UNIVERSITE DE YAOUNDE I

CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCE DE LA VIE, SANTE ET ENVIRONNEMENT

UNITE DE RECHERCHE ET DE FORMATION DOCTORALE SCIENCES DE LA VIE

DEPARTEMENT DE BIOCHIMIE

THE UNIVERSITY OF YAOUNDE I

CENTRE FOR RESEARCH AND TRAINNING IN GRADUATE STUDIES IN LIFE, HELTH AND ENVIROMENTAL SCIENCES

RESEARCH AND DOCTORATE TRAINNING UNIT IN LIFE SCIENCES

DEPARTMENT OF BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY

DEPARTEMENT DE BIOCHIMIE

LABORATORY OF MOLECULAR PARASITOLOGY AND VECTOR RESEARCH

LABORATOIRE DE PARASITOLOGIE MOLECULAIRE ET DE RECHERCHE VECTORIELLE

Genetic diversity of the tsetse fly microbiome from Campo, Southern Cameroon: implications in new strategies to control trypanosomiases.

THESIS

Presented as a partial requirement for the

Doctorate/Ph.D in Biochemistry

Speciality: Biotechnology and Development

By

BOUAKA TSAKENG Calmes Ursain

Matricule: 18W4601

Master in Clinical Biochemistry

Co-directed by :

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Academic Year: 2022/2023

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FACULTE DES SCIENCES
FACULTY OF SCIENCE

25 MARS 2024

ATTESTATION DE CORRECTION

Conformément à l'autorisation de soutenance de la thèse de Doctorat/Ph.D N°24-0126/UYI/VREPDTIC/DAAC/DRD/SR/TCL du 12 janvier 2024 de Monsieur le Recteur de l'Université de Yaoundé I, la thèse intitulée **« Genetic diversity of the tsetse fly microbiome from Campo, Southern Cameroon: implications in new strategies to control trypanosomiases »** a été présentée et soutenue publiquement le **vendredi 26 janvier 2024** par l'étudiant **BOUAKA TSAKENG Calmes Ursain**, Matricule **18W4601**. Le document final a été corrigé suivant les recommandations du jury.

En foi de quoi la présente attestation est délivrée pour servir et valoir ce que de droit.

Fait à Yaoundé, le

Le Président du Jury

Les Examinateurs

Le Chef de Département

Paul F. MOUNDIPA
Professor
Pharmacology & Toxicology

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LISTE DES ENSEIGNANTS PERMANENTS		**LIST OF PERMANENT TEACHING STAFF**

LISTE DES ENSEIGNANTS

ANNÉE ACADEMIQUE 2022/2023

(Par Département et par Grade)

DATE D'ACTUALISATION 31 MAI 2023

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5- DÉPARTEMENT DE CHIMIE ORGANIQUE (CO) (37)			
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	GUIDZAVAI KOUCHERE Albert	Assistant	En poste
	MANN MANYOMBE Martin Luther	Assistant	En poste
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	NYOUMBI DLEUNA Christelle	Assistant	En poste
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8- DÉPARTEMENT DE MICROBIOLOGIE (MIB) (24)

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	EZO'O MENGO Fabrice Télésfor	Assistant	En poste
	EHETH Jean Samuel	Assistant	En poste
	MAYI Marie Paule Audrey	Assistant	En poste
	NGOUENAM Romial Joël	Assistant	En poste
	NJAPNDOUNKE Bilkissou	Assistant	En poste

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	HONA Jacques	Professeur	En poste
	NANA ENGO Serge Guy	Professeur	En poste
	NANA NBENDJO Blaise	Professeur	En poste
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	BODO Bertrand	Maître de Conférences	En poste
	ENYEGUE A NYAM épse BELINGA	Maître de Conférences	En poste
	EYEBE FOUDA Jean sire	Maître de Conférences	En poste
	FEWO Serge Ibraïd	Maître de Conférences	En poste
	MBINACK Clément	Maître de Conférences	En poste
	MBONO SAMBA Yves Christian U.	Maître de Conférences	En poste
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	MVOGO ALAIN	Maître de Conférences	En poste
	NDOP Joseph	Maître de Conférences	En poste
	SIEWE SIEWE Martin	Maître de Conférences	En poste

	SIMO Elie	Maître de Conférences	En poste
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DÉPARTEMENT	**Professeurs**	**Maîtres de Conférences**	**Chargés de Cours**	**Assistants**	**Total**
BCH	8 (01)	15 (11)	13 (03)	7 (05)	**43 (20)**
BPA	14 (01)	16 (09)	18 (04)	4 (02)	**52 (16)**
BPV	6 (01)	12 (02)	13 (07)	3 (00)	**34 (10)**
CI	7 (01)	15 (04)	5 (01)	1 (00)	**28 (06)**
CO	6 (01)	18 (04)	11 (04)	2 (00)	**37 (09)**
IN	2 (00)	2 (00)	14 (01)	4 (00)	**22 (01)**
MAT	1 (00)	8 (00)	17 (01)	7 (02)	**33 (03)**
MIB	2 (01)	7 (03)	8 (04)	7 (02)	**24 (10)**
PHY	15 (01)	15 (04)	11 (01)	2 (00)	**43 (06)**
ST	8 (00)	17 (03)	15 (04)	3 (01)	**43 (08)**
Total	**69 (07)**	**125 (40)**	**125 (30)**	**40 (12)**	**359 (89)**

Soit un total de **359 (89)** dont :

Professeurs **69 (07)**

Maîtres de Conférences **125 (40)**

Chargés de Cours **125 (30)**

Assistants **40 (12)**

( ) = Nombre de Femmes **89**

DEDICATION

I dedicate this work:

To my parents

BOUAKA Emmanuel and MAFFO Jeannette

ACKNOWLEDGEMENTS

This work was a part of the PIIVeC (Partnership for Increasing the Impact of Vector Control) project funded by the UK government through the Medical Research Council (MR/P027873/1), in collaboration with the Centre for Research in Infectious Diseases (CRID) and the University of Yaoundé I (Laboratory of Molecular Parasitology and Vector Research). I would like to thank all these organisations.

This work could never have been done without the contribution of several people of whom I would like to thank sincerely. I am thinking in particular of:

- **Professor Charles WONDJI** who opened the doors of CRID to me at the end of my Master's degree. As a true teacher, supervisor, with his constructive criticism, his scientific and moral rigour, he was able to guide the researcher I have become today. He spared no effort in the success of this work. May you find here the expression of my deepest gratitude;
- **Professor Jude BIGOGA**: Despite your numerous occupations, you have shown dedication in accepting to supervise this work. Your human qualities, practical sense and rigour for a job well done have aroused in me admiration and respect. Thus, your advice and criticism have been precious for the scientific quality of this work. Please find here the expression of my deepest gratitude;
- **Dr Trésor Tito MELACHIO TANEKOU**, my mentor. You initiated this thesis project and relented no effort to guide and supervise it step by step. I thank you for your great availability, kindness and precious advice. Through your scientific rigour, you always pushed me to be a better scientist, as you used to say, "if I train you and you are not better than me, then I have failed in your training". I would never thank you enough for the quality of time spent during this thesis, both scientifically and humanly. You have not only been a mentor; you are an elder brother and you will remain an example for me;
- **Professor Gustave SIMO**, whose precious advice has been of great help to me in the beginning of this thesis. I thank him greatly;
- **Dr Elvis OFON,** for the corrections made which allowed the improvement of this work. I sincerely thank him;
- All the Lecturers of the Department of Biochemistry of the University of Yaoundé I: Through your contribution to my academic training, you have made me the Biochemist I am today. You have helped me to build a good knowledge base in order to enter the

world of research being sufficiently equipped. Please accept the expression of my gratitude and deep respect

- The members of the PhD office at CRID with whom I spent extraordinary moments in the laboratory, both scientifically and humanly. Our various discussions and sharing of the daily good mood, were essential to dissipate the stress of the thesis, and very beneficial. Thank you for your presence whenever I needed and for the sincere friendship you showed in the laboratory and outside. This is about: **Claudine MAFFO**; **Francine SADO**; **Yvan FOTSO**; **Amelie WAMBA**; **Marilene MAE**; **Fred ASHU;**
- The laboratory seniors and all the members of the CRID, who have repeatedly examined this work during the laboratory presentations, their corrections and suggestions have made this work better. All those I had to work with during these years of thesis, and whose multiple exchanges and discussions allowed me to make this work always better. I thank them;
- My brothers and sisters **Liliane MEKANANG**, **Belange SONFACK**, **Patrick WOUAFACK**, **Piaget DONFACK**, **Junior GUETSA** and **Ocean DONFANG**. Without the support they made for me, I would not have been able to finish this work;
- My beloved **Roseline MEFFO**, who always supported me in difficult moments, you knew how to cheer me up every time I despaired, find here dear the expression of my deep gratitude.

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LIST OF ABBREVIATIONS

AAT: Animal African Trypanosomiases

BBB: Blood-brain barrier

bp: Base pair

CI: Confidence Intervale

DNA: Deoxyribonucleic acid

dNTPs: Deoxynucleoside triphosphate

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme Linked Immunosorbent Assay

HAT: Human African trypanosomiasis

IgG: Immunoglobulin G

IL: Interleukin

INF-γ: Interferon gamma

KCl: Potassium chloride

$MgCl_2$: Magnesium chloride

NO: Nitrogen monoxide

NCP: National Control Program

NSSCP: National Sleeping Sickness Control Program

ORF: Open Reading Frame

OTU: Operational Taxonomic Unit

PATTEC: Pan African Tsetse and Trypanosomiasis Eradication Campaign

PBS: Phosphate Buffer Salin

PCR: Polymerisation Chain Reaction

RFLP: Restriction Fragments Length Polymorphism

RNA: Ribonucleic acid

SDS: Sodium Dodecyl Sulfate

Taq: Thermus aquaticus

TBE: Tris-Borate-EDTA

THA: Human African Trypanosomiasis

Tris: Tris hydroxymethyl aminomethane

TLTF: Trypanosome Lymphocyte Triggering Factor

TNF-α : Tumor Necrosis Factor- α

UV : Ultraviolet

VSG : Variant Surface Glycoprotein

WHO: World Health Organisation

ABSTRACT

The interest of vector control in the fight against African Trypanosomiases has been reinforced in recent years by the development of small insecticide-impregnated screens, known as "Tiny Targets". In addition, impairing the development of the parasite in the vector and therefore reducing the vectorial competence of tsetse flies was shown to be an alternative or additive method to stop disease transmission. To improve knowledge in developing this new vector control strategies, study on the characterisation of bacterial communities hosted by tsetse flies, their association with trypanosome establishment and their potential implication in the flies' fitness during vector control with "Tiny Targets" was carried out in Campo in Southern Cameroon.

The study site was divided into two areas, the South-West experiment area with vector control and the eastern control area. Tsetse flies were collected in December 2018 and July 2019 as baseline data prior to the Tiny Targets installation. Then, around 2000 targets were deployed in the South-West area and replaced every six months covering 2-year periods. Post-intervention surveys were conducted every six months and apparent tsetse densities were calculated, compared, and mapped. The different trypanosome species and the origin of *Glossina palpalis palpalis* flies blood meals were identified by PCR. Amplification of the highly variable V3-V4 region of the 16S rRNA bacterial gene and sequencing with Illumina Miseq® platform were carried out, followed by meta-genomic analysis to identify the different bacterial communities, to determine the impact of certain taxa in flies' infectivity or fitness.

Our study revealed the presence of four species of trypanosomes in *Glossina palpalis palpalis*. From two collection periods (December 2018 and July 2019), a total of 18.52% tsetse was infected with at least one trypanosome species respectively. Specifically, 13.75% were infected by *Trypanosoma congolense*, 4.51% by *T. brucei s.l.*, 1.51% by *T. vivax* and 0.32% by *T. simiae*. From all the flies infected with *T. brucei s.l.*, 4 were infected with *Trypanosoma brucei gambiense* subspecies which is the parasite responsible for sleeping sickness. Blood meal analysis revealed 2 out of 85 tsetse flies analysed fed on humans. Following the tsetse control intervention, tsetse densities decreased by 61% after six months and up to 73% after twelve months (pre-intervention: 2.48 flies/trap/day, 95%CI [1.92-3.14]; 12-months post-intervention: 0.66 tsetse/trap/day, 95%CI [0.42-0.94]). This decrease was not sustained after 18 months where the 12-months density doubled, and 24 months where the density still increased by 17% (18 months: 1.45 tsetse/trap/day, 95%CI [1.07-1.90] and 24 months: 1.71 tsetse/trap/day, 95%CI [1.27-2.24]). In the control area, variation of tsetse densities was

observed during the two years, with a general increase from 2.43 [0.73-5.77] to 3.64 [1.47-7.70] tsetse/trap/day. In addition, trypanosome infection rates dropped by 75% in both areas (P-value < 0.001) from 21.20% to 5.06% and from 13.14% to 3.45% in intervention and control areas respectively. Characterisation of the bacterial flora of the tsetse examined shown a total of 4 phyla and 31 bacterial genera present in tsetse flies. The primary symbiont *Wigglesworthia* was present in almost all specimens with an overall relative abundance of 47.29%. However, this symbiont seems to be replaced by *Serratia* or *Burkholderia* in some flies of the species *G. tachinoides*. Overall, significant differences were observed in bacterial diversity between tsetse species ($p<0.001$), between teneral and non-teneral flies ($p<0.001$) and between flies with and without mature trypanosome infections ($p<0.031$). Moreover, differential abundance tests shown some bacteria taxa associated with trypanosome maturation in tsetse flies, the most important being *Dechloromonas* (*log2 Fold Change* = -21.44; $p<0.001$), *Ralstonia* (*log2 Fold Change* = -10.59; $p<0.001$), *Listeria* (*log2 Fold Change* = -5.41; $p<0.001$), *Serratia* (*log2 Fold Change* = 3.39; $p<0.001$) et *Staphylococcus* (*log2 Fold Change* = 5.48; $p= 0.003$). Significant increase was observed in bacteria diversity and composition in tsetse flies during the vector control (Before vector control, Shannon H = 0.45; six months H = 0.44; twelve months H = 1.24; eighteen months H = 0.99; $p = 4{,}9 \times 10^{-05}$). Assessment of the relative abundances of the different phyla before and after the installation of Tiny targets showed a significant reduction in the abundance of *Firmicutes* ($p < 0.02$) and a concomitant increase in *Proteobacteria* ($p < 0.001$). In addition, differential abundance testing showed several bacteria genera contributing to differences between tsetse flies collected before and after 18 months of vector control. Phylogenetic analyses of bacterial genera represented by several operational taxonomic units (OTUs) showed that these OTUs exhibited some degree of intra-generic genetic polymorphism, strengthening the hypothesis that vector competence may be related to given bacteria genotypes.

This study shown bacteria taxa associated with trypanosome infection maturation in flies, which therefore need further studies for an understanding of their mechanism of action and alternatively, transformed and used to block trypanosome development in tsetse flies.

Key words: Trypanosomiases; Tiny Targets; Microbiome; Vector competence; Vector control.

RESUME

L'intérêt porté au contrôle des vecteurs dans la lutte contre les trypanosomoses Africaines a été renforcé ces dernières années grâce au développement de petits écrans imprégnés d'insecticide, appelés « Tiny Targets ». De plus, des études récentes montrent que certaines bactéries peuvent être utilisées pour bloquer le développement du trypanosome chez les glossines. Dans le but de contribuer au développement de ces nouvelles stratégies, une étude sur la caractérisation des communautés bactériennes hébergées par les glossines et leur association avec l'établissement des trypanosomes, ou leur implication dans la capacité de survie de ces mouches pendant la lutte antivectorielle avec les « Tiny Targets » a été réalisée à Campo, au Sud Cameroun.

A cet effet, la zone d'étude a été divisé en deux polygones (la zone traitée dans la partie Sud-Ouest et zone témoin dans la partie Est). Les tsé-tsé ont été collectées en décembre 2018 et juillet 2019 pour déterminer les données de base sur leur densité, leur taux d'infection aux trypanosomes et la composition de leur microbiome bactérien. Par la suite, environ 2000 « Tiny Targets » ont été déployés dans la zone d'intervention, et remplacés par de nouveaux tous les six mois, couvrant une période de 2 ans. Des enquêtes post-intervention ont été menées tous les six mois et les densités apparentes des tsé-tsé ont été déterminées et cartographiées. Les différentes espèces de trypanosomes et l'origine du repas sanguin chez *Glossina palpalis palpalis* ont été identifiés par PCR. L'amplification de la région hautement variable V3-V4 du gène 16S de l'ARNr des bactéries et le séquençage de nouvelle génération Illumina Miseq® ont été effectués, suivis d'une analyse métagénomique afin de déterminer la composition du microbiome bactérien des tsé-tsé, et l'impact de certains taxons bactériens sur l'infectivité de ces vecteurs aux trypanosomes, ou leur aptitude de survie pendant la lutte antivectorielle.

Les analyses ont révélé la présence de quatre espèces de trypanosomes chez *Glossina palpalis palpalis*. Pendant les deux périodes de collecte avant l'intervention, 18,52% de glossines étaient infectées par au moins une espèce de trypanosomes. Spécifiquement, 13.75% étaient infectées par *Trypanosoma congolense*, 4.51% par *T. brucei* s.l., 1.51% par *T. vivax* et 0.32% par *T. simiae*. Parmi toutes les mouches infectées par *T. brucei* s.l., une et trois étaient infectées par la sous-espèce *T. brucei gambiense*, le parasite responsable de la maladie du sommeil, respectivement pour les deux périodes. En outre, 22,60 % des mouches infectées de décembre 2018 portaient des infections matures de *T. congolense*. L'analyse des repas sanguins a révélé que 2 mouches se sont nourries sur des humains. Pendant la lutte antivectorielle, les densités de tsé-tsé ont diminué de 61% après six mois et jusqu'à 73% après douze mois (avant l'intervention : 2,48 mouches/piège/jour, 95%IC [1,92-3,14] ; 12 mois après l'intervention : 0,66

tsé-tsé/piège/jour, IC95% [0,42-0,94]). Cette diminution n'a pas été maintenue après 18 mois où la densité à 12 mois a doublé, et à 24 mois où la densité observée à 12 mois a encore augmenté de 17% (18 mois : 1,45 tsé-tsé/piège/jour, IC95% [1,07-1,90] et 24 mois : 1,71 tsé-tsé/piège/jour, IC95% [1,27-2,24]). Dans la zone contrôle, une augmentation des densités de tsé-tsé a été observée au cours des deux années, allant de 2,43 [0,73-5,77] à 3,64 [1,47-7,70] tsé-tsé/piège/jour. En outre, les taux d'infection par les trypanosomes ont chuté de 75 % dans les deux zones, allant de 21,20 % à 5,06 % et de 13,14 % à 3,45 % dans les zones traitées et contrôle respectivement (valeur P < 0,001). La caractérisation de la flore bactérienne des glossines a montré la présence de 4 phyla et 31 genres bactériens. Le symbiote primaire *Wigglesworthia* était présent dans presque tous les spécimens avec une abondance relative globale de 47,29%. Dans l'ensemble, des différences significatives ont été observées dans la diversité bactérienne entre les espèces de tsé-tsé, entre les mouches ténérales et non ténérales et entre les mouches ayant ou non des infections matures à trypanosomes (p<0,031). En outre, des tests d'abondance différentielle ont montré que certains taxons étaient associés à la maturation des trypanosomes chez les mouches. En ce qui concerne les mouches non infectées, le genre *Dechloromonas* était 21,44 fois moins abondant (p<0,001), le genre *Ralstonia* était 10,59 fois moins abondant (p<0,001) et le genre *Listeria* était 5,41 fois moins abonadnt (p<0,001). Cependant, *Serratia* était 3,39 fois plus abondant (p<0,001) et *Staphylococcus* 5,48 plus abondant (p= 0,003). Les résultats ont montré une augmentation significative de la diversité bactérienne chez les glossines au cours de la lutte antivectorielle (Avant la lutte antivectorielle, l'indice de Shannon H allant de 0,45 avant, à 0,44, 1,24 et 0,99 après six mois, douze mois et dix-huit mois de lutte antivectorielle ; p < 0.001). Les analyses phylogénétiques des genres bactériens représentés par plusieurs unités taxonomiques opérationnelles (UTOs) ont montré que ces UTOs présentent un certain degré de polymorphisme génétique intra-générique qui renforce l'hypothèse selon laquelle la compétence vectorielle peut être liée à des génotypes bactériens donnés.

Cette étude a mis en évidence des taxons bactériens associés à la maturation de l'infection aux trypanosomes chez les glossines pouvant faire l'objet d'études approfondies pour comprendre leur mécanisme d'action afin de les utilisées pour bloquer le développement du trypanosome chez les mouches tsé-tsé.

Mots clés : Trypanosomoses, « *Tiny Targets* » ; Microbiome ; Compétence vectorielle ; Lutte antivectorielle.

INTRODUCTION

African trypanosomiases are vector-borne tropical diseases caused by protozoan parasites of the genus *Trypanosoma* and transmitted to susceptible hosts via the bite of blood-sucking tsetse flies of the genus *Glossina* (Büscher *et al*., 2017). These diseases occur in 36 countries throughout sub-Saharan Africa with about 60 million people, 55 million cattle and 70 million small ruminants at risk of the disease (Cecchi & Mattioli, 2009; Kennedy, 2013). In humans, the disease is known as sleeping sickness or human African trypanosomiasis (HAT) while in livestock, it is called nagana or animal African trypanosomiasis (AAT). However, there are two forms of HAT, the more common (chronic form) found in West and Central Africa, caused by *Trypanosoma brucei gambiense*, responsible for about 97% of HAT cases, and the less common (acute form) found in Eastern and Southern Africa, caused by *Trypanosoma brucei rhodesiense*, responsible for about 3% of cases (Brun *et al*., 2010). Nagana cost USD 4.5 billion lost yearly to the African agriculture (Morrison *et al*., 2016) and therefore remains one of the main constraints for the development of livestock and agriculture in sub-Saharan Africa (Diall *et al*., 2017).

Large outbreaks of HAT occurred in the first half of the 20th century with 30,000 officially reported cases each year. These epidemics were largely brought under control by large-scale control programmes of active detection and treatment of cases (Kennedy, 2019). However, as a result of major efforts by WHO and partner agencies, with governments in improving both human case detection and treatment implemented by National Control Programmes (NCPs), as well as few vector control, HAT declined over the 3 past decades to a lowest record of ~2164 new cases in 2016 (Kennedy & Rodgers, 2019) and 977 in 2018 (Franco *et al*., 2020). Therefore, WHO aimed to interrupt all transmission by 2030. In Cameroon, Campo located in the South forest Region is an historical sleeping sickness focus where the disease has been mostly detected these later 10 years with around 7 new cases each year between 2012 and 2018 (NSSCP records). In 2019, a drastic increase was observed in the incidence, with 20 new cases detected (Franco *et al*., 2022) and therefore making Campo an important focus where HAT persists for decades. So far, there is no vaccine against trypanosomiasis, mainly due to the ability of trypanosomes to continually alter their surface glycoprotein layer through expressing distinct antigenic variants for immune evasion (Lumbala *et al*., 2018). Currently, control of HAT relies essentially on the active detection and treatment of cases, which often reaches less than 75% of the affected population (Tirados *et al*., 2015). Moreover, the use of chemotherapy is limited by chemotoxicity and increasing levels of resistance to the available drugs (Nerima *et al*., 2007; Chitanga *et al*., 2011). Nevertheless, some

efforts are being done to overcome these challenges, with new therapies like nifurtimox-eflornithine combination treatment and the recent fexinidazole. In addition, the possibility of there being animal reservoir hosts (Njiokou *et al*., 2006; Funk *et al*., 2013) may maintain circulation of parasites even if all human cases are detected, which may make complete elimination of gHAT difficult (Njiokou *et al*., 2006; Funk *et al*., 2013; Simo *et al*., 2014).

Vector control intervention strategies which aim to reduce the tsetse fly population density and/or their ability to transmit trypanosomes (vector competence) are better complementary methods to help curbing the disease transmission. However, vector control has long been considered infeasible on a large scale due to the costs associated with available vector control methods (Tirados *et al*., 2015; Lehane *et al*., 2016). In response to this, cheaper control methods have recently been developed; the most effective being small insecticide (deltametrin) impregnated screens of approximately 25 × 50 cm, called 'Tiny Targets' attracting tsetse and exposing them to the lethal dose of insecticide. These screens are easy to deploy, relatively cheap (Shaw *et al.*, 2015) and have increased interest in vector control for trypanosomiasis.

Other promising vector control methods under development rely on the use of the microbial symbionts of tsetse flies. To be transmitted, trypanosomes must first establish themselves in the tsetse fly midgut following a blood meal, and thereafter, mature in salivary glands or mouth parts, depending on the species (Van Den Abbeele *et al*., 1999). This phenomenon is influenced by several factors, among which the microbiome harboured by the tsetse vector, which are known to play diverse roles in their hosts. Studies have shown that symbiotic bacteria in insects, especially in the gut, endow the host with improved environmental adaptability (Tsuchida *et al*., 2014), enhanced immunity against external pathogenic microorganisms and increased detoxification rates (Oliver *et al*., 2003; Vorburger & Rouchet, 2016). Therefore, symbiotic microorganisms are considered as a potential tool to reduce or stop sleeping sickness and nagana transmission. They can be used to reduce the life span of tsetse flies or reduce infection rates by trypanosomes, either through natural competition mechanisms or through the production of genetically introduced anti-parasitic molecules by paratransgenesis (De Vooght *et al*., 2014; Romoli & Gendrin, 2018). Therefore, understanding the role of the tsetse microbiome in vector competence as well as in disease transmission is important as it could improve knowledge in initiatives to manage or develop these new vector control strategies.

Three major symbionts are described in tsetse flies: the intracellular primary symbiont *Wigglesworthia glossinidia*, necessary for the fly's fertility and immune response (Aksoy, 1995); *Wolbachia* sp, which acts on the reproductive process of tsetse flies by inducing

cytoplasmic incompatibility (Alam *et al*., 2011), and the secondary symbiont *Sodalis glossinidius*, present in gut and other tissues of the fly (Geiger *et al*., 2007). This latter was found to be involved in trypanosome establishment in the fly midgut through a complex biochemical mechanism involving inhibition of trypanocidal lectins specific to the procyclic forms of the parasite by N-acetyl glucosamine, resulting from hydrolysis of pupae chitin by endochitinases produce by *Sodalis* (Welburn & Maudlin, 1999; Dale *et al*., 2001). Beyond these three major symbionts, *Kosakonia cowanii* have been show to impairs trypanosome establishment in tsetse, while protecting this vector from the entomopathogenic *Serratia marcescens* (Weiss *et al*., 2019). This later has been shown to render mosquitoes resistant to plasmodial infection through the activation of immune response and also inhibits *Trypanosoma cruzi* in triatomine vectors (Bai *et al*., 2019; Da Mota *et al*., 2019).

Recent studies have shown a great diversity in the bacterial flora of *Glossina* species in different sleeping sickness foci in Cameroon (Jacob *et al.*, 2017; Kame-Ngasse *et al.*, 2018;Tsagmo *et al.*, 2019; Ngambia Freitas *et al.*, 2021). However, in these descriptive studies, no association was found between the presence of *Sodalis glossinidius* and trypanosome infection in flies, suggesting that vector competence might rather be linked to given bacteria genotypes or the abundance of the symbiont (Geiger & Frutos, 2005). Although previous studies that described the bacterial communities in tsetse flies have mainly focused on the midgut compartment (Hamidou *et al*., 2013; Jacob *et al*., 2017; Ngambia *et al*., 2021), other insect tissues could harbour some bacteria taxa not yet described in the tsetse, or could be a key localization for other important bacteria detected in the gut. Thus, the bacterial content of the whole fly has not yet been fully assessed. Therefore, studies are needed to make a complete inventory of bacterial communities associated with tsetse flies in order to characterize and provide a more comprehensive overview of their composition and association with trypanosome establishment or other traits.

Research Questions

- What is the composition of bacterial communities associated with the whole tsetse fly?
- Does the establishment or maturation of trypanosome infections in tsetse flies depend on the composition of their microbiome?
- Is vector control using insecticide impregnated tiny targets affect the composition of bacteria communities of remaining tsetse flies?
- Is vector competence linked to given genotypes of the bacteria?

Hypotheses

- Other insect tissues are key organs for bacterial development;
- The maturation of trypanosomal infection in tsetse flies depends on the composition of their microbiome;
- Bacterial diversity of flies varies during vector control;
- The vector competence of flies is linked to given bacterial genotypes rather than to the simple presence/absence of a taxon;

General objective

To improve the fight against trypanosomiases by studying the different bacterial communities present in tsetse flies and their association with fly's vector competence.

Specific objectives

- To identify the different trypanosome species circulating in tsetse flies, as well as flies' blood meal origin;
- To characterise the different bacterial communities present in some tsetse species and their association with the establishment of trypanosomes in *Glossina palpalis palpalis*;
- To study the variation of the bacterial composition of *Glossina palpalis palpalis* during vector control with "Tiny Targets" and their association with the potential tsetse fitness;
- To study the phylogenetic diversity of some bacteria of interest.

