of vector has rendered biological control methods such as male sterility approaches highly ineffective (Barrett et al., 2012). With more advanced technologies, the decline in public health vector control programmes and vector-borne specialists remains a contributing factor. Socioeconomic issues such as urbanization and population growth are also taken into account (Gratz, 1999; WHO, 2014). The key roles played by insects in the food chain do not also make it desirable to eliminate these arthropod vectors (Durvasula et al., 1997). This calls for novel and effective control interventions to combat infectious diseases transmitted by arthropod vectors.

Symbiosis control has proved successful in preventing the spread of Chagas disease, a protozoan infection caused by *Trypanosoma cruzi* and transmitted by the insect vector, Triatomine (kissing) bug (Beard et al., 2001; Durvasula et al., 1997). The symbiotic bacteria *Rhodococcus rhodnii* in the gut of the kissing bug was employed to limit Chagas disease transmissions via the

para-transgenesis process. Another bacterium making great news with promising results is an obligate intracellular bacterium, *Wolbachia*. *Wolbachia* is an alpha-bacterium known for its endosymbiotic relationship with arthropods and nematodes (Bonneau et al., 2018; Jeyaprakash & Hoy, 2000; Taylor et al., 2005; Werren, 1997; Werren et al., 2008). The unique characteristics of *Wolbachia* in insects range from reproductive manipulation to control of insect vectorial capacity and mutualism. Due to the several roles played by these gut microbes in their hosts, many scientific investigations have focused on exploiting them as a means to control the spread of infectious pathogens as well as their use in other industrial and biotechnological inventions.

Insect evolution has benefited enormously from the myriad endosymbiotic relation with its gut microbial community (Engel & Moran, 2013; Ricci et al., 2012). The bacteria in an insect's gut influence its vectorial abilities and the duration it takes for parasites to develop (McMeniman et al., 2009; Ricci et al., 2012). This opens the door to disease control in disease vector insects known as symbiotic control. As explained by Ricci et al., (2012), symbiotic control is a new approach that takes advantage of the symbiotic associations between microorganisms to control insect pests or reduce vector competence. Three of the strategies used in symbiosis control to reduce vector competence are the disruption of microbial symbionts required by disease vector, the manipulation of symbionts that can express anti-pathogen compounds within the host and the introduction of endogenous microorganisms that impact the lifespan and vector capability of new hosts in insect populations (Ricci et al., 2012).

Currently, no data exist on the gut microbiota of potential vectors involved in the transmission of cutaneous leishmaniasis in the endemic communities of Ghana. This study will therefore provide primary information on the gut microbiota of biting midges and sandflies. It will further elucidate their role in disease transmission. The goal of this study is to add knowledge to vector biology and emphasize the role of the insect gut microbiota in disease parthenogenesis. The study will also provide data on possible vector control measures for cutaneous leishmaniasis using the microbiome as a target.

Delimitations

Sandflies and biting midges, which are the potential vectors, were collected from an endemic community; Dodome Awuiaso using mouth and hand aspirators, sweep nets, and overnight traps. For this investigation, only female sandflies and biting midges were used. The male biting midges and sandflies captured were not included in the study since they are not involved in the transmission cycle.

Limitation

There was no classification of the potential vectors based on their species. This won't reveal the specific species that harbour particular bacteria or parasites in their gut and their prevalence per species.

Definition of Terms

Neglected Tropical Diseases: According to WHO, these are diseases that are predominately prevalent in tropical areas and affect mostly impoverished and underdeveloped communities.

Microbiome: A community of microorganisms including fungi, viruses, protozoa, bacteria and parasites that are present in a particular environment.

Gut Microbiome: The bacteria, archaea, viruses and eukaryotic microbes and their collective genetic material present in the digestive system.

Organization of Study

There are five chapters in this write-up. The first chapter gives a brief introduction and background to the study. The problem statement aims and specific objectives, hypothesis, the significance of the study, delimitation and limitations of this study and definition of specific terms are all presented in the first chapter. Important literature underlying this study is reviewed in the next chapter. Chapter two presents literature on the disease leishmaniasis and the several bacteria (*Wolbachia, Ochrobactrum, Ehrlichia* and *Tsukamurella*), and the *Leishmania* parasite screened in this study. The Methodology is covered in Chapter 3. The systematic methodical approach used to accomplish this study's aim is thoroughly described, starting with the collection of insect vectors, followed by laboratory work, and then analysis of the results. The results and discussion are presented in Chapter 4, and the conclusion is made in Chapter 5 (which also highlights challenges and makes recommendations for further studies).

Chapter Summary

This chapter gave a general overview of the study. A brief description of the microbiome and cutaneous leishmaniasis was provided. The main purpose of the study was stated followed by the hypothesis. The relevance of this study, as well as the knowledge gap this study aims to breach, was further stated.

CHAPTER TWO

LITERATURE REVIEW

Introduction

Cutaneous leishmaniasis (CL) is endemic in the Volta and Oti regions of Ghana. Biting midges instead of the known vector, phlebotomine sandflies are being implicated to transmit the CL in Ghana. The *Leishmania*-vector interactions are influenced by the vector's gut microbiome. The presence of certain bacteria in the gut could inhibit the growth of the parasite in the vector or vice versa. Additionally, some of the bacteria present in the gut when transmitted together with the *Leishmania* parasites can cause secondary infections during transmission and exacerbate disease outcomes. This study sought to detect the presence of selected bacteria; *Wolbachia*, *Ehrlichia*, *Ochrobactrum* and *Tsukamurella* in the two potential vectors, biting midges and sandflies, caught in a *Leishmania*-endemic community in Ghana. The selected bacteria are known to play important roles in vector competence as well as pathogenic to humans. Furthermore, the study sought to detect the presence of *Leishmania* parasite in the putative vectors. This chapter will provide a review of leishmaniasis and the two potential vectors, bacteria and parasites under study.

Overview of Leishmaniasis

Neglected tropical diseases (NTDs) are diseases caused by bacteria, parasites, viruses, fungi and toxins that are particularly common in tropical areas and the world's poorest communities. According to WHO, there are 20 conditions that are classified as neglected tropical diseases. These conditions include lymphatic filariasis, Chagas disease, human African trypanosomiasis

(sleeping sickness) and leishmaniasis. Leishmaniasis is caused by the protozoan *Leishmania*. According to WHO, leishmaniasis ranks among the top seven NTDs and public health threat to the world due to the disease burden and severity of clinical outcomes (Andrade-Narváez et al., 2001; Torres-Guerrero et al., 2017). Leishmaniasis is regarded as neglected because it predominantly affects vulnerable societies in developing nations (do Rosário et al., 2022). It is also associated with deforestation, malnutrition, weak immune system, extractive industries, population displacement, poor sanitation, and poverty (do Rosário et al., 2022; WHO, 2022). Although the disease is pantropical and primarily affects rural areas, it has now become common in peri-urban and urban areas (do Rosário et al., 2022). The geographical distribution of leishmaniasis continues to expand its frontiers (Kwakye-Nuako, 2016). The disease is now endemic in areas where it was previously absent (Faiman et al., 2013) with cases documented in Australia, Europe, and the United States (Azami-Conesa et al., 2020; de Almeida et al., 2021; Panahi et al., 2020). Except for Oceania, leishmaniasis has been found in every continent (Reithinger et al., 2007; Torres-Guerrero et al., 2017).

Clinical forms of Leishmaniasis

There are three main clinical manifestations of leishmaniasis; cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (kala-azar).

Cutaneous Leishmaniasis

CL has been in existence since antiquity (Manzur, 2005). In Egypt as far back as 2000 years BC, medical documents referred to CL as "EbersPapyrus" (Rab et al., 1986). In 1885, Cunningham, described observing microorganisms

in macrophages from lesions of "DelhiBoil" in India (Grevelink & Lemer, 1996). As early as the first century, Old World CL was first described while New World CL was later described around 400 – 900 AD (Lainson & Shaw, 1987; Peters, 1988). Based on geographical locations, CL has gained numerous names such as Aleppo evil, Rose of Jericho and forest yaws (Grevelink & Lemer, 1996). The geographical distribution of CL keeps increasing as well as the evolution of new causative species. Although the geographical distribution of CL is mainly determined by the sandfly vectors (Manzur, 2005), other factors such as population surge and displacement, urbanization, anthropogenic environmental modifications, drug resistance, and new agricultural practices happen to influence the increasing spread (Ashford, 1996; Daszak et al., 2001; Jeddi et al., 2011; Patz et al., 2000).

CL primarily affects the exposed parts of the body at the site of the sandfly bite (Savoia, 2015). CL is the most common form of the disease and typically causes skin sores on exposed body regions like the face, arms, and legs (WHO, 2022). There could be 200 or more skin lesions on different body parts, which could cause severe impairment (WHO, 2022). CL is the most prevalent clinical form and is endemic in 92 countries (WHO, 2023a). However, 75% of CL cases are from only 10 of these countries (Alvar, 2012). There are two distinct CL epidemiology types in some geographic areas. These are anthroponotic CL (ACL) and zoonotic CL (ZCL), which are caused by *L. tropica*, and *L. major* respectively (Minodier & Parola, 2007). Also, the subtypes of CL range from localized cutaneous leishmaniasis (LCL) to more serious and disseminated cutaneous leishmaniasis (DL) or diffuse cutaneous leishmaniasis (DCL) (Zijlstra, 2014).

Localized cutaneous leishmaniasis (LCL) mostly occurs at unclothed parts of the body where the vector bites. In the old world, *L. major, L.tropica, L. infantum*, and *L. aethiopica* are the parasites that cause LCL while in the new world, LCL is caused by *L. braziliensis* and *L. mexicana*: two separate species or "complexes" of the *Leishmania* parasites (Kubba et al., 1987). The clinical ulcers that result from each specie are similar with slight variations (Kubba et al., 1987). Red papules resembling furuncles first emerge less than three months after the start of the incubation phase (Bryceson et al., 1992). Over a few weeks, the papule steadily grows in size, and eventually, the lesion develops a crust in the centre. When the crust is scraped off, a shallow ulcer is seen, frequently with elevated and slightly indented edges. Lesions caused by *L. major* or *L. tropica* are frequently wet or dry (Shoaib et al., 2007).

Diffuse cutaneous leishmaniasis (DCL) is characterized by widely disseminated nodules, an abundance of parasites throughout the infection, a lack of a cell-mediated immune response specific to the parasite, and a poor response to antimonial treatment (Manzur, 2005). In the New World, *L. amazonensis, L. mexicana*, and *L. pifanoi* cause DCL, while *L. aethiopica* causes DCL in the Old World (Grevelink & Lemer, 1996). The difference between DL and LCL and DCL is that DL is characterized by the presence of more than 10-800 mixed-type lesions (e.g., acneiform, papular, nodular, and/or ulcerated), localized in more than two body parts (head, trunk, arms, and legs) (Couppie et al., 2004; Zijlstra, 2014). These lesions can be brought on by *L. braziliensis, L. amazonensis*, or *L. guyanensis*, and they are thought to have spread from a single initial lesion within 3 days to 8 weeks (Couppie et al., 2004; Turetz et al., 2002).

Mucocutaneous Leishmaniasis

Mucocutaneous leishmaniasis (MCL) predominantly affects the mucous membrane. The mucous membranes of the nose, mouth, and throat cavities, as well as the surrounding tissues, can be completely or partially destroyed by lesions caused by MCL (Grevelink & Lemer, 1996; WHO, 2022). Treatment is therefore a necessity for patients because it is life-threatening and can be fatal. *Leishmania* species of the *Viannia* subgenus - *L. (V) braziliensis, L. (V) amazonensis, L. (V) panamensis*, and *L. (V) guyanensis* - which are commonly found in the Americas, cause MCL (David & Craft, 2009). The development of MCL is dependent on parasite virulence and host cell-mediated immunity (Machado-Coelho et al., 2005). In an infected population, the MCL spreads to the mucosa in about 1-10% of the population (Konecny & Stark, 2007; Weigle & Saravia, 1996). Patients with LCL in areas where *L. (V.) braziliensis* is endemic have about 1-10% go on to develop MCL (Scorza et al., 2017). The size and frequency of LCL lesions, lesions above the belt, increased age, malnutrition, and sex (male > female) are known risk factors (Jones et al., 1987; Machado-Coelho et al., 2005; Marsden, 1986). Despite the possibility of healing, most lesions leave behind permanent scars, which can alter a person's physical appearance and lead to stigmatization and psychological effects (Handler et al., 2015).

Visceral Leishmaniasis

Visceral leishmaniasis (VL) is also known as kala-azar and can be lethal if untreated. It affects the vital internal organs like the spleen, liver and bone marrow (WHO, 2022). According to WHO, it is one of the top parasitic illnesses with potential outbreak and mortality. Symptoms of VL include irregular bouts

of fever, significant weight loss, enlargement of the spleen and liver and low blood counts (anaemia, leukopenia and thrombocytopenia) (CDC, 2020). A complication of VL is post-kala-azar cutaneous leishmaniasis (PKDL). PKDL typically manifests 6 months to 1 or more years after VL seems to have been cured. It is typically seen in patients who have recovered from VL and is characterized by a discoloured (hypopigmented), flat skin (macular) rash mixed with some slightly elevated (maculopapular) or elevated (nodular) rash. Most cases of PKDL are found in South-East Asia and East Africa (WHO, 2022).

Leishmaniasis is reported to affect about 12 million people in 102 nations across 5 continents with 350 million people at risk of infection (Alidosti et al., 2021; Alvar, 2012; Niño et al., 2023). Specifically, cutaneous leishmaniasis and visceral leishmaniasis (VL) are endemic in 92 and 83 countries, respectively (WHO, 2022). The epidemiology is influenced by the parasite and sandfly species, the local ecological features of the transmission locations, the human population's historical and present exposure to the infection, and human behaviour (WHO, 2022). Every year, 1.3 million new cases of human leishmaniasis are reported, with 20,000–30,000 fatalities recorded (Tabbabi et al., 2022). It is believed that between 700,000 and 1.2 million CL cases are recorded annually, with 95% of those cases occurring in the Middle East, Central Asia, the Americas, and the Mediterranean basin (CDC, 2020; Mann et al., 2021). Less than 100,000 cases of VL are thought to be reported each year, with more than 95% of those cases occurring in Brazil, China, Ethiopia, India, Kenya, Nepal, Somalia, and Sudan (CDC, 2020; Mann et al., 2021). According to WHO, VL is one of the top parasitic illnesses with the potential for outbreak and mortality (Mann et al., 2021; WHO, 2023b). The

polymorphic nature of the disease is expressed in the clinical features and evolution, depending on the species or strain of the *Leishmania* parasite the susceptible host is exposed to, as well as the host's genetic makeup and immune status (Andrade-Narváez et al., 2001).

The *Leishmania* Parasite

More than 30 distinct *Leishmania* species cause leishmaniasis (Bates, 2007). At least 10 of these species are of medicinal and veterinary relevance (Bates & Ashford, 2010; Lainson, 1996). The parasite species are separated into Old World and New World regions according to their geographical origin. The species, *L. donovani, L. infantum, L. tropica, L. major* and *L. aethiopica* are all found in the Old World whereas L. *infantum chagasi*, *L. mexicana*, *L. amazonensis, L. brazilensis, L. peruviana, L. guyanensis* and *L. panamensis* are New World species. *L. tropica*, *L. major*, *L. donovani*, *L. mexicana* and *L. braziliensis* are the five species that are most significant to humans. The only vector known to transmit the *Leishmania* parasite to humans or other animals is the female phlebotomine sandfly although other vectors are now being implicated in some areas. The female phlebotomine sandfly can support the development of the *Leishmania* parasite after a blood meal to the infective metacyclic stage.

The life cycle of *Leishmania*

The lifecycle of *Leishmania* begins when a female phlebotomine sandfly feeds on an infected mammalian host during a blood meal and picks up the amastigote form of the parasite which is already present in the skin (Bates, 2007). The sandflies' cutting mouthparts help them capture the parasites in the skin during the blood meal (Bates, 2007; Lane, 1993). When the parasite leaves

the mammalian host into the sandfly midgut, it begins to develop in the vector due to changes in the surrounding condition (decrease in temperature and increase in pH) (Bates & Rogers, 2004; Kamhawi, 2006).

Procyclic promastigote is the first stage of the amastigotes' transformation into motile promastigotes (Bates, 2007). The procyclics have a weakly motile but highly replicative form that multiplies in the bloodmeal. This first bloodmeal phase is restricted by the peritrophic matrix, chitin and protein mesh secreted by the midgut epithelium that encloses the blood being digested within (Secundino et al., 2005). The parasites slow down their replication after a few days and transform into elongated, highly motile nectomonad promastigotes (Bates, 2007). These forms migrate, assemble at the anterior end of the peritrophic matrix, and emerge from the bloodmeal (Bates, 2007). This escape is made easier by a parasite's secretory chitinase (Schlein et al., 1991; Shakarian & Dwyer, 2000) and possibly by endogenous chitinase from sand flies (Ramalho-Ortigão et al., 2005). When they reach the stomodeal valve (cardia), which protects the connection between the foregut and midgut, they continue to migrate in the direction of the anterior midgut, some of them attaching to the microvilli of the midgut epithelium (Bates, 2007). These nectomonad promastigotes mediate the phase of infection known as establishment, which is characterized by persistence past the bloodmeal and resistance to expulsion after feces (Bates, 2007). Thus, *Leishmania* (*Leishmania*) promastigotes' capacity to attach is a crucial trait (Sacks & Kamhawi, 2001). The nectomonad promastigotes transform into the shorter leptomonad promastigotes, which resume replication, once they reach the stomodeal valve (Gossage et al., 2003). These are responsible for the production

of promastigote secretory gel (PSG), which is essential for transmission (Rogers et al., 2002). Some nectomonad/leptomonad promastigotes can develop into haptomonad promastigotes by adhering to the cuticle-lined surface of the valve (Killick-Kendrick et al., 1974). Finally, the leptomonads differentiate into the mammal-infective stages known as metacyclic promastigotes (Rogers et al., 2002). The promastigote form is injected into the skin of the mammalian host when the sand fly takes another blood meal.

Vector for *Leishmania* transmission

Female sandflies are the known traditional vectors for leishmaniasis (Maroli et al., 2013). They are tiny blood-sucking insects approximately 2-3 mm in length, ranging from silver to black in colour, and fold their wings into a V shape while at rest (Showler & Boggild, 2017; Young & Duran, 1994). Sandflies are most active at night and rest during the day in buildings, cellars, caves, and gaps in rocks (Young & Duran, 1994). They feed on blood to produce eggs and their bites are typically painful. Sandflies of the genus *Phlebotomus and Lutzomyia* are responsible for the transmission of leishmaniasis in the Old World and New World respectively (Bates, 2007). Each sand fly species transmits only one specific species of parasite and this leads to a particular clinical syndrome.

Five major genera, *Phlebotomus* (94 species) and *Sergentomyia* (258 species) in the Old World, *Lutzomyia* (379 species), *Brumptomyia* (23 species), and *Warileya* (5 species) in the New World, account for almost 800 species of sand flies (Munstermann, 2004). *Phlebotomus* and *Lutzomyia* are the only two genera out of which about 70 species have been identified and proven as *Leishmania* vectors (Bates, 2007; Young & Duran, 1994). Additionally, each

type of vector is capable of fostering the growth and development of one particular species of *Leishmania* and its transmission. This is due to the numerous barriers that the parasite must overcome in order to establish and develop inside the vector host.

Leishmaniasis in Ghana

In 1999, chronic ulcers which later turned out to be cutaneous leishmaniasis were observed among residents in towns in the then Volta Region of Ghana, (now Volta and Oti regions), which shares borders with the Republic of Togo. An epidemic broke out from 2002 to 2003 in the Ho municipality, with the majority of cases in 2003. A total of 8,876 possible cases were recorded from nine sub-districts in the Volta area. Until 2018, no reported cases of CL were recorded in the Oti area. Recent studies have confirmed cases of CL in the Oti Region following reports of skin ulcers (Akuffo et al., 2021a). In 2018, out of 595 samples of skin ulcers from different individuals, nested PCR confirmed 25.2% (150 samples) of the samples collected were *Leishmania* positive (Akuffo et al., 2021a). In a similar study, the prevalence of *Leishmania* infection among 3,071 participants from three communities in the Oti region using a *Leishmania* skin test was found to be 41.8% (Akuffo et al., 2021b).

The *Leishmania* species responsible for causing CL in Ghana has been found to be novel (Kwakye-Nuako et al., 2023; Kwakye-Nuako et al., 2015). This was first reported after a study conducted to identify the type of parasite was negative for *Leishmania* major and *Leishmania infantum* (Villinski et al., 2008). The SSU rRNA and ITS1 sequences showed high similarity between the *Leishmania* isolate from Ghana obtained from patient samples as well as *Leishmania* sequences in both the Old world and New world species (Villinski

et al., 2008). The Ghana *Leishmania* species are more related to species within the *Leishmania enrietti* complex (Kwakye-Nuako et al., 2015). Studies conducted by Kwakye-Nuako et al., (2015) first isolated the new species of *Leishmania* species in Ghana from samples of three different CL patients. Out of the three isolates, detailed information on the isolate's description, biology and medical significance of MHOM/GH/2012/GH5:LV757 was recently published and named *Leishmania (Mundinia) chancei* (Kwakye-Nuako et al., 2023). The vector for the transmission of this novel species remains unknown.

In Ghana, sandflies have been caught and identified in both the *Leishmania*-endemic (i.e. Volta and Oti) and Northern regions. These sandflies caught belong to the old world, genus *Sergentomyia* and *Phlebotomus* with the majority belonging to the genus *Sergentomyia* (de Souza, Addo, et al., 2023; de Souza, Desewu, et al., 2023; Doe et al., 2020; Kwakye-Nuako et al., 2015). *Sergentomyia* sandflies are proven vectors for the transmission of *Sauroleshmania* in reptiles and not the human pathogenic species (Ready, 2013). The high prevalence of *Sergentomyia* species in the *Leishmania*-endemic previous studies carried has been reported in other studies (Boakye et al., 2005; Fryauff et al., 2006; Kweku et al., 2011; Mba-tihssommah, 2016).

It has previously been reported that the possible vectors of *Leishmania* in Ghana were *Phlebotomus rodhaini* and *Phlebotomus duboscqi*, however, due to their lower numbers in the endemic communities, this could not be concluded (Fryauff et al., 2006). According to Ready (2003), *Leishmania* DNA has been isolated from species of *Sergentomyia* sandflies from the endemic regions but this does not provide conclusive evidence for vector incrimination. The reason is that the insect must satisfy the vector incriminating criteria as outlined by

Dougall et al. (2011) and Killik-Kendrick (1999). Thus; (1) the suspected vector species or fly and the reservoir host (human or animal) must be present in the same environment; (2) the vector species bites the reservoir host; (3) the suspected vector species or fly must have the capacity to sustain the development of parasite life stages after the infecting blood meal has been digested to the transmissible infective metacyclic stage; (4) the parasitic isolates from wild-caught vectors and the reservoir host are identical; (5) the vector is able to transmit the parasite by a bite to the reservoir and other hosts.

Diagnosis, Treatment and management of leishmaniasis

Leishmaniasis is diagnosed using a variety of techniques. History, epidemiology, clinical symptoms, and physical exam findings are important aspects that may help a clinician make a diagnosis. For example, cutaneous Leishmaniasis should be suspected if a person has recently travelled to or moved away from an endemic location and develops a painless, non-purulent skin ulcer on their face or extremities. In contrast, persistent fevers, exhaustion, weight loss, anaemia, leucopenia, and hepatosplenomegaly may indicate visceral Leishmaniasis in a patient from an area where the disease is prevalent, like Africa. Methods for diagnosis depending on the sample (blood or other bodily tissues) and clinical form include microscopy, antibody detection (indirect fluorescent Antibody test and direct Agglutination test), and DNA detection (Polymerase chain reaction) (Mba-tihssommah, 2016). **Table 1** shows the various diagnostic methods (Mann et al., 2021).

Table 1: Diagnostic Methods for Leishmaniasis

Disease pattern	Direct vs indirect	Diagnostic method	Comments
Cutaneous Leishmaniasis	Direct	Biopsy, scraping, aspirate	Sensitivity is dependent on the expertise of the pathologist and the quality of the specimen. Obtain from the edge of the ulcer and base
		Microscopy	Giemsa-stained microscopy
		Culture	Amastigotes are typically 2–4 μm in diameter, round to oval in shape with a nucleus and kinetoplast
		Histology	Special media, as the organism is fastidious and it can take weeks to become positive.
		PCR	Most sensitive and identifies species which helps exclude ML-associated species. PCR is also helpful in cases with low parasite burden.
	Indirect	CL Detect	Immunochromatographic assay for the rapid detection of *Leishmania* species antigen in ulcerative skin lesions
			Sensitivity 96%, specificity 90%
		Serologic tests (see below)	Not recommended for diagnosis of CL
Visceral Leishmaniasis	Direct	Splenic aspirate (parasite isolation, culture, histology, and PCR per above)	Most sensitive (93–99%) compared to bone marrow and lymph node aspirate for diagnosing VL but the risk of splenic haemorrhage
		Bone marrow aspirate	Bone marrow sensitivity (52–85%) sensitivity.
			Safer to perform than splenic aspirate
		LN Aspirate	Lymph node aspirate sensitivity (52%–58%)
		Peripheral blood	Assess blood for buffy coat, in vitro culture, and molecular analyses. Helpful in the diagnosis of immunocompromised and HIV patients
	Indirect	Serological tests:	Cannot distinguish active from prior infection. Not helpful for CL. Often non-reactive in immunocompromised hosts.

Table 1: Cont'D

		Rapid Diagnostic Test (rK-39)	Detect specific antibodies against antigens present in *L. donovani, chagasi-infantum* Results available in 20–25 min Easy to perform, quick and cheap- particularly helpful in underserved areas Sensitivity varies depending on region and parasite species Can cross-react with other infections—for example, Chagas disease
		Direct Agglutination Test (DAT)	Uses whole organisms to look for antibodies. Gives antibody tires ranging from 1:100 up to 1: 151200. Sensitive (>95%) and specific (>85%) tests when performed correctly Needs well-trained technician to perform over 2-3 days Not available in North America
		Other antibody tests, ELISA, Indirect immunofluorescence, indirect agglutination test, antigen test	Serologic antigens and urine antigens are available Sensitivity and specificity varies based on the test False positive results in persons with Chagas, leprosy, tuberculosis, malaria
Mucosal Leishmaniasis	Direct	Biopsy, scraping, aspirate of mucosal lesion/LN (culture, histology, and PCR per above)	Direct diagnosis is preferred.
		Serological tests per above	Not as helpful for ML as for VL. Direct diagnosis is preferred
	Indirect	Leishmanin Test	Delayed type hypersensitivity response Also known as the Montenegro test, works similarly to the tuberculin skin test Most useful in the diagnosis of ML Negative in diffuse CL, active VL False positives with other skin diseases

Microscopy

In pertinent tissue aspirates or biopsies, such as bone marrow, spleen, lymph nodes, liver, and skin split smears, microscopy is employed to detect *Leishmania* amastigotes. Using the Giemsa stain, amastigotes could be detected from the infected patient sample (Singh, 2006) while a dissecting microscope could be used to identify *Leishmania* parasites in *Leishmania* vectors. Culturing of parasite isolates from the sand fly's midgut is possible, although some species can be challenging to isolate and culture due to reasons like they only grow on particular media. Novy-MacNeil-Nicole medium, Grace's medium, and Schineider's Drosophila media may be initially used for parasite isolation, however, M199 is employed for parasite culture maintenance.

Indirect Fluorescent Antibody Test (IFA)

IFA is the ideal test for diagnosis of CL, MCL, and PKDL (Assimina, 2008). Using fixed promastigotes, the IFA test is frequently used to identify anti-*Leishmania*l antibodies. Antibody detection is shown during the early stages of illness and disappears six to nine months after cure. Using *Leishmania* amastigotes as the antigen rather than promastigotes will improve the test's lower sensitivity.

Leishmanin skin test

Human cutaneous Leishmaniasis is characterized by delayed hypersensitivity, and can be detected with the leishmanin skin test, commonly known as the Montenegro reaction (Singh, 2006). Leishmanin Skin Test is effective for determining the distribution of human infections are occurring and identifying immune from nonimmune infections.

Polymerase chain reaction (PCR)

Among the molecular techniques employed for diagnosis, PCR happens to be the most sensitive and specific technique (Mba-tihssommah, 2016). PCR is appropriate because its conserved region target specificity for certain gene amplification requirements. When compared to conventional methods, PCR has numerous benefits including being extremely sensitive, quick, and able to work with a wide range of various clinical specimens. PCR assays can detect the presence of parasites up to a few weeks before any symptoms manifest (Mba-tihssommah, 2016). Different types of PCR are suitable for various clinical forms. According to Singh et al. (2006), nested PCR has demonstrated its predictive values in the diagnosis of PKDL. Real-time PCR can also be used to determine parasite load analysis quantitatively or qualitatively (Bell & Ranford-Cartwright, 2002; Bossolasco et al., 2003; Reithinger & Dujardin, 2007) while multiplex PCR may be used to detect mixed infections, such as those in probable AIDS patients (Mba-tihssommah, 2016; Singh, 2006).

The Gut Microbiota

Insect gut microbiomes are composed of bacteria, fungi, archaea, and protozoa (Siddiqui et al., 2022). The microbes in an insect's gut are more than the total number of cells of the insect (Rajagopal, 2009). The microorganisms invade the insect gut through food and play a crucial part in metabolism and digestion. While the majority of the microorganisms in the stomach are parasites or commensals, some have been found to have positive effects on their hosts (Munoz-Benavent et al., 2021). They can aid in the digestion of food, the production of energy and vitamins, and even the development of the body's defense mechanisms (Cheng et al., 2019). It has been demonstrated that

microbial symbionts have a wide range of effects on insect health and behaviour (Sampson & Mazmanian, 2015).

The gut microbiota of insects impacts the host's development, resistance to disease, digestion, and physiology (Douglas, 2015; Engel & Moran, 2013). The efficacy of disease vectors and the duration of development may be influenced by symbiotic bacteria (Siddiqui et al., 2022). For instance, *Asaia* symbionts contribute to the growth of *A. stephensi* larvae (Chouaia et al., 2012). Various stages of *A. stephensi* larvae were treated with rifampicin (through their feed and water), an antibiotic that works against *Asaia spp*. of the wild type. A delay in larvae development was observed when treated as well as the absence of concurrence in duration of development. On the other hand, *Enterobacter sp*. (Esp Z) isolated from wild mosquito populations in Zambia inhibited ookinete, oocyst, and sporozoite development of *Plasmodium falciparum* by the generation of reactive oxygen species (ROS) (Cirimotich, Dong, et al., 2011).

A thorough understanding of the interactions between the vector, its microbiota, and the pathogens they carry is crucial to understand the mechanisms underlying pathogen-vector interactions, disease biology as well as identify possible novel targets for controlling or preventing the spread of disease or disrupting the pathogen life cycle. The endosymbiotic bacteria, *Wolbachia* has been successfully introduced in *Aedes aegypti* to block the transmission of the dengue virus (Joshi et al., 2017; Moreira et al., 2009). For example, the presence of bacteria *Serratia marcescens* (Y1 strain) in the mosquito gut, has been found to render *A. stephensi* resistance to *Plasmodium berghei* infection (Bai et al., 2019). Similarly, *in vitro* studies with *Serratia marcescens* bacteria in *Rhodnius prolixus* have also been found to exhibit

trypanolytic activity on *Trypanosome cruzi* (Azambuja et al., 2004). **Table 2** shows varied interaction between various mechanisms through which the insect vector microbiota impacts vector competence especially in mosquitoes. The microbiota, specifically the bacteria are inevitably linked to leishmaniasis (Amni et al., 2023).

Table 2: The impact of gut bacteria on vector competence.

Bacteria	The impact upon vector competence	Reference
EspZ	Generation of ROS results in increased resistance to *Plasmodium* infection in *A. gambiae*	(Cirimotich, Ramirez, et al., 2011)
Serratia marcescens	Blocks sporogonic development of *P. vivax* parasites in *A. albimanus*	(Gonzalez-Ceron et al., 2003)
	Intra-specific diversity determines the level of *P. berghei* inhibition in *A. stephensi*	(Bando et al., 2013)
	Reduces susceptibility of *A. stephensi* to *P. falciparum* infection	(Pumpuni et al., 1996)
Enterobacteriaceae spp.	*P. falciparum* infected *A. gambiae* harbor a greater abundance of *Enterobacteriaceae*	(Boissière et al., 2012)
Enterobacter cloacae	Elevates the expression of *A. stephensi* SRPN6 leading to a heightened immune response to *P. falciparum* infection	(Eappen et al., 2013)
Proteus spp.	Introduction of a *Proteus* species into *Ae. aegypti* results in an increased resistance to DENV, potentially as a result of increased AMP expression.	(Ramirez et al., 2012)
Acinetobacter spp.	Increases resistance of *A. gambiae* to *Plasmodium* development, in part through induction of Imd pathway anti-plasmodium factors	(Bahia et al., 2014)

Table 2: Cont'D

Wigglesworthia glossinidia	PGRP-LB mediated tolerance of *W. glossinidia* impacts the tsetse fly anti-trypanosome immune response	(Wang et al., 2009)
	Presence of the tsetse fly symbiont *W. glossinidia* is required for proper immune function in adulthood	(Pais et al., 2008; Weiss et al., 2011)
Xanthomonas maltophilia *Pseudomonas aeruginosa* *Escherichia coli* *Cedecea lapagei* *Ewingella americana*	Gram-negative bacteria reduce *P. falciparum* infection of *A. stephensi*	(Pumpuni et al., 1993; Pumpuni et al., 1996)
Enterobacter cloacae *Enterobacter amnigenus*	Inhibits *P. vivax* infection in *A. albimanus*	(Gonzalez-Ceron et al., 2003)
Bacillus pumilus *Comamonas spp.* *Escherichia coli* *Enterobacter spp.* *Pseudomonas stutzeri* *Serratia marcescens*	Exposure to bacteria isolated from *A. gambiae* in Cameroon results in a decreased susceptibility to *P. falciparum* infection	(Tchioffo et al., 2013)
Enterobacter ludwigii *Pseudomonas rhodesiae* *Vagococcus salmoninarium*	Exposure to the bacteria results in inhibition of La Cross virus *in vitro*	(Joyce et al., 2011)
Wolbachia – wMel	Increases *Ae. aegypti* resistance to DENV infection	(Rancès et al., 2012; Walker et al., 2011)
	Increases *Ae. aegypti* resistance to CHIKV	(Van Den Hurk et al., 2012)
Wolbachia – wAlbB	Induces ROS mediated activation of the TOLL pathway leading to reduced DENV titers in *A. aegypti*	(Pan et al., 2012)
	Decreases *A. stephensi* susceptibility to *P. falciparum*, potentially through ROS generation	(Bian et al., 2013)
	Increase susceptibility to *P. berghei* infection in *A. gambiae*	(Hughes et al., 2012)
Wolbachia - wMelPop	Decreases *P. berghei* infection level in *A. gambiae*	(Hughes et al., 2012; Kambris et al., 2010)

Table 2: Cont'D

Wolbachia - wMelPop-CLA	Reduces susceptibility of *Ae. aegypti* to DENV, CHICKV and *P. gallinaceum*	(Moreira et al., 2009)
	Reduces *Ae. Aegypti* susceptibility to DENV	(Walker et al., 2011)
	Reduces *Ae. Aegypti* susceptibility to YFV	(Van Den Hurk et al., 2012)-

Pathogen (*Leishmania*) – Vector microbiome interactions

The interaction between *Leishmania* parasites and the gut microbial community of the vector is a crucial determinant of the fate of the parasites within the host. Each time the *Leishmania* parasites encounter the vector's microbiota, a complex interplay occurs, shaping the outcome of their development – whether they progress to the infective metacyclic stage or succumb to death (Telleria et al., 2018). The microbiota profoundly influences the vector's nutrition, digestion, and the maturation of the innate immune system, exerting significant effects on these physiological processes (Dillon & Dillon, 2004; Weiss & Aksoy, 2011). As such, any alteration of the microbiota has the propensity to disrupt the life cycle of the *Leishmania* parasite in the vector's gut (Kelly et al., 2017; Louradour et al., 2017). Similarly, eliminating or changing the gut microbiota can also activate the innate immune mechanisms of the vector which may either permit or inhibit the establishment of an infection by the parasite (Diaz-Albiter et al., 2012).

Researchers are investigating the potential use of either *Bacillus subtilis* or *Brevibacterium linens* to prevent the transmission of *L. donovani* in the sandfly vector *Phlebotomus argentipes*. When sandflies were raised on larval chow containing *B. subtilis*, it was observed that the emergence rates of the flies remained unaffected. However, significant amounts of these bacteria were

found in the gut lumens of the emerging flies (Hillesland et al., 2008; Hurwitz et al., 2011). Further investigations revealed that the sandfly larvae and pupae as well as the adult sandflies that developed in larval chow mixed with PBS had *B. cereus* and *Lys fusiformis* at each developmental stage, suggesting a transstadial passage of indigenous bacterial flora. These symbiotic bacteria have shown interactions with the *Leishmania* parasite during its development (Guernaoui et al., 2011; Kelly et al., 2017).