CHAPTER I: LITERATURE REVIEW

I.1. Human African Trypanosomiasis (HAT)

I.1.1. Epidemiological situation

Two subspecies of trypanosome are pathogenic to humans: *T. brucei gambiense* and *T. brucei rhodesiense*. However, biologically, clinically, therapeutically, geographically and epidemiologically, these parasites represent two distinct entities and cause two different diseases (Brun *et al*., 2010). In Eastern and Southern Africa, *T. b. rhodesiense* causes a rapidly progressive acute infection, while *T. b. gambiense* infection, which occurs in Western and Central Africa progresses more slowly. Limited to sub-Saharan Africa between latitudes 14°N and 29°S (Figure 1), there are around 250 foci of sleeping sickness in 36 countries (Kennedy, 2004). Around 60 million people are at risk of HAT, of which only 4 million are monitored with 25,000 patients followed up annually (Kennedy, 2013). In 2014, the number of reported cases was reduced to 3796 cases, in 2016 the figure was further reduced to 2184 new cases per year (Kennedy, 2019) and to 977 in 2018 (Franco *et al*., 2020). HAT cases have been documented in Europe and the United States, usually in recently returned travellers from East or West Africa. Between 2000 and 2010, 94 cases were reported in non-endemic countries (Simarro *et al*., 2012). Of these, 43% were diagnosed in Europe and 23% in the USA (Blum *et al*., 2012).

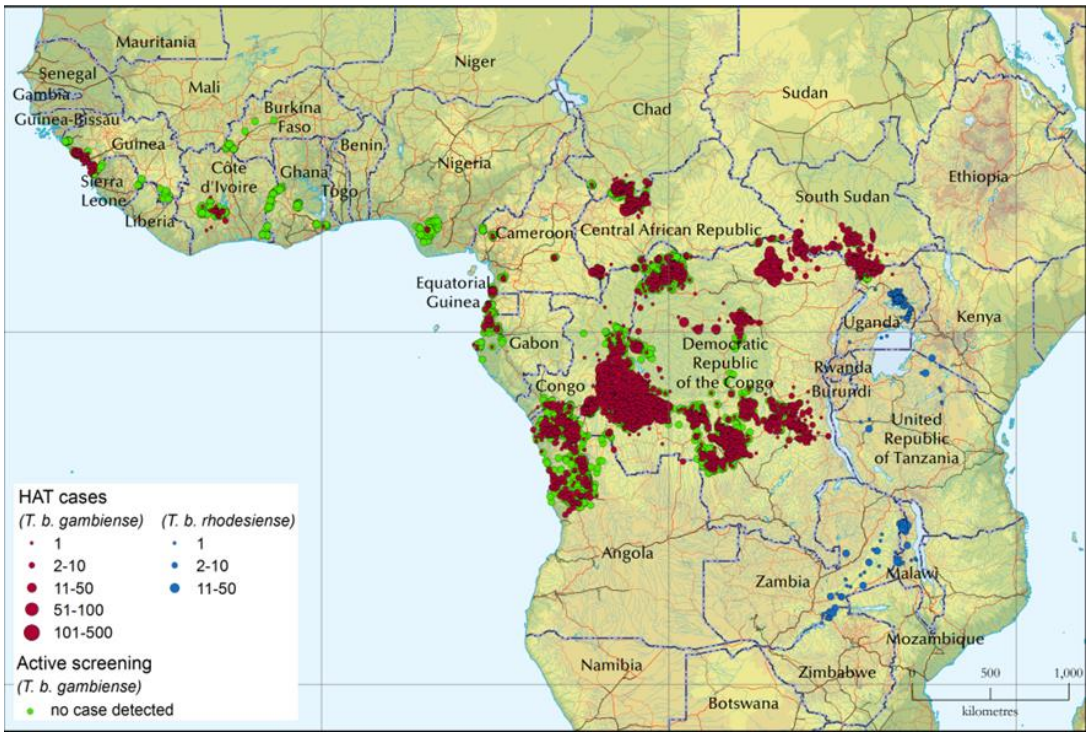

Figure 1. Geographical distribution of human African trypanosomiasis. Period of 2017-2018 (Franco *et al*., 2020).

According to the WHO Sleeping sickness assessment report 2010, Cameroon is considered as a meso-endemic transmission country with less than 100 new cases detected every year (WHO, 2010). Five active foci of sleeping sickness are described in Cameroon including the Fontem and Mamfe foci in the South-West Region, the Bipindi and Campo foci in the South Region and the Doumé foci in the East Region (Figure 2). In 2006, 15798 people were screened and 15 new cases diagnosed, while 24 new cases were observed in 2009 (Simarro *et al*., 2010). However, the disease has mainly been detected in the last 10 years in Campo, in the Southern Forest Region, where about 7 new cases were recorded each year between 2012 and 2018. In 2019, a drastic increase in incidence was observed, with 20 new cases detected (NSSCP records).

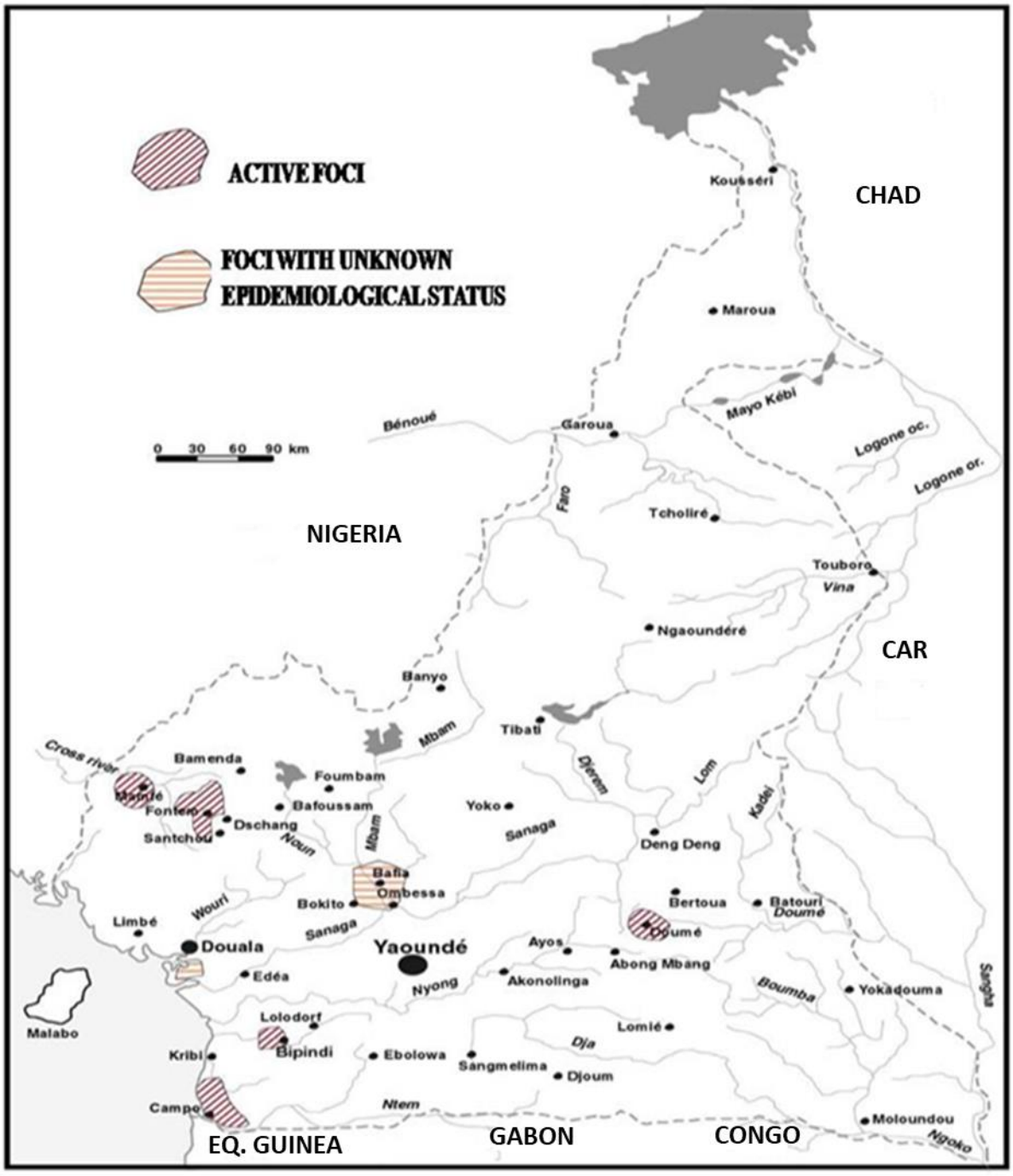

Figure 2. Different human African trypanosomiasis foci in Cameroon (Simo, 2009).

I.2.2. Biological manifestation and disease evolution

The disease has two phases or stages, the haemo-lymphatic phase corresponding to the presence of trypanosomes in the blood and lymphatic- systems, and the meningo-encephalitis phase, which begins when the trypanosomes invade the central nervous system (CNS). However, the clinical signs and symptoms lack specificity, and their frequencies vary from one individual to another.

After the bite and inoculation of trypanosomes, the host reaction is initially local with the development of an inoculation chancre. This corresponds to cellular infiltration of neutrophils and T and B lymphocytes with a predominance of CD8+ T lymphocytes (Mwangi et al., 1996) in response to the secretion of a T CD8+ lymphocyte activator, the Trypanosome Lymphocyte Triggering Factor (TLTF) or trypanin (Olsson *et al*., 1991). The activated T CD8+ cells produce interferon-γ (IFN-γ) which have been suggest to be a growth factor for the trypanosome. The IFN-γ produced also enables the activation of monocyte-macrophages which in turn produce nitric oxide (NO) and Tumor Necrosis Factor-α (TNF-α) toxic to the trypanosome. The macrophagic system thus represents the first body's defence barrier. When this barrier is breached, the trypanosome reaches the lymphatic-sanguineous system (stage 1). It will then be detectable in the blood and/or lymph nodes and a state of armed peace may be established for several months to several years between the host's defence reactions and the parasite's escape mechanisms. The infected individual thus unknowingly becomes a reservoir of parasites, perpetuating transmission (Koffi *et al*., 2006). The main mechanism used by the trypanosome is antigenic variation by periodically changing its surface antigens (VSG for "variant surface glycoprotein"), which are highly immunogenic. During this haemo-lymphatic phase, four main clinical signs can be distinguished: persistent fever (between 38 °C and 38.5 °C) along with peaks of parasitaemia; adenopathy and hepatosplenomegaly (signs of inflammatory reaction produced by the host), and cutaneous signs (oedema and pruritus) (Dumas *et al*., 1999). After a non-defined period, often several months to several years for *Trypanosoma brucei gambiense* infection, the trypanosomes penetrate the blood-brain barrier (BBB) and cause a central nervous system (CNS) infection resulting in mesenchymal meningoencephalitis with perivascular infiltration (stage 2). The mechanisms of this passage are not fully understood. However, it is assumed that BBB passage is an active phenomenon mediated by a multifactorial system depending on diffusible substances from the parasite (mainly VSGs, hydrolases, proteases, saturated fatty acids and aromatic amino acid derivatives) and/or substances secreted by the host (endotoxins, prostaglandins, NO, TNF-α, IFN-γ) that promote parasite passage (Keita *et al*., 1997; Pentreath *et al*., 1997; Nikolskaia *et al*., 2006). In addition a parasite-derived protein (trypanosome apoptotic factor) has been shown to induce apoptosis in human endothelial cells, which may be a key mechanism for parasite passage into the CNS (Stiles *et al*., 2004). Furthermore, while the signs of stage 1, except fever, regress, sensitivity, psychic, motor, neuroendocrine, and sleep disorders appear. The latter are the best known and potentially the earliest signs. They are characterised by a succession of episodes of wakefulness and sleep beginning abnormally with a paradoxical sleep phase. The evolution of

the disease in the absence of treatment is towards a state of permanent sleep cachexia with encephalitis, which is sometimes irreversible.

Human African trypanosomiasis with *Trypanosoma brucei rhodesiense* has a similar but much more rapid evolution with potentially lethal acute clinical manifestations (Koten & De Raadt, 1969).

I.2. Animal African Trypanosomiasis (AAT)

I.2.1. Epidemiological situation

AAT is an infectious disease mainly caused by *T. congolense*, *T. vivax*, *T. simiea* and *T. brucei brucei*. The evolution of the disease varies from acute to chronic depending on the parasite species involved. The clinical picture of AAT is characterised by intermittent fever, anaemia, occasional diarrhoea, and rapid loss of body weight. If left untreated, the disease ends in death (Itard, 2000).

Today, there are about 36 endemic countries including Cameroon (Coustou *et al*., 2010). The prevalence of AAT varies between countries or regions within the same country. Recent studies have shown that AAT remains a threat to animals in many rural communities in Africa with high prevalence of different trypanosome species (Kouadio *et al*., 2014; Alemu & Alemneh, 2017; Malekani, 2017). In Cameroon, all the ten regions are likely to be endemic. Moreover, the regions of Adamawa, the North and the Far North are the most affected areas because they are infested with many tsetse fly species of veterinary importance and constitute a high cattle and small ruminant breeding area (PATTEC, 2010). Studies conducted in the southern forest of Cameroon have revealed the presence of many trypanosomes species (*T. congolense*, *T. brucei* s.l., *T. vivax*, *T. simiae*) in domestic animals, wildlife and tsetse *(*Morlais *et al*., 1998; Herder *et al*., 2002; Njiokou *et al*., 2004; Simo *et al*., 2006; Nimpaye *et al*., 2011). The AAT annual losses are estimated at US$ 4750 million (Vreysen *et al*., 2013) including losses of 10-50% of livestock, 2-10% of agricultural products and 10-40% of milk (Duvallet, 2015). This disease thus has devastating effects on the livelihoods of rural local farmers, for whom livestock represent not only a source of food (meat and milk) but also a source of savings to fulfil certain social obligations (Mungube *et al*., 2012). Because of the adverse effects of AAT, areas with high agricultural potential that are very often infested with tsetse are increasingly abandoned in favour of less productive but trypanosomiasis-free areas, thus, resulting in a reduction of agricultural yields and the massive importation of meat and dairy products.

I.2.2. Biological manifestation and disease evolution

The disease usually begins with a phase of hyperthermia, corresponding to the first peak of parasitaemia. In the acute form, lethality is high and occurs within 2 to 3 weeks after the onset of symptoms. The chronic form evolves over two to three months, with a high fever (40 to 41°C), progressive weight loss to the point of cachexia, weakness, prickly hair, lacrimation, pale mucous membranes due to anaemia, and lymph node hypertrophy (Sidibé, 1996).

The host response is initially through the activation of T-CD4 lymphocytes by antigenic variants (specifically VSG). These immune reaction lead to the production of cytokines IFN-γ, IL-2, IL-4 and TNF-α and also reactive oxygen molecules NO (nitric oxide) (Vincendeau & Bouteille, 2006) which will stimulate the production of immunoglobulins and cytotoxic cells. Several of these molecules are responsible for the immunosuppression observed in animals with infections. TNF-α, whose secretion is induced by soluble VSGs on the surface of trypanosomes, first activates the production of suppressor macrophages in the ganglia. These macrophages will inhibit the activation of T lymphocytes (responsible for the synthesis of IL-2) by producing prostaglandins. In a second step, they contribute to the activation of CD8 lymphocytes for the hyper production of IFN-γ which will inhibit the expression of IL-2 receptors at the lymphocyte level. In addition, these IFN-γ stimulate macrophage proliferation and lead to the release of NO (Dagenais *et al*., 2009). In synergy with prostaglandin, NO inhibits IL-2 production. IL-10, which is produced during infection has an inhibitory effect on the Th1 response and macrophage activation (Moore *et al*., 2001).

In West Africa, *T. vivax* are the most common species. However, *T. congolense* is the most pathogenic species for cattle, usually with a chronic form of the disease. *T. brucei brucei* infection usually manifests itself in a chronic form, with a slow onset with intermittent febrile state, nasal and ocular discharge, digestive disturbances, progressive anaemia, and pitted hairs that subsequently fall out (Itard and Frézil 2003).

I.3. Aetiological agent: Trypanosome

Trypanosomes are small, flagellate protozoa classified as kinetoplastids (Hoare, 1972). There are about 20 species infecting various vertebrates.

I.3.1. Morphology

Trypanosomes are slender, spindle-shaped cells with an undulating membrane more or less wrapped around the body. Their size varies according to species and measure between 20-40 µm long and 2-3 µm wide (Vickerman *et al*., 1988). Trypanosomes have a central nucleus

containing the bulk of the genome and a specific organelle: the kinetoplast, which contains the extranuclear DNA (Matthews, 2009) and whose function is analogous to that of the mitochondria. The undulating membrane has a filament at the outer end that forms the flagellum, which in some species extends beyond the body into a free flagellum (Figure 3).

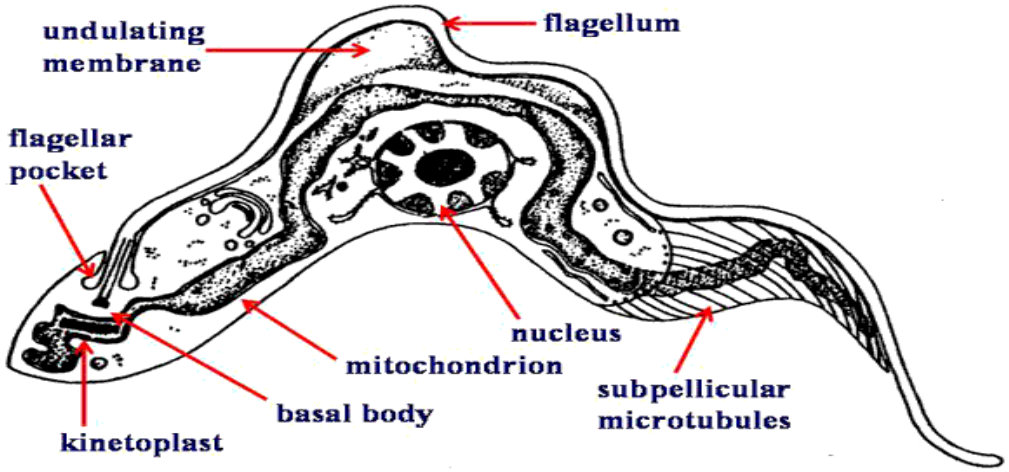

Figure 3. Morphology of *Trypanosoma brucei* s.l. (Modified from Laveissiere, 2005)

I.3.2. Classification of trypanosomes

According to the classification of Hoare (1972) reviewed by Levine *et al*. (1980), mammalian trypanosomes are classified into several species on the basis of morphological characteristics, geographical distribution, transmission pattern, host specificity and pathology caused. The genus *Trypanosoma* is subdivided into two sections: *Stercoraria* with a posterograde developmental cycle and *Salivaria* which has an anterograde developmental cycle (Figure 4).

I.3.2.1. *Stercoraria*

This group includes mostly trypanosomes that are non-pathogenic to humans and livestock. In vectors, the development cycle of *Stercoraria* takes place entirely in the digestive tract and transmission to the vertebrate host is by contaminating droppings of the vector on the skin. From this group, only *T. cruzi* of the subgenus *Schizotrypanum* causes a human disease called Chagas disease in Latin America (Breniere *et al*., 1989).

I.3.2.2. *Salivaria* group

The *Salivaria* section includes all pathogenic trypanosomes that are transmitted by tsetse flies and few other blood-sucking insects. Transmission occurs by inoculation of the infective forms during the insect bite. This group is divided into five subgenera with some morphological

similarities but differ in their evolutionary cycles in tsetse and in their vertebrate hosts. The three subgenera that are most widespread in Africa with considerable economic importance are described below.

a. Subgenus *Duttonella*

Within this group, *T. vivax* is the most prevalent trypanosome species. It was first described in Africa, specifically in a goat in Cameroon by Ziemannen (1905). In central and western Africa, *T. vivax* is generally less pathogenic than *T. congolense* (Trail *et al*., 1990). This species can be transmitted cyclically by tsetse flies and mechanically by other biting insects. *T. vivax* is not exclusively an African parasite (Usman *et al*., 2008); they are also found in tropical Latin America, India and in the Sahelian regions where they are responsible for epizootics of bovine trypanosomiasis. Also included in this group is *T. uniforme*, which is very similar to *T. vivax*, but not pathogenic to animals.

b. Subgenus *Nannomonas*

The subgenus *Nannomonas* consists of several species of trypanosomes including *T. congolense*, *T. godfreyi* and *T. simiae*. Of these species, *T. congolense* is known to be the most virulent for several domestic animals and have consequences on productivity. It is endemically present in all tsetse-infested areas and infects a wide range of mammals (Van den Bossche *et al*., 2006; Coustou *et al*., 2010; Masumu *et al*., 2012). The development of molecular tools has made possible to differentiate several subspecies of *T. congolense* corresponding to distinct eco-geographical zones: *T. congolense* forest found in forest areas of Central Africa; *T. congolense* kilifi in East Africa and *T. congolense* savannah found mainly in the savannah areas of tropical Africa. The forest and savannah variants are more virulent in domestic animals than the kilifi variant which is otherwise pathogenic for mice (Bengaly *et al*., 2002).

c. Subgenus *Trypanozoon*

This group includes *T. equiperdum*, *T. evansi* and *T. brucei* s.l.. *T. brucei* s.l is the most studied of all trypanosomes because of its ability to infect both humans and animals. On the basis of geographical distribution, pathogenicity, host specificity and clinical evolution, *T. brucei* is divided into three subspecies (Levine *et al*., 1980): *T. b. rhodesiense* and *T. b. gambiense*, which are responsible for acute and chronic forms of human sleeping sickness, respectively, and *T. b. brucei*, which is responsible for AAT.

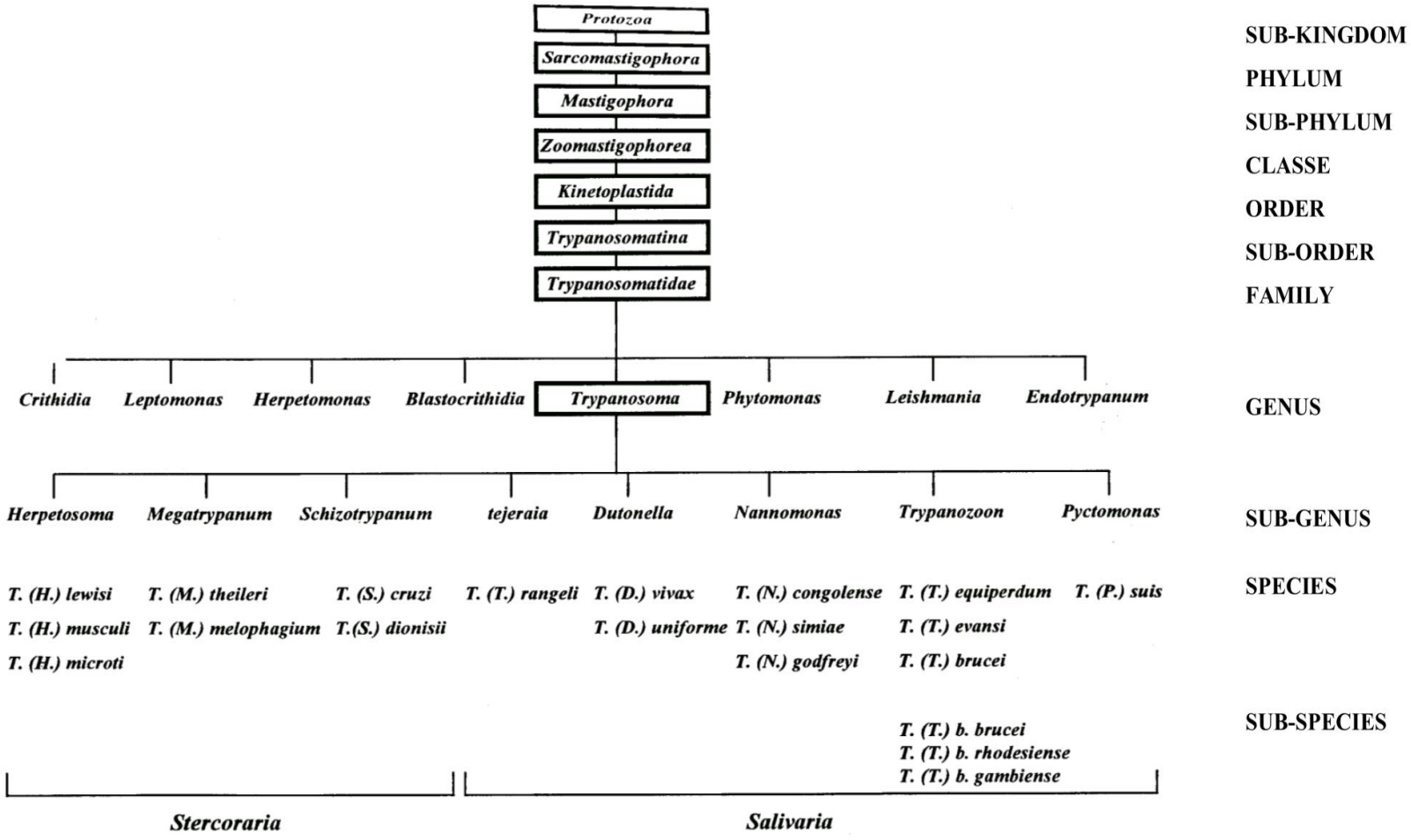

Figure 4. Taxonomy of mammalian trypanosomes (Desquesnes, 2003)

I.3.3. Life cycle of the parasite

Trypanosomes life cycle involve two hosts: invertebrate hosts that are their vectors and a vertebrate host (Figure 5).

a. In the vector

During a blood meal on an infected vertebrate, the tsetse fly ingests blood forms of the parasite (trypomastigotes). In the fly's midgut, these forms will undergo both the attack of the intestinal environment (proteases) and nutrient depletion (particularly in glucose) which will favour their differentiation into procyclic forms that are not infectious for the mammalian host. This latter will acquire a coat of proteins, the procyclins, on their membrane surface, which will protect them from the fly's intestinal proteases (Acosta-Serrano *et al*., 2001). The procyclic forms multiply in the midgut of the fly and then migrate to the salivary glands (*T. b. brucei*) or to the mouthparts (*T. congolense*) where they differentiate into epimastigote forms that adhere to the epithelial tissues of the insect (Vickerman *et al*., 1988). These forms eventually differentiate into infectious forms known as metacyclic. They become infectious to the mammalian host by the acquisition of an antigenic coat of Variable Surface Glycoprotein (VSG) and by the transformation of mitochondrial energy metabolism (insect forms) to glycolysis (blood forms).

It should be noted that the cycle of *T. vivax* does not include a procyclic stage; blood forms ingested by the insect attach and differentiate into epimastigote forms in the proboscis, pharynx and oesophagus and finally differentiate into infectious metacyclic forms (David Barry & McCulloch, 2001)

b. In the mammalian host

During a new blood meal, the insect inoculates metacyclic trypanosomes at the biting site. The parasites change their surface antigen resulting of antigenic variations that were restricted in the metacyclic forms to a much more complex system characteristic of bloodstream trypomastigotes (David Barry & McCulloch, 2001; Matthews *et al*., 2004). These parasites migrate to the draining lymph node where they are detectable in few days before detection in the blood, which occurs two to three weeks after infection (David Barry & McCulloch, 2001). Trypomastigotes proliferate in mammalian blood to the non-proliferative squat forms. Only these squat forms are infectious to the insect (Matthews *et al*., 2004). These short trypomastigote forms undergo modifications that prevent their transition into long blood forms and therefore, are destined to be transmitted to the insect vector during the blood meal or to be destroyed by the immune response of the mammalian host.

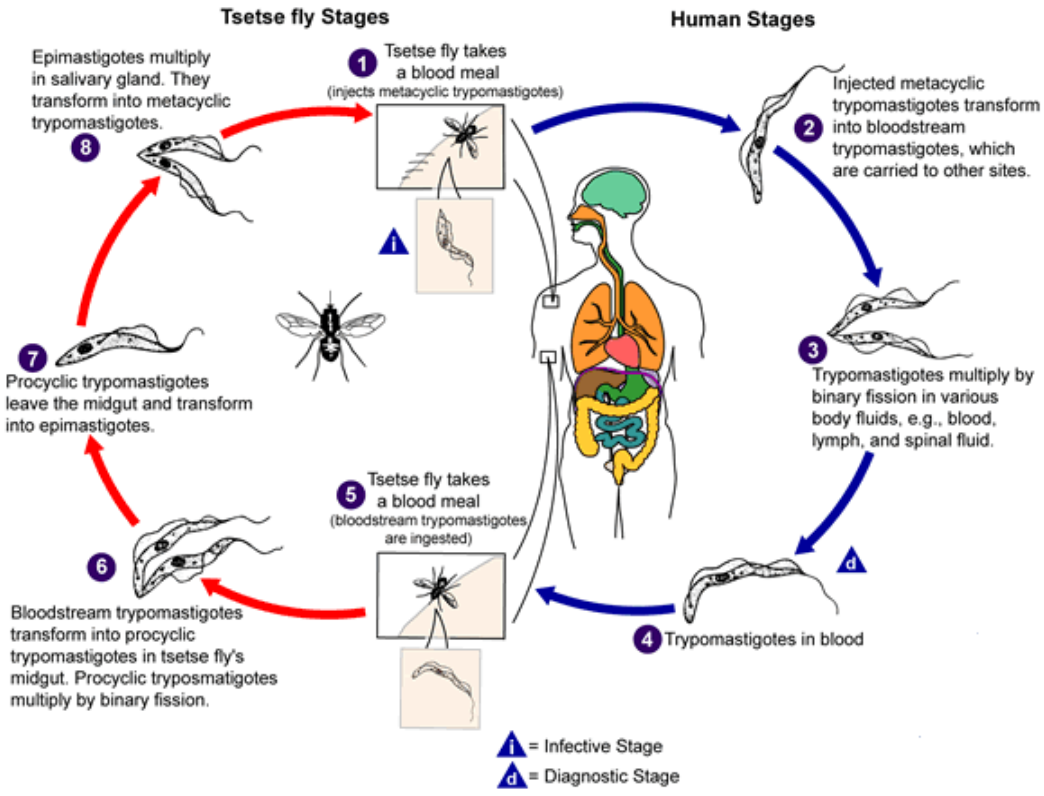

Figure 5. Development cycle of *Trypanosoma brucei gambiense* in tsetse and mammals (Langousis & Hill, 2014)

I.4. Vectors

Depending on whether the vector allows the biological development of the trypanosome, a distinction is made between mechanical and cyclic transmission vectors (Marc Desquesnes & Dia, 2003; Matthews *et al.*, 2004).