According to a study that employed a VL mouse model of vector-transmitted *L. donovani*, the bacteria present in the sandfly gut which are egested into the host skin during blood meal triggered a cascade of immunological response necessary for the spread of the *Leishmania* parasite to internal organs (Dey et al., 2018a). *Enterobacter cloacae* and *Bacillus subtilis* complexes are examples of commensal bacteria present in the gut of sandflies (Fraihi et al., 2017; Oliveira et al., 2000). *E. cloacae* and *B. subtilis* possess the capability to regulate an insect's immunological response (Eappen et al., 2013; Zhang et al., 2021). These two bacteria also produce secondary metabolites that show activity against microorganisms harboured by insects (Caulier et al., 2019). Their presence in sandflies depicts the likelihood of a crucial role in the ability of sandflies to transmit *Leishmania* parasites (Louradour et al., 2017). In a study by Amni et al. (2023), the possibility of *L. major* causing an infection wass greatly influenced by the bacteria *E. cloacae* and *B. subtilis*. In this investigation, a needle infection model was used to mimic transmission of *Leishmania* by sandflies in order to examine the variations from the start to the development of *L. major* infection initiated by inoculation with "low" or "high" concentrations of *E. cloacae* and *B. subtilis* bacteria. Amni et al. (2023),

observed that the bacterial co-infection had a significant effect on the expression balance of pro- and anti-inflammatory cytokines at the start, middle, and end of the infection course. In addition, a variation in the local expression of pro- and anti-inflammatory cytokines was seen in mice depending on the type and amount of bacteria inoculum. *L. major* burden remained constant after injecting the two bacteria containing Leishmania parasites in the low-dose group (Amni et al., 2023). However, an increased thickness of the ear pinna and enhanced tissue chronic inflammatory cells were seen. Also, there was a multifold increase in the expression of IL-4 and IL-1 and a decrease in the expression of iNOS (Amni et al., 2003).

In an *in vitro* study, the introduction of *Serratia marcescens* resulted in the lysis of the cell membrane of *L. infantum chagasi* and *L. braziliensis* (Moraes et al., 2009; Moraes et al., 2008). In another *in vivo* study using *Lu.* longipalpis sand flies, there was a reduction in *L. mexicana* infection rate when flies fed on *Pseudozyma sp.*, *Asaia sp.*, or *Ochrobactrum intermedium* (Sant'Anna et al., 2014). In this study, *Lu.* longipalpis sand flies having their gut colonised by *L. mexicana* and fed with *Serratia marcescens* had a significantly higher survival rate compared to the flies without *Leishmania* infection. The presence of the *Leishmania* parasite somewhat protected and offered an extended survival rate to the sandflies. In another study, the richness and diversity of the gut microbiota of *P. evansi* was observed to have decreased when treated with antibiotics however the *Leishmania* infection in the gut increased (Vivero et al., 2021). This supports the theory that suggests that any decline in the size and/or diversity of the microbial community of the sandflies

tend to enhance its vectorial capacity and the ability of the *Leishmania* to establish infections (Dong et al., 2009).

Biting midges

Biting midges are amongst the world's tiniest hematophagous flies, belonging to the, family *Ceratopogonidae* and Order *Diptera* with sizes ranging from 1 to 3 mm (Mellor et al., 2000). In various English-speaking jurisdictions, they are called gnats, midges, punkies or no-see-ums (Hill & MacDonald, 2008). Classification and differentiation of biting midges are done based on their wing patterns. They have distinctive wings of grey and white patterns (Boorman, 1993; Meiswinkel et al., 2004). With the exception of Antarctica and New Zealand, over 1400 species of biting midges belonging to thirty-eight (38) subgenera have been identified in the world (Borkent, 2005; Mellor et al., 2000; Venter, 2007). About ninety-six per cent (96%) of these identified midges are obligatory blood feeders. They suck the blood of mammals including humans, reptiles, avians, and even blood-engorged mosquitoes (Mellor et al., 2000). Midges are best known as arbovirus vectors although they can also transmit protozoans and filarial nematodes to mammals, avians and other animals (Linley, 1985; Meiswinkel et al., 2004; Mellor et al., 2000). Some midge species cause seasonal recurring dermatitis (sweet itch) upon biting (Braverman, 1988).

Description and Life Cycle of Biting Midges

Biting midges go through the standard holometabolous life cycle of egg, larvae, pupa, and adulthood except for females. The females are haematophagous and hence require blood to complete the gonotrophic cycle (Venter, 2007). Most biting midges species breed in only moist low-lying areas (Venter, 2007). Environments with enough moisture must exist because this

affects the distribution, abundance, and seasonal occurrence of the midges (Mellor et al., 2000). Their eggs are laid on different moist soil because of their susceptibility to drying out (Hill & MacDonald, 2008; Venter, 2007). The eggs measure about 0.25mm in length and are shaped like bananas. When laid, the eggs are originally white but eventually turn black or brown. Depending on the species and the climate, eggs can hatch in 2 to 10 days (Hill & MacDonald, 2008; Nevill, 1969; Nevill, 1967; Venter, 2007). The 2 to 5mm long larvae have a worm-like form and a creamy white colour. They go through four developmental instars which takes place in various semi-aquatic or aquatic habitats based on the species (Dyce & Marshall, 1989; Hill & MacDonald, 2008; Meiswinkel et al., 2004; Nevill, 1968; Nevill et al., 2007). The development of the larva may take two weeks to a year to complete depending on the temperature and availability of food (Hill & MacDonald, 2008). The pupa is 2 to 5mm in length and either light/dark brown or pale yellow. The pupal stage is mostly found at the last larval stage (Hill & MacDonald, 2008). Within 2-3 days after the pupa stage, the adults emerge. The pupa of all the species; except *Culicoides imicola* wriggle free of the breeding medium and happen to float to the surface (Venter, 2007). The pupa also possesses a pair of respiratory horns on its unsegmented cephalothorax, which may have spines or wrinkles on them. The fly's spiny integument at this stage allows for species-level identification. Adult midges have two wings covered in dense hair and a grey appearance. The dense hair gives rise to the distinctive coloured patterns in the wings that are used for identification and taxonomical classification. Adjacent to the bases of the 15-segmented antennae are the large compound eyes. The Johnston's organ is located in the pedicel of the males' antennae. The mouthparts

are well-developed with cutting teeth on extended mandibles in the proboscis that are specialized for sucking blood in females but not in males. The abdomen is nine-segmented and tapering at the end, while the thorax extends just over the head. The female adult biting midge can live up to 63 days and is dependent on the temperature of its habitat (Hill & MacDonald, 2008; Nevill, 1971).

Evidence suggesting biting midges as potential vectors for *Leishmania* transmission

There is a common criterion that must be met in order to implicate a vector for leishmaniasis transmission as outlined by Dougall et al., (2011) and Killick-Kendrick, (1999). Briefly, the suspected vector must meet the following criteria: (1) the suspected vector species or fly and the reservoir host (human or animal) must be present in the same environment; (2) the vector species bites the reservoir host; (3) the suspected vector species or fly must have the capacity to sustain the development of parasite life stages after the infecting blood meal has been digested to the transmissible infective metacyclic stage; (4) the parasitic isolates from wild-caught vectors and the reservoir host are identical; (5) the vector is able to transmit the parasite by a bite to the reservoir and other hosts (Dougall et al., 2011; Killick-Kendrick, 1999).

Numerous studies have been conducted to implicate arthropods such as midges, ticks, fleas, and other flies as alternative vectors for leishmaniasis (Coutinho et al., 2005; Coutinho & Linardi, 2007; Otranto & Dantas-Torres, 2010), despite the fact that phlebotomine sandflies are the disease's traditional vector. After a blood meal, these suspected vectors are typically unable to support the development of parasite life stages (Coutinho et al., 2005; Coutinho & Linardi, 2007; Otranto & Dantas-Torres, 2010). This is due to the intricate

and unique vector-parasite relationship between *Leishmania* species and its vector (Panahi et al., 2020). After a blood meal, the parasite goes through several developmental stages within the vector before reaching the infective stage, which is transmitted at the subsequent blood meal (Bates, 2007; Kamhawi, 2006).

Evidence suggests that the growth of Leishmania life stages is supported by several species of biting midges following a blood meal (Panahi et al., 2020; Seblova et al., 2014b). Studies conducted to suggest an alternative vector have failed to provide sufficient evidence of parasite transmission via biting to satisfy the fifth Killick-Kendrick criterion; the alternate vector transmits the parasite by a bite to the reservoir and other hosts (Seblova et al., 2014c). The discovery of an alternate *Leishmania* vector is very important and changes the current knowledge and understanding of the *Leishmania* life cycle (Dougall et al., 2011). Many biting midges have been found during entomological surveys in regions where leishmaniasis transmission is active (Rebêlo et al., 2016). This brings on board the possibility that biting midges may contribute to the spread of leishmaniasis (Rebêlo et al., 2016).

Two different genera of biting midges; *Forcipomyia* (*Lasiohelea*) and *Culicoides* have been implicated to possess vectorial capacity for the transmission of leishmaniasis. Dougall et al., (2011) and Panahi et al (2020) have conducted studies in Northern Australia to identify the probable vector for *Leishmania* transmission. The presence of *Leishmania* DNA was screened for in both the known vector, sand flies, and the putative vectors, day-feeding midges of the genus *Forcipomyia* (*Lasiohelea*), and night-feeding midges (*Culicoides* spp.) (Dougall et al., 2011). Both the day and night-feeding midges

had parasite DNA that had been amplified. Only the day-feeding midge, however, was able to meet almost all of the requirements for vector incrimination. The day-feeding midges in the endemic area tested positive for *Leishmania* DNA, and several of them had significant parasitemia. Using microscopy, *Leishmania* promastigotes and several artefacts resembling promastigote secretory gel, including parasites with metacyclic-like shapes, were visible after dissection of the day-feeding midges. The *Leishmania* parasite that was isolated from the midges was the same as the isolated *Leishmania* species from the sandflies (Dougall et al., 2011). Panahi et al. (2020) were able to offer proof in support of satisfying the fourth (the parasitic isolates from wild-caught vectors and the reservoir host are identical) and fifth requirements (the vector is able to transmit the parasite by a bite to the reservoir and other hosts). To demonstrate that the infected day-feeding midge deposits the *Leishmania macropodum* during feeding, FTA cards were employed. Additionally, parasites have a maximum survival time of 7 days in the vector. These data demonstrate that the biting midges may be able to promote parasite growth following a blood meal.

Infection studies using *L.* (*Mundinia*) spp. have been successfully established in *Culicoides* (Diptera: *Ceratopogonidae*) models (Chanmol et al., 2019; Kwakye-Nuako, 2016; Seblova et al., 2014c; Seblova et al., 2015). According to studies by Kwakye-Nuako (2016), midges can serve as vectors for the *Leishmania* parasite and may successfully transmit *L. chancei* species in Ghana. In the membrane feeding apparatus, *Lutzomyia longipalpis* and midges (*Culicoides sonorensis*) were made to feed on a prepared chicken membrane that had been filled with 2ml of blood meal. To establish an infection in the

potential vectors within 7 days, the flies were examined 1 – 10 days post-blood meal. After the third day, *Lutzomyia longipalpis* showed no signs of infection. However, *Culicoides sonorensis* were severely infected with the human pathogenic *L. chancei* (without metacyclic promastigotes), colonizing the midgut and stomodeal valve up to and even past day 10 of infection. The evidence provided by Kwakye-Nuako (2016) was reinforced by studies by Becvar et al. (2021). Following a blood meal through a chicken skin membrane, five different *Mundinia* species; *L. enrietti, L. marcropodum*, *L. orientalis, L. chancei* and four strains of *L. martiniquensis* (MAR1, CU1, Cu2 and Aig1), were exposed to *Culicoides sonorensis*. All *Mundinia* species evaluated at post-blood meal had a high rate of infection established, were able to successfully colonize the stomodeal valve, and produced a larger percentage of metacyclic forms in *C. sonorensis*. In addition, a bite from *C. sonorensis* introduced three parasite species; *L. martiniquensis, L. orientalis*, and *L. chancei* to the host mouse's ear (Becvar et al., 2021).

In a study using *C. nubeculosus* and *C. sonorensis*, *C.nubeculosus* was not susceptible to infection by parasites (*Leishmania enriettii* and *Leishmania* sp. AM-2004) from the *L. enriettii* complex (Seblova et al., 2015). Contrarily, *C. sonorensis* produced late-stage infections with *L. enrietti* and colonized the stomodeal valve and thoracic midgut. Up to 80% of the *C. sonorensis* carrying infection with *L. enrietti* that was made to feed on guinea pigs' ears and noses developed an infection.

In Thailand, human leishmaniasis is caused by *L. (Mundinia) orientalis* (Chanmol et al., 2019). Under laboratory conditions, *C. sonorensis* was infected with *L. (Mundinia) orientalis* and after 7 days of the post-blood meal,

metacyclic promastigotes had colonized the stomodeal valve and mixed with leptomonad promastigotes producing what resembled a promastigote secretory gel (Chanmol et al., 2019). In cutaneous leishmaniasis, endemic areas in the Brazilian Amazonia, the DNA of *Leishmania amazonensis* and *Leishmania braziliensis* has been found in several *Culicoides* species (Rebêlo et al., 2016). In Tunisia, there is a report on the detection *of Leishmania infantum* DNA in wild-caught *Culicoides spp.* for the first time (Slama et al., 2014). Out of the 259 midges collected, belonging to 7 different *Culicoides* spp., *Leishmania* DNA was detected in only 2 of *Culicoides* spp, 14 *C. imicola* and one *C. circumscriptus*. In Ghana, the biting midges are very abundant in CL endemic communities and are known for their nuisance biting.

Wolbachia

The Gram-negative alphaproteobacteria, *Wolbachia*, is of the order *Rickettsiales* and family *Anaplasmataceae* (Dumler et al., 2001). Based on gene sequence data, there are 13 supergroups -(A-F and H-N) of *Wolbachia* (Augustinos et al., 2011). Ten of these major clades are found in arthropods whereas three are in nematodes (Augustinos et al., 2011; Casiraghi et al., 2005; Haegeman et al., 2009). In arthropods, the two most prevalent *Wolbachia* clades are supergroups A and B (De Oliveira et al., 2015). Several reports have indicated that *Wolbachia* is a part of the natural microbiome of many insects from various geographical regions.

Wolbachia as an endosymbiotic bacterium has been identified in several insects (do Rosário et al., 2022). According to studies, *Wolbachia* is present in as many as 76% of insects (do Rosário et al., 2022; Hilgenboecker et al., 2008; Jeyaprakash & Hoy, 2000; Werren, Windsor, et al., 1995). Species belonging to

the order *Coleoptera*, *Diptera*, *Hymenoptera*, *Lepidoptera*, and *Orthoptera* have been identified to have the bacteria. About 17% of the examined insect species from Panama belonging to the *Coleoptera*, *Diptera*, *Hemiptera*/*Homoptera*, *Hymenoptera*, *Lepidoptera*, and *Orthoptera* were found to be infected with *Wolbachia* (Werren, Windsor, et al., 1995). Similar investigations conducted in the UK and North America reported 22% and 19.3% of insects from various orders that were *Wolbachia*-infected (De Oliveira et al., 2015; West et al., 1998).

It is well known that *Wolbachia* has a variety of symbiotic relationships with its host, especially in insect vectors. (O'Neill et al., 1992; Werren et al., 2008). It has the capacity to act as a biological control for vectors of several diseases and has been widely explored for disease prevention. For example, Infection in *C. sonorensis* (biting midges) with *w*AlbB *Wolbachia* significantly reduced the replication of bluetongue and epizootic hemorrhagic fever viruses (Matthews et al., 2022). Varied hosts have different interactions with the various *Wolbachia* strains (Dennison et al., 2014; Matthews et al., 2022). In some cases, the *Wolbachia*–host interaction results in an extended lifespan of flies allowing the vectors to sustain the developmental stages of the pathogen they carry (Bi & Wang, 2020). In other instances, the presence of *Wolbachia* has been noted to decrease the lifespan of adult female vectors curtailing their ability to transmit infections (Bruner-Montero & Jiggins, 2023). Other studies carried out in insect vectors revealed that Wolbachia-vector interactions result in increased host resistance to infectious pathogens by offering host defence against RNA viruses and lead to cytoplasmic incompatibility (CI) (Boakye et al., 2005; Hedges et al., 2008; Moreira et al.,

2009; Teixeira et al., 2008). Due to its capacity to cause a variety of reproductive phenotypes in arthropods, *Wolbachia* is known as a reproductive parasite (Werren, 1997; Werren et al., 2008).

Wolbachia has also been associated with male-killing (Hurst & Jiggins, 2000), cytoplasmic incompatibility (O'Neill et al., 1992), phenotypic feminization of genetic males (Rousset et al., 1992) and parthenogenesis (Weeks & Breeuwer, 2001) in insects. *Wolbachia*'s prevalence and method of interspecies transmission determine how important it is in reproductive processes (Stouthamer et al., 1999). It is believed that among arthropods, transmission is largely maternal within species (De Oliveira et al., 2015), although there is evidence of horizontal transmission between species over longer evolutionary timeframes (Schilthuizen & Stouthamer, 1997; Werren, Zhang, et al., 1995). They are also believed to be essential in local adaptation and speciation (Brucker & Bordenstein, 2012). *Wolbachia* is important for the maintenance of the ecosystem, serves as agricultural pests and disease vectors, is useful in medicine and scientific study, and in some cultures, serves as a food with a high commercial value (De Oliveira et al., 2015).

***Wolbachia-Leishmania* interactions**

Wolbachia has been found in leishmaniasis-approved vectors as well as potential vectors. In sandflies, it has been identified in the genera *Phlebotomus* and *Lutzomyia*. In studies by Ono et al. (2001), 53 samples of sandflies from 15 different species were examined for the presence of *Wolbachia* using wsp gene primers. A total of 7 samples from 4 species: *P. papatasi, P. perniciosus, Lutzomyia shannoni*, and *Lutzomyia whitmani*, were positive for *Wolbachia* (Ono et al., 2001). Sandflies of the genus *Lutzomyia*

collected from a leishmaniasis hotspot on the Caribbean coast of Colombia were screened using the wsp gene primer (Vivero et al., 2017). The endosymbiotic *Wolbachia* were detected in 3 species of sandflies: *Lutzomyia c. cayennensis, Lutzomyia dubitans,* and *Lutzomyia evansi* and was absent in *Lu. Rangeliana, Lu. Trinidadensis, Lu. Gomezi,* and *Lu. Atroclavata*.. Of the 3 species positive for *Wolbachia*, a relatively low *Wolbachia* infection (8.5%) was observed in *Lu. dubitans* and *Lu. c. cayennensis* (3 positives; out of 11 pools for both species) (Vivero et al., 2017). In *Lu. evansi*, there was only 1 positive pool; 2.8%. In addition, two *Wolbachia* strains (genotypes) from the B Supergroup, wLcy and wLev, were discovered in *Lutzomyia spp.*, that was in *Lu. c. cayennensis*. In this same study, 171 individual insects were screened for *Leishmania* infection, and they were all negative (Vivero et al., 2017). Sandflies captured in a region of Brazil where visceral leishmaniasis is endemic were examined for *Wolbachia* (Da Rocha et al., 2018). A total of 5 (2.5%) sandflies, consisting of 3 males and 2 females, tested positive for *Wolbachia* out of the 200 sandflies (62 females and 138 males) classified as *Lu. longipalpis*. In some studies where the infection rates of *Wolbachia* were low, it was attributed to the endosymbionts now adapting to the sandfly host (Da Rocha et al., 2018). Also, insufficient geographical sampling may result in an underestimated rate of infection (Duron et al., 2008). Another possible explanation was the host characteristics differences such as immunology, physiology and microbiota (Da Rocha et al., 2018).