I.4.1. Vectors ensuring mechanical transmission

The vectors in this group are tabanids, stomoxes and more rarely hippoboscids (Figure 6). These vectors mainly transmit *T. vivax* and *T. evansi* in tsetse-free areas. *T. congolense* is also transmitted mechanically by tabanids (Marc Desquesnes & Dia, 2003). Transmission occurs via a blood meal that is interrupted on an infected host and completed within a few moments on an uninfected host. As the parasite can only survive in this vector for a very short time (a few minutes), the interval between these two blood meals must be as short as possible (Desquesnes & Dia, 2004).

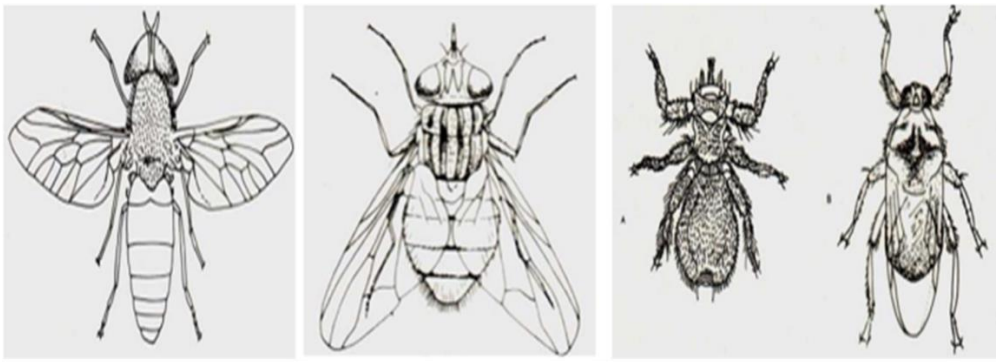

Figure 6. Morphology of mechanical vectors (Troncy *et al*., 1981).

I.4.2. Vectors ensuring biological transmission: Tsetse fly

I.4.2.1. Taxonomy

Tsetse flies are the main biological vectors of trypanosomes in Africa. They are cyclorrhapha dipteran insects that differ from other insect species by having a pair of wings that overlap each other at rest like scissor blades (Figure 7). These Diptera belong to the family *Glossinidae*, which has a single genus *Glossina*. Varying in size from 6 to 16 mm, tsetse flies are classified in thirty species and subspecies divided into three subgenera or groups according to their morphological and bio-ecological characteristics (Morlais *et al*., 1998):

- **The sub-genus *Nemorhina*** Robineau-Desvoidy, 1930 **(*palpalis* group)**. This includes small species that live near wetlands (river flies). This group includes the major vectors of human sleeping sickness such as *Glossina fuscipes*, *G. palpalis* and their subspecies. The species of this group are generally less mobile and remain confined to their habitats.
- **The sub-genus *Glossina*** Zumpt, 1935 **(*morsitans* group)**. This includes the medium-sized species, which are highly mobile and occupy the savannah. To this group belong the major vectors of animal trypanosomiases such as *G. pallidipes*, *G. morsitans spp.*, *G. longipennis* and *G. austeni*.
- **The sub-genus *Austenina,*** Townsend, 1921 **(*fusca* group)**. **This** includes the large tsetse flies of woodland areas (forest flies) that feed on animals. They are of no medical interest and are considered less important vectors as their natural habitat is less frequented by humans and livestock.

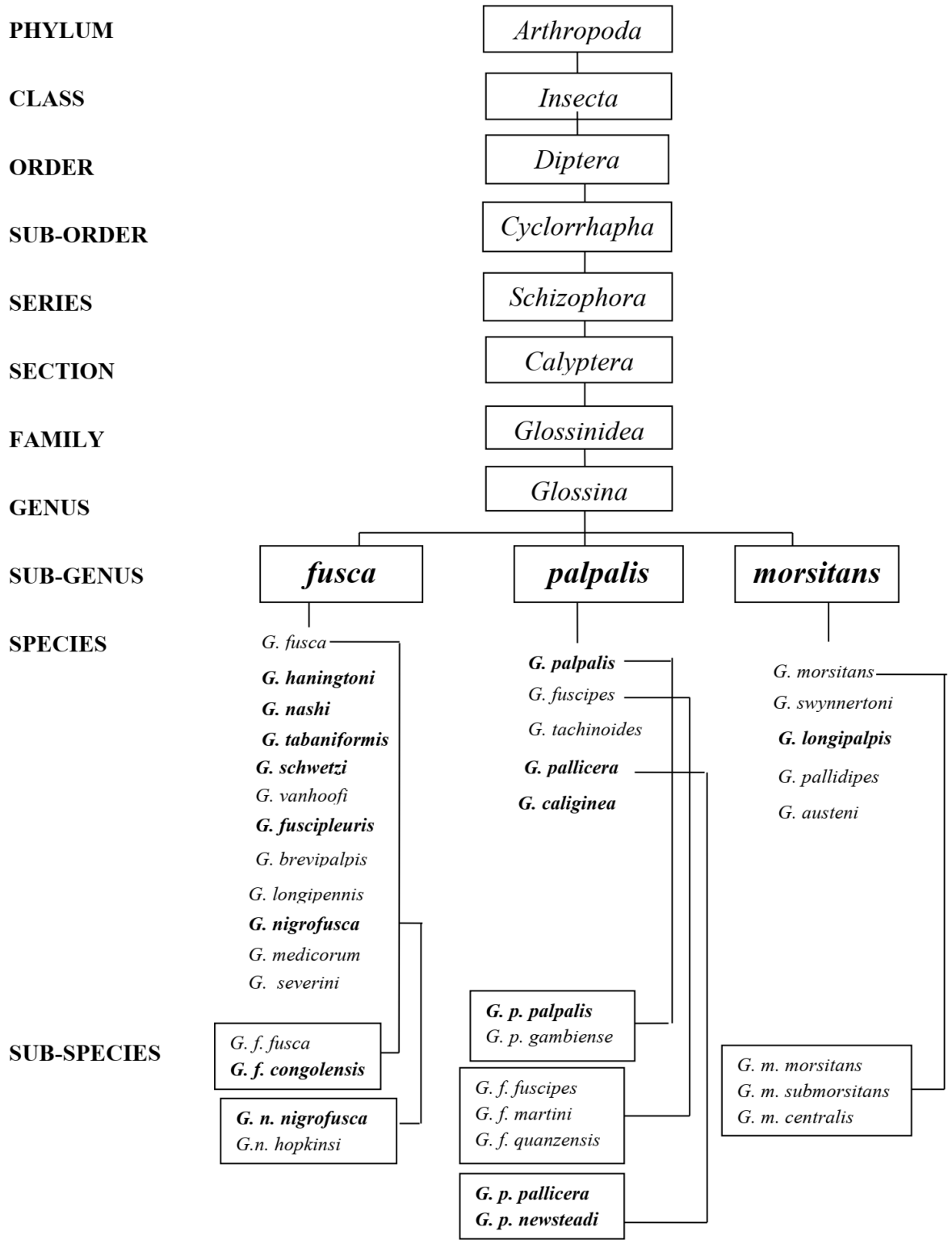

Figure 7. Taxonomy of the tsetse fly (WHO, 1986)

I.4.2.2. Morphology

Tsetse flies are elongated, blackish brown to light brown in colour and consist of three main parts: the head, the thorax (to which the wings and legs are attached) and abdomen (Pollock, 1982). Tsetse size range from 6 to 16 mm without the proboscis (Figure 8). Males are generally smaller than females. Adult weights depend on the species: the lightest weighing 7-14 mg, are found in *Glossina tachinoides*. The heaviest weighing 50 to 80 mg are found in *G. brevipalis*. Adults of medical and veterinary interest such as *G. palpalis*, *G. fuscipes* and *G. morsistans* have an intermediate weight ranging from 21 to 25 mg (Launois *et al.* 2004).

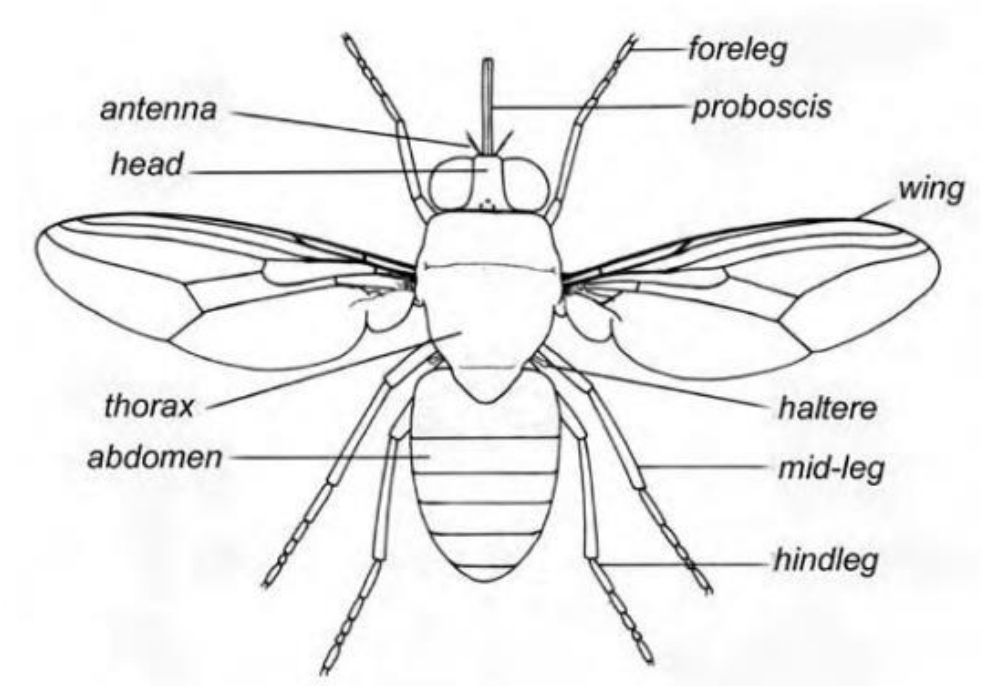

Figure 8. Diagram of tsetse fly (Pollock, 1982)

The ventral side of a male tsetse is characterised by a rounded structure at the posterior end of the abdomen called the hypopygia. Just in front of the hypopygia is a plate with black bristles called hectors. These two structures make it easy to recognise the male fly (Figure 9). The hypopygia and the hectors help the male to cling to the end of the female's abdomen during mating. The abdomen consists of 8 segments, 7 of which are visible dorsally. The eighth carries the male or female genitalia, the characteristics of which are used to identify the species and subspecies (Pollock, 1982).

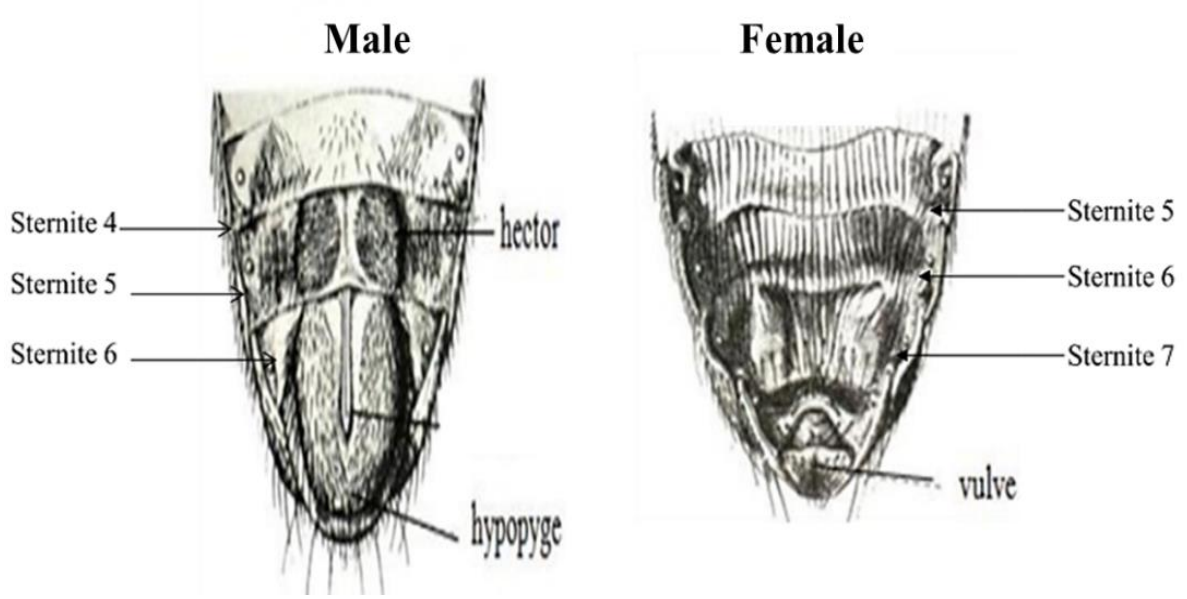

Figure 9. Posterior end of the abdomen: difference between males and females (Pollock, 1982)

I.4.2.3. Life cycle of tsetse fly

Most females are fertilised just after hatching from the pupa, often before the first blood meal. There is usually only one mating in the life of the tsetse, with sperm stored in the spermathecae. Mating is long and lasts from 30 to 180 minutes. After fertilisation, the egg gives rise to a first stage larva which develops in 1 to 2 days to a second stage larva. A second moult results in a third stage larva with fully developed respiratory lobes. This development takes place in the intrauterine environment (tsetse flies are larviparous) in 9 to 10 days (Figure 10) and the first larviposition occurs in around 18 days (De La Roque & Cuisance, 2005). The female deposits the larva in a favourable environment where it quickly buries itself by crawling in the soil. In less than 2 hours, the larva immobilises and becomes a pupa: its tegument hardens and turns brown. These pupation sites correspond to clayey-sandy soils that are heavily covered (aerial roots, dense undergrowth) and where shade and humidity are important. Pupation lasts from 20 to 80 days depending on the soil temperature; it lasts 30 days at a temperature of 25°C. From the pupa emerges a "teneral" fly (with a soft body) (Cuisance & De La Roque, 2003).

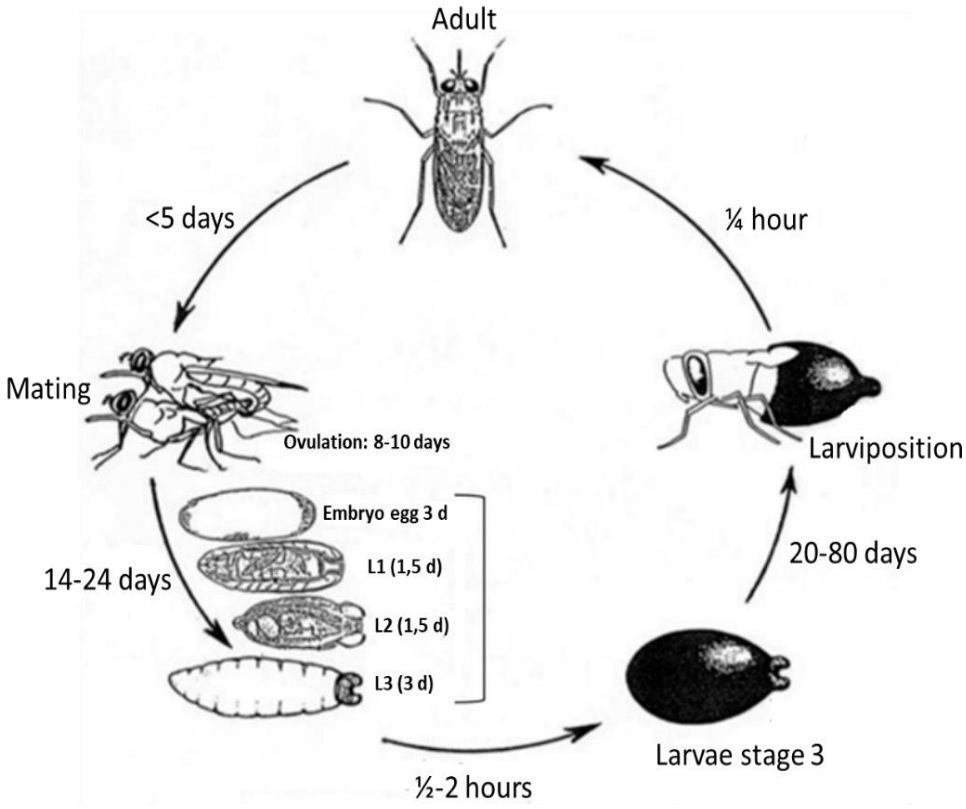

Figure 10. Live cycle of the tsetse fly (Cuisance, 1989)

I.4.2.4. Geographical distribution of tsetse fly

A total of 33 species and subspecies are found to transmit trypanosomes (Gooding & Krafsur, 2005). The total surface infested by tsetse flies throughout Africa is estimated at 11 million square kilometres ranging between latitude 14°N and 29°S. The groups' distribution is related to their habitat preferences. Geographical dispersal of tsetse is limited by climates that are too hot (above 35°C) or too cold, altitudes above 2000m, low rainfall, sparse vegetation, and soil unsuitable for larval burial (Launois *et al*., 2004). Tsetse flies are rare or absent in North Africa and are abundant in the humid tropics of equatorial Africa. In Eastern Africa, they are fragmented with isolated populations. They are absent in the Far East (Somalia) and the South of the continent (South Africa, Botswana, Namibia).

I.5. Diagnosis of trypanosomiases

As no clinical signs are specific to the disease, the diagnosis of HAT and AAT necessarily requires laboratory techniques. In the field, active screening of infected patients is recommended by the WHO expert committee (WHO, 1998). The detection of specific antibodies allows the selection of patients for parasitological examination for the parasite

identification. However, biological diagnosis can be made in the first few weeks after infection by microscopic observation of a thick or thin blood smear, or swollen lymph node swab.

I.5.1. Biological signs and symptoms

The biological signs of HAT are non-specific and may include disturbed liver and kidney functions. The usual markers of inflammation such as C-reactive protein (CRP) or sedimentation rate may be increased. The blood count reveals anaemia and hyperleukocytosis with monocytosis and especially plasmacytosis. Some plasma cells develop into very large cells full of vacuoles: Mott cells. Thrombocytopenia can also be observed (Talabani & Ancelle, 2010). The study of proteins reveals a decrease in albumin levels and above all a very significant increase in gamma globulins and especially IgM in the blood and Cerebrospinal fluid (CSF), with levels that are sometimes very high, from 8 to 16 times the usual concentrations. Non-specific polyclonal activation of B lymphocytes can sometimes lead to a delay in diagnosis because of the increase in various antibodies specific to other pathogens (*Toxoplasma gondii*, *Cytomegalovirus*, *Borrelia burgdorferi*, etc.) or the increase in auto-antibodies (Asonganyi *et al*., 1989).

The clinical diagnosis of AAT is equally difficult to make because of the absence of the characteristic signs of the disease. Nevertheless, hyperthermia, anaemia, oedema, lymph node enlargement, pyrexia (febrile state), abortion and weight loss, increased pulse and respiratory rate are observed in the animal (Desquesnes *et al*., 2022). In the final phase of a chronic infection, the animal becomes cachectic. All these clinical signs attributed to trypanosomiasis can be confused with those due to other haemoparasitoses and certain helminthiases; hence the need for a diagnosis of certainty such as parasitological, molecular, and immunological diagnostics.

I.5.2. Indirect diagnosis

Serological tests for trypanosome-specific antibodies are initial methods of diagnosis and the positivity of these tests must be confirmed by direct search for the parasite. Many tests have been developed for mass screening in the field. The sensitivity and specificity of these tests depend on the antigens used and the type of sample (blood, plasma, serum). However, their use only provides a presumptive diagnosis; they do not differentiate between a past cured infection and a present infection. Indeed, the antibodies appear 3 to 4 weeks after infection and can persist for up to 3 years after treatment (Paquet *et al*., 1992).

The commonly used serological test for mass screening of human trypanosomiasis is the card agglutination test for trypanosomiasis (CATT) (Magnus *et al*., 1978). It is an agglutination test using variable LiTat 1.3 antigens from *T. b. gambiense*. One drop of this reagent is mixed with one drop of blood and shaken for 5 minutes. The result is visible to the naked eye. This test has the advantage of being directly applicable in the field, easy to use and inexpensive. Its sensitivity varies from 87% to 98% according to studies (Talabani & Ancelle, 2010). "False positive" reactions can sometimes be encountered in cases of brief passage of animal forms of trypanosomiasis, or infection with some filarial helminths. "False negatives" can also be observed in case of zonal phenomena (sera with high antibody titres) or when certain strains, notably in Cameroon and Nigeria, lack the gene coding for the LiTat 1.3 protein (Dukes *et al*., 1992). To overcome this problem, a latex test consisting of a freeze-dried suspension of latex particles sensitised with a combination of 3 purified surface antigens LiTat 1.3, 1.5, 1.6 was studied. This technique appears to be more specific with a sensitivity of 71-100% (Büscher *et al*., 1999; Jamonneau *et al*., 2000). Indirect immunofluorescence has been used successfully in Equatorial Guinea, Gabon and the Republic of Congo (Lejon *et al*., 1998). This technique which uses fixed trypanosomes is very specific and sensitive and can be used on serum or eluate obtained from blood on filter paper. Finally, the ELISA technique is currently widely used in many laboratories for the serodiagnosis of many pathogens, notably because of its simplicity and its possibility of automation. For the diagnosis of trypanosomiasis, the ELISA technique can be used on serum, eluate obtained from blood on filter paper and CSF (Lejon *et al*., 2006).

In addition, for the diagnosis of AAT, ELISA test kits have been developed based on an invariant parasite antigen (HSP70 protein), which is found in *T. congolense*, *T. vivax* and *T. brucei brucei* at all parasite stages (Boulangé *et al*., 2002).

I.5.3. Direct diagnosis

The definitive method of diagnosis is the detection of trypanosomes in peripheral blood, lymph node fluid or CSF centrifugation pellet. The diagnosis is simpler for *T. rhodesiense* because the parasitaemia is usually higher (Talabani & Ancelle, 2010).

- **Blood examination**

The examination of fresh blood allows the detection of trypanosomes by their mobility. This technique is simple and inexpensive but not highly sensitive (threshold of 10,000 trypanosomes/mL). It is important to do quickly to avoid immobilisation and lysis of trypanosomes. An alternative to this technique is the smear of a drop of blood stained with May Grunwald Giemsa. Due to the very low parasitaemia, it is often necessary to use concentration

techniques. Therefore, centrifugation in capillary tubes or Woo technique based on the separation of different blood elements according to their specific gravities are required. The blood, collected in heparinised capillary tubes, is centrifuged at 3,000g for 10 minutes. Microscopic observation allows the visualisation of trypanosomes at the plasma-red blood cell interface. The QBC (Quantitative Buffy Coat) is a variant of capillary tube centrifugation that uses more blood, thus increasing the detection threshold. QBC is highly sensitive and is also suitable for the diagnosis of malaria (Bailey & Smith, 1994). The mini anion exchange column chromatography (m-AECT) technique also increases the sensitivity of direct diagnosis (Lumsden *et al*., 1979). Blood is passed through a diethyl amino-ethyl (DEAE)-cellulose column. Figurative elements in the blood are more negatively charged than the trypanosomes and are therefore adsorbed onto the ion exchange column, while the trypanosomes are eluted with full viability. The eluate is centrifuged and examined under a microscope.

- **Cerebrospinal fluid (CSF) examination**

CSF examination is essential to determine the stage of the disease which will define the treatment to be administered. The presence of trypanosomes in the CSF allows classification of the patient in the neurological phase (second phase of the disease). This technique is rapid and inexpensive but lacks sensitivity. A patient is also considered to be in the neurological phase when a cytorachy greater than 5 elements/µL and the presence of trypanosomes in the haemo-lymphatic system; a proteinorachy greater than 450 mg/L or a cytorachy greater than 20 elements/µL are found (WHO, 1998). Lymphocytes and sometimes Mott cells are found, which are highly suggestive of HAT. Trypanosome testing is done by single or double centrifugation. Hyperproteinorachia is a consequence of high intrathecal IgM synthesis (Lejon *et al*., 2002).

I.5.5. Molecular diagnosis

The identification of trypanosomes usually based on various microscopic observations has been improved since the 1980s with the advent of molecular biology tools which are essentially based on the detection of parasite DNA (Moser et al., 1989; Masiga et al., 1992). These new identification techniques offer high sensitivity and specificity. In this context, trypanosomes species-specific DNA probes or primers were developed and used in diagnostic tests for trypanosomiases (Dickin & Gibson, 1989; Moser *et al*., 1989; Masiga *et al*., 1992). These primers are used to amplify DNA fragments that are specific to each species of trypanosomes; thus making possible to detect, in the same host, mixed infections due to several species or subspecies of trypanosomes with a detection threshold in blood of 1 to 40 trypanosomes/mL according to the studies (Truc *et al*., 1999; Becker *et al*., 2004). A new

method, the loop mediated isothermal amplification (LAMP) technique has recently been used to amplify several DNA targets of *T. b. rhodesiense*. This promising technique appears to be very sensitive with a detection threshold of 1 trypanosome/mL in blood and does not require expensive thermocycling equipment (Njiru *et al*., 2008). In addition, the nucleic acid sequence-based amplification (NASBA) technique coupled with an oligochromatography technique to amplify and detect parasite RNA have been recently developed. This technique has a detection threshold of 10 parasites/ml of blood and would be an interesting alternative in the field (Mugasa *et al*., 2009).

I.6. Trypanosomiases control methods

Many control strategies have been developed to fight again trypanosomiases and are mainly based on the control of the parasite and to a lesser extent the control of the vectors.

I.6.1. Control of the parasite

This consists of using drugs to treat infected individuals or animals. The treatment aims to destroy the trypanosomes in the infected individuals and thus reduce the risk of spreading the disease.

I.6.1.1. HAT treatment

The therapy used for the treatment of sleeping sickness varies according to the form and stage of the disease. Accurate identification of the parasite and determination of the stage of the disease are two essential steps in making a therapeutic decision. Up to 1918, atoxyl and tryparsamide, derivatives of arsenic acid, were used as trypanocides. However, both molecules were found to be toxic to the optic nerve. Consequently, the sulphated naphthylamine derivative called suramin was developed around 1920 (WHO, 2013). Until 1922 it was used in combination with tryparsamide in the second stage of the *gambiense* form of the disease. Currently, this molecule is used against *Trypanosoma brucei rhodesiense* in the first period. Pentamidine, a diamidine derivative, was discovered in 1939. It is used against *Trypanosoma brucei gambiense* in the early period. During the 1950s, it was commonly used as a prophylactic agent in West Africa. Melarsoprol (derived from arsenic acid), discovered in 1940, is effective in the second period for both forms of the disease. However, 3 to 10% of patients treated with melarsoprol have developed encephalopathy (convulsion, progressive coma). It was found to cause brain damage in those who survive the encephalopathy. However, due to its effectiveness, melarsoprol has still been used till the years 2000 (Burri *et al*., 2000; Brun *et al*., 2010). Eflornitine (difuoromethylornithine or DFMO), developed in the 1970s to 1980s, represented an alternative to melarsoprol in the treatment of the second phase of chronic sleeping sickness

(Van Nieuwenhove *et al*., 1985; Priotto *et al*., 2008). Besides the problem of the limited number of drugs approved for the treatment of HAT and the high toxicity of melarsoprol, another problem faced by the treatment of sleeping sickness is the development of drug resistance by trypanosomes (Matovu *et al*., 2001; Ollivier & Legros, 2001; Kibona *et al*., 2006). Thus, in the 1990s, the arrival of new drugs against HAT led researchers to focus on drug combinations. A range of approved and experimental drug combinations were tested in animals (Jennings, 1990) and several clinical trials of combinations of eflornithine, melarsoprol and nifurtimox were conducted. In all of these trials, the combinations were more effective than any of these drugs used alone, but all those containing melarsoprol often caused severe adverse reactions (Bisser *et al*., 2007; Priotto *et al*., 2007) and were soon abandoned. Finally, the combination of nifurtimox and eflornithine (NECT) was compared with the usual eflornithine therapy alone in a multicentre trial in the Republic of Congo and the Democratic Republic of Congo (Chappuis, 2007), which showed a reduction in the number of eflornithine infusions required from 56 to 14 and a reduction in the duration of hospitalisation by one third (Priotto *et al*., 2009). Based on these good results, the combination of nifurtimox and eflornithine (NECT) was included in the WHO list of essential medicines for the treatment of second-stage *T. b. gambiense* HAT in May 2009 (OMS, 2009).

Regarding the mode of action of these different trypanocides registered for the treatment of HAT, pentamidine, melarsoprol and eflornitine act mainly by blocking the synthesis of polyamines (Bitonti *et al*., 1986), essential for the growth and cell differentiation of trypanosomes. Their actions have the effect of modulating ribosomal structure, decreasing membrane deformability and stabilising the cytoskeleton. Melarsoprol acts at the end of synthesis on the enzyme trypanotione reductase (the key enzyme in the redox trypanosomatid Metabolism), and also inhibits pyruvate kinase (enzyme of glycolysis, the main energy-producing pathway in blood forms). Pentamidine inhibits S-adenosylmethionine decarboxylase which is one of the enzymes in the polyamine synthesis sequence. It also binds selectively to mitochondrial DNA and inhibits the activity of ribosomal RNA polymerase (Penchenier *et al*., 2003; Steverding, 2010) Suramin inhibits many enzymes, including L-α-glycerophosphate oxidase (Fairlamb & Bowman, 1977), glycerol-3-phosphate dehydrogenase (Fairlamb & Bowman, 1980), RNA polymerase and kinases (Hawking, 1978), thymidine kinase (Chello & Jaffe, 1972), dihydrofolate reductase (Jaffe et al., 1972), hyaluronidase, urease, hexokinase, fumarase, trypsin (Pépin & Milord, 1994) and also blocks the uptake of low density lipoproteins at trypanosome receptors (Vansterkenburg *et al*., 1993).