A study was conducted to detect *Wolbachia* and *Leishmania* infections in about 437 sandflies collected in Panama (Azpurua et al., 2010). Twenty (20) different species of sandflies were morphologically identified. Two species, (*Lu.*

vespertilionis and *Lu. sp. nr vespertilionis*) were infected with only *Wolbachia*. Also, *Leishmania* infection was detected in only a single species, *Lu. gomezi*. However, *Lu. Trapidoi* species showed infection with both *Wolbachia* and *Leishmania*. The infection rate of *Wolbachia* in this study was low compared to other studies. This was linked to reduced vertical maternal transmission of the bacteria. However, the coinfection of *Lu. trapidoi* with *Wolbachia* and the *Leishmania* parasite proposes a vector target for the introduction of *Wolbachia* populations into sandflies as a disease control measure (Azpurua et al., 2010).

Spanish *Culicoides spp*. populations were examined for the presence of *Wolbachia* endosymbionts in both livestock premises and natural environments using single specific PCR by amplifying specific regions of the wsp gene (Muñoz-Muñoz et al., 2017). *C. imicola, C. obsoletus (s.s.), C. pulicaris (s.l.), C. vexans, C. kibunensis* and *C. heteroclitus* were all positive for the presence of Wolbachia. *C. imicola, C. obsoletus (s.s.)* and *C. pulicaris (s.l.)* are of major epidemiological relevance as they are potential Palaearctic vectors for the bluetongue and Schmallenberg diseases. The infection prevalence of *Wolbachia* in the *Culicoides* species was very low in *C. imicola* (6 out of 256 samples) and *C. obsoletus* (2 out of 466 samples) but relatively high in *C. pulicaris* (56 out of 309). Low levels of infection with *Wolbachia* were observed in 10 species of *Culicoides* in a similar study that screened 20 species of *Culicoides* using both traditional PCR and more sensitive quantitative PCR (qPCR) techniques (Mee et al., 2015). qPCR was used to detect *Wolbachia* in all 10 of the 20 species that tested positive for *Wolbachia*. This implies the sensitivity of the technique used in screening is important.

The interaction between *Wolbachia* and *Leishmania* remains to be studied in detail. For example, to find out whether *Wolbachia* could offer some protection and reduce the number of adherent parasites in sand fly cells, a study with *L. infantum* was conducted (da Silva Gonçalves et al., 2019). Much difficulty was encountered with the introduction and establishment of *Wolbachia* into sand fly cells. Nevertheless, the wMelPop strain was successfully introduced and maintained in Lulo and LL-5 cell lines. *Wolbachia* provided a superior model in Lulo cell during in vitro studies in sandflies. Also, in using Lulo cells to study the interaction with *L. infantum*, it was observed that *Wolbachia* did not have a negative impact on *L. infantum* adhesion in *in vitro* infection experiments.

Ehrlichia

Ehrlichia,the genus name for several obligatory intracellular Gram-negative bacteria species that can cause infection in humans and other mammals such as dogs. The species of *Ehrlichia* that cause infection in humans are *Ehrlichia chaffeensis* and *Ehrlichia ewingii* which cause human monocytic ehrlichiosis (HME) and human ewingii ehrlichiosis respectively (Dumler et al., 2007). The two other species *Ehrlichia canis* and *Ehrlichia ruminatium* are primarily veterinary pathogens but can occasionally cause infection in humans (Murphy et al., 1998; Snowden et al., 2017). Tick is the vector known to transmit the intracellular Gram-negative bacteria *Ehrlichia* through its bite during a blood meal (Dumler et al., 2007). *Ehrlichia* has a wide variety of hosts, including humans, dogs, rodents, deer, cattle, sheep and goats (Snowden et al., 2017). Replication of *Ehrlichia* occurs in both the vector; ticks and infected hosts (Snowden et al., 2017).

Ehrlichia-Leishmania interactions

There are remarkable similarities between the bacteria *Ehrlichia* and the *Leishmania* parasite. Both organisms result in human infections (causes zoonotic infections; leishmaniasis and ehrlichiosis), are transmitted by arthropods (vector-borne) and have similar reservoir hosts (Atanaskova et al., 2011; Dantas-Torres, 2007; Dumler et al., 2007; Snowden et al., 2017). *Ehrlichia* is transmitted by ticks via a bite and is very common in dogs. *L. infantum* and *L. braziliensis* are the two most common forms of the *Leishmania* parasite in dogs but also cause human infection (Atanaskova et al., 2011; Dantas-Torres, 2007).

The clinical and haematological symptoms of leishmaniasis and ehrlichiosis are remarkably similar. For example, *Ehrlichia canis* and *Leishmania infantum*, are intracellular pathogens that infect the monocyte/macrophage cells (Atanaskova et al., 2011). The antibodies against *E. canis* cross-react with the *Leishmania sp*. antigen found in serological tests used to identify canines infected with *Leishmania sp*. This makes establishing a differential diagnosis challenging (Stefanovska et al., 2012). A case report indicated that a female Samoyed was found infected with canine monocytic ehrlichiosis (CME) and canine leishmaniasis caused by *E. canis* and *Leishmania infantum* respectively (Atanaskova et al., 2011). This showed that dogs could be coinfected by *Ehrlichia* and *Leishmania*. Leishmaniasis and ehrlichiosis are zoonotic and vector-borne diseases.

With humans and dogs both serving as reservoir hosts for both organisms, sandflies can pick up the *Ehrlichia* together with or without the *Leishmania* parasite during a blood meal. This could result in ehrlichiosis if the

bacteria are transmitted by the sandfly at the subsequent blood meal. Also, microbiota interaction between the bacteria and the *Leishmania* parasite can affect the development of the parasite as they both fight for space and nutrients. Currently, there are no reports on the detection of *Ehrlichia* in sandflies and biting midges. However, considering that dogs serve as reservoir hosts for both leishmaniasis and ehrlichiosis, it is crucial not to underestimate the potential for *Leishmania* vectors to acquire the bacteria from dogs during a blood meal. Such transmissions may result in an alteration in the gut microbiota of the vectors, parasite development and ultimately lead to the possibility of transmitting both pathogens during a subsequent meal. Furthermore, whether *Ehrlichia* can be potentially used as a biological control for *Leishmania* parasites within the vectors remain to be elucidated.

Ochrobactrum

The genus *Ochrobactrum* are Gram-negative, non-fermenting and opportunistic bacteria. The *Ochrobactrum* can colonize an incredibly wide variety of habitats including water, soil, plants and animals (Bathe et al., 2006; El-Sayed et al., 2003; Handschuh et al., 2017; Möller et al., 1999). There are about 21 accepted species of the genus *Ochrobactrum* including *O. anthropi, O. intermedium, O. grignonense, O. tritici, O. gallinifaecis, O. lupini, O. oryzae, O. cytisi, O. pseudintermedium, O. haematophilum, O. pseudogrignonense, O. cicero, O. daejeonse, O endophyticum, O. teleogrylli* and *O. pecoris* (Ryan & Pembroke, 2020). *O. anthropi, O. intermedium,* and *O. pseudintermedium* — have been isolated in clinical samples and are clinically relevant (Kämpfer et al., 2007). In healthy people, the strains of *O. anthropi* and *O. intermedium* have been reported to cause severe systemic infections that mimic a Brucella

infection. They have also been linked to invasive medical interventions like indwelling catheters in patients with an underlying disease. Both *O. anthropi* and *O. intermedium* are classified as biosafety level 2 agents. They cause serious infections such as endocarditis and septicemia in immunocompromised individuals (Kettaneh et al., 2003; Ozdemir et al., 2006). Recent studies show a variety of infections resultant from *Ochrobactrum* species and also resistant to β – lactams and many antibiotics (Ryan & Pembroke, 2020). *O. anthropi*, one of the clinically relevant species, is gaining attention as a potentially dangerous, opportunistic, and nosocomial pathogen (Chain et al., 2011; Romero Gomez et al., 2004).

***Leishmania- Ochrobactrum* interactions**

Ochrobactrum sp, known to be pathogenic to humans has been found in both Old and New world sandflies (Louradour et al., 2017; Monteiro et al., 2016; Volf et al., 2002). Studies suggest *Ochrobactrum* exhibiting transstadial passage in *Phlebotomus duboscqi* (Volf et al., 2002). This was observed in a study where *Ochrobactrum sp* was isolated from the gut of different developmental stages of *Phlebotomus duboscqi* from the fourth instar larvae just before pupation, the gut of females ready to emerge from pupae as well from midgut and hindgut of newly emerged adult females (Volf et al., 2002). In the study, the majority of the isolates were phenotypically identified as *Ochrobactrum sp*. and genotypically, they belonged to a single strain. This was observed more in the blood-fed sandflies than in the sugar-fed sandflies. In unfed wild and laboratory-reared female sandflies in Turkey, *Ochrobactrum* has been found to be present in their guts (Karakuş et al., 2017). *Ochrobactrum* has also been found in the American sandfly, *Lutzomyia intermedia* under different

physiological conditions including blood-fed, non-blood fed and the presence of developed ovaries (Monteiro et al., 2016). In a culture-dependent analysis of the gut bacteria of *P. duboscqi*, the Gram-negative *Ochrobactrum* sp. was very dominant (Louradour et al., 2017). *O. intermedium* has also been identified in wild-caught and laboratory-reared female *Lutzomyia longipalpis* (Sant'Anna et al., 2014). In this study, the colonization resistance of *Leishmania* parasite in the presence of O. *intermedium* in the gut of *L. longipalpis* was assessed. This was done by feeding the flies with chick membranes infected with the bacteria/parasite. After 72 hours of *Leishmania* infection, the sandflies were dissected and examined for the number of promastigotes and a decline in parasite survival was observed.

Tsukamurella

Tsukamurella is a genus of aerobic actinomycetes containing unsaturated mycolic acid and a series of long chains (Safaei et al., 2018b). They are environmental saprophytes which are isolated from soil, arthropods, water, sludge foam, and sponges (Safaei et al., 2018b). *Tsukamurella* is a genus of aerobic actinomycetes (Safaei et al., 2018b). and was originally isolated from the bedbug in 1941 (Steinhaus, 1941). *Tsukamurella* spp. are a type of saprophyte bacterium, that rarely infect humans. The majority of the knowledge about human infections comes from case reports (Safaei et al., 2018b). There are now eleven (11) species in the genus *Tsukamurella* (Safaei et al., 2018b). Nine of the species; *T. inchonensis, paurometabola, strandjordii, tyrosinosolvens, pulmonis, hongkongensis,* and *sinensis*, have been isolated from infection in humans (Cheung, 2014). *Tsukamurella* infections are classified as nosocomial or sporadic infections. According to the many case

reports, bacteremia, meningitis, peritonitis, keratitis, cutaneous infections, conjunctivitis, brain abscesses, respiratory tract infections, catheter-related bloodstream infections, and acute otitis media are the most often documented sources of infection (Prinz et al., 1985; Safaei et al., 2018b; Schwartz et al., 2002; Sheng et al., 2009). They can lead to colonization in immunocompromised people (Dworkin, Falkow, Rosenberg, Schleifer, et al., 2006). *Tsukamurella* infection has long been linked to lung disease in persons with immune deficiencies (Safaei et al., 2018b). Amikacin, ciprofloxacin, imipenem, doxycycline, linezolid, and sulfamethoxazole are all effective treatments for *Tsukamurella* (Conville & Witebsky, 2015). *Tsukamurella* spp. are resistant to penicillin, oxacillin, piperacillin/tazobactam, and cephalosporins (Savini et al., 2012). A combination of β -lactam or macrolide antibiotics with aminoglycoside antibiotic drugs for a prolonged therapy period produced effective outcomes. spp (Safaei et al., 2018b).

***Leishmania* - *Tsukamurella* interacactions**

There are very few studies caried out on the bacteria *Tsukamurella* in insect vectors. A study was conducted in Campo and Bipindi, two southern Cameroonian towns where trypanosomiasis is endemic (Tsagmo Ngoune et al., 2019). The bacterial diversity of *Glossina palpalis palpalis*, a vector for the transmission of human African trypanosomiasis and African animal trypanosomiasis, was investigated. Although some tsetse flies caught in the campo region had *Tsukamurella* found in them, the prevalence overall was very low (<0.1%). The existence of *Tsukamurella* was linked to the fact that tsetse flies are not exclusively hematophagous because they can feed on plant nectar hence the possibility of harbouring bacteria related to plants.

Leishmaniasis is one of the topmost NTDs. Unfortunately, there are no viable vaccines available but very limited anti-leishmaniasis treatments for human leishmaniasis. Until vaccinations are developed, vector control techniques will remain one of the most effective preventative methods (Tabbabi et al., 2022). The vector's microbiome happens to be one of the promising fields to explore. This study therefore sought to investigate the presence of selected bacteria; *Wolbachia*, *Ehrlichia*, *Ochrobactrum* and *Tsukamurella* in the two potential vectors, biting midges and sandflies, caught in a *Leishmania*-endemic community in Ghana. In addition, the study sought to detect the presence of *Leishmania* parasite in the putative vectors. This will provide data on possible bacteria targets for biological vector control as well as the possibility of bacteria coinfection with *Leishmania* or bacterial secondary infection after CL infection.

CHAPTER THREE

METHODOLOGY

Introduction

This study sought to identify the presence of selected bacteria; *Wolbachia*, *Ehrlichia*, *Tsukamurella* and *Ochrobactrum* as well as *Leishmania* parasite from potential vectors; sandflies and biting midges, responsible for CL transmission in Ghana. This chapter covers the materials and methods used to reach the objectives of this study. It describes the sampling site and explains the sampling procedure, laboratory activities and data analysis procedure.

Sampling Site

Biting midges and sandflies were collected from the Dodome-Awuiasu community (6°49'46.5"N 0°28'51.7" E) in Ho Municipality in the Volta region of Ghana, one of Ghana's sixteen administrative regions. The community is endemic for leishmaniasis with several cutaneous leishmaniasis cases identified (Doe et al., 2019; Doe et al., 2020; Kwakye-Nuako, 2016). Dodome-Awuiasu is a rural area located about 30 kilometres away from the region's capital, Ho. It is sandwiched between two mountains and surrounded by semi-deciduous forests. It is located at an altitude of 960m above sea level with an average temperature between 22-30°C. The community has an estimated population of 2,000. Residents of Dodome-Awuiasu are known for their engagement in cash crop production, especially palm nut oil, bananas and plantain. There is also a clinic and two basic schools in the town.

The Volta region is also located in the country's southeast and is bordered to the east by the Volta Lake, south by the Atlantic Ocean, west by Togo, and north by the Oti region. It is situated 150m/490ft above sea level,

between latitudes 6°36'43"N and 0°28'13"E. The Ho municipality serves as the region's capital and has 25 districts. The region covers a total land area of 2,361 square kilometres. The region is divided into three different climate and vegetation zones: the northern Savannah, mangrove swamps, and adjacent arid coastal plains. Agriculture, fishing, tourism, and animal husbandry make up the majority of the region's economic activity.

Figure 1: Pictures of different areas in the Dodome-Awuiasu community located in the Volta region of Ghana (A) Signpost of Dodome Awuiasu community located at the entrance to the community (B) Major road that link the community to Ho, the capital of the Volta region (C) the main and only community health centre that serves the health needs of the inhabitants in the community (D-F) represents other locations within the community.

Collection of sandflies and *Culicoides* biting midges

Biting midges and sandflies were collected using CDC light traps, aspirators (handheld and mouth aspirators) and sweep nets. *S*ampling using either the handheld/mouth aspirators were done twice daily, at dawn and dusk whiles the CDC light traps were set overnight from dusk to dawn (5.00 pm -

5:30 am). The overnight CDC light traps were set outdoors in residents' homes, pens of livestock belonging to the inhabitants, tap areas where inhabitants sourced water and school premises. These locations and duration were selected because it is believed that the putative vectors may feed while the host (human or animals) are sleeping or undertaking routine activities such as fetching water and late-night discussions outdoors. For dawn and dusk sampling, the handheld aspirators were used to collect sandflies in residents' homes, community eating area (drinking bar), health facility and pens of livestock belonging to the inhabitants. The sweep nets and mouth aspirators were used for collection of biting midges at tap areas where inhabitants sourced water, school premises, clinic and open space where people usually gathered. These sampling times were used because it was observed that the flies were most abundant at dusk (4.30 pm -6.00 pm) and at dawn (4.30 am -5.30 am) in the community. The collected insects were freeze-killed at -4°C, sorted into labelled 1.5ml Eppendorf tubes containing 70% ethanol and then transferred from the field to the laboratory for further analysis. The insects were placed in ethanol to keep them sterile and also for preservation.

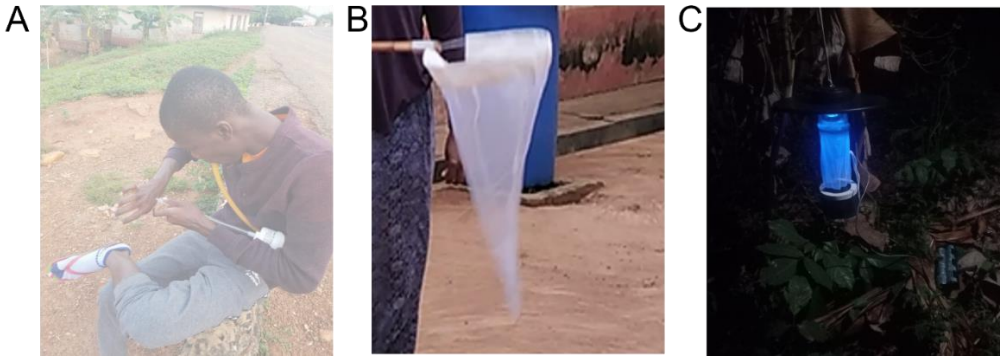

Figure 2: Pictures of equipment used for sample collection: (A) Mouth aspirator being used for human landing catches of biting midges, (B) Sweep net being used for collection of biting midges in an open area such as the forecourt of the

clinic or tap area where inhabitants of the community gather, (C) CDC UV traps set in the farm close to a goat pen to trap insects overnight.

Extraction of DNA from flies

Prior to DNA extraction, only the female sandflies were sorted from their male counterparts because only the female flies are known to feed on humans/animals and transmit leishmaniasis. Sandflies or biting midges, in ethanol, were placed on a glass slide, viewed under a microscope and sorted according to their sex. The female sandflies were differentiated from the males under the dissection microscope based on their size. The female sandflies are slightly larger than the males and have thick rounded abdomen while the males are thinner and possess distinct genital claspers (Doe et al., 2020). The biting midges were identified based on their distinctive wing pattern. The females were also identified from the males based on their distint antennae. The antennae of male midge possess many flagellomeres (segments) and whorls of lengthy setae giving them a plumose appearance, as opposed to the antennae of female midges, which have fewer, shorter flagellomeres and more sparse setae (Langton & Pinder, 2007). The sandflies and biting midges were put into pools of five and ten respectively in labelled 1.5 ml Eppendorf tubes containing phosphate-buffered saline (PBS). The flies were pulled in pools in order to increase the concentration and quantity of DNA due to their small size which will in turn increase the probability of molecularly detecting the bacteria or parasite of interest if present. The number of sandflies to midges in groups was because of the sizes; the sandflies are larger compared to the biting midges. The flies were then washed in the phosphate-buffered saline (PBS) to wash off the ethanol in order to prevent any inference with the extraction process. DNA was

extracted from the insects using the Qiagen extraction kit following the manufacturer's protocol with a few minor modifications.

The samples were washed twice with 150µl of PBS. 180µl of buffer ATL was added to the samples after the second wash and homogenized using a plastic pestle. Subsequently, 3µl of Proteinase K was added to the mixture and homogenized again using a plastic pestle. The homogenate was then vortexed vigorously for 20 seconds and incubated at 56°C for 5 hours in order to ensure complete lysis of tissue. The samples were vortexed intermittently during the 5-hour incubation period. Following incubation, the sample was vortexed for 15 seconds. 200µl of buffer AL was then added and mixed thoroughly by vortexing. 200µl of absolute ethanol (100%) was added to the mixture and vortexed thoroughly for 20 seconds. The entire mixture was pipetted onto a 2ml collection tube containing a DNeasy mini spin column, and centrifuged at 13,000 rpm for 1 minute. The flow-through and collection tube were discarded, and the DNeasy mini spin column was placed in a new collection tube. Afterwards, 500µl of buffer AW1 was pipetted into the DNeasy mini spin column and centrifuged at 13,000rpm for 2 minutes. Again, the flow-through and collection tube was discarded, and the DNeasy mini spin column was placed in a new collection tube. A total of 500µl of buffer AW2 was pipetted into the DNeasy mini spin column and centrifuged at 13,000 rpm for 3 minutes. The spin column was air-dried for 10 minutes at room temperature after which extracted DNA was eluted with 50 µlof molecular-grade water and incubated overnight. Arbitrary quantification of 10 different samples gave a concentration of 10-25ng of DNA per µl hence no further dilution was conducted and we proceeded to PCR analysis.

The extracted DNA was stored at -20ºC for further analysis.

Polymerase chain reaction and agarose gel electrophoresis

Five different 16s bacteria primers were used to amplify different bacteria. The *Wolbachia-Ehrlichia* (W-E F/R) primer amplifies *Wolbachia*, *Ehrlichia* and other intermediate bacteria. The other primers were for specific bacteria; *Wolbachia* (ftsZF1/R1), *Ehrlichia* (EHRl6SDF/R), *Tsukamurella* (LPW 57/58) and *Ochrobactrum* (OchroF1/R1). Also, the primers AM1/2 and BN1/2 were used to amplify the *Leishmania* DNA in the samples. The PCR was carried out in a 18µl reaction mixture containing 10µl of Platinum Superfi PCR Masrermix, 4µl of nuclease-free water and 1µl each of primers, Dimethyl sulfoxide (DMSO) and DNA template. Table 1.1 provides the cycling conditions and expected band size for the respective primers.

Following the PCR, 6µl of the amplicons were separated on 1.5% agarose gel. Agarose powder was used for the preparation of gels with 1X TAE buffer. The 1.5% agarose gel was stained with ethidium bromide (0.5µg/ml). 6µl of the amplified PCR products was loaded with its corresponding gene ruler (50bp, 100bp or 1kb), and gel electrophoresis was carried out at 90V for 45 minutes to get a good separation of the amplified products. Following the gel electrophoresis, the migrated DNA was visualized and photographed under UV illumination.

Table 3: List of primers and cycling conditions employed in the PCRs.

Primers	Sequences	Size/bp	Cycling conditions	Reference
ftsZF1	GTTGTCGCAAATACCGATGC	1043-1055	Initial denaturation at 94°C for 2min, followed by 40 cycles of 94°C for 1min, 55°C for 1min and 72°C for 3min. Final extension at 72°C for 10 min	(Werren & Windsor, 2000)
ftsZR1	CTTAAGTAAGCTGGTATATC			
OchroF1	AGAGTTTGATC(A/C)TGGCTCAG	1391	Initial denaturation at 94°C for 3min, followed by 35 cycles of 94°C for 30s, 55°C for 1min and 72°C for 1min. Final extension at 72°C for 10 min	(Hagiya et al., 2013)
OchroR1	AAGGAGGTGATCCAGCC			
OchroF2	ATGTCTCAAAATTCATTGCGAC	1023	Initial denaturation at 94°C for 2min, followed by 35 cycles of 94°C for 30s, 62°C for 30s and 72°C for 1min. Final extension at 72°C for 7min	
OchroR2	AGCATCTTCTTCCGGTCCGC			
EHRl6SDF	GGTACC(C/T)ACAGAAGAAGTCC	345	Initial denaturation at 94°C for 2min, followed by 40 cycles of 94°C for 1min, 57°C for 1min and 72°C for 3min. Final extension at 72°C for 10 min	(Parola et al., 2000)
EHRl6SR R	TAGCACTCATCGTTTACAGC			
LPW57-F	AGTTTGATCCTGGCTCAG	1300	Initial denaturation at 94°C for 2min, followed by 40 cycles of 94°C for 1min, 55°C for 1min and 72°C for 3min. Final extension at 72°C for 10 min	(Safaei et al., 2018a; Woo et al., 2003)
LPW58-R:	AGGCCCGGGAACGTATTCAC			
W-EF	CAGACGGGTGAGTAATG(C/T)ATAG	1025	Initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30s, 63°C for 30s and 72°C for 1min. Final extension at 72 °C for 10 min	(Werren & Windsor, 2000)
W-ER	TATCACTGGCAGTTTCCTTAAA			

Table 3: Cont'D

AM1	CGCGTGTCGTTCGGCTTTATGTG	1100-1400	Initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30s, 63°C for 30s and 72°C for 1min. Final extension at 72 °C for 10 min	(Kwakye-Nuako, 2016)
AM2	CTTACGGAGCTTGCTGAGGTGAGG			
BN1	GAAGGTCAACACCCTGATCC	595	Initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30s, 60°C for 30s and 72°C for 90s. Final extension at 72 °C for 10 min	
BN2	CTTCTTGGCGGTCTTCTGAG			

Chapter Summary

This chapter outlines the systematic approach used to obtain results for the study. From the sampling of the insects; biting midges and sandflies from Dodome-Awuiasu to laboratory analysis. It describes how the insects were collected and processed to acquire DNA for the detection of various bacteria and *Leishmania*.

CHAPTER FOUR

RESULTS AND DISCUSSION

This study sought to examine the gut microbiome of biting midges and sandflies caught in the CL-endemic communities in Ghana and their possible interactions with *Leishmania* parasites in the vectors. Briefly, the insects were collected using CDC light traps and aspirators. The collected insects were identified, characterized and sorted under the microscope after which DNA was extracted. Using PCR, the bacteria of interest were amplified with 16s bacteria primers and amplicons migrated on agarose gel and visualized/photographed under UV illumination. The data were entered into SPSS version 25. Frequencies and percentages for entomological data and molecular analysis data were calculated using SPSS version 25.

Results

A total of 885 of the desired insects were collected and sorted. Specifically, 135 sandflies and 750 biting midges were identified. The sandflies were combined into pools of 27 with each pool containing 5 individuals, whereas the biting midges were grouped into 75 pools with each, pool containing 10 individuals. The difference in the number of insects per pool between sandflies and biting midges was due to the relatively larger size of sandflies. Additionally, the difference aimed at increasing the DNA concentration of biting midges due to their small size during extraction. **Figure 3** shows pictures of biting midges while **Figure 4** shows pictures of sandflies all collected from the endemic community, Dodome-Awuiasu, located in the Volta region of Ghana.

Figure 3: Pictures of biting midges collected from the Dodome-Awuiasu community located in the Volta region of Ghana.

Figure 4: Pictures of sandflies collected from the Dodome-Awuiasu community located in the Volta region of Ghana.

PCR products and gel electrophoresis for bacteria detection

A total of 5 bacteria primers were used in this study. The 16S rRNA primers for; *Wolbachia-Ehrlichia* (W-E F/R), *Wolbachia* (ftsZF1/R1), *Ehrlichia* (EHRl6SDF/R), *Tsukamurella* (LPW 57/58) and *Ochrobactrum* (OchroF1/R1) were used to amplify the DNA extracted from the sandflies and biting midges. The five primers yielded a product of different band sizes as follows: W-E F/R ≈ 1025bp, ftsZF1/R1, ≈ 1023bp, EHRl6SDF/R ≈ 345bp, LPW 57/58 ≈ 1200bp and OchroF1/R1 ≈ 1391bp.

All the twenty-seven (27) pools of sandflies were amplified by *Wolbachia-Ehrlichia* and *Ehrlichia* primers yielding a product size of 1025bp and 345bp respectively (Table 4.). Only one sandfly pool (1) was amplified by *Ochrobactrum* primers whereas five (5) pools were amplified by *Wolbachia* primers (Table 4.0). Of the 27 pools of sandflies, nineteen (19) were amplified by the *Tsukamurella* primers (Table 4.0). The initial primer, *Wolbachia-Ehrlichia* (W-E F/R), is designed to amplify *Wolbachia, Ehrlichia* and other intermediate bacteria. This was initially used to confirm the presence of either *Wolbachia* or *Ehrlichia* in the samples before subsequently using the more sensitive and specific independent primers for further analysis. Figure 5 indicates the results for sandflies, showing the images of the bands generated from the PCR reaction along with the controls. The results were visualized and photographed under UV illumination after gel electrophoresis.

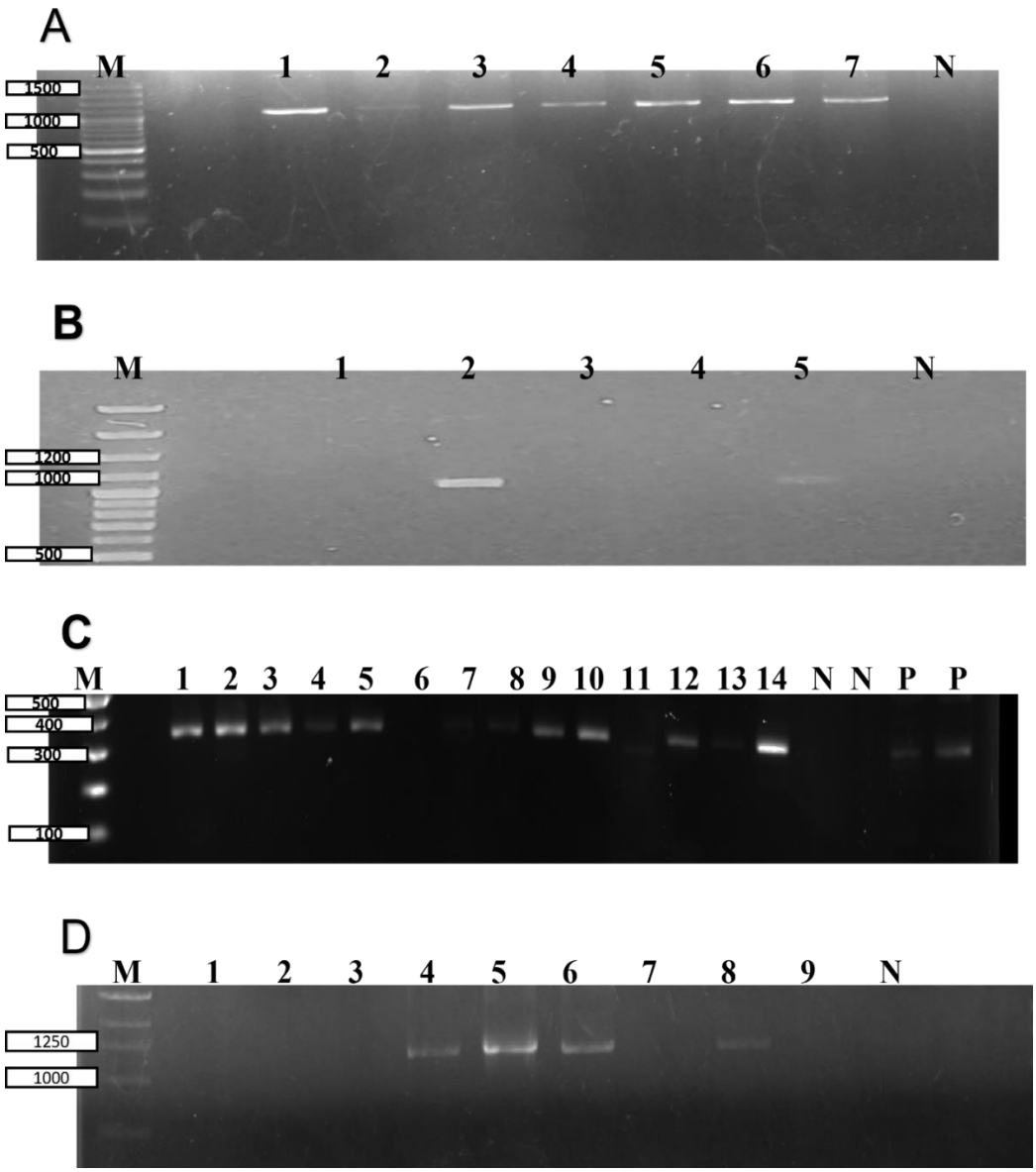

Figure 5: Gel photograph of the amplicons for sandflies for various primers: A - *Wolbachia-Ehrlichia* (W-E F/R), B – *Wolbachia* (ftsZF1/R1), C - *Ehrlichia* (EHRl6SDF/R) and D - *Tsukamurella* (LPW 57/58). PCR amplicons were run on a 1.5% agarose gel stained with ethidium bromide and visualized by UV illumination revealing the different band sizes. (M – Gene rule, N – Negative Control, P – Positive control). Gel image A, bands 1-7 are all positive samples which gave a product size of approximately 1025bp for W-EF/R primer of DNA extracted from sandflies. Gel image B, only bands 2 and 5 are positive samples with a product size of approximately 1023bp using ftsZF1/R1 primer for the bacteria *Wolbachia* only in sandflies. Gel image C shows results for bacteria *Ehrlichia* amplification in sandflies with bands 1-14 (excluding band 6) being positive samples with a product size of approximately

325bp. Gel image D shows results for *Tsukamurella* with bands 4,5,6and 8 being PCR products positive with a product size of approximately 1200bp using primer LPW 57/58 in sandflies.

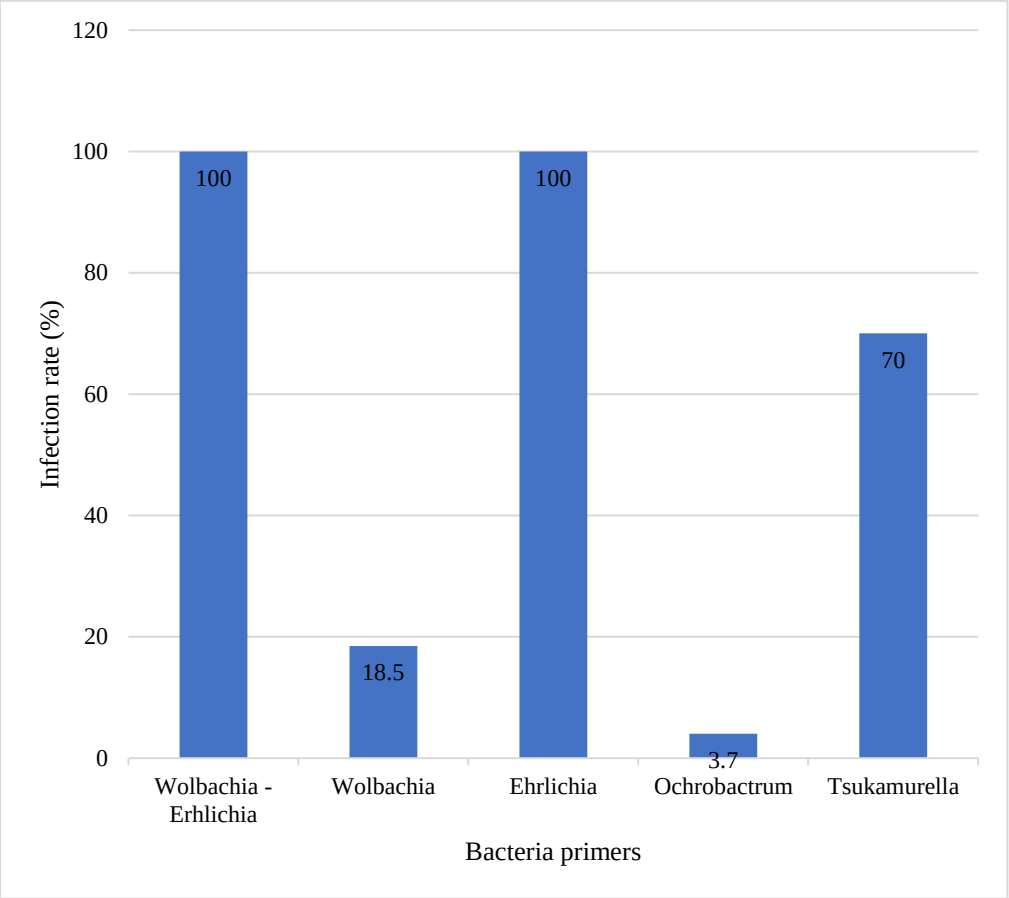

Figure 6: Infection rate of bacteria amplified in the sandflies.