I.6.1.2. AAT treatment

The therapy used for the treatment of AAT is mainly based on the use of 3 trypanocides including isometamidium chloride, homidium bromide and diminazene aceturate (Barrett *et al*., 2004).

- Diminazene aceturate is an aromatic diamidine mostly marketed in combination with phenyldimethyl pyrazolone. It is known under various names (Berenil®, Veriben®, Azidine®, Ganaseg®, Ganasegur® and Diamyl®). It acts by binding to the kinetoplastic DNA of the trypanosome. This binding occurs by specific interaction between the molecule and regions rich in A-T base pairs. In addition, it specifically inhibits the action of mitochondrial topoisomerase II (Shapiro & Showalter, 1994).
- Isometamidium chloride is used for both preventive and curative purposes. Isometamidium chloride (ISM) is an aromatic phenanthridine amidine known by various names: Trypamidium®, Samorin® and Veridium®. It acts by blocking nucleic acid synthesis because of its intercalation between DNA low pairs. It can also inhibit RNA and DNA polymerase (Marcus & Smith, 1981) and thus the incorporation of nucleic acid precursors into DNA and RNA molecules. Their effects also include modulation of glycoprotein biosynthesis, lipid metabolism and selective cleavage of kinetoplast DNA minicircles (Casero *et al*., 1982).
- Homidum belongs to the phenanthridine class and is marketed as two salts: homidium bromide (Ethidium®) and homidium chloride (Novidium®). They are generally recommended for curative treatment. The mechanism of action of homidium is like that of isometamidium. It inhibits DNA and RNA replication and synthesis, inhibits mitochondrial topoisomerase and polyamine synthesis (Bacchi *et al*., 1980).

However, the emergence of resistance in currently used therapies is a major problem in the treatment of African trypanosomiases.

I.6.1.3. Drug resistance in the control of African trypanosomiases

Resistance to parasite therapies most often emerges as a result of a genetic change (mutations) that alters the uptake of the drug, its metabolism or its interaction with its target. The trypanosome feeds on the host and therefore has transporters on its cell surface and in its flagellar allowing the passage of nutrients. The molecules administered during an antiparasitic treatment can thus enter the parasite cell via these transporters. This is particularly the case for the transporters named P2 (purine transporter 2) because of their physiological substrates, adenine and adenosine, both of which compete with trypanocides (Delespaux *et al*., 2008; Baker

et al., 2013). A deletion in the TbAT1 and TcoAT1 genes encoding the P2 transporter in *T. brucei* s.l. and *T. congolense* and mutations leading to loss of function have been reported in strains resistant to melarsoprol, pentamidine and diminazene aceturate (Nerima *et al*., 2007; Delespaux *et al*., 2007). Indeed, when the P2 transporters are defective, the parasite no longer ingests drug molecules and survives (Baker *et al*., 2012). Furthermore, type 2 aquaglyceroporins (AQPs), which are proteins that allow the passage of water and small solutes across the membrane, are also believed to be melarsoprol and pentamidine transporters in *Trypanosoma brucei* s.l.. Mutations affecting the AQP2 transporters are thought to be linked to resistance phenomena (Baker *et al*., 2013). In addition, cases of multidrug resistance to diminazene aceturate and isometamidium have been reported in trypanosome populations in ten African countries (Tsegaye *et al*., 2015).

In view of these various problems in the treatment of trypanosomiases, vector control is therefore of major interest.

I.6.2. Vector control

The presence of wild or domestic reservoirs that are often uncontrollable (De La Rocque and Dia, 2001) as well as the existence of trypanosome strains resistant to the usual trypanocides have given new importance to vector control. This vector control can be direct or indirect, aiming at modifying the living environment of tsetse fly (Laveissière & Penchenier, 2005), aiming at directly destroying the insect or aiming at reducing the vectorial competence of these latter. This control is based on:

- Action on the tsetse fly habitat through forest thinning (also called agronomic prophylaxis or ecological control)
- Action on the feeding hosts by destroying the game. The aim here is to eliminate the animal reservoirs of trypanosomes. In addition, the elimination of wild animals on which the insect takes its blood meals can make it possible to rapidly reduce or eradicate its populations (WHO, 2013).
- Insecticides are used, sprayed (endosulfan, synthetic pyrethroids, Dichlorodiphenyltrichloroethane) by land or air and the impregnation of the body of domestic animals with insecticides (Maclennan & Aitchison, 1963). Indeed, these ground insecticide sprays have been widely used to control fly populations in many countries such as Botswana, Kenya, Nigeria, Somalia, Zambia and Zimbabwe. In Nigeria, between 1955 and 1978, about 200,000 km^2 of land were cleared of tsetse by

ground application of 570 tons of DDT, 176 tonnes of dieldrin and 77 tonnes of endosulfan (Jordan AM, 1986).

- Traps installation or screens impregnated with insecticides (Challier and Laveissière, 1973): the study of the visual responses of tsetse to different target patterns has led to the design of cost-effective devices for the control of sleeping sickness and animal trypanosomiasis in Africa called "Tiny Targets" (Rayaisse *et al*., 2011). These are small blue-black fabric made screens impregnated with deltamethrin that attract and kill tsetse flies that come in contact with them.
- Use of sterile males and tsetse fly hyperparasites: viruses, fungi and bacteria were shown capable of causing up to 100% mortality in tsetse flies (Kaaya *et al*., 1993). However, the sterile insect release technique is probably the most effective when tsetse fly densities are low, whereas most of the methods described above are more effective on dense populations (Mangan, 2005).
- Transgenic and paratransgenic approaches have been considered for the control of parasite transmission by arthropod vectors. This is the introduction of a gene of interest, under the control of a well-defined promoter, into the genome of the vector (transgenesis) or through a symbiotic bacteria (paratransgenesis) to express molecule that impair parasite development (De Vooght *et al*., 2012). Transgenesis has already been used to transform germ lines of *Anopheles* and *Aedes* mosquitoes, using the transposon technique. However, this technique is not applicable to tsetse flies in the long term, because of their viviparity (embryos and larvae develop in utero), in which the induced trait in the tsetse fly cannot be transmitted to its offspring (Hagan, 1951). The technique of paratransgenesis aims at reducing vector competence by genetically modifying the symbionts of the vectors. This technique has been demonstrated for *Rhodnius prolixus*, the triatomine vector of Chagas disease (Durvasula *et al*., 1997), *Anopheles gambiae*, the main vector of malaria in Africa (Wang *et al*., 2012) and *Glossina morsitans morsitans* (De Vooght *et al*., 2014).

I.7. Generalities on tsetse fly vector competence

Vector competence of tsetse flies is the ability to acquire the trypanosome, to allow its maturation and to transmit it to a vertebrate host. This is influenced by several factors.

I.7.1. Extrinsic factors

I.7.1.1. Climatic conditions

Climatic conditions modulate the density, longevity, and dispersal of tsetse flies and consequently the chances of contact with hosts. In the rainy season, the temperature and relative humidity are optimal for tsetse life, resulting in increased densities and dispersal, favourable to increased disease transmission ability (Ford & Leggate, 1961). Temperature influences the development of tsetse flies and their role in trypanosome transmission. Studies have shown a positive correlation between the susceptibility of tsetse flies to infection and the temperature at which they are kept in the laboratory. Indeed, subspecies *G. morsitans morsitans*, which emerge from pupae incubated at 30°C, generate higher infection rates with *T. b. rhodesiense* or *T. congolense* (Ndegwa *et al*., 1992) than insects from pupae incubated at temperatures of 23-25°C. In the field, there is a positive correlation between increases in mean annual atmospheric temperature and increases in the infection rate of collected tsetse (Ford & Leggate, 1961). Other studies suggest that exposure of tsetse flies to a low temperature of 0-5°C after the infecting meal affects the infection rate of the gut but not the mature infection rate of male tsetse flies (Otieno *et al*., 1983). Macleod *et al*. (2007) found that brief chilling had no effect on the gut infection rate but increased the parasite maturation rate in females. These discrepancies show that the effect of temperature on tsetse fly susceptibility is still a poorly understood factor, although it is agreed that it has a definite influence on trypanosome infection and tsetse fly vectorial competence (Cuisance, 1989).

I.7.1.2. Fasting status and frequency of blood meals

The nutritional status of the tsetse at the time of its first infecting meal has a considerable influence on its vectorial competence. Indeed, a period of fasting of varying length (3 to 4 days for adults) leads to a decrease in the hostility of the gut towards trypanosomes (Cuisance, 1989). This suggests that the physiological factors involved in the establishment of the trypanosome barrier are suppressed or diminished under nutritional stress. In the tsetse fly, an infected blood meal triggers the synthesis of antimicrobial peptides by the insect's immune system. The adipose tissue of the tsetse fly represents the energy reserves, key to metabolism and the biochemical control of the innate immune response. Triggering the defence machinery involves a significant expenditure of energy. Thus, when the tsetse fly is exposed to a nutritional stress of a level that may result in the depletion of this reserve, the consequence is a decrease in the immune response, notably through a decrease in the secretion of lectins, and a decrease in the levels of attacin and cecropin peptides (anti-microbial peptides), which may contribute to an

increased susceptibility of the tsetse fly to trypanosomes (Akoda *et al*., 2009). The origin of the infecting blood meal influences the differentiation and multiplication of trypanosomes in the tsetse fly gut (Olubayo et al., 1994; STEVE Mihok et al., 1995). In addition, Olubayo *et al*. (1994) demonstrated that goat blood favoured the establishment of *T. congolense* in *G. morsitans morsitans* than buffalo blood.

I.7.2. Intrinsic factors

I.7.2.1. Effect of tsetse species

The tsetse flies of *morsitans* group includes the species considered to be the best vectors of *T. congolense* and *T. vivax* for cattle, but also of *T. b. rhodesiense* for humans in East Africa. The tsetse of *palpalis* group are better vectors of *T. brucei* (*T. b. gambiense* in West Africa and *T. b. rhodesiense* partly in East Africa) (Walshe *et al*., 2009). However, between species and within the same group, vector competence vary. Savannah tsetse flies infect more easily than "riparian" flies (Moloo, 1993). Variation in the susceptibility of tsetse flies to trypanosomes has also been observed in laboratory-bred tsetse flies (Maudlin *et al*., 1990; Baker *et al*., 1990). Welburn *et al*. (1989) shown 100% infection rate a strain of *T. congolense* in *G. morsitans morsitans*, whereas with the same trypanosome stock, such high infection rates were rarely recorded in *G. palpalis palpalis*. One of the responses of tsetse to invasion by trypanosomes is agglutination of the parasites; this response depends on the vector species.

I.7.2.2. Effect of Longevity

For a tsetse that remains infective throughout its life, its vectorial capacity will depend on its longevity, which in turn depends on many factors, mainly climatic. However, it has been shown that in *G. m. morsitans*, the longevity of tsetse flies infected with *T. congolense* was significantly reduced, compared to that of uninfected flies (Nitcheman, 1988). It has also been shown that infected tsetse flies are more sensitive to insecticides than uninfected flies, particularly with endosulfan and pyrethroids including deltamethrin in the laboratory (Golder *et al*., 1982).

I.7.2.3. Influence of age

It is generally known that tsetse fly can only become infected at the time of its first meal. Indeed, the midgut of teneral tsetse flies contains little lectins (Maudlin & Welburn, 1988). A first non-infecting meal lowers the chances of infection by a second infecting meal because of a peritrophic membrane that is formed and covers the intestinal wall preventing trypanosomes to cross (Mwangelwa *et al*., 1987). However, infections have been obtained with non-tenereal

adults of *G. morsitans morsitans* fed 3-10 times on rabbits infected with *T. brucei brucei* (Gooding, 1977).

I.7.2.4. Influence of sex

The disparity of observations between localities in the field is very large. Infection rates are reported higher in females than in males by some authors (Clarke, 1965; Distelmans *et al*., 1982), but are shown identical elsewhere (Diallo, 1985). Other laboratory studies have shown males more susceptible than females to infection by *Trypanosoma simiae* in *Glossina brevipalpis*, *G. morsitans* and *G. pallidipes* (Moloo, 1993). With *T. congolense* in *G. morsitans morsitans*, *G. morsitans submorsitans* and *G. tachinoides*, sex did not seem to have any influence on trypanosome infection rates, whereas in *G. palpalis gambiensis*, infection rate was shown higher in the proboscis in males than in females (Reifenberg, 1995). In *G. morsitans morsitans*, *G. morsitans centralis* and *G. pallidipes*, trypanosome maturation is twice as high in males as in females (Mihok *et al*., 1992). This trend was also found in *G. morsitans morsitans* infection with *T. congolense* (Maudlin & Ellis, 1985) and in *G. palpalis palpalis* infection with *T. brucei brucei*. Maudlin *et al*. (1990) showed in the laboratory that males of different species (*G. morsitans morsitans*, *G. morsitans centralis*, *G. pallidipes*, *G. fuscipes fuscipes*) infected with *Trypanozoon* had much higher infection rates in the salivary glands than females, while the infection rates in the midgut were identical. This is thought to be due to intestinal lectins whose synthesis and level of activity is regulated in part by a gene linked to the male sex (Maudlin *et al*., 1995). With *T. vivax*, males infect less than females in *G. morsitans centralis* in the laboratory (Moloo *et al*., 1992a). In *G. pallidipes* infected with *T. congolense* from the same locality (Nguruman) in Kenya and maintained in the laboratory, males were found to be more susceptible than females, whereas in *G. pallidipes* from another locality (Shimba Bills), both sexes showed the same susceptibility to trypanosome infections (Moloo *et al*., 1992b).

I.7.2.5. Levels of intestinal proteases, trypanolysin and trypanoagglutinin

Chymotrypsin and related trypsin-like compounds are thought to impair the establishment of trypanosome infections. They have a destructive action on intestinal trypanosomes (Borovsky & Schlein, 1988). A certain concentration of Trypsin enzyme was shown to be necessary to induce the transformation of ingested blood trypanosomes into procyclic forms (Yabu & Takayanagi, 1988). In return, trypanosomes (*T. b. brucei*) were shown to have an inhibitory activity on these proteolytic enzymes as demonstrated in vitro. This partial inhibition of digestive enzymes would allow the parasite to settle in the intestine while preserving its capacity for further maturation (Cuisance, 1989). Females have higher levels of

digestive proteases than males (Gooding, 1977) hence higher rates of trypanosome maturation in males (Maudlin & Ellis, 1985). Interestingly, *T. congolense* and *T. brucei* avoid proteolysis by remaining in the anterior part of the midgut (without proteases) until the level of proteases is lowest in the posterior part (Akov, 1972; Gooding, 1974). However, protease activity does not appear to be correlated with infection rates, nor is it different in tsetse flies with very different vector competence such as *G. moesitans morsitans* and *G. morsitans centralis* (Mihok *et al*., 1995). However, the important role of lectins produced in response to the blood meal seems to be the most important determinant of the ability of flies to be infected by trypanosome (Welburn *et al*., 1989). Agglutination and lysis of *T. congolense* and *T. brucei brucei* have been shown to occur in the midgut of *G. palpalis palpalis*, but only agglutination occurs in *G. palpalis gambiensis* (Stiles *et al*., 1990). Agglutination and lysis follow a low-level cycle immediately after ingestion of the blood meal with a peak at 48-72h after the blood meal and a drop after 120h. Their release is synchronous with the blood meal, which would explain the ability of fasting tsetse flies to acquire infection. Lectins are agglutinins secreted by midgut cells in response to ingested blood or trypanosome infection. They are said to be glycoproteins of non-immune origin, with specific receptors for certain carbohydrate configurations. They agglutinate or precipitate cells or carbohydrate complexes. These lectins have been shown in *G. morsitans morsitans* (Molyneux *et al*. 1986), *G. austeni* (Ibrahim *et al*. 1984), *G. palpalis gambiensis* and *G. tachinoides* (Ingram *et al*. 1988), *G. fuscipes fuscipes* (Ingram *et al*. 1990), *G. p. palpalis* (Welburn *et al*., 1989). Lectins exert cytotoxicity towards procyclic forms of the parasite.

I.7.2.6. Role of the tsetse immune system

The insect immune system relies on a battery of response mechanisms ranging from phagocytosis to the activation of proteolytic cascades (coagulation) plus the production of antimicrobial peptides (Dimopoulos *et al*., 2001). The innate humoral response of the tsetse fly, and insects in general, relies on two pathways: the immunodeficiency pathway (IMD) and the Toll pathway (Lehane *et al*., 2003). The Toll and IMD pathways respond to different classes of microbes and are expressed differently in different tissues of the insect (De Gregorio *et al*., 2002). The IMD pathway is mainly involved in the regulation of epithelial immune responses and is most strongly activated by infections with Gram-negative bacteria. The Toll pathway is involved in systemic immune responses and is activated by most fungal infections and by Gram-positive bacteria (Hu & Aksoy, 2005). The epithelial response appears to be most involved in the elimination of procyclic trypanosomes, which establish themselves in the tsetse fly gut in

close proximity to host epithelial cells (Dyer *et al*., 2013). The proventriculus is involved in the production of reactive oxygen compounds, such as NO and H_2O_2, which serve as immunological signals for communication between different compartments in the tsetse fly, but also as a signal for the production of antimicrobial peptides (AMPs) by lipids (Hao *et al*., 2001). The activity of the enzyme "Nitric oxide synthase" is directly correlated to the production of nitric oxide which has a trypanocidal effect (Bogdan, 2001).

The role of AMPs in inhibiting trypanosome establishment in tsetse fly was highlighted after investigations on attacin, defensin, cecropin and diptericin. High initial levels of attacin transcripts were observed in the proventriculus and intestine of infection-resistant tsetse species. These levels increase dramatically in fat bodies 6 hours after the tsetse fly infection (Nayduch & Aksoy, 2007). Hu et Aksoy, (2005) also showed that this peptide is more active and effective against trypanosomes when blood meals were supplemented with purified peptide during the course of parasite infection. Thus, it is easier to significantly reduce infection rates in teneral tsetse (0%) and aged tsetse (12%). In contrast, when tsetse flies are already infected, administration of these peptides only results in a 50% reduction in infection rates. The immune response in tsetse flies is specific because it differentiates between bacteria and trypanosomes and between bloodstream and procyclic forms of trypanosomes. Indeed, bloodstream trypanosomes do not stimulate the production of attacin and defensin (Hu & Aksoy, 2005) ; the transcription of these peptides starts when bloodstream forms differentiate into procyclic forms. In tsetse flies that develop infection, the transcription of the genes for the antimicrobial peptides, attacin and defensin, induced by the procyclic forms, remains high for about twenty days. This level decreases in tsetse flies that have eliminated the parasite (Hao *et al.*, 2001). The specificity of the immune response towards the bloodstream forms of trypanosomes compared to the procyclic forms could be explained by the differences in their surface coating. Indeed, the blood forms are coated with VSG while procyclin is present in the procyclic forms (Hao *et al*., 2001).

I.7.2.7. Role of symbionts in vector competence

Symbiotic micro-organisms play a vital role in the host's metabolic processes, fecundity, and immune system development (Douglas, 2011). Most of these symbionts are bacteria, which provide to their hosts nutritional supplements that are absent from their diet and that they are unable to synthesise. Indeed, tsetse flies serve as hosts for numerous micro-organisms, including three main symbionts and a taxonomically diverse collection of commensal bacteria (Wang *et al*., 2013).

The obligate enterobacterium *Wigglesworthia glossinidia* is the primary symbiont of tsetse. It is a gram-negative bacterium present intracellularly in bacteriocytes, specialised cells of the gut (Aksoy, 1995). This bacterium is involved in fertility, nutrition and the development of the immune system of the tsetse fly (Akman *et al*., 2002; Weiss *et al*., 2011). The *Wigglesworthia* genome encodes a high number of putative pathways for the biosynthesis of certain vitamins and metabolites such as biotin, thiazole, lipoic acid, FAD (riboflavin, B2), folic acid, pantothenate, thiamine (B1), pyridoxine (B6), protoheme, and nicotinamide, which are either missing or present in low amounts in the tsetse fly blood diet (Akman et al., 2002; Rio *et al*., 2012). Furthermore, the presence of *Wigglesworthia* is essential for tsetse fly reproduction, as its elimination by antibiotics (tetracycline or rifampicin) renders females sterile (Pais *et al*., 2008). Additionally, flies that undergo intrauterine larval development in the absence of this bacterium have a severely compromised immune system when they become adults. Under these conditions, the *Wigglesworthia*-free fly is exceptionally susceptible to infection by *E. coli* K12 and normally non-pathogenic trypanosomes (Weiss *et al*., 2011, 2013; Wang *et al*., 2013).

Sodalis glossinidius is the secondary symbiont of the tsetse fly. It is a gram-negative bacterium closely related to the *Enterobacteriacaea*. Unlike *Wigglesworthia*, *Sodalis* exhibits extensive tissue tropism and can be found both intra- and extracellularly in a variety of tissues, including the midgut, adipose tissue and salivary glands (Balmand *et al*., 2013). *Sodalis* has also the ability to synthesise a complete flagella structure, which may be important in the colonisation of intrauterine tsetse fly larvae. This bacterium can be grown in cell-free medium, which further indicates its recent association with tsetse and its intermediate status between free-living and obligate intracellular bacteria. However, it does not have a clearly defined functional role within tsetse. Furthermore, several natural tsetse populations lack this bacterium, suggesting that it has a truly commensal phenotype within its host. However, several studies indicate that *Sodalis* may play a role in the ability of tsetse flies to acquire trypanosomes. *Sodalis* has been shown to increase the risk of trypanosome contraction by impairing the trypanocidal activity of host midgut lectins (welburn *et al*., 1993).

Some tsetse populations also harbour bacteria of the genus *Wolbachia*. *Wolbachia* is a widespread alpha-proteobacterial symbiont, infecting about 70% of insects (Hilgenboecker *et al*., 2008). This bacterium manipulates the reproductive biology of its host through various mechanisms, including cytoplasmic incompatibility (CI), feminisation and parthenogenesis (Werren *et al*., 2008). Expression of CI occurs when a *Wolbachia*-infected male mates with an uninfected female, causing developmental disturbance during embryogenesis. In contrast,

Wolbachia-infected females can mate with uninfected males or with a male infected with the same *Wolbachia* strain and produce viable offspring. In tsetse flies, *Wolbachia* is located exclusively in germline tissues, intracellularly, and can be detected in oocytes, embryos and larvae (Cheng *et al*., 2000; Balmand *et al*., 2013). In contrast to *Sodalis* and *Wigglesworthia*, which are transmitted by secretions from milk glands, *Wolbachia* has a transovarian transmission by germ cells.

In addition to the three symbionts described above, other bacteria colonise the tsetse fly digestive system (Geiger *et al.*, 2010). However, their involvement in tsetse fly vectorial competence or any other biological trait is not yet assessed. Thus, increasing fundamental knowledge of the role of the microbiome in disease transmission could lead to the development of control strategies which aims at reducing tsetse fly populations and/or its vector competence.

CHAPTER II: MATERIALS AND METHODOLOGY

II.1. Study area

This study was conducted in Campo (2° 22'N; 9° 49'E), an active sleeping sickness focus located in the Ocean Division of the Southern Cameroon (Figure 11). Considered as a sleeping sickness focus since 1902 (Penchenier *et al*., 2003), Campo is located on the Atlantic coast on the border with Equatorial Guinea and at the mouth of the Ntem River. Campo has equatorial climate with four seasons, two dry and two rainy. The climate is equatorial, with four seasons: the heavy and light rainy seasons (September to November and March to May, respectively) and the heavy and light dry seasons (December to February and June to August, respectively). The environment is composed of a dense hydrographic network with several rivers, a coastal plain along the ocean, a mangrove swamp along the Ntem River in the southern part of the area, marshes, and evergreen forest. Rainfall is high and varies from 2500 mm to 2800 mm per year with temperatures and relative humidity averages of 25° C and 87% respectively. The inhabitants of this locality practice agriculture, artisanal hunting, fishing, and the breeding of domestic animals such as pigs, goats, and sheep. This area is rich in flora and fauna and is a part of the "Campo Ma'an" National Park. Campo is a HAT hypo-endemic focus were several tsetse species and subspecies are found including *Glossina palpalis palpalis*, *G. pallicera*, *G. caliginea* and *G. nigrofusca* (Simo *et al*., 2008). For the study of the impact of impregnated screens, the study site was divided into two polygons: The South-West polygon where HAT cases were reported in the two last decades was chosen as intervention area with implementation of vector control, and the Eastern polygon located around 10 km from there was used as the control area with no vector control.

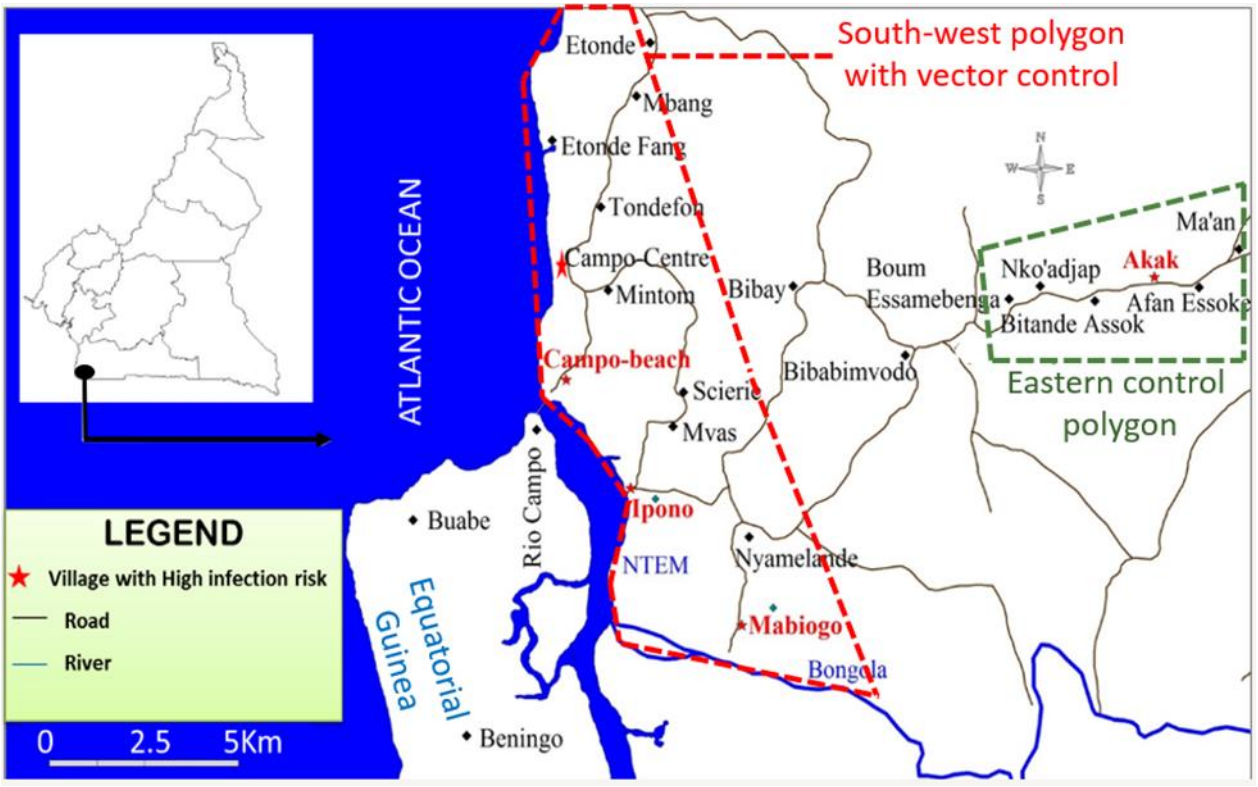

Figure 11. Map of Campo HAT focus (Extracted and modified from National Institute of Cartography Yaoundé Cameroon, 1976 topographic map).

II.2. Entomological surveys and sample collection

Tsetse flies were collected using pyramidal traps (Figure 12) placed in suitable biotopes (Gouteux & Lancien, 1986) where tsetse are likely to concentrate: water points, rivers banks, behind dwellings, along the roads, farmlands. The trap positions were geo-referenced with a Global Positioning System (GPS). Two pre-intervention entomological surveys were conducted using 100 and 99 respectively during the heavy and light dry seasons (December 2018 and July 2019 respectively). These surveys were intended to assess the tsetse flies population densities, their infection rates with trypanosomes and their microbiome composition before the start of vector control. During the vector control, entomological data monitoring was carried out using sentinel traps installed according to the geographical coverage of the area., Pyramidal traps were deployed to monitor changes in tsetse captures, infection rates and microbiome composition over the vector control with tiny targets. These surveys were conducted at six-month intervals after the initial deployment of the tiny targets (August 2020, January 2021, August 2021 and January 2022). Once the traps were installed, they were visited every day for four consecutive days for the recovery of captured flies. Flies were then identified using morphological criteria to determine species and sex (Pollock, 1982). The relative

abundance of tsetse flies was estimated by determining the apparent density per trap per day (ADT), calculated using the formula below:

ADT = (Number of tsetse flies caught)/ (Number of traps used x Number of days of capture)

The subspecies *Glossina palpalis palpalis* was selected for subsequent analyses, as it is the most abundant subspecies in Campo (more than 95%) and it is the one responsible for the transmission of HAT. These tsetse flies were separated into groups of teneral (never took a blood meal) and non-teneral tsetse. Tsetse flies were then sterilized twice with 5% sodium hypochlorite and rinsed twice with distilled water to eliminate potential contaminants from the environment, as recommended in previous similar studies (Jacob *et al*., 2017; Ngambia Freitas *et al*., 2021). The head and legs of the flies were then separated from the rest of the bodies and the different parts conserved separately in well labelled microtubes containing ethanol 95°, for the determination of trypanosome infections (bodies), mature trypanosome infections (heads) and tsetse genetic population structure studies (legs). On the field, samples were conserved at room temperature and once in the laboratory, these samples were stored at −20°C until DNA extraction for further analyses.