Regarding biting midges, seventy-two (72) of the seventy-five (75) pools were amplified by *Wolbachia-Ehrlichia* primers, thirty-one (31) by *Ehrlichia* primers, thirty-five (35) by *Ochrobactrum* primers, eight (8) by *Wolbachia* primers, and forty-eight (48) by *Tsukamurella* primers (Table 4.0). Figure 8 shows the gel images of the bands generated from the PCR reaction along with the controls when visualized and photographed under UV illumination.

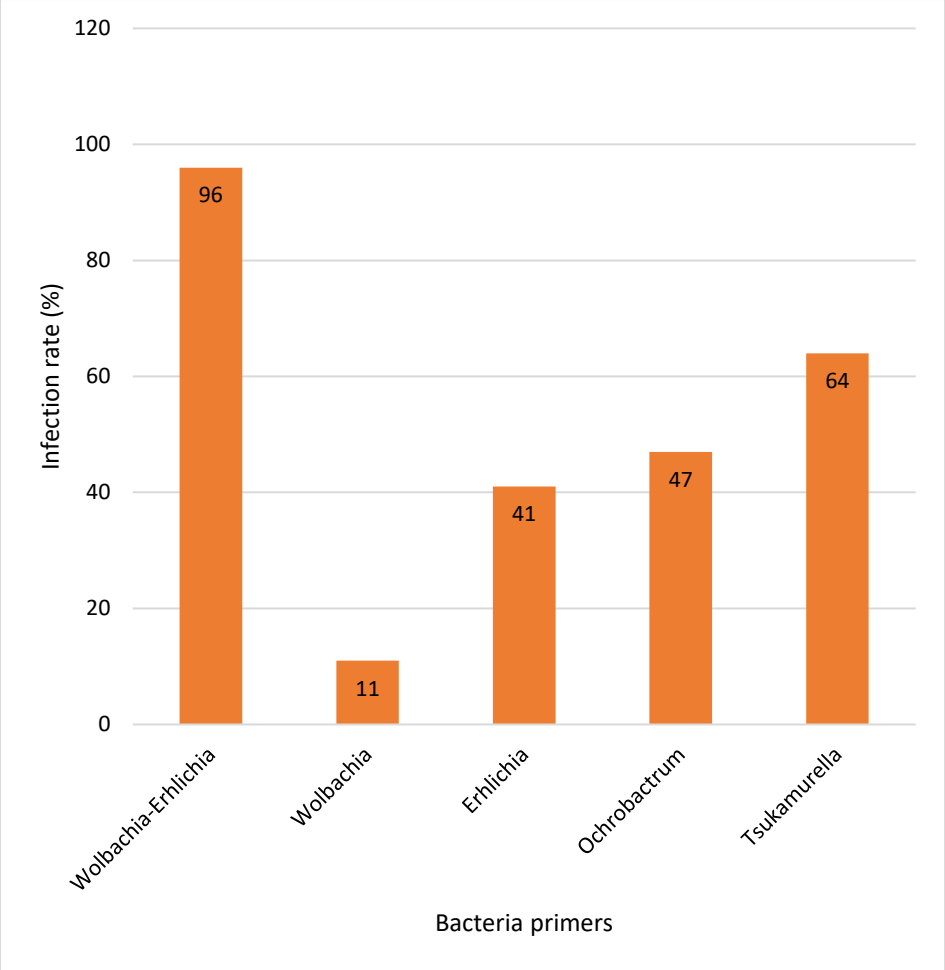

Figure 7: Infection rate of bacteria amplified in the biting midges.

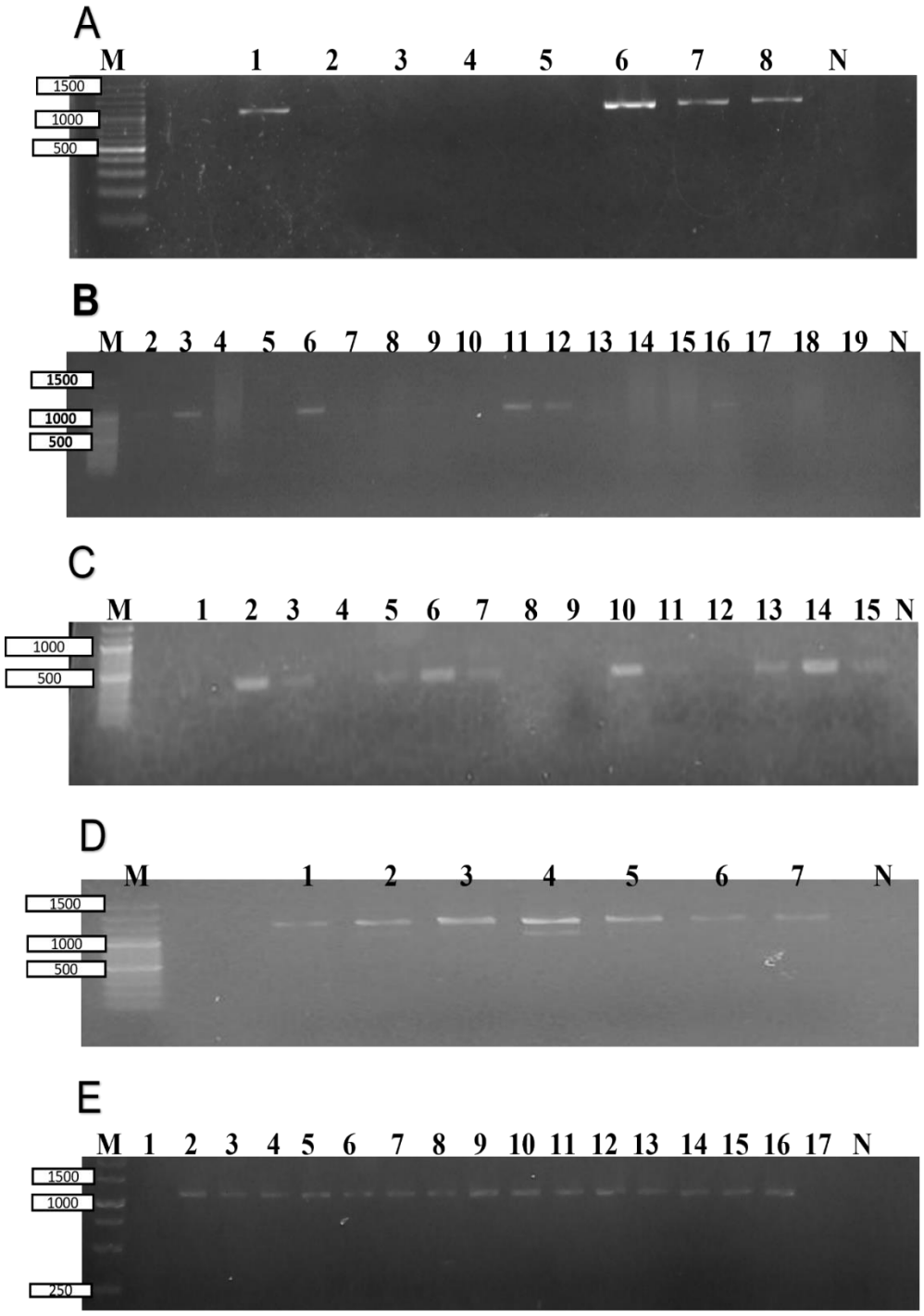

Figure 8: Gel photograph of the amplicons for biting midges for various primers: A - *Wolbachia-Ehrlichia* (W-E F/R), B - *Wolbachia* (ftsZF1/R1), C - *Ehrlichia* (EHRl6SDF/R) and D – Ochrobactrum (OchroF1/R1), and E - *Tsukamurella* (LPW 57/58). PCR amplicons were run on a 1.5% agarose gel stained with ethidium bromide and visualized by UV illumination revealing the different band sizes. (M – Gene rule, N – Negative Control, P – Positive control). Gel image A, bands 1, 6, 7 and 8 are all positive samples which gave a product size of approximately 1025bp for the W-

EF/R primer of DNA extracted from biting midges. In gel image B, only bands 2, 5, 7, 10,11 and 16 are positive samples with a product size of approximately 1023bp using ftsZF1/R1 primer for the bacteria *Wolbachia* only in biting midges. Gel image C shows results for bacteria *Ehrlichia* amplification in biting midges with bands 2, 3, 5, 6, 7, 10, 11, 13, 14 and 15 being positive samples with a product size of approximately 325bp. Gel image D, bands 1 - 8 are all positive samples which gave a product size of approximately 1391 bp for the OchroF1/R1 primer of DNA extracted from biting midges. Gel image E shows results for *Tsukamurella* with bands 2-16 being PCR products positive with a product size of approximately 1200bp using primer LPW 57/58 in biting midges.

Table 4: Bacteria detected in the potential vectors.

		Insects		
		Sandflies	Biting midges	Total
	Number of flies pools analysed	27	75	102
Bacteria primers	*Wolbachia-Ehrlichia*	27 (100%)	72 (96%)	99 (97%)
	Wolbachia	5 (18.5%)	8 (11%)	13 (13%)
	Ehlrichia	27 (100%)	31 (41%)	58 (57%)
	Ochrobactrum	1 (3.7%)	35 (47%)	36 (5%)
	Tsukamurella	19 (70%)	48 (64%)	67 (66%)

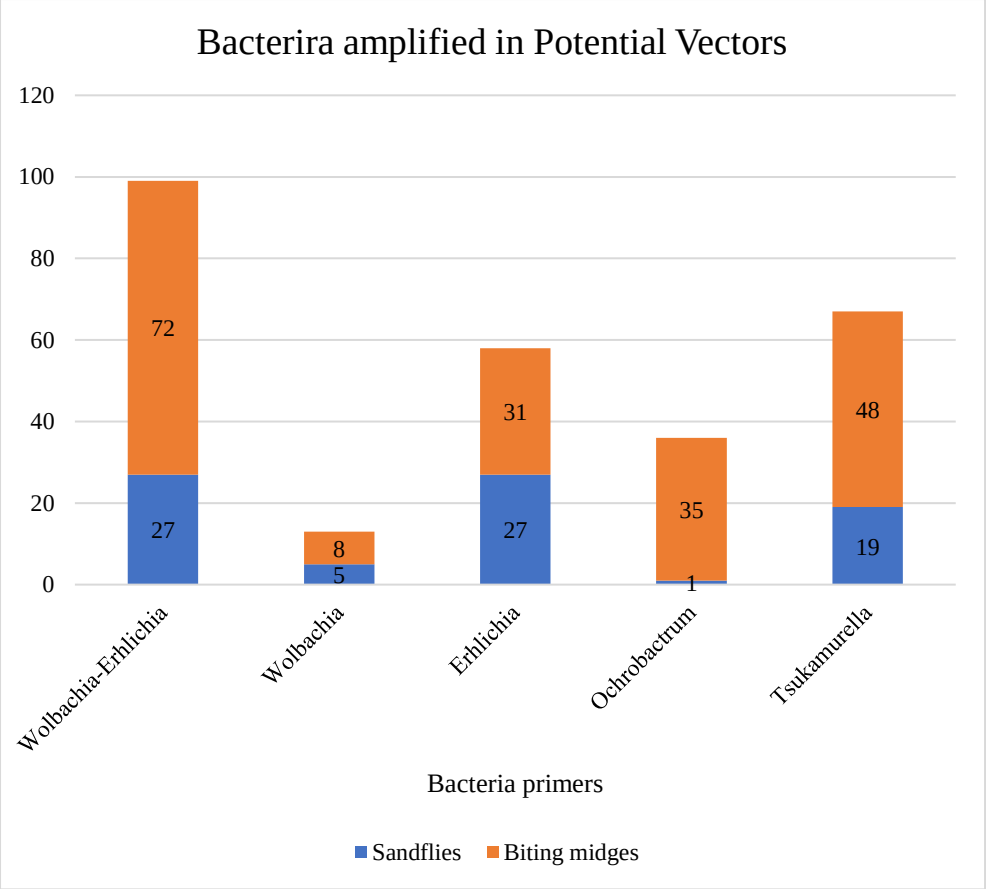

Figure 9: Bacteria amplified in Potential Vectors.

Detection of *Leishmania* parasite in sandflies and biting midges

To test for the presence of *Leishmania* in the sandflies and biting midges, two different sets of primers (AM1/AM2 and BN1/BN2) were used to enable speciation. This was because they both amplify different regions of the *Leishmania* parasite DNA and have different levels of sensitivity. Also, while AM1/AM2 can be used for restriction enzyme analysis, BN1/BN2 can be used for sequencing the entire genome of the parasite. Seven (7) pools of sandflies and two pools of (2) biting midges were amplified by AM1/AM2 primers yielding a product size between 1100 – 1400bp **(Figure 10)**. Using BN1/BN2, ten (10) pools of sandflies and thirty-eight (38) pools of biting midges were positive and produced a size of ≈595 bp (**Figure 10**). Only two (2) pools of

sandflies and one (1) pool of biting midges tested were amplified by both AM1/2 and BN1/2 (Table 5).

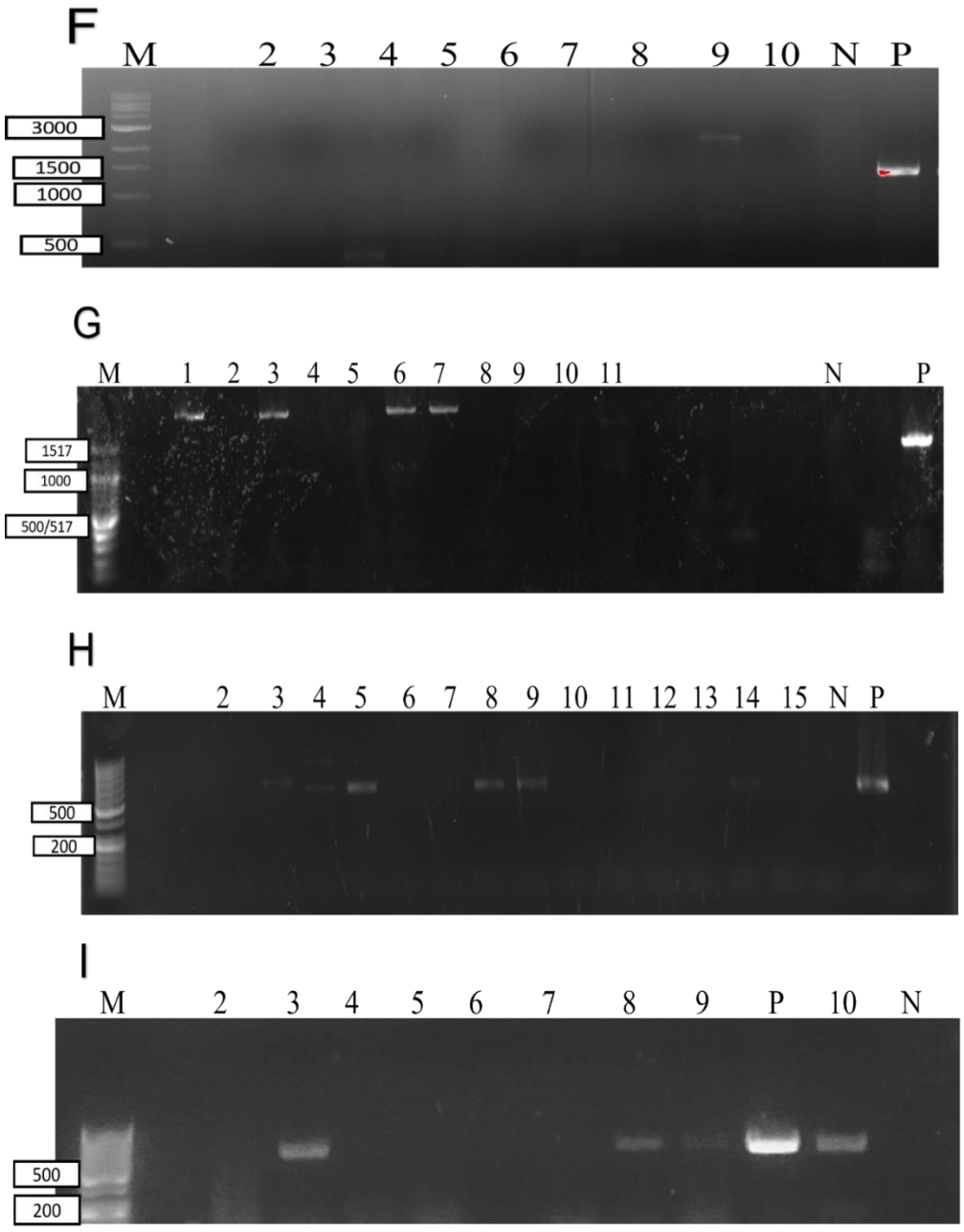

Figure 10: Gel photograph of PCR amplicons for the *Leishmania* parasite using AM1/AM2 and BN1/BN2 primers. PCR amplicons were run on a 1.5% agarose gel stained with ethidium bromide and visualized by UV illumination revealing the different band sizes. (F) Gel Image of AM1/AM2 for biting midges pools which were amplified by AM1/AM2 primers. Lanes 2-10 represent PCR products with amplification observed in lane 9 only giving a band. Positive

control; P – *L. major*, N - negative control. (G) Gel image of AM1/AM2 for sandflies pools. Lane 1-11 represents PCR products amplified with AM1/AM2 primers (1100-1400bp) with bands observed in lanes 1, 3, 6 and 7. Lane P, *L. major* positive control and lane N1-N2, negative control. (H) Gel Image of BN1/BN2 primer (≈595bp) for biting midges pool. Lanes 2-16, PCR products with bands in lane 3. 4, 5, 8, 9 and 14.; Lane P, L. *major* positive control; lane N, negative control. (I) Gel Image of BN1/BN2 primer (≈595bp) for sandflies pool. Lanes 2-10, PCR products with bands in lane 3, 8, 9 and 10.; Lane P, L. *major* positive control; lane N, negative control.

Table 5: Detection of *Leishmania* parasite in vectors.