Figure 12. Image of a pyramid trap and trapping cage containing tsetse flies caught (Bouaka, 2020)

II.3. Tiny Targets installation and monitoring

Tiny Targets are small screens made of a blue polyester (LSTM, 2016) attached to a thin black polyethylene net measuring 25 x 50 cm (Figure 13) both impregnated with insecticide (Deltamethrin 300 mg/m^2) manufactured by Vestergaard-Frandsen (Lausanne, Switzerland).

These screens were deployed between January 2020 and August 2021, at intervals of 6 months, (corresponding not only to the dry seasons, but also to a period of optimum activity of the screens) for a total of 4 deployments covering a period of 2 years. Prior to the deployment, a sensitization campaign was conducted to explain the activities to the local communities and the role of tiny targets in their environment. The deployments were carried out during the dry season, which allows easy access to fly breeding sites and is the season favourable for the flies' biological activities (feeding and reproduction), thus facilitating their contact with the screens. The location of each screen was recorded using a GPS. The installation was carried out by our teams in association with local people that we recruited and trained. The screens were placed near rivers, watering holes, paths, behind houses, cultivated fields and along the Cameroun's side of the Ntem River bank, and the surrounding vegetation was cut within a 1.5m radius of each screen to ensure optimum visibility. The impact of this vector control was assessed by analysing changes in tsetse fly densities before and during the intervention.

Figure 13. Image of a "Tiny target" installed on River Ntem bank (Bouaka, 2020)

II.4. Detection of trypanosomes in tsetse flies by polymerase chain reaction (PCR)

II.4.1. Extraction of total tsetse DNA

Total DNA was extracted from fly heads and the rest of the bodies separately, for detection of trypanosome infections. DNA was extracted using the LIVAK protocol (Livak, 1984). The tubes containing individual fly bodies or heads were left opened at room temperature for evaporating the alcohol used for conservation. Five hundred microliters of filtered and sterilized LIVAK buffer were introduced into each tube (LIVAK: 1.6 mL NaCl 5M; 5.48g Sucrose; 1.57g Tris; 10.16 mL EDTA 0.5M; 2.5 mL 20% SDS; distilled water to 100 mL total volume). The contents of each tube were crushed and homogenized using a tube pestle and the

tubes were incubated in a water bath at 65°C for 30 minutes. Then, 70 µL of potassium acetate were added, followed by an incubation on ice for 30 minutes and centrifugation at 13,500 rpm for 20 minutes. The aqueous upper phase containing the nucleic acids was transferred into new Eppendorf tubes. One millilitre of absolute ethanol was then added for precipitation of the nucleic acids; the tubes were homogenized and centrifuged at 13,500 rpm for 15 minutes. The pellet obtained was washed twice with 200 µL of 70% ethanol. The alcohol was completely removed, and the tubes were air-dried for about 1 hour. The pellet was finally suspended in 30 µL and 100 µL of sterile water respectively for heads and bodies and stored at -20°C for subsequent molecular analyses.

II.4.2. Principle of PCR

PCR is a molecular technique that allows amplification of specific region of a nucleic acid in vitro to obtain an exponential quantity of copies for detection and study. Therefore, a series of reactions allowing the replication of a double-stranded DNA template is repeated. During the PCR reactions, the products obtained at the end of each cycle serve as a template for the next cycle.

The first step consists of initial denaturation of the double-stranded (90-98°C) to obtain single-stranded templates, follow by cycles of three steps:

- denaturation of the double-stranded DNA (90-98°C);
- hybridization, during which the primers (oligonucleotides) attach to single-stranded sequences;
- polymerisation (72°C), during which complementary strands are synthesised from the free 3'OH ends of the hybridised primers;

and a final step of elongation.

II.4.3. Identification of trypanosome species by polymorphism of the ITS1 region

The ITS1 (Internal transcribed spacer) region of the ribosomal DNA is a non-functional region with specific sequences highly conserved in all trypanosome species (Delespaux *et al*., 2003). The rRNA gene is a locus with 100-200 copies per genome and each transcribed copy consists of 18S and 5.8S rRNA, separated by the ITS1 region (Desquesnes & Dávila, 2002). In trypanosomes, these intergenic fragments vary from one species to another. Thus, the size of the amplified fragments makes possible to differentiate them.

Trypanosomes were detected by a nested PCR (double PCR in which the product from the first serves as template for the second) as described by Desquesnes *et al*. (Desquesnes *et al*., 2001), amplifying the ITS1 of the rRNA gene, using primers TRYP18.2C (5'-GCAAATTGCCCAATGTCG-3') and TRYP4R (5'-GCTGCGTTCTTCAACGAA-3') for the first round, then, IRFCC (5'-CCTGCAGCTGGATCAT-3') & TRYP5RCG (5'-ATCGCGACACCTTGTG-3') for the second reaction. Reactions were performed in a final volume of 20 µL consisting of 2 µL of TBE buffer 10X (10 mM Tris–HCl; 1,5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.56 µL of each primer (10 µM), 0.4 µL of dNTPs (10 mM), 0.2 µL of Taq DNA polymerase (5U/µL), 14.28 µL H_2O and 2µL of DNA extract. In the negative and positive control tubes, 2µl of sterile water and *T. congolense*, *T. brucei* s.l., and *T. vivax* DNA were introduced respectively. The amplification program consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 amplification cycles each consisting of denaturation at 94 °C for 30 s, primer hybridization at 58°C for 1 min, elongation at 72 °C for 1min and one final elongation step at 72 °C for 5 min. The composition of the reaction medium as well as the amplification program were the same for both reactions except that amplicon for the first PCR were diluted 1/10 and used as template for the second PCR.

II.4.4. Identification of the *Trypanosoma brucei gambiense* subspecies

Trypanosoma brucei gambiense, the human parasite was identified from all samples that were positive to *T. brucei* s. l. using a nested PCR with specific primers, through the amplification of the TgsGP (*T. brucei gambiense*-specific glycoprotein) gene, involved in its resistance to human serum. The first amplification reaction was performed using the primers TsGP1 (5'-GCTGCTGTGTTCGGAGAGC-3') and TsGP2 (5'-GCCATCGTGCTTGCCGCTC-3). Reactions were performed in a final volume of 20 µL consisting of 2 µL of TBE buffer 10X (10 mM Tris–HCl; 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.8 µL of each primer (10 µM), 0.4 µL of dNTPs (10 mM), 1 µL of MgCL2 (25Mm), 0.1 µL of Taq DNA polymerase (5U/µL), 11.5 µL H_2O and 4µL of DNA extract. In the negative and positive control tubes, 2µl of sterile water and *T. brucei gambiense* DNA were introduced respectively. The reaction mixture was placed in a thermal cycler for the PCR reaction. The amplification programme consisted of an initial denaturation step at 95°C for 5 min followed by 45 amplification cycles each consisting of denaturation at 95°C for 1 min, primer hybridisation at 63°C for 1 min, elongation at 72°C for 1 min and one final elongation at 72°C for 5 min.

The second amplification reaction was performed using 4 µL of the amplification product from the first reaction (diluted 1:10) as DNA template. This second amplification was

performed with the primer pair TgsGPs (5-TCAGACAGGGCTGTAATAGCAAGC-3) and TgsGPas (5-GGGCTCCTGCCTCAATTGCTGCA-3). For this second amplification, the reaction medium was the same as for the first PCR. The amplification program consisted of an initial denaturation step at 95°C for 5 min followed by 25 amplification cycles each consisting of a denaturation step at 95°C for 1 min, a primer hybridization step at 63°C for 1 min, an elongation at 72°C for 1 min and a final elongation at 72°C for 5 min.

The amplification products were separated on a 2% agarose gel and visualised under ultraviolet light using a trans-luminator.

Table I. Primers used and expected size of different trypanome bands

Trypanosome species	PCR name	Expected sizes (bp)	References
T. congolense	ITS-PCR	680	(Desquesnes *et al*., 2001)
T. brucei s.l.	ITS-PCR	400	(Desquesnes *et al*., 2001)
T. vivax	ITS-PCR	150	(Desquesnes *et al*., 2001)
T. simiea	ITS-PCR	250	(Desquesnes *et al*., 2001)
T. brucei gambiense	TgsGPas-PCR	270	(Morrison et al., 2008)

II.5. Separation of amplified products by agarose gel electrophoresis

The products obtained after PCR reactions were separated by electrophoresis on a 2% agarose gel. The separation of negatively charged nucleic acids is done under the effect of an electric field created by a power generator and a buffer. Smaller molecules (of low molecular weight) move faster and will migrate further than larger molecules on a gel.

The agarose gel was prepared from a mixture of 2g of agarose and 100 ml of TAE 1X (Tris-Acetate-EDTA) buffer. The mixture was boiled in a microwave for 3 minutes to complete melting of agarose. After cooling, 0.8 µL of "Midori green" is added and the solution is poured into a mould with a comb to form wells. After solidification of the gel, the comb is removed, the gel placed in the buffer contained in the electropheris tank, and the samples placed individual wells after being mixed with a loading buffer (6X loading buffer: 0.03% bromophenol blue; 0.03% xylene cyanol; 60 mM EDTA pH 7.6; 60% glycerol in distilled water). The loading buffer allow the amplicons to be densified and to sink to the bottom of the

wells, and also to monitor the migration. A molecular weight marker was deposited in one of the wells to estimate the size of the amplicons obtained. Electrophoresis was performed using a generator at 100 volts and 35 milliamperes for 35 minutes. At the end of the electrophoresis, the gel was visualised under ultraviolet light of a transilluminator and photographed. Positive samples were those with a band of the same size as the positive control.

II.6. Tsetse blood meal origin determination

The origin of the remaining tsetse blood meals was determined by the amplification of a portion of the Cytochrome b gene from DNA extracts from fly bodies, using the universal vertebrate primers CYTBF (5'-CCATCCAACATCTCAGCATGATGAAAA-3') and CYTBR (5'-GCCTCAGAATGATTTGTCCTCA-3'). Amplification was done in a final volume of 20 µL consisting of 2 µL TBE buffer (10X), 0.4 µL of dNTPs (10 mM), 0.8 µL of each primer (10 µM), 1 µL of $MgCL_2$ (25 mM), 0.08 µL Taq DNA polymerase (5U/µL), 12.92 µL H_2O and 2 µL DNA extract. In the negative and positive control tube, the DNA extract was replaced with 2 µL of sterile water and purified DNA for pig respectively. The amplification program consisted of an initial denaturation step at 94 °C for 3 min and 30 s, followed by 40 cycles each containing a denaturation step at 94 °C for 30s, a primer hybridization step at 58 °C for 30s and an extension step at 72 °C for 1 min, plus a final extension step at 72 °C for 5 min. The amplification products were separated on a 2% agarose gel and visualised under ultraviolet light using a transilluminator as described in part II.5

Cytochrome b PCR positive products were purified, sequenced using the Sanger method, and species identification was performed using the BLASTn algorithm to align and compare the sequences obtained to reference sequences in GenBank DNA Sequence Database (http://www.ncbi.nlm.nih.gov/).

II.6.1. Sanger sequencing method

DNA sequencing aims to determine the linear alignment of bases in a DNA fragment. The Sanger method was described in 1977 and is still the most widely used method in laboratories today (Lamoril *et al*., 2008).

The target DNA is firstly denatured to obtain a single-stranded DNA. Using a primer that is specific and complementary to the strand under study (forward or reverse), usually identical to the one used for PCR, a DNA polymerase then synthesises complementary DNA from this primer. From the 5′ end to the 3′ end, this enzyme adds complementary deoxyribonucleotide triphosphates (dNTPs) and randomly and inconsistently adds dideoxyribonucleotide triphosphates (ddNTPs) (e.g. a ddGTP will sometimes be added instead

of a dGTP). As the reaction takes place in a single tube for each dNTP, the ddNTPs (ddATP, ddGTP, ddCTP and ddTTP) are labelled with fluorophores (Heller *et al*., 2008). When a ddNTP is incorporated in place of a dNTP, the DNA polymerase cannot continue its polymerisation. The extension reaction stops (indeed, the dideoxynucleotide does not have a 3′-hydroxyl group that is essential for the polymerisation reaction of the enzyme). Statistically, during the reaction, for each "base" of the target DNA, at least once, a complementary ddNTP will be incorporated in place of a dNTP. Therefore, at the end of the reaction, fragments of different sizes are obtained. Analysis of the reaction is then carried out. Different analysis methods are possible. Today, capillary electrophoresis performed on a sequencing machine is the method of choice. During migration, each fragment (containing a ddNTP labelled with a fluorophore) is excited by a laser and the signal obtained is analysed by specific software. Computer analysis of the signals provides the sequence under study in the form of an electrophoregram (Figure 14). In general, this technique more efficient for sequences between 400 and 850 bp (Heller *et al*., 2008).

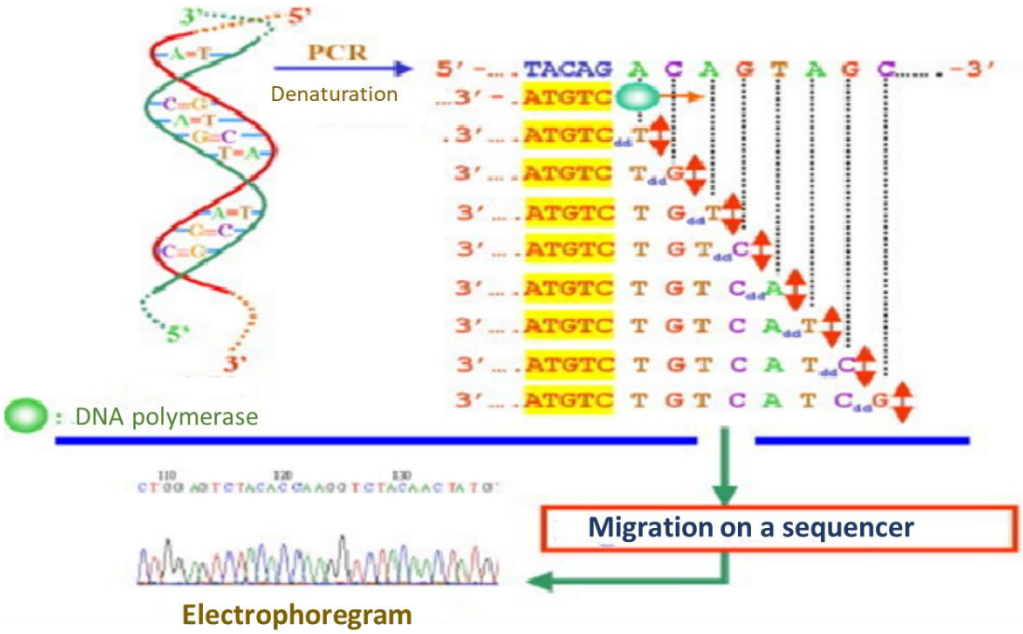

Figure 14. Principle of Sanger sequencing (Lamoril *et al*., 2008)

II.6.2. Sequence identity determination

To identify vertebrate host species, the obtained sequences were compared with those already deposited in the GenBank database using the BLAST program. BLAST (Basic Local Alignment Search Tool) is a set of programs designed to perform similarity searches against a database of sequences. Sequences of a given pair-wise alignment with the lowest E-value and

the maximum identical and query coverage at least 98% were selected as the most likely species of host.

II.7. Determination of bacterial composition of tsetse flies by metagenomics approaches

The bacterial composition of tsetse flies was determined though the high-throughput sequencing of the highly variable V3-V4 region of the bacteria 16S rRNA gene, followed by the alignment of the sequences against a reference database for their identification. Different groups of tsetse flies were constituted for this purpose, in order to evaluate the impact of certain bacteria present on some biological parameters of the tsetse flies:

- 40 flies containing trypanosome infections in the gut,
- 40 flies containing no trypanosome infection, for the search for bacteria associated with the infection with trypanosomes,
- 20 teneral flies for bacteria associated with feeding,
- 40 flies containing mature trypanosome infections (presence of trypanosomes in the mouthparts) for the search of bacteria associated with the establishment and maturation of trypanosomes,
- A group of 20 *Glossina tachinoides*, 20 *Glossina pallicera* and 20 *Glossina caliginea* for the research of bacterial communities of the different tsetse species,
- A group of flies constituted of *Glossina palpalis palpalis* collected before (73) the screen implementation, after six months (45), after twelve months (45) and after eighteen months (45) of vector control for the identification of bacteria associated with potential flies' fitness.

DNA samples from these different flies were sent to the Infravec group (https://infravec2.eu/) for sequencing.

II.8.1. Principle of Illumina next-generation sequencing

The Illumina next-generation sequencing (NGS) method is based on sequencing-by-synthesis (SBS), and reversible dye-terminators that enable the identification of single bases as they are introduced into DNA strands (Figure 15).

- **Step 1. Library preparation**

Library preparation is the first step of next generation sequencing which allows DNA to adhere to the sequencing flow cell and thus, allows the sample to be identified. Through ultrasonic fragmentation, the genomic DNA is fragmented to 200-500bp portions. The 5’ and 3’ adapters are added to the two ends of these small segments to tag them with unique

codes and allow multiplexing. Adapter-ligated fragments are then amplified with PCR and purified on a gel. The concentration of each purified sample is then measured, and samples are pooled in equivalent quantities to construct the library.

- **Step 2. Cluster generation**

A cluster generation is an amplification reaction that occurs on the surface of an Illumina flow cell. In this step, libraries are loaded in the flow cell of Illumina equipment, a channel for adsorbing mobile DNA fragments and core sequencing reactor vessel. The DNA fragments in the sequencing library randomly attach to the lanes on the surface of the flow cell and pass through it. Adapters attached to the surface of the flow cell match the adapters added at the ends of the DNA fragments in the library building process, and bind to the sequences as primers. Each sequence is then amplified to produce millions of copies, forming a cluster.

When cluster generation is complete, those templates are ready for sequencing.

- **Step 3. Sequencing**

The sequencing method used here is sequencing-by-synthesis (SBS). DNA polymerase, connector primers and the 4 dNTPs with base-specific fluorescent markers are added to the reaction system. The 3′-OH end of these dNTP are protected, so that only one base is added at a time during the sequencing process. All unused free dNTP and DNA polymerase are eluted after the synthesis reaction. Then, buffer solution needed for fluorescence excitation are added, the fluorescence signal is excited by laser, and fluorescence signal is recorded by optical equipment.

Finally, the optical signal is converted into sequencing base by computer analysis. When the fluorescence signal is recorded, a chemical reagent is added to quench the fluorescence signal and remove the dNTP 3′-OH protective group, so that the next round of sequencing reaction can be performed.

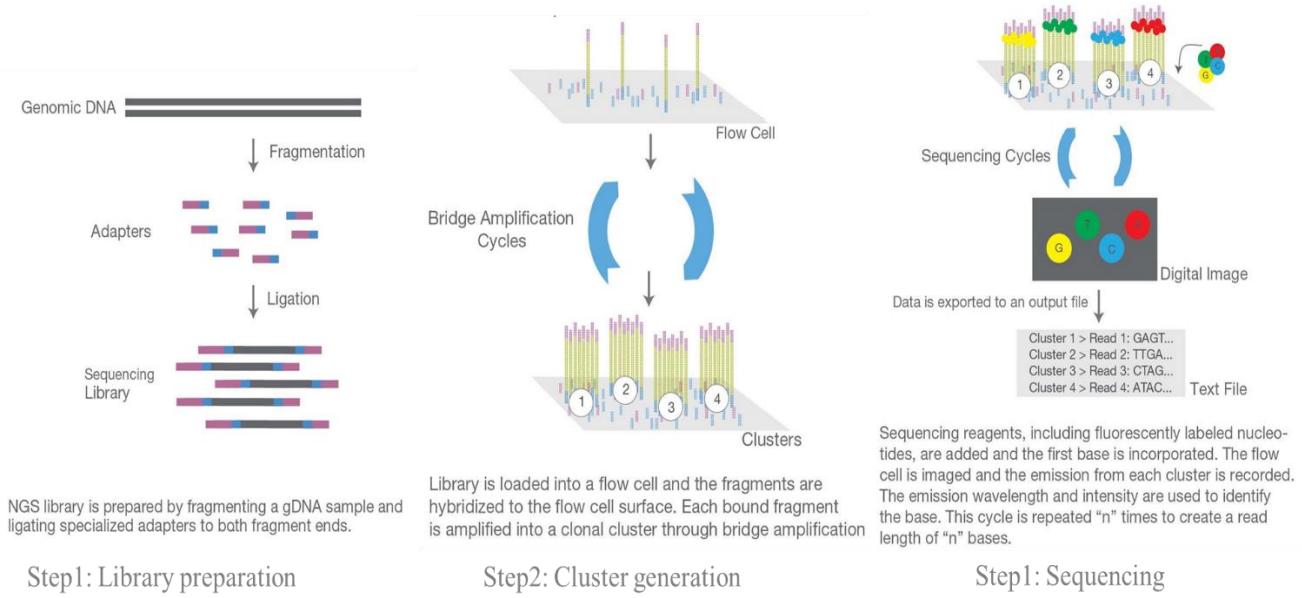

Figure 15. Diagram of Illumina Miseq sequencing procedure (https://www.cd-genomics.com/blog/principle-and-workflow-of-illumina-next-generation-sequencing/, accessed on Monday, 12 December 2022)

II.8.2. Libraries preparation and Illumina Miseq sequencing

Two Sequencing sets were performed with DNA extracted from 192 and 148 individual flies for the first and second sequencing respectively, using the Illumina MiSeq system ("Polo d' Innovazione di Genomica Genetica e Biologia"). In this protocol, two specific primers targeting the V3-V4 region of the bacteria 16S rRNA gene, and flanked with the Illumina overhang adapters were used for the sequencing; namely, V3F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA G-3′ and V4R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATC TAATCC-3′ (Klindworth *et al*., 2013).

Amplicons were generated using a 2X KAPA HiFi HotStart Ready mix (KAPA Biosystems), through PCR reactions performed in a total volume of 25 µL, containing 2.5 µL DNA (5 ng/µL), 5 µL of each primer (1 µM), and 12.5 µL 2X KAPA HiFi HotStart Ready mix. The amplification program consisted of an initial denaturation step at 94 °C for 3 min, followed by 25 amplification cycles, each consisting of denaturation at 94 °C for 30 s, primer hybridization at 55 °C for 30 s, elongation at 72 °C for 30 s and final elongation at 72 °C for 5 min. The expected sizes of the PCR products were verified by running 1 µL of the PCR product on a bioanalyzer (expected size ~550 bp). PCR products were then cleaned up using AMPure XP beads (Beckman Coulter Genomics) to remove free primers and primer dimers. Then, 5 µL of purified products were used in the second PCR round to attach dual multiplexing indices (i5

and i7) and Illumina sequencing adapters using the Nextera XT Index Kit (Illumina catalogue), as recommended by the manufacturer. After this step, a second clean-up was performed using AMPure XP beads, and 1 μL of a 1:50 dilution of each sample was analysed on a bioanalyzer to verify the final size (~630 bp). Libraries were then normalized, and 5 μL of each were pooled. Finally, pooled libraries were denatured with NaOH diluted with hybridization buffer, heat-denatured, and loaded on the Illumina MiSeq flow-cell. Each run included 5% PhiX solution (Illumina catalogue) to serve as an internal control (Illumina, 2013).

II.9. Data exploration and analyses

II.9.1. Entomological and parasitological data

Entomological and parasitological databases were generated using Microsoft Excel 2016. Average tsetse fly densities per trap (ADT) were calculated after a logarithmic transformation of the numbers of tsetse captured in each trap, to avoid the trap effect. The comparison of tsetse catches during the different entomological monitoring surveys was subjected to an analysis of variance using GraphPrism8 software. These ADT data were then mapped using QGIS 3.16 (QGIS Development Team, 2020).

Tsetse fly infection rates to trypanosomes were calculated by dividing the numbers of tsetse infected by the numbers of tsetse examined, and then the Chi-square test was used to compare fly infection rates between different trypanosome species and between sampling periods using R software. Differences were considered significant for $P < 0.05$.

II.9.2. Metagenomic data processing and bacteria taxa identification

Illumina MiSeq reads were analysed using Mothur v.1.44.3 (Schloss *et al*., 2009), following a modified pipeline previously described for the same kind of analyses (Kozich et al., 2013). Briefly, forward and reverse demultiplexed paired-end reads were merged to contiguous sequences for each individual fly, and primers were trimmed, followed by quality filtering that removed all merged reads containing ambiguous bases. The dataset was reduced to unique sequences and a count-file summarizing the number of sequences of each type for all the flies. The unique sequences were then aligned to the V3-V4 region of the 16S rRNA gene sequences from the SILVA v.123 reference database and filtered to remove the overhangs at both ends. Then, the dataset was filtered to eliminate unique sequences with an abundance lower than 0.01%, probably issued from sequencing errors, and for the remaining sequences, highly similar samples (up to 1 difference each 100 base pairs) were pre-clustered for further denoising of the data. Another filtering step was performed to remove chimeric sequences, as well as those classified as eukaryote or mitochondria (probably from fly DNA), chloroplast, or

unknown after alignment in the SILVA database. A distance matrix was generated between remaining clean sequences, and these were later clustered and classified into operational taxonomic units (OTUs). Finally, using the count-file, an OTU table was generated containing each individual fly, with all the OTUs it harbours, as well as their abundances.

II.9.3. Statistical analyses

Statistical analyses and plots were performed in the R environment (R Core Team, 2020). Several packages were used for the different types or analyses performed: phyloseq (McMurdie & Holmes, 2013) and microbiome (Callahan *et al*., 2016) for the exploration and analysis of 16s microbiome data, taxonomic profiling, and association tests; ggplot2 (Wickham, 2008) and ggpubr (Kassambara, 2020) for graphical plots visualization; vegan (Oksanen *et al*., 2019) for communities ecology (diversity analysis, community ordination, and dissimilarity analysis); DESeq2 (Love *et al*., 2014) for differential OTU abundance testing between groups; ape (Paradis & Schliep, 2019) and dendextend (Galili, 2015) for the analyses of phylogenetic trees and hierarchical clustering; knitr (Xie, 2021) for dynamic reports generation. Rarefaction curves were performed prior to comparative analyses to ensure the sequencing depth was sufficient to describe all present taxa in all individual flies. Alpha diversity metrics were estimated using the Shannon diversity index (H) and compared between groups (fly species, trypanosome infected or not, teneral or not, between sampling periods) using the Wilcoxon signed rank test. Principal components analysis (PCoA), using the Bray–Curtis dissimilarity index and ordination plots, was performed to determine differences in bacterial communities across samples of different groups, and the differences were quantified using permutational multivariate analysis of variance (PERMANOVA). Finally, differential abundance testing was performed to search potential taxonomic groups that can serve as biomarkers associated with a specific condition, mainly fly infectivity with trypanosomes, or ability to carry mature trypanosome infections in mouthparts or fly's fitness during the vector control. All tests considered statistical significance threshold of 0.05.

CHAPTER III:
RESULTS AND DISCUSION

III.1. Results

III.1.1. Entomological data before vector control

A total of 1915 and 1300 tsetse flies were caught using 100 and 99 pyramidal traps yielding apparent densities of 3.13 [2.92-3.33] and 2.47 [2.28-2.66] flies/trap/day in heavy and light dry seasons respectively. In those captures, four tsetse fly species or sub-species were identified, namely *Glossina palpalis palpalis*, *G. pallicera*, *G. caliginea* and *G. nigrofusca. G. palpalis palpalis* which is the sub-species responsible of the transmission of the human sleeping sickness in Campo was largely dominant, with a relative abundance of 93.42 % and 92.85 % for the two seasons (Table 2). Regarding the other species present, *G. pallicera* accounted for 3.82% and 5.31%, *G. caliginea*, 2.45% and 1.15%, and *G. nigrofusca*, 0.26% and 0.62% for heavy and light dry seasons, respectively. Although more females were caught in the light dry season (around 61% of catches), the sex-ratio was balanced in the heavy dry season; the teneral flies accounted for 5.07% and 5.85% respectively for the two seasons.

Table II. Composition of tsetse flies by village during the long and short dry seasons (December 2018 and July 2019)

Season	Sex	*G. caliginea*	*G. nigrofusca*	*G. pallicera*	*G. p. palpalis*	NI	Teneral	Total
December 2018	F	29 (2.50%)	4 (0.34%)	34 (2.93%)	1092 (94.15%)	1 (0.09%)	82 (7.07%)	**1160 (60.57%)**
	M	18 (2.38%)	1 (0.13%)	39 (5.17%)	697 (92.32%)	-	15 (1.99%)	**755 (39.63%)**
	Total	**47 (2.45%)**	**5 (0.26%)**	**73 (3.82%)**	**1789 (93.42%)**	**1 (0.05%)**	**97 (5.07%)**	**1915**
July 2019	F	4 (0.61%)	5 (0.76%)	35 (5.33%)	612 (93.15%)	1 (0.15%)	61 (9.28%)	**657 (50.54%)**
	M	11 (1.71%)	3 (0.47%)	34 (5.29%)	595 (92.53%)	-	15 (2.33%)	**643 (49.46%)**
	Total	**15 (1.15%)**	**8 (0.62%)**	**69 (5.31%)**	**1207 (92.85%)**	**1 (0.07%)**	**76 (5.85%)**	**1300**

NI: non identified

The major vector of the Human sleeping sickness in Cameroon, *G. palpalis palpalis*, displayed high densities in the different sampling points in Campo, with mean ADT values of 3.87 95%CI [3.84-3.91], and 2.59, 95%CI [2.49-2.53] flies/trap/day in heavy and light dry seasons respectively. The Figure 16 present the distribution of fly's ADT in the Campo focus.

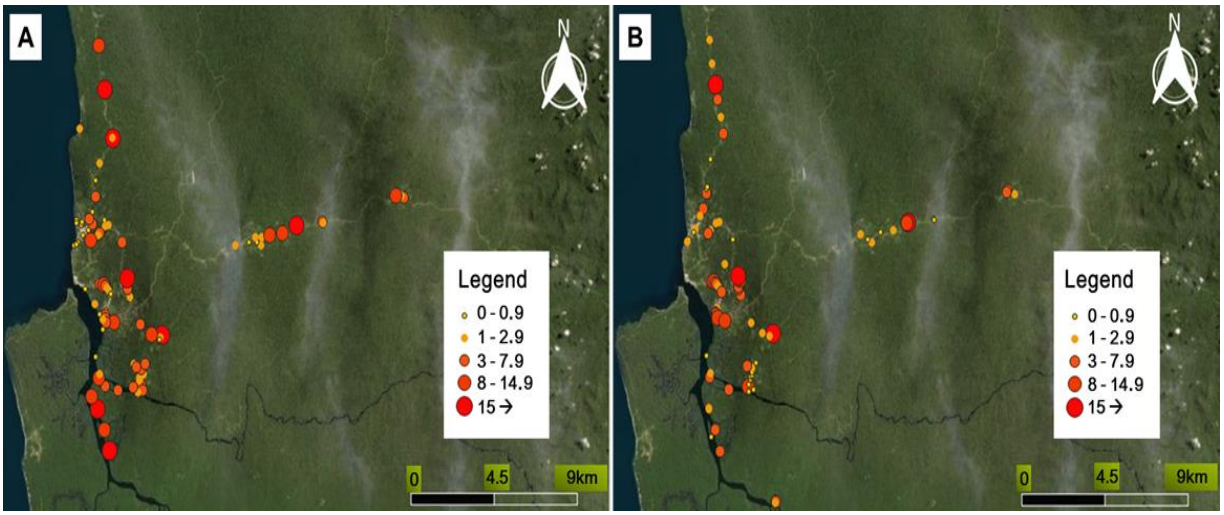

Figure 16. Map of the distribution of the apparent density per trap of tsetse flies (ADT) during December 2018 heavy dry season (A) and July 2019 light dry season (B) in Campo

III.1.2 Trypanosomes infection rates in *Glossina palpalis palpalis* before vector control

The Figure 17 illustrates the resolution on a 2% agarose gel of the Trypanosomes ITS1 amplified products with the presence of 680 bp, 400 bp, 250 bp and 150 bp bands specific to *T. congolense*, *T. brucei* s.l., *T. simiea* and *T. vivax* infections respectively.

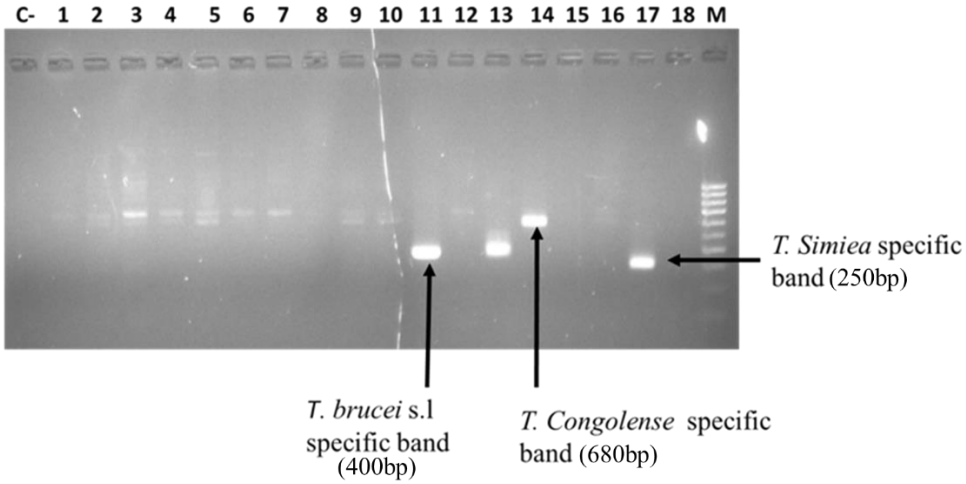

Figure 17. Agarose gel showing amplification of *T. congolense*, *T. brucei* s.l and *T. simiea* specific DNA bands (M: marker; C-: negative control; 14: *T. congolense* positive sample; 11 and 13: *T. brucei* s.l. positive samples; 17: *T. simiea* positive sample)

III.I.2.1. Trypanosome infection rates in tsetse according to sampling periods

- **Heavy dry season (December 2018)**

After PCR-based analyses of 1054 randomly selected flies of the sub-species *G. palpalis palpalis* captured in December 2018, we found 177 flies (16.79%) harbouring at least one

trypanosome species in their midguts (Table 3). The most frequent trypanosome species was *Trypanosoma congolense* (15.37%), followed by *T. brucei* s. l. (1.52%), *T. vivax* (0.47%) and *T. simiae* (0.47%). Amongst the 177 tsetse flies with trypanosome infections, 11 (6.21%) were mixed infection, including 5 double infections with *T. congolense* and *T. brucei brucei*, 1 of *T. congolense* and *T. vivax*, 1 of *T. brucei brucei* and *T. vivax* and 4 of *T. congolense* and *T. simiae*. Also, 40 (22.60%) of those infected flies harboured mature infections, with trypanosome DNA detected in their mouthparts (heads), and all these were of the species *T. congolense*.

T. brucei gambiense, the human parasite responsible of the sleeping sickness, were identified in l sample among the *T. brucei* s. l. infections.

- **Light dry season (July 2019)**

A total of 1132 flies captured during the light dry season (July 2019) were tested and 229 (20.23%) midguts were found infected by at least one *Trypanosoma* species. As in December 2018, *T. congolense* (12.10%) were the most common (table 3), followed by *T. brucei* s. l. (7.51%), *T. vivax* (2.56%) and *T. simiae* (0.18%). Amongst the 229 infected tsetse flies, 23 (10.04%) were mixed infections, including 18 double infections of *T. congolense* and *T. brucei*, 4 of *T. congolense* and *T. vivax* and 1 triple infection of *T. congolense*, *T. brucei* and *T. vivax*. No mature infections were identified in these infected flies.

Out of the 85 samples infected with *T. brucei* s. l., 3 (1.31% of the total *Trypanosoma* infections, and 3.53% of *T. brucei* s. l.) were identified as *T. brucei gambiense*.

Of the 100 and 99 traps deployed in December 2018 and July 2019, 58 (58%) and 80 (80.8%) caught at least one tsetse fly infected by trypanosomes respectively. Although the traps that caught at least one infected fly were widely distributed in different area, infection rates were significantly different between sampling periods ($X^2 = 2.06$; p-value = 0.01) suggesting a dynamic in the transmission.

Table III. Trypanosome infection rates in *Glossina palpalis palpalis* according to sampling periods.

	Sample size	TC (%)	95% IC	TB (%)	95% IC	TV (%)	95% IC	TS (%)	95% IC	Tbg (%)	Total (%)	95% IC
December 2018	1054	162 (15.37)	[13.2-17.6]	16 (1.52)	[0.8-2.4]	5 (0.47)	[0.1-1.1]	5 (0.47)	[0.1-1.1]	1 (0.09)	177 (16.79)	[15.5-20.2]
July 2019	1132	137 (12.10)	[10.2-14.1]	85 (7.51)	[6-9.2]	29 (2.56)	[1.7-3.6]	2 (0.18)	[0 - 0.6]	3 (0.26)	229 (20.23)	[19.9-24.8]

P-value	**-**	**0.03**	**<0.001**	**<0.001**	**0.39**	**0.01**

TC: *Trypanosoma congolense*; TV: *Trypanosoma vivax;* TB: *Trypanosoma brucei* s. l.; Tbg: *Trypanosoma brucei gambiense*.

II.1.2.2. Trypanosome infection rates in villages

- **Heavy dry season (December 2018)**

High infection rates were observed in Ipono (41/87; 47.13%), Doumassi (6/13; 46.15%), Okanbiloun (2/5; 40%), Mabiogo (32.64%) and Nazareth (7/22; 31.82%). Apart from Assok, Enyengenamanga and Mintomb, *T. congolense* was identified in tsetse flies captured in all other villages with high infection rates in Doumassi (46.15%), Ipono (44.83%) and Nazareth (31.82%). These infection rates varied significantly between villages (X^2 = 15.04; p-value < 0.001). In contrast to *T. congolense* identified in almost all villages, *T. brucei* s. l. was only identified in 6 villages (out of 20) with relatively low rates, the highest rates being observed in Ipono (4.60%) and Campo beach (3.45%). However, no significant difference was observed in the infection rates to *T. brucei* between the different villages (X^2 = 1.97; p-value = 0.05). Of the flies infected with *T. brucei* s. l., the only fly identified as carrying *T. b. gambiense* (sleeping sickness species) infection was found in Mabiogo. Moreover, *T. vivax* and *T. simiea* showed a similar pattern as they were only found in 3 villages with relatively low rates (an average of 0.47%). Although the traps that caught at least one infected fly were widely distributed in the different sampling point, infection rates were significantly different between villages (X^2 = 16.99; p-value < 0.001) (Table 4)

Table IV. Trypanosome infection rates in *G. palpalis palpalis* according to villages during the great dry season (December 2018)

Villages	TC (%)	TB (%)	TV (%)	TS (%)	Total (%)
Afan essokie	8 (3.79)	2 (0.95)	0 (0)	0 (0)	9 (4.27)
Akak	1 (1.85)	0 (0)	0 (0)	0 (0)	1 (1.85)
Assok	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Bokombe centre	-	-	-	-	-
Campo beach	15 (17.24)	3 (3.45)	0 (0)	2 (2.30)	17 (19.54)
Campo ville	1 (20)	0 (0)	0 (0)	0 (0)	1 (20)
Doumassi	6 (46.15)	0 (0)	0 (0)	0 (0)	6 (46.15)
Enyengenamanga	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ipono	39 (44.83)	4 (4.60)	1 (1.15)	0 (0)	41 (47.13)
Itonde	5 (11.63)	0 (0)	0 (0)	1 (2.33)	6 (13.95)
Maan	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Mabiogo	42 (29.17)	4 (2.78)	1 (0.69)	2 (1.39)	47 (32.64)
Mintomb	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Monakak	-	-	-	-	-
Mvass	9 (12.68)	1 (1.41)	0 (0)	0 (0)	10 (14.08)
Nazareth	7 (31.82)	0 (0)	0 (0)	0 (0)	7 (31.82)
Nkoalong	4 (6.06)	0 (0)	0 (0)	0 (0)	4 (6.06)
Nkouandjop	-	-	-	-	-
Ntem bank	24 (10.53)	1 (0.44)	3 (1.32)	0 (0)	26 (11.40)
Okanbiloun	1 (20)	1 (20)	0 (0)	0 (0)	2 (40)
Total	**162 (15.37)**	**16 (1.52)**	**5 (0.47)**	**5 (0.47)**	**177 (16.79)**
P -value	**< 0.001**	**0.05**	**0.04**	**0.04**	**< 0.001**

TC = *Trypanosoma congolense;* TV = *Trypanosoma vivax;* TB = *Trypanosoma brucei s. l.;* Tbg = *Trypanosoma brucei gambiense.*

- **Light dry season (July 2019)**

In general, the tsetse fly from Campo Ville, Mintomb, Assok and Bekombe Centre were the most infected with prevalence of 80%, 45%, 33.33% and 33.33% respectively. *T. congolense*, the predominant species was found in almost all villages except Okanbiloun, with rates varying significantly ($X^2 = 14.01$; p-value < 0.001). *T. brucei* s.l. followed almost the same pattern, being found in all villages except Akak and Maan also with rates varying significantly ($X^2 = 15.62$; p-value < 0.001). Of the flies infected with *T. brucei* s. l., the 3 flies identified as carrying *T. brucei gambiense* infection (species responsible for sleeping sickness) were found

in Nazareth, Mvass and on the banks of the Ntem River. As in December 2018, trypanosome infection rates varied significantly between villages ($X^2 = 26.81$; p-value < 0.001) (Table 5).

Table V. Trypanosome infection rates in *G. palpalis palpalis* according to villages during the light dry season (July 2019)

Villages	TC (%)	TB (%)	TV (%)	TS (%)	Total (%)
Afan essoke	31 (13.14)	18 (7.63)	9 (8.31)	1 (0.42)	54 (22.88)
Akak	1 (16.67)	0 (0)	0 (0)	0 (0)	1 (16.67)
Assok	1 (33.33)	1 (33.33)	0 (0)	0 (0)	1 (33.33)
Bokome centre	1 (16.67)	2 (33.33)	0 (0)	0 (0)	2 (33.33)
Campo beach	6 (7.50)	2 (2.50)	4 (5)	0 (0)	12 (15)
Campo ville	1 (20)	3 (60)	0 (0)	0 (0)	4 (80)
Ipono	15 (13.89)	2 (1.85)	4 (3.70)	0 (0)	18 (16.67)
Itonde	16 (13.91)	10 (8.70)	1 (0.87)	0 (0)	27 (23.48)
Maan	1 (25)	0 (0)	0 (0)	0 (0)	1 (25)
Mabiogo	7 (10.94)	1 (1.56)	2 (3.13)	0 (0)	8 (12.50)
Ntem banks	13 (11.82)	8 (7.27)	2 (1.82)	0 (0)	23 (20.91)
Mintoumb	3 (15)	7 (35)	0 (0)	0 (0)	9 (45)
Mvass	13 (9.15)	10 (7.04)	3 (2.11)	1 (0.70)	26 (18.31)
Nazareth	24 (13.41)	12 (6.70)	4 (2.23)	0 (0)	31 (17.32)
Nkoelong	2 (6.06)	4 (12.12)	0 (0)	0 (0)	5 (15.15)
Nkouandjop	2 (11.76)	4 (23.53)	0 (0)	0 (0)	6 (35.29)
Okanbiloun	0 (0)	1 (25)	0 (0)	0 (0)	1 (25)
total	**137 (12.10)**	**85 (7.51)**	**29 (2.56)**	**2 (0.18)**	**229 (20.23)**
P-value	**< 0.001**	**< 0.001**	**0.006**	**0.088**	**< 0.001**

TC = *Trypanosoma congolense;* TV = *Trypanosoma vivax;* TB = *Trypanosoma brucei s. l.;* Tbg = *Trypanosoma brucei gambiense*.

III.1.3. Determination of tsetse blood meal sources

Figure 18 illustrates the resolution on a 2% agarose gel of amplified vertebrate cytochrome b gene fragment products from DNA extracted from tsetse flies. This picture shows the length of ~340 bp bands corresponding to the expected size.

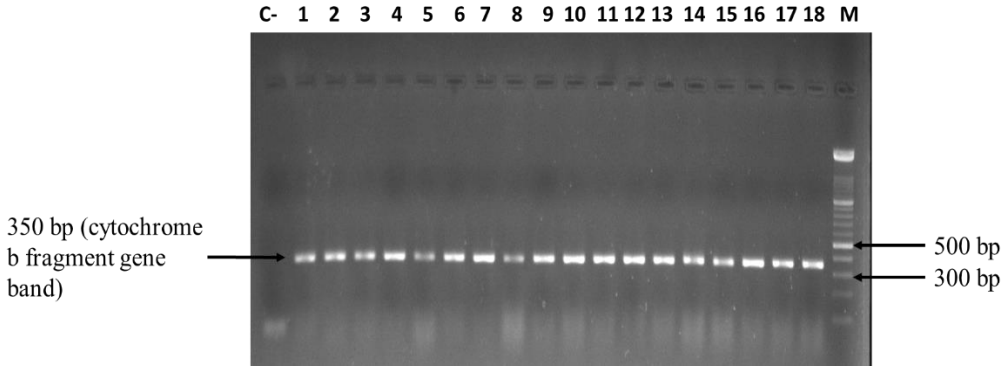

Figure 18. 2% agarose gels showing amplification of DNA bands specific to the cytochrome b gene fragment

(M: 100bp lanes molecular weight marker; C-: negative control; 1-18: cytochrome b gene positive samples)

Vertebrates' cytochrome b DNA was successfully amplified in 40 and 45 *G. palpalis palpalis* samples from December 2018 and July 2019 surveys respectively. After sequencing and aligning to reference databases (in Genbank), 2 out of 40 (from December) aligned perfectly with Human DNA, showing that the tsetse concerned fed on human. The 38 remaining from that trip, plus the 45 of July corresponded to four different profiles but didn't align with any known vertebrate existing in the database.

III.1.4. Entomological and parasitological data during vector control

III.1.4.1. Tiny targets installation

Around 2000 targets were deployed in Campo, especially on the Ntem bank river, places of population activities, behind dwellings and along the roads (Figure 19). These targets were replaced by new ones every 6 months covering period of 2 years (August 2020, January 2021, August 2021 and January 2022) in order to maintain optimal trap effectiveness.

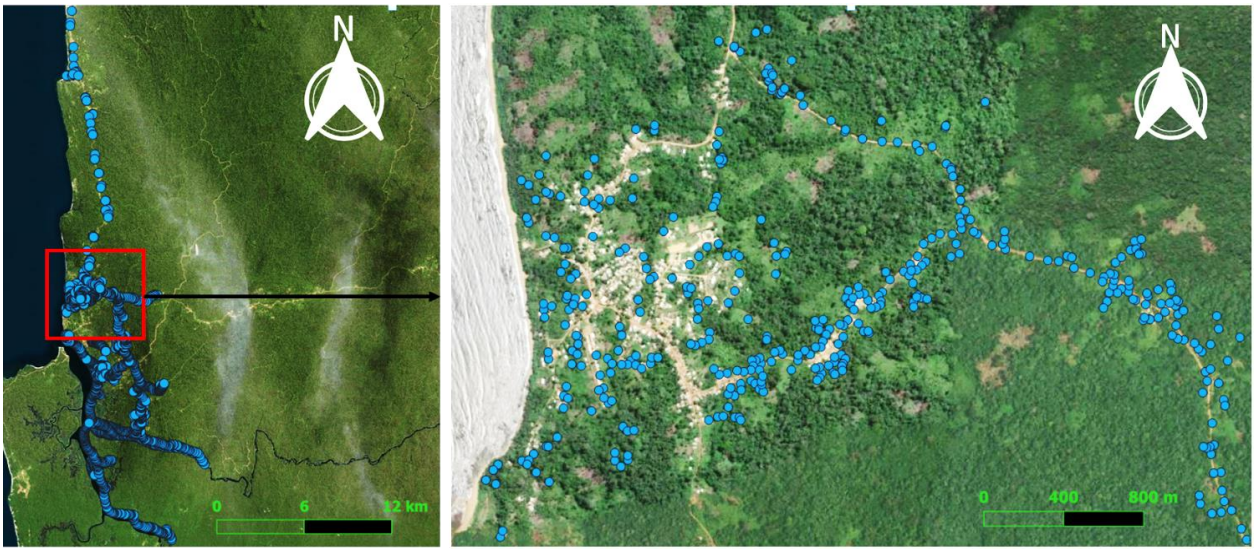

Figure 19. Location and distribution of tiny targets in Campo.

III.1.4.2. Assessment of the impact of screens on tsetse fly densities

- **Variation of tsetse densities in the treated area**

Post-intervention surveys showed a great reduction of tsetse densities in the treated area in the first twelve months of vector control, followed by a partial recovery in the next twelve other months. Indeed, the overall *Glossina palpalis palpalis* density decreased from 2.48 [1.92-3.14] tsetse/trap/day in July 2019 to 0.95 [0.69-1.35] tsetse/trap/day in August 2020, showing significant reduction of 62.50% ($p < 0.0001$). During the second evaluation after twelve months of vector control (January 2021), the mean catches continued decreasing to 0.66 [0.42-0.94] tsetse/trap/day, for an overall reduction of 73.39%. During the third and the fourth surveys, we observed a recovery in tsetse densities from 0.66 in January 2021 to 1.45 [1.07-1.90] tsetse/trap/day in August 2021, and 1.71 [1.27-2.24] tsetse/trap/day in January 2022 (Table 6; Figure 20).

Table VI. Summary of the number of flies captured during tiny targets implementation in treated area

Tsetse sp	*G. palpalis palpalis*	*G. caliginea*	*G. nigrofusca*	*G. pallicera*	Non identified	Total	*G.p.p.* ADT
December 2018	1218 (92.84)	38 (2.90)	2 (0.15)	53 (4.04)	1 (0.08)	1312	2.15 [1.60 -2.81]
July 2019	909 (93.23)	12 (1.23)	6 (0.62)	47 (4.82)	1 (0.10)	975	2.48 [1.92-3.14]
August	471	7	0	4	0	482	0.95

2020	(97.72)	(1.45)	(0)	(0.83)	(0)		[0.69-1.35]
January	403	12	0	8	0	423	0.66
2021	(95.27)	(2.84)	(0)	(1.89)	(0)		[0.42-0.94
August	572	1	5	12	0	591	1.45
2021	(96.79)	(0.17)	(0.85)	(2.03)	(0)		[1.07-1.90]
January	662	14	1	29	0	726	1.71
2022	(91.18)	(1.93)	(0.14)	(3.99)	(0)		[1.27-2.24]

G.p.p.: *Glossina palpalis palpalis*; Numbers in brackets are relative abundances of each tsetse taxon.

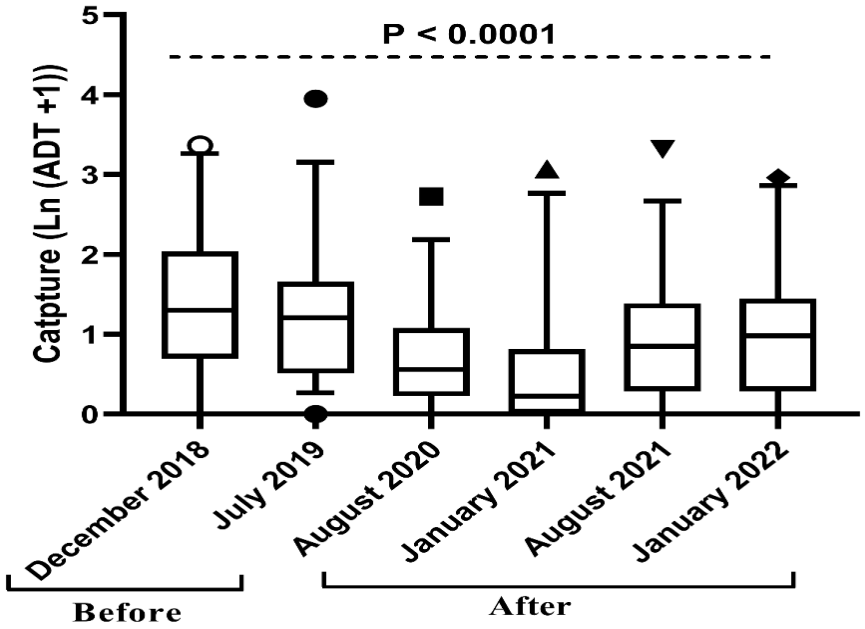

Figure 20. Boxplot of the *Glossina palpalis palpalis* apparent densities per trap before and over vector control with tiny targets

- **Evolution of tsetse densities in the control area**

A total of 928 tsetse flies were caught in the control area during tsetse pre-intervention surveys, from which 603 in the heavy dry season (December 2018) and 325 in the light dry seasons (July 2019). As in the treated area, four tsetse fly species or sub-species were identified, namely *Glossina palpalis palpalis* with relative abundances 94.68% and 91.69%, *G. pallicera* (3.32% and 6.77%), *G. caliginea* (1.49% and 0.92%), and *G. nigrofusca* (0.50% and 0.62%) for heavy and light dry seasons, respectively (table 7). *Glossina palpalis palpalis* displayed high densities in the different sampling points in the control area, with mean ADT values of 3.98 95%CI [1.48-8.09], and 2.43, 95%CI [0.73-5.77] flies/trap/day in heavy and light dry seasons respectively.

Although *G. palpalis palpalis* densities varied in the control area, it generally increased by 33.24%, i.e., from 2.43 [0.73-5.77] tsetse/trap/day in July 2019 to 3.64 [1.47-7.70] tsetse/trap/day over the 2 years of the study (Table 7; Figure 21).

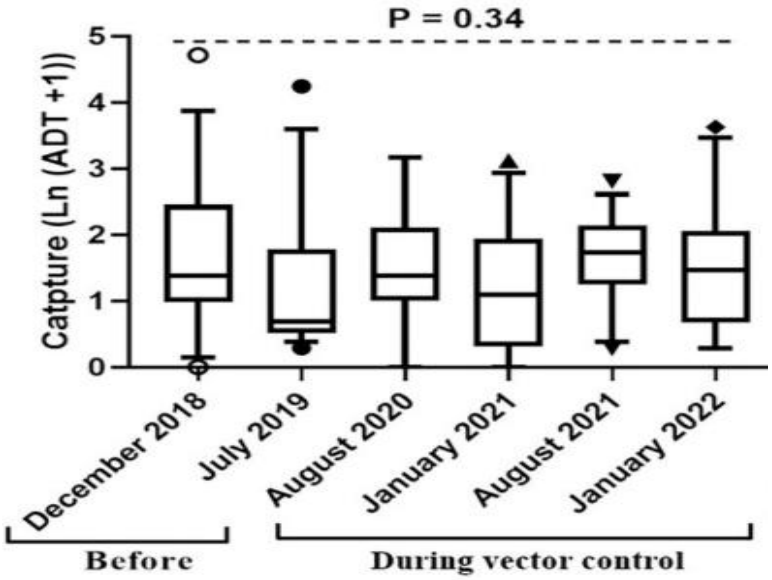

Figure 21. Boxplot of *Glossina palpalis palpalis* apparent densities per trap in the control area

Table VII. Summary of the number of flies captured during tiny targets implementation in control area

Tsetse species	*G. palpalis palpalis*	*G. caliginea*	*G. nigrofusca*	*G. pallicera*	Non identified	Total	*G.p.p.* ADT
December 2018	571 (94.68)	9 (1.49)	3 (0.50)	20 (3.32)	0 (0)	603	3.98 [1.48-8.09]
July 2019	298 (91.69)	3 (0.92)	2 (0.62)	22 (6.77)	0 (0)	325	2.43 [0.73-5.77]
August 2020	314 (95.73)	8 (2.44)	2 (0.61)	4 (1.22)	0 (0)	328	3.74 [1.73-7.21]
January 2021	219 (89.02)	14 (5.69)	6 (2.44)	7 (2.85)	0 (0)	246	2.40 [0.87-5.19]
August 2021	214 (86.64)	10 (4.05)	1 (0.40)	21 (8.50)	1 (0.40)	247	4.13 [2.31-6.96]

January	258	10	2	11	0	281	3.64
2022	(91.81)	(3.56)	(0.71)	(3.91)	(0)		[1.47-7.70]

G.p.p.: *Glossina palpalis palpalis*; Numbers in brackets are relative abundances of each tsetse taxon.

III.1.4.3. Impact of tiny targets on trypanosomes circulating in Campo

- **Trypanosomes' circulation in the intervention area**

From 713 and 844 randomly selected flies of the sub-species *Glossina palpalis palpalis* captured in December 2018 and July 2019 pre-intervention surveys, we identified a total of 162 (22.72%) and 166 (19.67%) flies harbouring at least one trypanosome species in their midguts respectively. The most frequent species was *Trypanosoma congolense* with infection rates of 20.90% and 11.97%, followed by *T. brucei* s. l. (1.96% and 7.23%), *T. vivax* (0.47% and 2.37%) and *T. simiae* (0.47% and 1.90%) for the heavy and light dry seasons respectively. Mixed infections occurred in 11 (1.54%) and 16 (1.90%) flies for the two seasons, and 36 flies (22.22%) from heavy dry season (December 2018) were harbouring mature infections to *T. congolense*, with trypanosomes' DNA detected in their mouthparts. *Trypanosoma brucei gambiense*, the human parasite responsible of the human sleeping sickness, were identified in 4 flies among the *T. brucei* s. l. infections.

After tiny target implementation, tsetse infection rates to trypanosomes remained high after the six first months (from 19.67% in July 2019 to 20.18 % in August 2020), but a significant drop was observed at the eighteenth month, with only 3.03% ($p < 0.001$) of flies infected in January 2021 (Figure 22). During the six last months of vector control, tsetse infection rates increased to 5.06%. However, no mature infection was identified in all those infected flies during the vector control.