		Insects		
		Sandflies	Biting midges	Total
	Number of flies analysed	27	75	102
***Leishmania* primers**	AM1/AM2	7 (30%)	2 (3%)	9 (9%)
	BN1/BN2	10 (37%)	38 (51%)	48 (47%)
	Positive for both AM1/2 & BN1/2	2 (7%)	1 (1)	3 (3%)
	Positive for either AM1/2 or BN1/2	15 (55.5%)	39 (52%)	54 (52.9%)

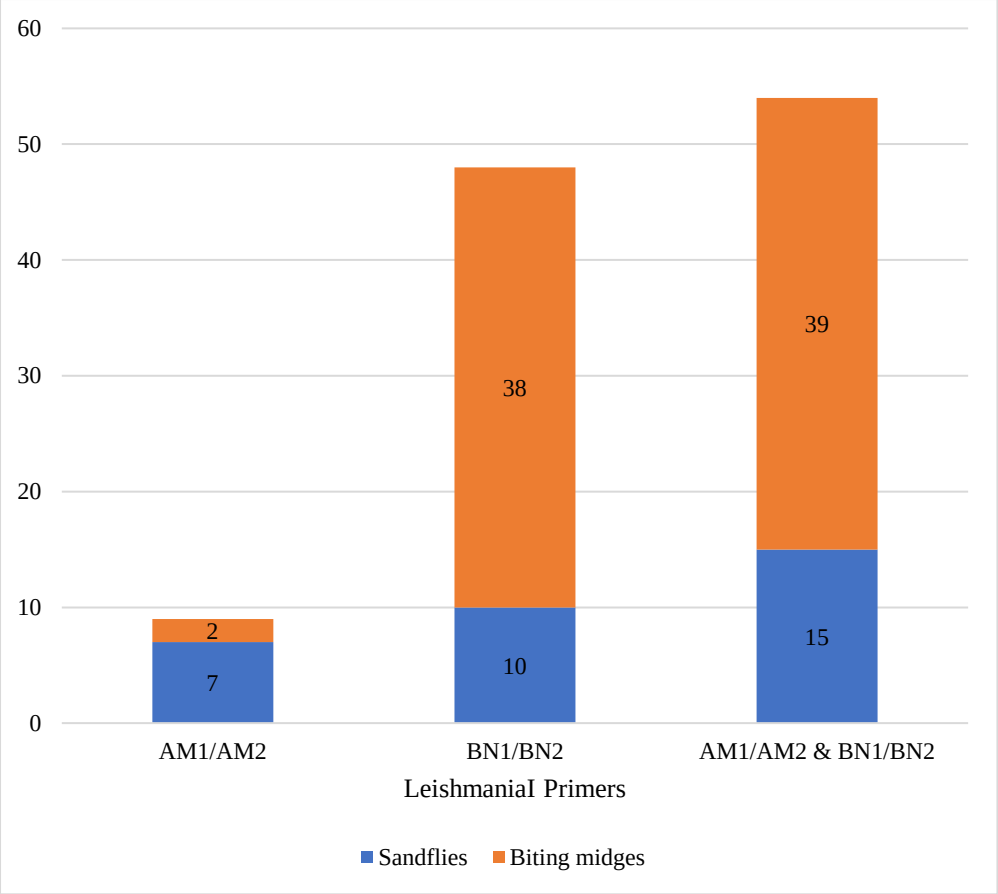

Figure 11: Leishmania Parasite amplified in Potential Vectors.

Co-existence of bacteria and *Leishmania* parasite in vectors

To investigate the potential coexistence of *Leishmania* with the bacteria of interest in sandflies and biting midges, DNA samples amplified by bacteria primers were subjected to a second PCR testing using *Leishmania*-specific primers, AM1/AM2 and BN1/BN2. The presence of the *Leishmania* parasite in a pool was based on whether the PCR amplicons were positive for either primers AM1/AM2 or BN1/BN2 or both. Among the 27 sandfly DNA samples amplified by *Wolbachia-Ehrlichia* primers, 15 of them were also amplified by either AM1/AM2 and BN1/BN2 primers. Among the *Wolbachia*-positive sandflies pools, 3 were amplified by AM1/AM2 and BN1/BN2 primers. A total of 15 *Ehrlichia*-positive sandflies were amplified by AM1/AM2 and BN1/BN2 primers and 9 *Tsukamurella*-positive sandflies were also amplified by

AM1/AM2 and BN1/BN2 primers. None of the *Ochrobactrum*-positive sandflies was amplified by the AM1/AM2 and BN1/BN2 primers (Table 6).

Regarding biting midges, 38 of the 72 *Wolbachia-Ehrlichia* positive pools were amplified by the AM1/AM2 and BN1/BN2 primers. Of the 8 *Wolbachia*-positive biting midges pools, 4 of them were also amplified by AM1/AM2 and BN1/BN2 primers. Similarly, the AM1/AM2 and BN1/BN2 primers amplified 17 of the *Ehrlichia*-positive biting midges pools, 21 of the *Tsukamurella*-positive pools and 18 of the *Ochrobactrum*-positive pools (Table 6). Figure 12 shows the frequency of sandflies and biting midges infected with both the bacteria and the *Leishmania* parasite.

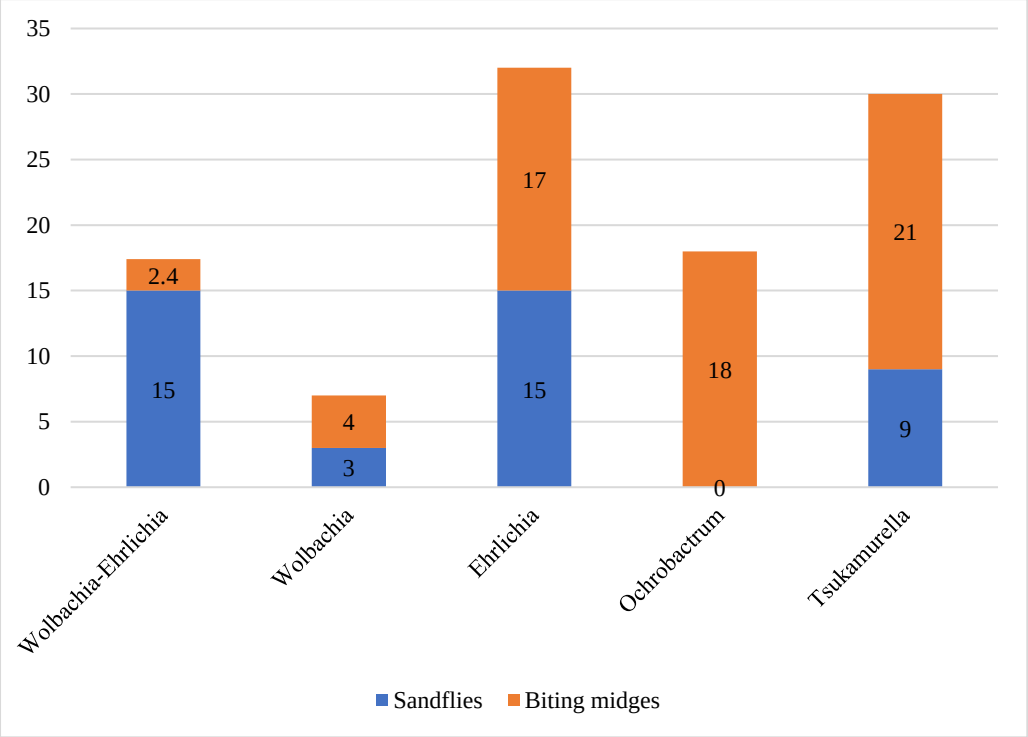

Figure 12: Potential vectors infected with both bacteria and the *Leishmania* parasite.

Table 6: Detection of both bacteria and *Leishmania* parasite in vectors.

Primers	Sandflies			Bitng midges		
	Number of insect pools amplified by bacteria primers	Number of bacteria-positive pools amplified by *Leishmania* primers	Percentage co-existence (%)	Number of insect pools amplified by bacteria primers	Number of bacteria-positive pools amplified by *Leishmania* primers	Percentage co-existence (%)
Wolbachia-Ehrlichia	27	15	55.56	72	38	50.67
Wolbachia	5	3	11.11	8	4	5.33
Elrichia	27	15	55.56	31	17	22.67
Ochrobactrum	1	0	0	35	18	24
Tsukamurella	19	9	33.33	48	21	28

Discussion

Cutaneous leishmaniasis (CL) has long been endemic in the Volta and Oti regions of Ghana, affecting the local communities. While the novel causative organism responsible for the infection has been identified and recently named *Leishmania chancei* (Kwakye-Nuako et al., 2023), the specific vector(s) responsible for its transmission remains unknown. Although sandflies have been observed in these endemic communities, biting midges have proven to support the various developmental stages of *L. chancei* (Becvar et al., 2021; Kwakye-Nuako, 2016). The gut microbiome of disease vectors is well recognized for its ability to either positively or negatively influence the vectorial capacity of the vectors and presents promising avenues for vector control and disease prevention (Douglas, 2015; Engel & Moran, 2013; Siddiqui et al., 2022). To leverage this potential for controlling leishmaniasis in Ghana, this study aimed at investigating the gut microbiota of the two potential vectors of *L. chancei*: sandflies and biting midges. Additionally, the study sought to explore any potential interactions between the microbiota and the parasites in the gut of the vectors.

The subgenus *Mundinia*, which was recently established, exhibits a broad geographic range and is made up of six distinct species (Kwakye-Nuako et al., 2023). Among these species, three, including *L. chancei* have the ability to cause human infections (Desbois et al., 2014; Jariyapan et al., 2018; Kwakye-Nuako et al., 2023). Unlike the other *Leishmania* subgenera that are transmitted by phlebotomine sandflies only, the transmission vectors for *Mundinia* remain elusive. In this study, 55.6% of the 27 pools of sandflies were amplified by either of the *Leishmania* primers used. This finding contradicts a previous study

also conducted in the Ho District of Ghana that found no *Leishmania* DNA in the 700 sandflies screened (Fryauff et al., 2006). In that study, *Sergentomyia* spp dominated the sandfly population, a genus associated with only reptile-pathogenic *Leishmania* (*Sauroleishmania*). Although this study did not identify the species of the wild-caught sandflies used, it has previously been established that the sandflies in the endemic community are also *Sergentomyia* spp (Doe et al., 2020). Previous studies have identified human-pathogenic *Leishmania* in some *Sergentomyia* sandflies. For example, *L. major* DNA was detected in *Sergentomyia minuta* caught in certain rural communities in Portugal (Campino et al., 2013) and *L. major* was also detected in *Sergentomyia* (*Spelaeomyia*) *darlingi* in Mali (Berdjane-Brouk et al., 2012). Pombi et al. (2020) similarly reported *L. infantum* in *S. minuta*, challenging the dogma that human-pathogenic *Leishmania* is exclusively transmitted by phlebotomine sandflies and *Sergentomyi*a is restricted to *Sauroleishmia* only. While the detection of parasite DNA in field-caught sandflies may suggest vectorial capacity, it fulfils only one of the vector incrimination criteria (Killick-Kendrick, 1979). Furthermore, the peripylarian nature of *Sergentomyia* (Lainson & Shaw, 1987; Maia & Depaquit, 2016) casts doubts on their competence as vectors in the study area. Our findings also corroborate those of Nzelu et al. (2014) and de Souza, Addo, et al. (2023) who detected *Leishmania* DNA in pools of sandflies but at a lower infection rate compared to our study. Despite the smaller sample size of sandflies investigated, the significantly high frequency of *Leishmania* infection raises more intriguing questions about the transmission dynamics of *Mundinia* spp. It will be imperative that future investigations on this study

identify the species of the sandflies caught as well as the species of parasite amplified to aid in making definitive conclusions.

The study revealed the presence of *Leishmania* DNA in 52% of biting midges pools, presenting the first evidence of *Leishmania* in biting midges from endemic communities in Ghana. Previous investigations have implicated biting midges as potential alternate vectors for members of the *Leishmania* (*Mundinia*) subgenus. Notably, none of the sandflies collected from kangaroo leishmaniasis endemic sites in northern Australia tested positive for *Leishmania*; however, day-feeding biting midges of the Forcipomyia subgenus had parasite DNA, with some midges showing high parasitaemia (Dougall et al., 2011). This provided evidence, which corroborates our findings, that biting midges could potentially be responsible for *Mundinia* spp transmission. Additionally, Seblova et al. (2015), demonstrated that *Culicoides sonorensis* biting midges were susceptible to *L. enriettii*, a member of *Mundinia* subgenus, and supported the late-stage development of *L. enriettii*. Becvar et al. (2021) further established, through an experimental transmission model, that *L. chancei* from Ghana had a high infection rate in *C. sonorensis* and could transmit the infection to mouse ears. These laboratory findings give credence to the detection of parasite DNA in midges in this study and provide additional evidence that biting midges may be the alternate vector for CL transmission in endemic communities in Ghana. To gain a more comprehensive understanding, it is necessary to identify biting midges species present in the endemic communities. Moreover, the presence of parasite DNA in both vectors could imply that leishmaniasis transmission in the endemic areas in Ghana, particularly Dodome-Awiausu, may involve multiple

arthropod genera as assumed in other studies (Azeredo-Coutinho et al., 2007; Coutinho et al., 2005).

In the current investigation, *Wolbachia*, *Ehrlichia*, *Ochrobactrum*, and *Tsukamurella* were naturally found to be present in the guts of sandflies and biting midges collected from Dodome-Awiausu, a *Leishmania*-endemic community in Ghana. *Wolbachia* was found in 18.5% of the 27 sandflies' pools. The detection of *Wolbachia* in the sandflies is consistent with other studies where *Wolbachia* was detected in sandflies using either wsp or ftszF1/R1 primers (Da Rocha et al., 2018; Ono et al., 2001). Previous studies that detected *Wolbachia* in sandflies found a relatively low infection rate of *Wolbachia* in the sandflies. The findings of previous studies are similar to this present study as a relatively low infection rate of *Wolbachia* was observed in the sandflies collected. The low prevalence of *Wolbachia* in sandflies was attributed to a reduced vertical transmission (Azpurua et al., 2010; Kassem & Osman, 2007) or the endosymbiotic bacteria, *Wolbachia* now adapting to the sandfly host (Da Rocha et al., 2018). Considering the low prevalence of sandflies in the endemic communities in this study, it could be suggested that the low infection of *Wolbachia* in the sandflies may be attributed to an ongoing adaptation of the bacteria to the sandflies. However, it is worth noting that the small sample size of sandflies and the limiting geographical sampling could potentially result in an underestimation of the presence or absence of the symbiont. Thus, increasing the number of sandflies and expanding the study to other endemic settings is likely to produce a better representation of the incidence of *Wolbachia* in sandfly vectors.

Wolbachia was detected in 11% of the 75 pools of biting midges examined. This infection rate is consistent with the findings of a previous study by Muñoz-Muñoz et al., (2017) where the prevalence of *Wolbachia* in the *Culicoides* species was notably low, with only 6 out of 256 samples from *C. imicola* and 2 out of 466 samples from *C. obsoletus* testing positive, while *C. pulicaris* showed a relatively higher infection of 56 out of 309 samples. Similarly, Mee et al., (2015), reported a low infection rate in biting midges, which was attributed to the limitations of the technique used. Traditional PCR could not detect the presence of *Wolbachia* due to the low concentration of *Wolbachia* DNA whereas qPCR successfully identified *Wolbachia* DNA. This discrepancy could also explain the low prevalence of *Wolbachia* infection in this study as the technique employed was conventional PCR. Additionally, the diverse strains of *Wolbachia* exhibit considerable variability, posing challenges for detection using a single universal primer (De Oliveira et al., 2015). This variability may have accounted for the observed low infection rate in our investigation. Consequently, the utilization of different *Wolbachia* primers other than the one used in the current study, and molecular detection techniques is imperative for future investigation.

Although the infection rate of *Wolbachia* among the potential vectors was low, sandflies exhibited a relatively higher infection rate compared to biting midges. The difference in infection rate observed between sandflies and biting midges could be associated with the variations in host characteristics including immunology, physiology and microbiota as stated by Da Rocha et al, (2018). The higher prevalence of *Wolbachia* in the sandflies, as observed in this study, along with the known effects of *Wolbachia* such as male killing, reduced vector

lifespan and cytoplasmic incompatibility, may contribute to the inability of sandflies to support the life cycle of *L. chancei*. Another possible explanation is the phenomenon observed in mosquitoes, where the presence of *Wolbachia* has been shown to enhance their resistance to pathogens such as dengue virus (Walker et al., 2011), yellow fever virus (Van Den Hurk et al., 2012) and chikungunya virus (Moreira et al., 2009) and has been used as a vector control method in mosquitoes (Ross, 2021; Ross & Hoffmann, 2021). As such, it is plausible that similar interactions occur within the sandflies, although the specific outcomes may vary depending on the particular strains of *Wolbachia* and their interactions with the host. This could have further contributed to the higher abundance of biting midges within the endemic community. The higher infection rate of *Wolbachia* in the sandflies may have shaped the population dynamics of the vectors, limiting the sandfly population size and thus allowing biting midges to dominate Dodome-Awuiasu. However, further investigations will be needed to elucidate the precise mechanisms underlying the differential abundance of these vectors and the role of Wolbachia in shaping their populations within the endemic community.

Wolbachia strains have the potential to significantly decrease or distort the development of infectious pathogens within host vectors (Joshi et al., 2017; Kambris et al., 2009) . While the use of *Wolbachia* as a vector control agent in *Leishmania* vectors lacks supporting evidence, this endosymbiont offers a promising avenue to devise innovative strategies for combating leishmaniasis. Karimian et al. (2018) suggest that co-infection of *Leishmania* and *Wolbachia* may be common in endemic areas, with *Wolbachia* impacting the clinical presentations and disease progression of leishmaniasis through the modulation

of host immune responses. In this study, *Wolbachia* and *Leishmania* co-infected 11.11% of sandflies contrasting the findings of Alipour et al. (2021) who found no *Leishmania* DNA in sandflies that had *Wolbachia* in the gut, suggesting an antiparasitic role of *Wolbachia* in their study area. Contrary to our findings, reports by Lozano-Sardaneta et al. (2023) also showed that none of the sandflies from Mexico infected with *Wolbachia* had coinfection with *Leishmania.* Similarly, Vivero et al. (2016) also observed no detection of *Wolbachia* and *Leishmania* in the gut of *Lutzomyia evansi* caught in Columbia. Another study that investigated coinfection of *Leishmania*, *Wolbachia* and filarial worms in dogs reported no *Leishmania* infection in dogs that were infected with *Wolbachia*. Inferring from these, our findings may suggest that Wolbachia may not prevent the establishment of *L. chancei* infection in sandfly vectors in Dodome-Awiuasu. This phenomenon could be attributed to the distinct properties and transmission dynamics of members of the *L.* (*Mundinia*) subgenus (Baneth et al., 2020; Espinosa et al., 2018), which might have acquired resistance mechanisms or undergone mutation to override the antiparasitic effects of *Wolbachia*. To gain further insights, it is important to identify the supergroups and subgroups of the *Wolbachia* species present in the sandflies using sequencing and phylogenetic analysis as not all strains of *Wolbachia* may possess antiviral or antiparasitic properties (Audsley et al., 2017; Osborne et al., 2009). Our present study did not extend to sequencing and phylogenetics analysis, preventing definitive conclusions regarding the possible reasons for the observed *Wolbachia-Leishmania* coinfection in the sandflies. Nonetheless, the findings of Azpurua et al. (2010) align with observations, as they reported co-infection of *Leishmania* and a single strain of *Wolbachia* in *Lu. trapidoi*

sandflies. Thus, the presence of *Wolbachia* alongside *Leishmania* in sandflies presents a unique opportunity to introduce disease-blocking transgenes within sandfly populations as a means of disease prevention. The coinfection rate of *Wolbachia* and *Leishmania* spp in biting midges was observed to be 5.33%. The potential role of alternative vectors, such as biting midges, in the transmission of *Leishmania* is gaining increasing attention. Several studies conducted in Australia, Brazil and Thailand have implicated different species of biting midges as potential vectors for transmitting members of the *Leishmania* (*Mundinia*) subgenus (Dougall et al., 2011; Seblova et al., 2015; Songumpai et al., 2022; Sunantaraporn et al., 2021). Notably, the vectorial life cycle of *L. chancei*, the parasite responsible for causing CL in Ghana, has been reported to be supported by biting midges (Culicoides spp.) and can be transmitted by these vectors to mammalian pitching these vectors as possible vectors of CL in Ghana (Becvar et al., 2021; Kwakye-Nuako, 2016). In this study, the amplification of *Leishmania* DNA in the wild-caught biting midges provides evidence, although inconclusive, that biting midges could transmit CL in Dodome-Awiausu. Emerging studies are investigating the use of Wolbachia for population replacement and population suppression approach to limit the transmission of diseases vectored by biting midges (Caragata et al., 2016; Dutra et al., 2017; Ghosh et al., 2019; Rasgon, 2008). While coinfections of *Wolbachia* and other pathogens, particularly arboviruses, in midges, have been explored (Matthews et al., 2022), there are currently no reports on *Wolbachia-Leishmania* coinfection in biting midges, making the findings of this study novel. Although *Leishmania* DNA was predominantly amplified in biting midges compared to sandflies, the coinfection rate with *Wolbachia* was lower in biting midges. This

observation is consistent with Mee et al. (2015), who reported low levels of *Wolbachia* DNA in wild-caught female *Culicoides* species. In contrast, a higher prevalence of *Wolbachia* infection was reported in medically important *Culicoides* species caught in Europe (Muñoz-Muñoz et al., 2017). Our observation may somewhat indicate that in the sampling area, whilst *Wolbachia* may confer a protective role for *Leishmania* in sandflies, the opposite may be true for the biting midges. However, considering that *Wolbachia* coexisted with *Leishmania* in the gut of some of the biting midges in our study, the possibility of exploring *Wolbachia*-induced mechanisms (e.g. transinfection) to modulate pathogen transmission, in this case, *Leishmania* (given that biting midges are the vectors), in biting midges is plausible. This possibility has been explored by Ghosh et al. (2019) who successfully transfected *Culicoides sonorensis* cell lines with *Wolbachia* and suggested that *Wolbachia* could be used for a population replacement and/or population suppression approach to limit the transmission of diseases vectored by biting midges.

The bacteria *Ochrobactrum* has been isolated and characterised in different species of laboratory-reared and wild-caught sandflies (Louradour et al., 2017; Monteiro et al., 2016; Volf et al., 2002). A study by Volf et al., (2002), found the bacteria *Ochrobactrum* at all the different phases of development of the *Leishmania* parasite in *Phlebotomus duboscqi* suggesting a transstadial passage of *Ochrobactrum* in sandflies (Volf et al., 2002). In the present study, *Ochrobactrum* was only found in 3.7% of the sandflies' pool. This infection rate is very low compared to other studies. *Ochrobactrum* is reportedly one of the most abundant bacterial genera isolated from sandflies (Louradour et al., 2017; Tabbabi et al., 2020). Although the exact role of *Ochrobactrum* in sandfly

physiology remains unknown, different studies have reported its contrasting roles on sandfly vectorial capacity (Louradour et al., 2017; Sant'Anna et al., 2014). On the other hand, 47% of the pools of biting midges were positive for *Ochrobactrum*, which is a relatively high infection rate when compared to the sandfly pools. Of note, was one of the predominant bacteria present in the gut of biting midges. At present, there has been no detection of *Ochrobactrum* in biting midges nor their potential impact on midges' physiology or vectorial capacity. However, symbiont midgut bacteria in biting midges have been noted to be important to maintain some physiological processes including lifecycle, fitness and vectorial capacity (Lewis et al., 2014; Nayduch et al., 2015) and can also dampen their pathogen transmission (Möhlmann et al., 2020).

Interestingly, none of the *Ochrobactrum*-positive sandfly pools was coinfected with *Leishmania*. *O. intermedium* has been reported to prevent the establishment of *Leishmania mexicana* infection within *Lutzomyia longipalpis* (Sant'Anna et al., 2014). In the study, pre-feeding sandflies with *Ochrobactrum* resulted in a reduced infection rate of sandflies and decreased *Leishmania* population in the gut of the vectors. Similar findings were reported by Campolina et al. (2020) who observed a reduction in infected sandflies and a decrease in *Leishmania* load when the vectors were pre-fed *O. intermedium.* Additionally, in their study, Hassan et al. (2014) demonstrated that *Phlebotomous papatasi* exhibited increased susceptibility to *L. major* infection following antibiotic treatment when compared to the untreated control. This suggests that the presence of symbionts like *Ochrobactrum* accounted for the sandflies' resistance to *Leishmania* infection. Thus, our findings support the notion that the presence of *Ochrobactrum* negatively affected the vector

competence of sandflies. In contrast, Louradour et al. (2017) demonstrated that diverse bacterial communities in *P. duboscqi* were essential for the development of metacyclic *L. major* promastigotes in the anterior midgut. Their study revealed that the gut microbiota of *P. duboscqi* was predominantly dominated by the Gram-negative *Ochrobactrum* spp. and antibiotic treatment significantly impaired *Leishmania* metacyclogenesis and reduced the vector competence of *P. duboscqi*. Based on these contrasting reports, it will be plausible to speculate that the effects of *Ochrobactrum* on sandfly vectorial capacity may differ depending on the species of sandfly. In the present study, the sandflies used were not characterized to the species level, however, previous studies have identified sandflies fauna in CL endemic communities to be predominantly *Sergentomyia* spp. (~99%) with less than 1% being *P. duboscqi* (de Souza, Addo, et al., 2023; Doe et al., 2020; Kweku et al., 2011).

Our findings also revealed a noteworthy occurrence of *Leishmania-Ochrobactrum* coinfection in biting midges, with a total of 18 pools (24%) having both infections, representing one of the highest coinfection rates observed in the study. This presents the very first documented report of *Leishmania-Ochrobactrum* coinfection in biting midges. Interestingly, the presence of *Ochrobactrum* in these midges did not impede their ability to host *Leishmania* parasites. This discovery challenges the theory of community ecology regarding insect gut microbiota, which suggests that a diverse range of bacteria species within the insect gut provides more resistance against *Leishmania* invasion compared to a species-poor gut (Dillon et al., 2005; Law & Morton, 1996; Robinson & Valentine, 1979; Sant'Anna et al., 2014). It is also worth noting that certain species of *Ochrobactrum* have been associated with

human respiratory tract infections, wound infections and several opportunistic diseases in humans (Brady & Leber, 2018). Therefore, the coexistence of *Ochrobactrum* with *Leishmania* in biting midges which are potential vectors of CL in Ghana raises public health concerns. The inoculation of both pathogens into hosts during blood meal could potentially lead to severe disease outcomes, although further investigations are required to ascertain this. However, given that some species of *Ochrobactrum* are known to induce cartilage degeneration and bone deformities in toads (Shilton et al., 2008), it is worth exploring the contribution of this endosymbiotic bacterium to the pathogenesis of CL and its possible association with the observed cartilage deformities in mucocutaneous leishmaniasis (El-Ayouty, 2012).

Ehrlichia are mainly tick-transmitted Gram-negative obligate intracellular bacteria that cause ehrlichiosis in humans and other animals. At present, very limited studies are available on the detection of *Ehrlichia* in sandflies but have been detected in both adult and juvenile mosquitoes responsible for transmitting malaria (Guo et al., 2016). Our study revealed a remarkable 100% infection rate of *Ehrlichia* in sandflies suggesting its prominent presence within the sandfly microbiota. Our findings are consistent with that of Fraihi et al. (2017)who first identified *Ehrlichia* DNA in the midgut of sandflies from the Western Mediterranean Basin. However, the infection rate of *Ehrlichia* in sandflies may vary across sandfly vectors and geographic regions (Rar & Golovljova, 2011) due to ecological factors, the presence of different reservoir hosts and host competence and susceptibility (Massung et al., 2002; Rar & Golovljova, 2011; Stuen et al., 2013). The acquisition of microbiota in sandflies is a multifaceted process, particularly among adult

individuals. This intricate process involves several distinct sources, including the acquisition of microbiota during blood meals from animal/reservoir hosts, assimilation of microbial elements from plants during feeding activities and exposure to various microorganisms in terrestrial habitats during their larval and pupal stages (Dillon et al., 1996; Hillesland et al., 2008; McCarthy et al., 2011; Sant'Anna et al., 2012). Reptiles and dogs have been implicated as important reservoirs of *Ehrlichia* (Andoh et al., 2015; Asgarali et al., 2012; Liddell et al., 2003; Omondi et al., 2017) and considering that *Sergentomia* sandflies responsible sauroleishmania in reptiles predominate in the endemic community, transmission of *Ehrlichia* from these reptiles to the sandflies during feeding is plausible. Additionally, dogs may serve as a potential source of some *Ehrlichia* for sandflies. In addition, the high prevalence of *Ehrlichia* in sandflies suggests the possibility of transstadial transmission, as observed in mosquitoes (Guo et al., 2016), potentially indicating a novel vector for Ehrlichiosis which may require further investigation.

Conversely, the infection rate of *Ehrlichia* in biting midges was relatively low (41%) contrasting with the 100% infection rate in sandflies. There is currently no evidence of the detection of *Ehrlichia* in biting midges or a possible relationship between *Ehrlichia* spp. and biting midges. However, since biting midges are not entirely hematophagous, the bacteria may have been acquired from both blood and sugar meal sources. Further research is needed to elucidate the relationship between *Ehrlichia* and biting midges and its implications on vectorial capacity.

Ehrlichia and *Leishmania* co-infection is primarily seen in canines, with *E. canis* and *L. infantum* being the most common zoonotic species involved.

Previous studies have indicated that *E. canis* infections often precede *L. infantum* infection in dogs (Attipa et al., 2018; Mekuzas et al., 2009; Toepp et al., 2019), suggesting that the presence of *Ehrlichia* increases the risk of *Leishmania* infection. In our investigation, we found *Ehrlichia* and *Leishmania* co-infection in 55.56% of sandflies and 22.67% of biting midges. The higher coinfection rate in sandflies supports the idea that the presence of *Ehrlichia* enhances the potential for *Leishmania* co-infection. Attipa et al. (2018) also reported an elevated risk of *E. canis* infection in dogs with leishmaniosis, further supporting a synergistic relationship between *Leishmania* and *Ehrlichia* (Mekuzas et al., 2009). This synergy is thought to be attributed to the shared pathogenesis and immune response mechanisms of both pathogens (Attipa et al., 2018). However, contrasting results were reported in another study where no coinfection was observed in foxes independently infected with *E. canis* and *L. infantum* (Cardoso et al., 2015). Given, the relatively low infection rate of *Ehrlichia* in biting midges, it is not surprising that the coinfection rate in this insect group was also low.

Tsukamurella species are saprophytic microorganisms found in various environmental habitats such as water, sludge, arthropods, soil, sponges and foam (Safaei et al., 2018a). Exhibiting opportunistic pathogenicity, they can be transmitted through clinical instruments, leading to various infections in humans such as cutaneous infections, meningitis and pulmonary diseases (Dworkin, Falkow, Rosenberg, Stackebrandt, et al., 2006). In this investigation, a significant incidence of *Tsukamurella* infection was observed in the potential vectors under study as seen in previous reports. Specifically, 70% of sandfly pools and 64% of the biting midges pools tested positive for *Tsukamurella*.

Tsukamurella has been recognized as an important member of the microbiota of *Lu. Longipalpis* (Dey et al., 2018b). In their study which corroborated our findings, Dey et al. (2018) showed that this bacterium was present in both sandfly bites and the midgut of the flies infected with *Leishmania*. Notably, they reported that the egestion of *Tsukamurella* by sandflies during blood feeding on the host skin triggered the activation of the inflammasome, resulting in elevated levels of IL-1β. This, in turn, led to the recruitment of neutrophils to the bite site, creating a protective environment that facilitated the successful establishment of *Leishmania* infection in the vertebrate host. The study by Dey et al. (2018) proposed that *Tsukamurella* could serve as a valuable indicator to differentiate between *Leishmania*-infected and uninfected midguts in sandflies. Furthermore, they found that the elimination of *Tsukamurella* from the gut microbiota disrupted the recruitment of neutrophils to the site of infection, suggesting its potential as a target for intervention strategies against *Leishmania* transmission. However, Cambronero-Heinrichs et al. (2022) reported a contrasting role of *Tsukamurella* in the gut of triatomine bugs, where its presence, along with other bacteria, suppressed the growth of *Trypanosoma cruzi*, inhibiting the protozoa's establishment in the vector's gut. Additionally, *Tsukamurella* was found in very low abundance in field-caught *Glossina palpalis palpalis* tsetse flies (Tsagmo Ngoune et al., 2019). These findings suggest that the role of *Tsukamurella* in the gut of disease vectors may vary depending on the type of pathogen, source of meal and possibly, the physiology of the vector.

The rates of *Tsukamurella-Leishmania* coinfection were notably high in both sandflies and biting midges. These substantial coinfection rates in both

vectors reinforce the findings of Dey et al. (2018), highlighting the significance of *Tsukamurella* as an important bacterium for *Leishmania* development within the vector and for the successful invasion of hosts. This observation potentially explains the high occurrence of coinfection in both vectors and suggests the bacterium as a target for the prevention of *Leishmania* transmission.

In this study, populations of sandflies and biting midges from the Dodome-Awiuasu community were found to be naturally infected with *Wolbachia*, *Ehrlichia*, *Ochrobactrum*, and *Tsukamurella*. The study also provided evidence of the detection of *Leishmania* parasites in biting midges which supports the hypothesis of their potential as alternate vectors for *Leishmania*. The study also revealed varying rates of *Leishmania* and bacteria coinfection in both vectors under investigation. These findings provide crucial baseline data, emphasizing the need for a comprehensive understanding of the unique interactions between these bacteria and the two vector hosts. This understanding will facilitate the identification of potential candidate bacteria for paratransgenic studies or other biological strategies aimed at that controlling the vector populations and curtailing the transmission of *Leishmania* within the endemic community.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

The novel species *Leishmania* (*Mundina*) *chancei* has been identified as the causative agent for cutaneous leishmaniasis in some communities in Ghana (Kwakye-Nuako et al., 2023). However, the specific vector(s) responsible for its transmission remains unknown. Among the proposed vectors are sandflies and biting midges, and for successful transmission, the parasite must undergo its developmental stages in the gut of the vector. This implies that the insect gut and its associated microbiome might play a crucial role in supporting parasite development. This study aimed to detect the presence of selected bacteria and the *Leishmania* parasite in the potential vectors, as these bacteria are known to either impede or support the vector's capacity and could potentially lead to co-infection in the host. It was also hypothesized that the presence or absence of *Wolbachia, Ochrobactrum, Ehrlichia* and *Tsukanurella* might affect the vectorial capacity of biting midges or sandflies in the transmitting *Leishmania* parasites. Secondly, the presence or absence of the selected bacteria in biting midges or sandflies could result in co-infection or secondary infections during the inoculation of the parasite into the hosts. To achieve the study's objective, DNA was extracted from the flies in pooled samples and specific primers were employed in PCR to detect the target bacteria and parasite. The resulting PCR amplicons were visualized using a gel dock after gel electrophoresis. The study's outcomes were presented in tables and figures to facilitate understanding. The findings from this study are expected to contribute to the exploration of the microbiome as a potential target candidate for disease

prevention, offering new insights into controlling the transmission of cutaneous leishmaniasis.

Conclusion

For this study, a total of 125 sandflies and 750 biting midges were examined and grouped into 27 pools of sandflies and 75 pools of biting midges for analysis. The selected bacteria, *Wolbachia*, *Ochrobactrum*, *Ehrlichia* and *Tsukamurella* were detected in both the sandflies and biting midges. The presence of these bacteria, particularly *Wolbachia* and *Ochrobactrum* in the vectors suggests that they may have diverse roles in either supporting or impeding *Leishmania* parasite development and hence warrant further in-depth investigation.

Moreover, the presence of *Wolbachia* in the sandflies aligns with the hypothesis that the endosymbiotic bacteria *Wolbachia* could serve as a potential biological vector control in sandflies. On the other hand, *Ehrlichia* and *Tsukamurella* could result in co-infection with the parasite or secondary infections. Additionally, the *Leishmania* parasite was identified in the vectors alongside coinfection with the aforementioned selected bacteria under investigation. These findings highlight the complexity of the interactions between vectors, microbiome and the *Leishmania* parasite, suggesting potential implications for disease transmission dynamics.

Overall, the study sheds light on the possible roles of specific bacteria in the sandflies and biting midges, providing valuable insights for future research and potential strategies for controlling *Leishmania* transmission.

Recommendations

The results of this study show the prevalence of biting midges in the endemic community. These midges are known for the transmission of viral pathogens as well as nuisance biting.

1. This call for a thorough study to identify the biting midges present in the endemic communities. Also, the biting midges should be screen for all viral pathogens they can transmit. Data from such a study will inform the Ghana Health service (GHS) the measures to put in place to prevent any possible disease outbreak in the communities and it surrounding towns.

2. An epidemiological survey should be carried out to assess the knowledge of residents in the CL endemic communities as well as surrounding communities on CL transmissions, impact of the potential vectors; biting midges and sandflies, and the local therapeutic approaches employed in these communities. The findings will inform the Government and other organizations the necessary measures needed to be put in place such as vector control measures, diseases management and prevent strategies and education of residents.

4. The detection of *Ehrlichia* and *Tsukanurella* in sandflies and biting midges calls for surveillance for the diagnosis of Ehrlichiosis in both humans. No data is available on Ehrlichiosis in these endemic communities hence any study carried out will inform health personnels and researchers whether the disease is prevalent or residence have developed immunity to Ehrlichiosis.

Suggestion for Further Research

Based on the findings from this study, further investigation to fully understand the role of the bacteria detected in the vectors in this current study. A study should also be carried out to investigate the entire microbial ecology of

the gut of the putative vectors in the endemic region. This will provide data on the pathogenic and non-pathogenic resident bacteria in the gut of the vectors as well as suggest new vector control targets to impede the cycle of disease transmission. *In vitro* studies could also be carried out to study the bacteria-*Leishmania* parasite interactions.

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