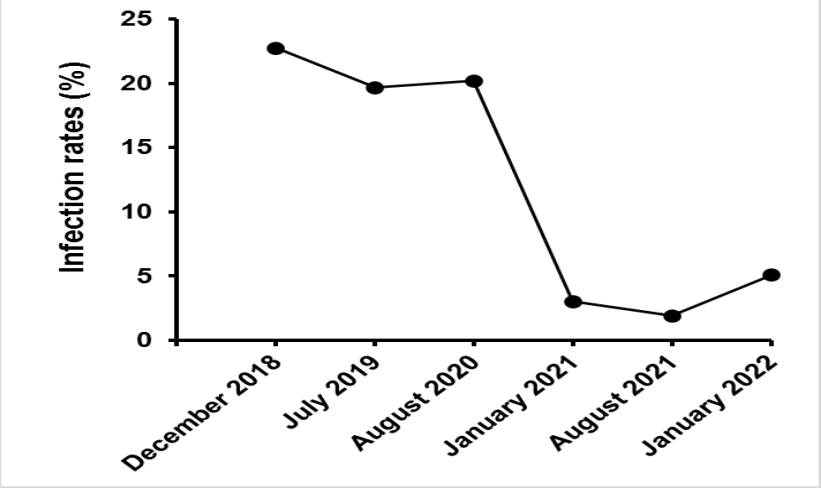

Figure 22. Trypanosome infections pattern during tiny targets implementation in the treated area

The vector control equally affected the different trypanosome species previously detected; indeed, the infection rates of the predominant species *Trypanosoma congolense* decreased from 11.97% before vector control (July 2019) to 4.30% after 2 years (January 2022), *T. brucei s.l.* from 7.23% to 0.41%, *T. vivax* from 2.37% to 0.25%, and *T. simiae* from 1.90% to 0% (Table 8).

Table VIII. Trypanosome infections rate before and during the target intervention in the treated area

Date	Number analyzed	TC (%)	TB (%)	TV (%)	TS (%)	Mixed infections	Total	95% CI in %
December 2018	713	149 (20.90)	14 (1.96)	5 (0.70)	5 (0.70)	11 (1.54)	162 (22.72)	[19.8-25.94]
July 2019	844	101 (11.97)	61 (7.23)	20 (2.37)	1 (0.12)	16 (1.90)	166 (19.67)	[17.13-22.49]
August 2020	337	19 (5.64)	47 (13.95)	2 (0.59)	2 (0.59)	2 (0.59)	68 (20.18)	|16.24-24.79]
January 2021	297	8 (2.69)	1 (0.34)	1 (0.34)	0 (0)	1 (0.34)	9 (3.03)	[1.6-5.6]
August 2021	366	5 (1.37)	2 (0.55)	0 (0)	0 (0)	0 (0)	7 (1.91)	[0.93-3.89]
January 2022	395	17 (4.30)	3 (0.76)	1 (0.25)	0 (0)	1 (0.25)	20 (5.06)	[3.48-8.09]

TC: *Trypanosoma congolense*; TV: *Trypanosoma vivax;* TB: *Trypanosoma brucei* s. l.; Tbg: *Trypanosoma brucei gambiense*. Numbers in brackets are the flies' infection rates.

- **Trypanosomes' circulation in *Glossina palpalis palpalis* in the control area**

From 341 and 288 randomly selected *G. palpalis palpalis* captured in December 2018 and July 2019 pre-intervention surveys, we identified a total of 15 (4.40%) and 63 (21.98%) flies harbouring at least one trypanosome species in their midguts respectively (Table 4). The most frequent species was *Trypanosoma congolense* with infection rates of 3.81% and 12.50%, followed by *T. brucei* s. l. which accounted for 0.59% and 8.33% for the heavy and light dry seasons respectively. *T. vivax* and *T. simiae* were found only in the light dry season where they accounted for 3.13% and 0.35% respectively. Amongst these infected flies, 7 (2.43%) were carrying mixed infections and none was having a mature infection.

After Tiny targets implementation, there was a significant decrease in the fly's infection rates from a mean of 21.88% in July 2019 to 3.45% after 2 years of intervention (Figure 23), showing reduction of 84.23% ($p < 0.001$). More specifically, infections rate shown significant decrease from 21.88% in July 2019 to 15.47% in August 2020, followed by drastically decrease to 0% in January 2021. However, we observed an increase to 1.67% in August 2021 and to 5.45% in January 2022 (Table 9).

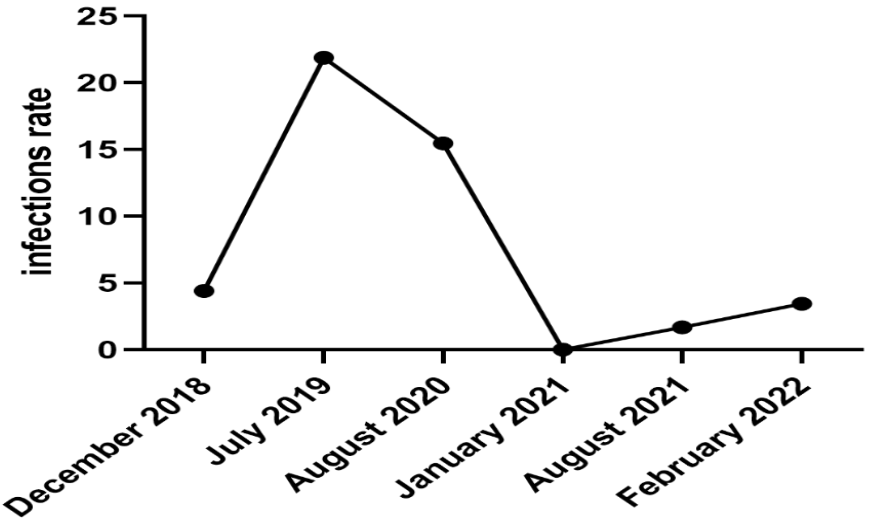

Figure 23. Trypanosome infections pattern in *Glossina palpalis palpalis* in the control area

Concerning trypanosome species, *Trypanosoma congolense* decreased from a rate of 15.50% July 2019 to 1.67% after 2 years (January 2022), showing a total reduction of about 89.22% ($p < 0.001$). Regarding other species, *T. brucei s.l.* infection rate reduced from 8.33% to 0% in January 2022, showing reduction of about 100% ($p < 0.001$). *T. vivax* and *T. simiae* rate passed from 3.13% and 0.35% to 0% respectively.

Table IX. Trypanosome infection rates before and during the target intervention in the control area

Date	Number analyzed	TC (%)	TB (%)	TV (%)	TS (%)	Mixed infections (%)	Total (%)	95% CI (%)
December 2018	341	13 (3.81)	2 (0.59)	0 (0)	0 (0)	0 (0)	15 (4.40)	[2.68 - 7.13]
July	288	36 (12.50)	24	9	1	7	63	[17.49 - 27.01]

2019			(8.33)	(3.13)	(0.35)	(2.43)	(21.88)	
Aug 2020	181	2 (1.10)	24 (13.26)	0 (0)	2 (1.10)	0 (0)	28 (15.47)	[10.99 - 21.57]
January 2021	103	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	-
August 2021	60	1 (1.67)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.67)	[0.3 - 8.86]
January 2022	116	5 (4.31)	0 (0)	0 (0)	0 (0)	1 (0.86)	4 (3.45)	[1.35 - 8.53]

TC: *Trypanosoma congolense*; TV: *Trypanosoma vivax;* TB: *Trypanosoma brucei* s. l.; Tbg: *Trypanosoma brucei gambiense*. Numbers in brackets are the flies' infection rates.

III.1.5. Identification of tsetse bacterial composition

III.1.5.1. Raw sequences data

The V3-V4 hyper-variable region of the bacterial 16S rRNA gene was analysed in 192 individual field-collected tsetse flies, for the general description of microbiome composition of tsetse flies. These tsetse flies included 132 *Glossina palpalis palpalis* from Campo (72 harbouring trypanosome infections in their midguts of which 32 were carrying a mature infection, 40 uninfected, and 20 teneral flies), 20 *G. caliginea*, and 20 *G. pallicera* (also captured in Campo), and 20 *G. tachinoides* (from the Dodeo animal trypanosome focus in the Adamaoua Region, Cameroon). A total of 96 million raw reads were obtained from the sequencing company, and after quality control and filtering, 24 million clean reads remained for subsequent analyses.

III.1.5.2. Rarefaction analyses

Rarefaction analyses carried out to check the depth of sequencing and thus ensure the description of almost all the taxa present in the samples showed curves rapidly reaching a plateau at around 6000 sequences while the sample with the lowest number of sequences had 40,000 sequences, showing that the depth of sequencing was sufficient to characterise most or all the taxa present (Figure 24).

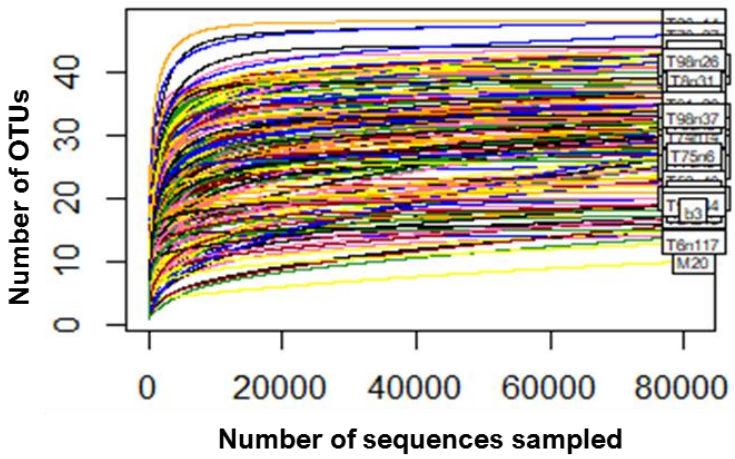

Figure 24. Sample rarefaction curves

III.1.5.3. General characterisation of bacterial phyla and their abundances in tsetse flies

A total of 85 bacterial OTUs were detected in the 4 tsetse species examined. These bacterial OTUs belonged to 4 phyla and 31 genera. Most of the sequences were identified as belonging to the phylum *Proteobacteria* (95.04%) and were present in all the 192 samples. The relative abundance of other bacteria phyla described was 4.45% for *Firmicutes*, 0.30% for *Chlamydiae*, 0.08% for *Acidobacteria*, and 0.13% of sequences could not be classified in a particular phylum.

The overall bacterial phyla identified were unevenly distributed in the different fly species, i.e., *Proteobacteria* represented 99.240% in *G. pallicera*, 96.219% in *Glossina caliginea*, 94.531% in *G. tachinoides*, and 94.656% in *G. palpalis palpalis* (Figure 25).

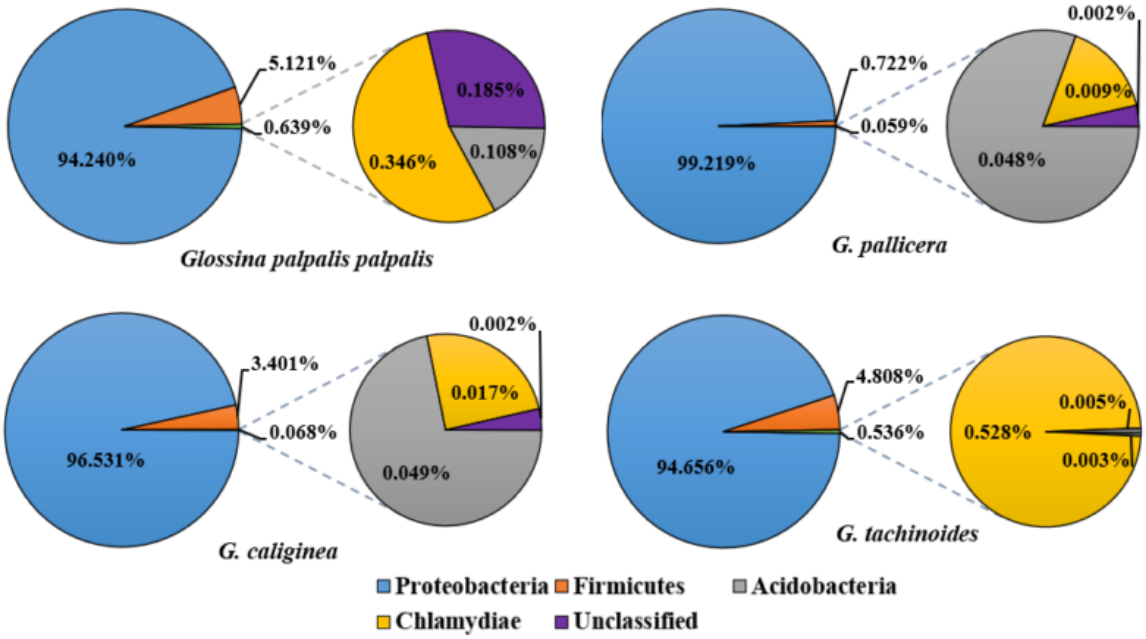

Figure 25. Relative abundance of bacterial phyla in tsetse flies

III.1.4.4. General composition of the bacterial communities of tsetse flies at genus level

The most abundant bacteria genus was *Wigglesworthia*, the primary symbiont of tsetse flies with a relative abundance of 47.29%. The other abundant genera found were *Serratia* (16.58%), *Pantoae_Klebsiella_Enterobacter_Kluyvera* (16.30%), which were highly similar in their V3-V4 sequences and could not be distinguished, *Pseudomonas* (6.16%), *Staphylococcus* (3.39%), *Acinetobacter* (2.77%), *Stenotrophomonas* (2.03%), and *Burkholderia* (1.38%). The 4.1% remaining were shared by other less-represented genera (Table 9), including 0.93% unclassified sequences.

The bacteria genera described were also unevenly distributed among different tsetse species and individual tsetse flies. *Wigglesworthia* displayed an overall abundance of 65.64% in *G. pallicera pallicera*, 62.61% in *G. tachinoides*, 47.61% in *G. caliginea*, and 42.13% in *G. palpalis palpalis* (Figure 26). However, this bacterium seemed to be replaced as the predominant symbiont by *Pantoae_Klebsiella_Enterobacter_Kluyvera* in some *G. palpalis palpalis* samples, as T19n12 (96.08%), T19n16 (95.61%), T18n2 (94.95%), and T11n5 (84.3%), or by *Burkholderia* in some *G. tachinoides* samples, including M17 (91.9%), M14 (69.9%), and M4 (47.18%), or by *Serratia* in other *G. palpalis palpalis* samples T40n2 (54.2%), T19n38 (42.21%), and T31n1 (40.3%).

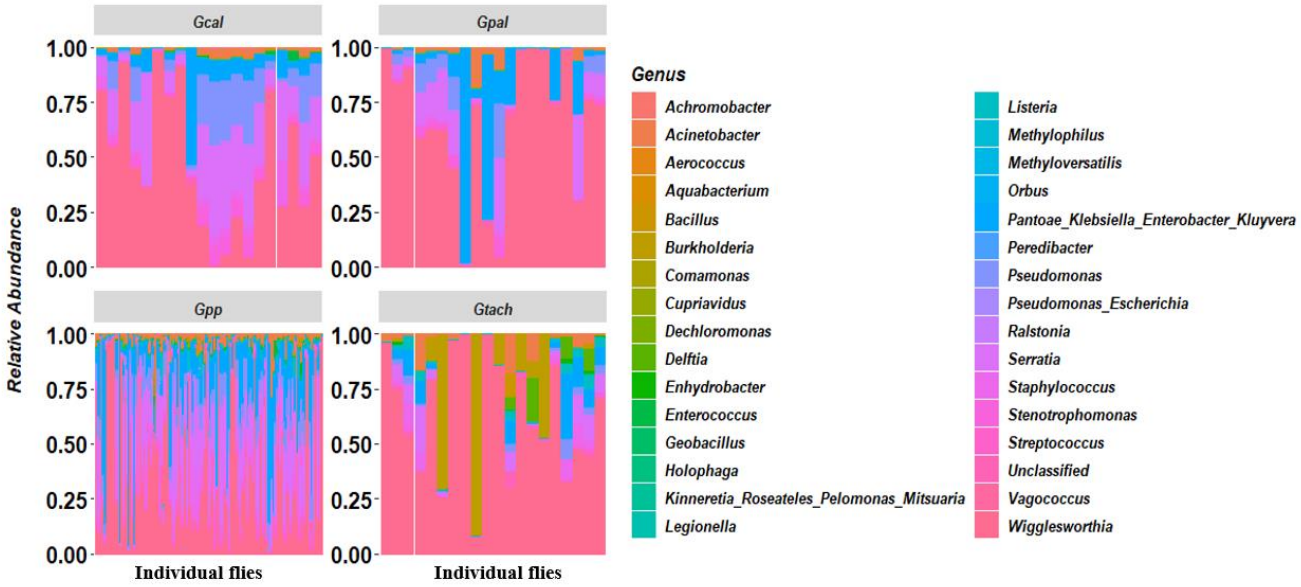

Figure 26. Map showing the relative abundance and distribution of the bacterial genera within different tsetse species (*Gtach*: *Glossina tachinoides*; *Gpp*: *G. palpalis palpalis*; *Gcal*: *G. caliginea*; *Gpal*: *G. pallicera*).

Globally, concerning the distribution of other abundant bacteria in tsetse fly species, *Serratia* was the second most abundant bacterium in *G. caliginea* (21.7%) followed by

Pseudomonas (11.95%), *Pantoae_Klebsiella_Enterobacter_Kluyvera* (8.10%), and Staphylococcus (2.96%). However, in *G. pallicera*, *Pantoae_Klebsiella_Enterobacter _Kluyvera* (14.81%) was the second most abundant bacterium, followed by *Serratia* (8.95%), *Pseudomonas* (4.80%), and *Acinetobacter* (2.35%). In *G. palpalis palpalis*, the trend was similar to *G. pallicera*, with *Pantoae_Klebsiella_Enterobacter_Kluyvera* (19.45%) in second, followed by *Serratia* (18.82%) and *Pseudomonas* (6.16%); however, here, *Acinetobacter* was replaced by *Staphyloccocus* (3.96%). Finally, in *G. tachinoides*, *Burkholdderia*, while completely absent in *G. pallicera* and very less represented in *G. palpalis palpalis* and *G. tachioides*, was the second most abundant bacteria (13.02%) followed by *Pantoae_ Klebsiella_ Enterobacter_ Kluyvera* (5.18%), *Serratia* (4.3%), and *Acinetobacter* (3.62%). Some bacteria, such as *Cupriavidus*, were only present in G. palpalis palpalis, while *Orbus*, *Vagococcus*, and *Dechloromonas* were only present in *G. palpalis palpalis* and *G. pallicera* (Table 10**).**

Table X. Composition and abundance of bacterial genera according to tsetse species

Genus	Gcal %	Gpal %	Gpp %	Gtach %	Total abundance %
Wigglesworthia	47.610182	65.642191	42.137893	62.611812	47.2936772
Serratia	21.702030	8.951618	18.823373	4.332136	16.5834137
Pantoae_Klebsiella_ Enterobacter_Kluyvera	8.108133	14.817094	19.456272	5.183636	16.3013454
Pseudomonas	11.958733	4.808630	6.165262	1.699745	6.1622939
Staphylococcus	2.969413	0.694511	3.968475	2.432323	3.3627958
Acinetobacter	1.780862	2.354777	2.856591	3.629718	2.7727156
Stenotrophomonas	3.976580	1.581399	2.064466	0.396511	2.0395646
Burkholderia	0.015110	0	0.031788	13.022083	1.3810743
Unclassified	0.564854	0.692730	1.012066	1.049295	0.9360245
Listeria	0.031633	0.017813	0.469581	1.554900	0.4899692
Methylophilus	0.028071	0.002641	0.643010	0.078872	0.4533113
Delftia	0.226040	0.112650	0.105101	2.418686	0.3597069
Orbus	0.000123	0	0.410778	0	0.2823053
Enhydrobacter	0.042137	0.014680	0.276657	0.341165	0.2316157
Achromobacter	0.416761	0.160007	0.173112	0.001167	0.1792226
Bacillus	0.002641	0.003378	0.160923	0.623914	0.1762639
Aquabacterium	0.009828	0.005282	0.216017	0.001536	0.1501858
Vagococcus	0.001106	0	0.193381	0	0.1330097
Enterococcus	0.388444	0.000369	0.112715	0.003256	0.1183375

Geobacillus	0.001966	0.000921	0.141567	0.000123	0.0976006
Legionella	0.006081	0.007248	0.075420	0.248103	0.0790860
Holophaga	0.047296	0.045392	0.075597	0	0.0616152
Streptococcus	0.005160	0.003378	0.055384	0.184772	0.0582145
Cupriavidus	0	0	0.082474	0	0.0566775
Dechloromonas	0.001106	0	0.071245	0	0.0490757
Pseudomonas_Escherichia	0.094654	0.070268	0.037836	0.000123	0.0432094
Ralstonia	0.000553	0.000061	0.045990	0.075862	0.0395782
Methyloversatilis	0.005344	0.004730	0.030995	0.096747	0.0324375
Comamonas	0.004914	0.006572	0.031033	0	0.0225237
Kinneretia_Roseateles_ Pelomonas_Mitsuaria	0	0.000061	0.030725	0	0.0211212
Peredibacter	0.000061	0	0.025283	0.004361	0.0178358
Aerococcus	0.000184	0.001597	0.018992	0.009153	0.0141918

III.1.4.5. Composition of the bacterial communities of *Glossina palpalis palpalis* according to infection and teneral status

Regarding the midgut infection status of flies, *Wigglesworthia* was the predominant genus with relative abundance of 39.06% and 50.02% respectively for midgut infected and non-infected flies. The other abundant genera were *Pantoae_Klebsiella_Enterobacter_Kluyvera* which accounted for 19.54% and 16.29%, *Serratia*, 19.54% and 16.29%, *Pseudomonas*, 6.41% and 7.12% and *Staphyloccocus*, 3.22% and 4.04% for midgut infected and non-infected flies, respectively.

Regarding the mature infection status of flies, *Wigglesworthia* was also the predominant genus with relative abundance of 45.98% and 34.86% respectively for mature and non-mature infected flies. The other abundant genera were *Pantoae_Klebsiella_Enterobacter_ Kluyvera* which accounted for 16.70% and 22.42%, *Serratia*, 17.54% and 20.82%, *Pseudomonas*, 6.17% and 6.57% and *Staphyloccocus*, 2.96% and 3.39% for mature and non-mature infected flies, respectively.

In non-teneral testes flies of the species *G. palpalis palpalis*, the top represented bacteria were *Wigglesworthia* (37.10%), *Serratia* (20.97%), *Pantoae_Klebsiella_Enterobacter_Kluyvera* (20.51%), *Pseudomonas* (6.99%), and *Staphyloccocus* (4.44%), while teneral flies were dominated by *Wigglesworthia* (70.30%), *Pantoae_Klebsiella_Enterobacter_Kluyvera* (13.52%), *Serratia* (6.79%), *Acinetobacter*

(3.21%), and *Pseudomonas* (1.52%). *Orbus* and *Burkholderia* were found only in non-teneral flies (Table 11). In summary, the abundance of *Wigglesworthia* in flies decreased, whereas the abundance of most of other bacteria genera increased through the process of blood feeding.

Table XI. Composition and abundance of bacterial genera in *G. palpalis palpalis* according to their teneral and infection status.

Genus	MInf (%)	MNInf (%)	InfM (%)	InfNM (%)	NT (%)	T (%)
Wigglesworthia	39.066157	50.020640	45.988990	34.865451	37.1085	70.3097
Pantoae_Klebsiella_ Enterobacter_Kluyvera	20.212214	13.473089	16.706950	22.421387	20.5158	13.5212
Serratia	19.548457	16.291457	17.547673	20.825939	20.9704	6.7966
Pseudomonas	6.417254	7.126770	6.178006	6.578372	6.9935	1.5258
Staphylococcus	3.226800	4.045775	2.961774	3.397441	4.4455	1.2962
Acinetobacter	3.025839	2.447747	3.150567	2.954917	2.7925	3.2154
Stenotrophomonas	2.288195	2.302296	1.868167	2.552487	2.4313	0.0098
Unclassified	1.180123	0.691701	1.193990	1.149876	1.0228	0.9521
Listeria	0.773025	0.230305	0.592645	0.886124	0.5533	0.0004
Orbus	0.645767	0.000030	0.000083	0.935185	0.4841	0
Burkholderia	0.592105	1.691628	0.011642	0.952248	0.0375	0
Methylophilus	0.560750	0.166929	0.841759	0.388134	0.5307	1.2719
Enhydrobacter	0.343575	0.137929	0.236354	0.410525	0.3148	0.0630
Vagococcus	0.303999	0.000257	0.021087	0.479570	0.2279	0.0001
Aquabacterium	0.271143	0.029256	0.630718	0.049420	0.2225	0.1795
Bacillus	0.224723	0.155918	0.279896	0.191095	0.1749	0.0824
Achromobacter	0.195832	0.210806	0.259968	0.156611	0.2037	0.0020
Geobacillus	0.179772	0.036808	0.317472	0.095074	0.1622	0.0260
Enterococcus	0.173893	0.095200	0.058249	0.175211	0.1327	0.0007
Delftia	0.125895	0.556777	0.089860	0.148464	0.1222	0.0093
Streptococcus	0.086166	0.040675	0.013009	0.131607	0.0628	0.0136
Dechloromonas	0.084405	0.000272	0.199026	0.013706	0.0633	0.1158
Cupriavidus	0.084053	0.000045	0.200186	0.012349	0.0630	0.1914
Holophaga	0.077469	0.046445	0.100632	0.063331	0.0768	0.0686
Legionella	0.070932	0.074749	0.105686	0.049599	0.0721	0.0942
Ralstonia	0.069478	0.017309	0.155525	0.016473	0.0541	0.0003
Pseudomonas_Escherichia	0.046206	0.042759	0.075774	0.028130	0.0376	0.0389

Comamonas	0.034540	0.008443	0.064630	0.015961	0.0302	0.0356
Kinneretia_Roseateles_ Pelomonas_Mitsuaria	0.032672	0.000740	0.075733	0.006149	0.0251	0.0624
Methyloversatilis	0.026670	0.035222	0.029208	0.025107	0.0287	0.0441
Aerococcus	0.016152	0.004199	0.029415	0.007993	0.0146	0.0437
Peredibacter	0.015739	0.017823	0.015329	0.016063	0.0245	0.0295

MGInf: midgut infected; MGNInf: midgut non-infected; HNinf: head non-infected; Hinf: Head infected; T: teneral; NT: non teneral

III.1.5.6. Bacterial Genera diversity in tsetse flies (alpha-aiversity)

III.1.5.6.1. Diversity in tsetse species

Bacterial genera richness and evenness were significantly different between tsetse species (Figure 27; *p*-value = 0.006). Briefly, the gut microbiota of *G. palpalis palpalis* (Shannon diversity index H = 1.58) was significantly richer in genera compared to that of *G. pallicera* (H = 0.89) (*p*-value = 0.007). In addition, significant difference was observed between *G. pallicera* and *G. caliginea* (H = 1.52) (*p*-value = 0.04). However, no significant difference was observed when comparing *G. palapalis palpalis* vs *G. caliginea* (*p*-value = 0.9554), *G. tachinoides* vs *G. caliginea* (*p*-value = 0.9554), *G. tachinoides* (Shannon H = 0.83) vs *G. pallicera* (*p*-value = 0.49), and *G. tachinoides* vs *G. palapalis palpalis* (*p*-value = 0.37).

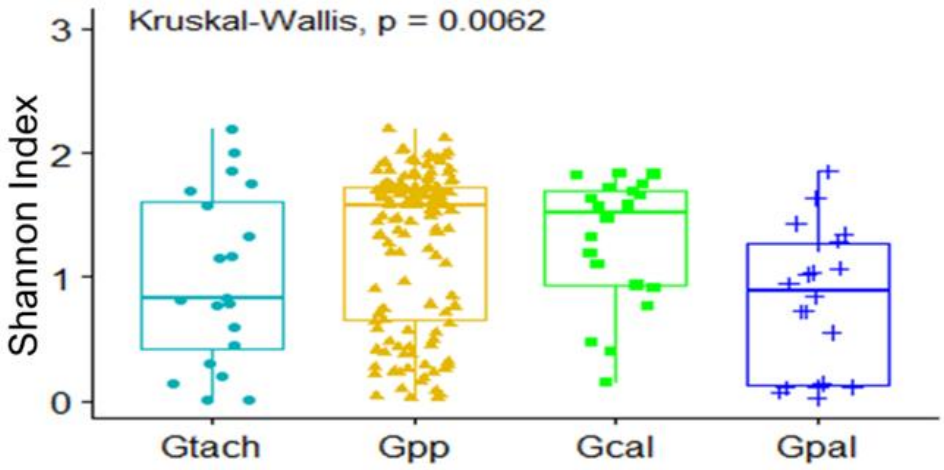

Figure 27. Bacterial diversity in different tsetse species (Gtach: *Glossina tachinoides*; Gpp: *G. palpalis palpalis*; Gcal: *G. caliginea*; Gpal: *G. pallicera*)

III.1.5.6.2. Bacteria diversity of *Glossina palpalis palpalis* between teneral and non-teneral, male and female flies and between villages

Comparison of the values of bacteria diversity using Shannon diversity index showed a significant difference between teneral (Shannon H = 0.52) and non-teneral (Shannon H = 1.63)

tsetse flies (*p*-value < 0.001, Figure 28a). However, no significant difference was observed in the bacterial flora composition of male (Shannon H = 1.66) and female (Shannon H = 1.52) tsetse (*p*-value = 0.34; Figure 28b). Similarly, no significant difference was observed when comparing the Shannon diversity index of tsetse flies between the different villages (*p*-value = 0.24; Figure 28c).

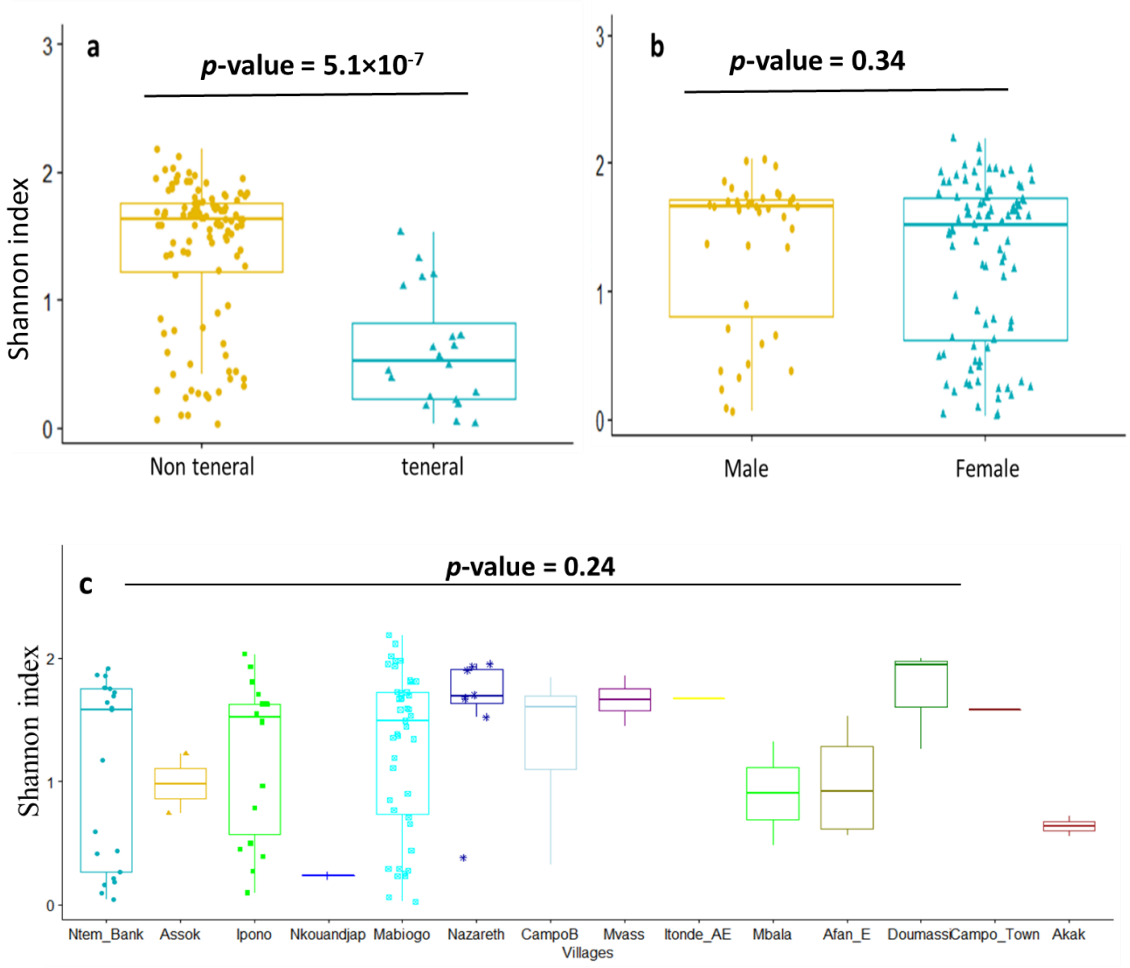

Figure 28. Bacteria diversity in non-teneral and teneral flies (a), male and female (b) and between villages (c)

III.1.5.6.3. Bacterial diversity of *Glossina palpalis palpalis* between infected and uninfected flies

Globally, no statistically significant difference was observed when comparing bacteria richness and evenness between *G. palpalis palpalis* flies harbouring trypanosomes in their midgut (Shannon H = 1.63) and non-infected (Shannon H = 1.60) flies (*p*-value = 0.8), as shown in the Figure 29. However, within the infected tsetse flies, a significant difference was observed

between samples harbouring a mature trypanosome infection (Shannon H = 1.45) and without infections (Shannon H = 1.60) in the mouthparts (*p*-value = 0.031).

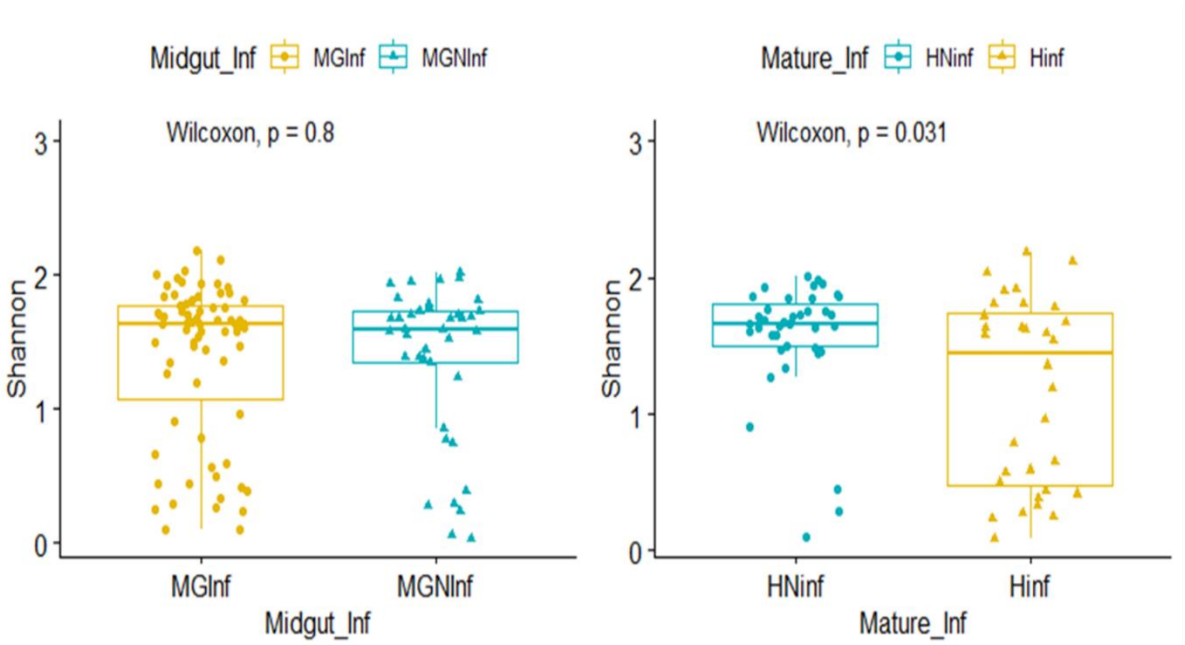

Figure 29. Tsetse bacteria alpha diversity regarding trypanosome infection status (MGInf: midgut infected; MGNInf: midgut non-infected; HNinf: head non-infected; Hinf: Head infected)

III.1.5.7. Multivariate Analysis (Beta Diversity)

III.1.5.7.1. Multivariate analyses in different tsetse species

No clear separation or clustering of flies' microbiome composition was observed according to their species, as shown by the principal coordinate analysis (PCoA) performed using the Bray–Curtis dissimilarity index (Figure 30). Nevertheless, the level of dissimilarity was significant as shown by the permutational analysis of variance (PERMANOVA), showing a difference in the composition of the fly microbiota in different tsetse species (R^2 = 0.088; *p*-value = 0.01). The community structure and composition of the microbiota of *Glossina palpalis palpalis* was substantially different from that of *G. caliginea* (*p*-value = 0.048) and *G. pallicera* (*p*-value = 0.012). However, *G. caliginea*, *G. pallicera*, and *G. tachinoides* had a similar pattern in their microbiota when compared to each other (*p*-value values of 0.64; 0.94 and 0.70, respectively).

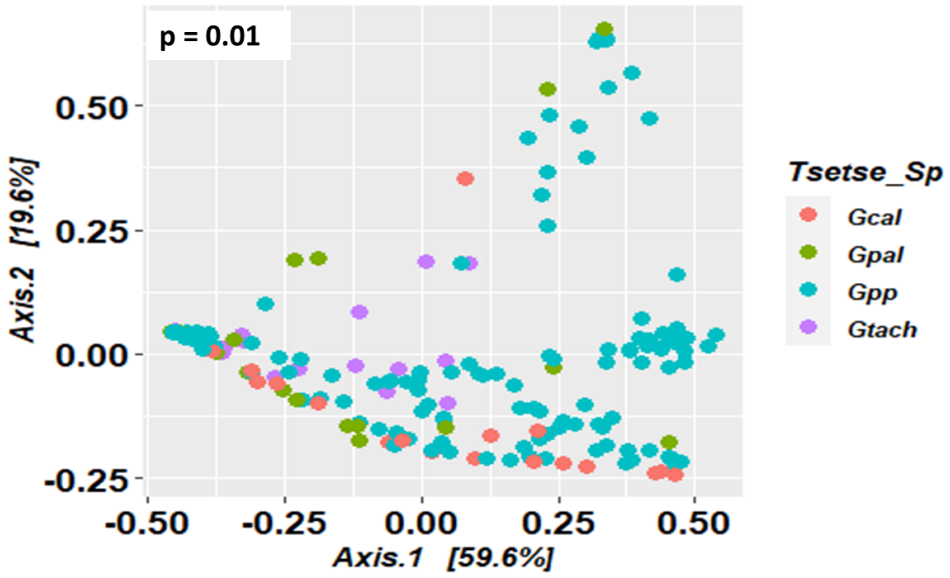

Figure 30. Distribution of the tsetse flies according to their bacterial composition, based on principal coordinates analysis using Bray–Curtis index (Gcal: *G. caliginea*; Gpal: *G. pallicera*; Gpp: *G. palpalis palpalis*; Gtach: *G. tachinoides*)

III.1.5.7.2. Multivariate analysis of *Glossina palpalis palpalis* between teneral and non-teneral flies, males and females and between villages

In *Glossina palpalis palpalis*, although a great variation was observed in the microbiome composition of non-teneral flies, most of the teneral flies clustered together on the PCoA plot, showing that they have a similar composition in the bacterial genera present, as well as their abundances (Figure 31a). Moreover, a significant difference in beta diversity was observed between non-teneral and teneral tsetse flies (R^2 = 0.09; *p*-value = 0.01). Similarly, there was a significant difference between villages (Figure 31b; R^2 = 0.16; p-value = 0.02). However, males and females were similar according to their bacteria composition (Figure 31c; R^2 = 0.006; *p*-value = 0.54).

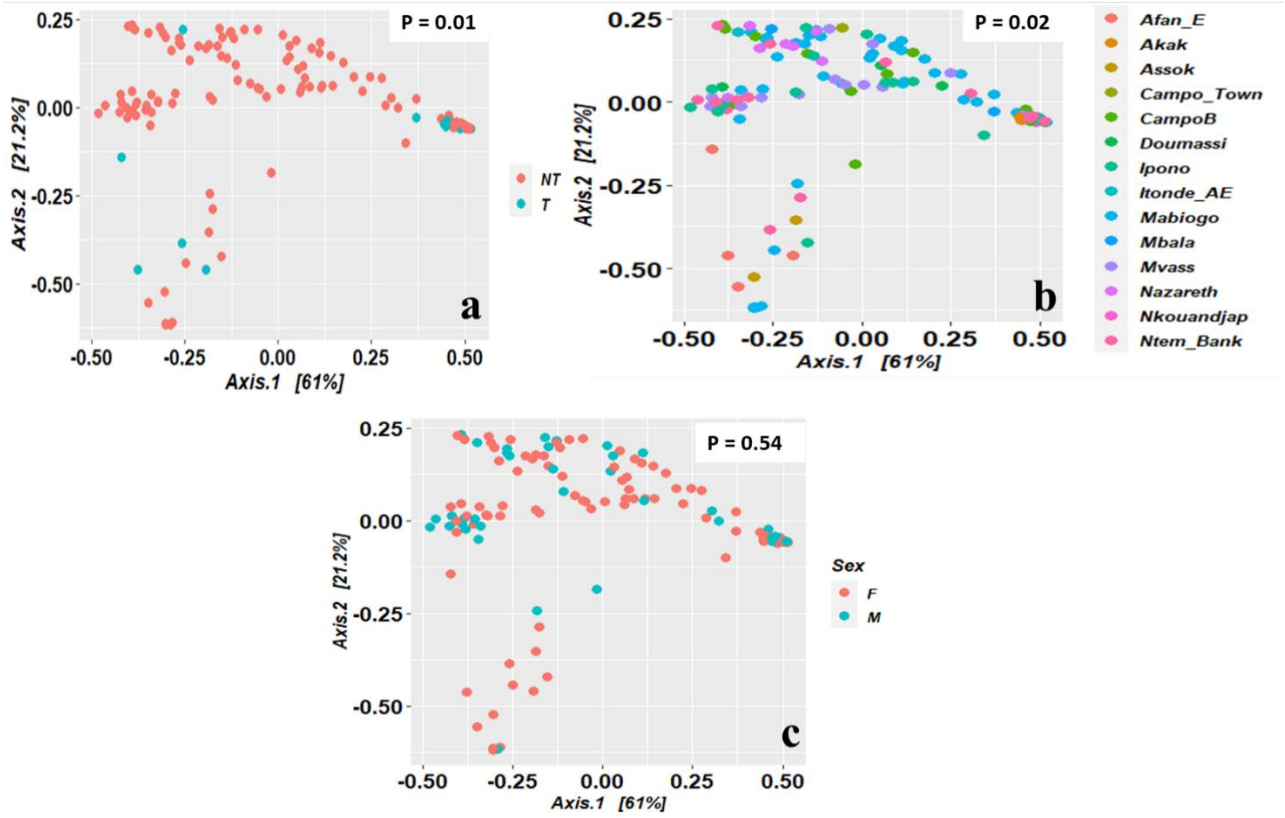

Figure 31. Distribution of tsetse fly samples according to their microbiome composition based on principal coordinate analysis using the Bray-Curtis index in non-teneral and general flies (a), male and female (c) and between villages (b). T: teneral; NT: non-teneral; M: male; F: female.

III.1.4.7.3. Multivariate analysis of *Glossina palpalis palpalis* between infected and non-infected flies

Regarding the beta diversity, the tsetse flies studied had similar microbiome composition (taxa richness and abundance) regardless of if they harboured trypanosome infections in their midguts or not, confirmed by the PERMANOVA test (Figure 32a; $R^2 = 0.003$ *p*-value = 0.84). However, comparing the microbiome composition of the flies harbouring a mature trypanosome infection and those only infected in the midgut, a significant difference was observed (Figure 32b; $R^2 = 0.12$; p-value = 0.02).

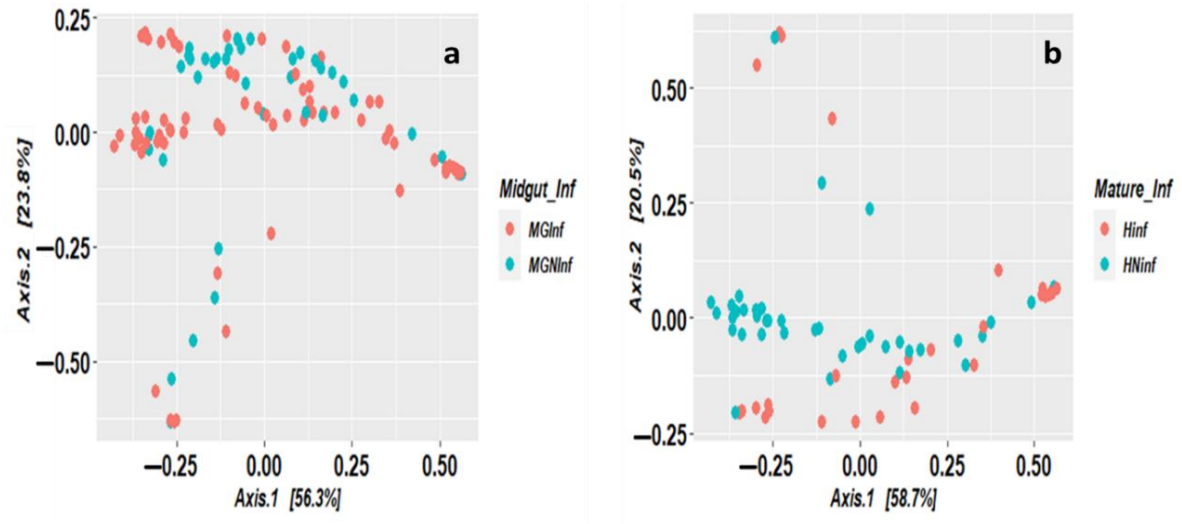

Figure 32. Distribution of tsetse fly samples according to their microbiome composition, based on principal coordinates analysis using the Bray–Curtis index, within trypanosome infection status: (a) midgut infection, (b) mature infection (MGInf: midgut infected; MGNInf: midgut non-infected; Hinf: head infected or mature infection; HNinf: head non-infected)

III.1.5.8. Hierarchical classification and differential abundance test according to infection status

The hierarchical clustering using the Bray–Curtis dissimilarity index clearly showed a similarity in microbial communities of flies according to infection status (midgut infection and mature infection), despite the high variability observed in the different groups and no clear "higher-level" clustering of infected flies on one hand, and uninfected ones on the other hand (Figure 33).

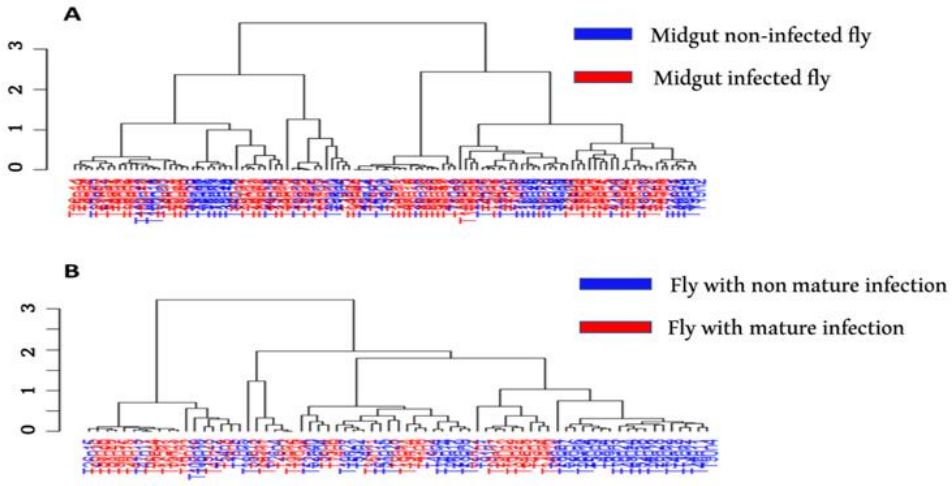

Figure 33. Hierarchical cluster dendrogram, based on Bray–Curtis Index values, showing the relationship between different tsetse microbiome communities and infection status. (A) midgut infection with trypanosomes, (B) mature infection with trypanosomes.

Looking deeply at the difference in the diversity of the tsetse flies harbouring trypanosome infections in their midguts or mature and non-mature infections, differential abundance testing showed many OTUs presenting significantly different abundances, as summarized in the Tables 12 and 13. Indeed, numerous genera and OTUs contributed to differences between samples with high fold change, the most important being *Dechloromonas* OTU37 (log2 Fold Change = -21.44; *p*-value < 0.001), *Ralstonia* OTU49 (log2 Fold Change = -10.59; *p*-value < 0.001), *Listeria* OTU14 (log2 Fold Change = -5.41 ; *p*-value < 0.001), *Serratia* OTU55 (log2 Fold Change = 3.39 ; p<0.001) et *Staphylococcus* OTU40 (*log2 Fold Change* = 5.48; *p*-value = 0.003).

Table XII. Operational Taxonomic Units displaying a significant difference in abundance between tsetse flies with midguts non-infected vs. infected with trypanosomes.

OTUs	Genera (Percentage / Number UTOs in the genus)	Base Mean	log2 Fold Change	lfcSE	statistic	*p*-value
OTU37	*Dechloromonas* (100 / 1)	21,44	-25,44	2,3	-11,06	0,0000
OTU49	*Ralstonia* (100 / 1)	19,88	-10,58	1,67	-6,33	0,0000
OTU14	*Listeria* (100 / 1)	283,11	-5,41	1,08	-5,01	0,0000
OTU28	*Aquabacterium* (79,12 / 2)	46,11	-7,76	1,99	-3,9	0,0001
OTU25	*Bacillus* (53,62 / 3)	38	-2,98	0,9	-3,31	0,0009
OTU24	*Methylophilus* (24,65 / 2)	11,6	-6,3	2,25	-2,8	0,0051
OTU70	*Kinneretia_ Roseateles_ Pelomonas_Mitsuaria* (100 / 1)	11,36	-4,37	1,59	-2,74	0,0061
OTU33	*Pseudomonas_ Escherichia* (100 / 1)	9,31	-2,98	1,19	-2,51	0,0120
OTU45	*Pseudomonas* (0,36 / 3)	4,66	-5	2,11	-2,37	0,0179
OTU63	*Cupriavidus* (40,19 / 2)	3,25	-4,82	2,18	-2,21	0,0273
OTU27	*Enterococcus* (70,41 / 3)	5,99	-5,36	2,52	-2,13	0,0334
OTU18	*Wigglesworthia* (0,25 / 2)	15,97	4,57	0,84	5,43	0,0000
OTU55	*Serratia* (0,14 / 2)	10,16	3,39	0,96	3,51	0,0004
OTU40	*Staphylococcus* (22,2 / 9)	44,63	5,48	1,89	2,9	0,0037

* Percentage of the OTU/number of OTUs for the genus concerned; lfcSE: log2 fold change standard error

Table XIII. Operational Taxonomic Units displaying a significant difference in abundance between tsetse flies with non-mature vs. mature infection with trypanosomes.

OTU	Genera (Percentage / Number UTOs in the genus)	Base Mean	log2 Fold Change	lfcSE	statistic	*p*-value
OTU29	*Staphylococcus* (6,19 / 7)	7,85	-24,84	2,92	-8,51	0,0000
OTU55	*Serratia* (0,14 / 2)	1,59	-3,02	1,32	-2,28	0,0224

OTU49	*Ralstonia* (100 / 1)	21,57	-3,88	1,77	-2,19	0,0286
OTU16	*Enhydrobacter* (100 / 1)	238,65	2,66	0,84	3,15	0,0016
OTU25	*Bacillus* (53,62 / 3)	37,31	2,35	1,07	2,19	0,0287

* Percentage of the OTU/number of OTUs for the genus concerned; lfcSE: log2 fold change standard error.

III.1.6. Evaluation of *Glossina palpalis palpalis* bacterial composition during vector control using Tiny targets

A total of 208 tsetse flies (73 before the vector control, 45 after six months, 45 after twelve months and 45 after eighteen months of control) were analysed for this part of the work. Illumina sequencing of the V3-V4 hypervariable region of the 16 S rRNA gene amplicons yielded a total of 15,607,674 raw sequence reads from 148 individual field-collected *Glossina palpalis palpalis* flies (13 before tiny targets implementation, 45 after six months, 45 after twelve months and 45 after heighten months of vector control). After removing chimeric and other non-bacterial sequences, and quality filtering to remove bacterial OTUs constituting < 0.01%, a total of 7,659,379 sequences were obtained. From the analysis of the 4 groups and removing outliers, the samples rarefaction curves showed that the sequencing depth was enough for subsequent analysis.

III.1.5.1. Summary of general composition of tsetse bacterial composition from the second Illumina sequencing

A total of 111 bacterial OTUs were detected and belonged to 5 phyla and 48 genera (Table 10). Most of the sequences were identified as belonging to the phylum *Proteobacteria* (96.69%) and were present in all the 148 samples. The relative abundance of other bacteria phyla described were 2.48% for *Firmicutes,* 0.16% for *Chlamydiae*, 0.05% for *Acidobacteria*, 0.01% of *Bacteroidetes* and 0.60% of sequences could not be classified in a particular phylum.

At the genus level, the most abundant bacteria genus was *Wigglesworthia,* the primary symbiont of tsetse flies with a relative abundance of 71.76%. This bacterium was predominant in the majority of the samples, except some sample like T126Cn19, T154Dn36 and T34En2 where it accounts for only 0.19%, 1.84% and 4.37% and replace by *Bacillus*, *Pseudomonas* and *Klebsiella* which accounted for 99.80%, 81.15% and 87.54% respectively. The other abundant genera found were *Curvibacter* (4.26%), *Pelomonas* (4.25%), *Stenotrophomonas* (2.95%), *Acinetobacter* (2.16%), *Klebsiella* (2.11%), *Bacillus* (1.54%), *Escherichia_Shigella* (1.17%) which were highly similar in their V3-V4 sequences and could not be distinguished, *Pseudomonas* (0.98%) and *Staphyloccocus* (0.40%) (Table 14). The 8.39% remaining were shared by other less represented genera including 5.91% being unclassified.

Table XIV. Summary of bacterial genera abundance according to sampling periods

Genus	Before (%)	6months (%)	12months (%)	18months (%)	Total (%)
Wigglesworthia	83.07	82.45	62.57	66.87	71.76
Curvibacter	0.57	0.65	4.73	8.57	4.26
Pelomonas	0.27	4.67	8.55	0.62	4.25
Stenotrophomonas	0.35	0.08	0.10	9.55	2.95
Acinetobacter	1.28	1.59	1.39	3.79	2.16
Klebsiella	1.26	0.57	3.23	2.79	2.11
Bacillus	2.67	3.43	0.21	0.65	1.54
Escherichia_Shigella	0.14	0.21	3.39	0.26	1.19
Pseudomonas	0.40	0.66	2.00	0.44	0.98
Staphylococcus	0.57	0.42	0.55	0.18	0.40
Unclassified	4.09	4.61	9.39	2.68	5.45
Others	5.34	0.66	3.89	3.60	2.94

III.1.6.2. Dynamics of variation in tsetse fly bacterial communities between sampling periods

Out of the five bacterial phyla identified, the relative abundances of *Firmicutes* shown significant reduction during the vector control from 4.53% before to 3.97%, 1.23% and 1.63% respectively after 6, 12 and 18 months of vector control (p-value = 0.02). These decrease in *Firmicutes* abundance were concomitant to an increase in relative abundance of *Proteobacteria* from 92.89% before to 95.78%, 97.34% and 98.08% respectively (p-value < 0.0001). There was no significant difference in the relative abundance of *Bacteroidetes* and *Chlamydiae* during the vector control intervention (p-value = 0.08 and 0.13 respectively). Bacteria genera showed differential compositions during the vector control at genus level with the core bacteria unevenly distributed among tsetse flies sampled at different periods. *Wigglesworthia* displayed an overall abundance of 83.07% before the targets installation and did not show any significant changes after six months of vector control (82.45%; p-value = 0.43). This abundance showed a significant drop to 62.57% (p-value < 0,001) 12 months later followed with a slight increase to 66.87% (p-value = 0.25) 18 months later. *Curvibacter* showed significant increase (P-value = 0.001) in abundance from 0.57% before the target to 0.65%, 4.73% and 8.57% after 6, 12 and 18 months of tsetse control respectively. During the vector control *Pelommonas* showed

different pattern with an increase from 0.27% before the intervention to 4.67% (p-value < 0,001) and 8.55% (p-value < 0,001) six and twelve months after, followed by a decrease to 0.61% (p-value < 0,001) 18 months later. Overall, the relative abundances of 11 of the 48 bacteria genera identified were significantly different between sampling periods (p-value < 0.05). Some bacteria taxa such as *Cupriavidus* and *Veillonella* were only found in tsetse flies collected before the targets' implementation, *Lactobacillus* found only in flies collected after 6 months of vector control, *Chromohalobacter* found only in flies collected after 12 months and *Alishewanella* and *Oxalobacter* after 18 months.

Of the 111 UTOs identified, 88 were found in flies collected before the installation of the screens, 88 were also found in flies collected six months later while 96 and 98 were found in flies collected twelve and eighteen months later respectively. Five were unique to flies collected before vector control, 1 was unique to flies collected 18 months after implementation of control and 71 were common in all sample groups (Figure 34).

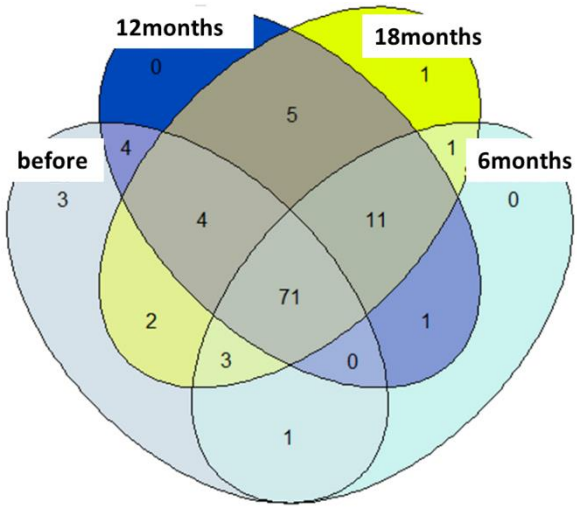

Figure 34. Venn diagrams comparing the number of OTUs present in different sampling periods.

III.1.5.3. Bacterial diversity during vector control (alpha and beta diversity)

The microbiome diversity in flies varied throughout the vector control with significant difference between some sampling periods (Figure 35A). Indeed, although the alpha diversity estimated with the Shannon index didn't vary after the six first months of control, (H = 0.45 before and H = 0.44 after six months, p-value = 0.35), the diversity significantly increased to H = 1.24 twelve months later (p-value < 0.001) followed by a slight decrease to 0.99 after eighteen months.

Regarding the beta diversity, the structure of the flies' microbiome composition showed a great heterogeneity between sampling periods as shown by clear separation or clustering of flies after principal component analysis (PCA) performed using the Bray-Curtis dissimilarity index (Figure 35B). This dissimilarity observed during the vector control implementation is supported by the permutational analysis of variance (PERMANOVA), showing a significant difference in the composition of the fly microbiota in different sampling periods (R^2 = 0.16; p-value = 0.001).

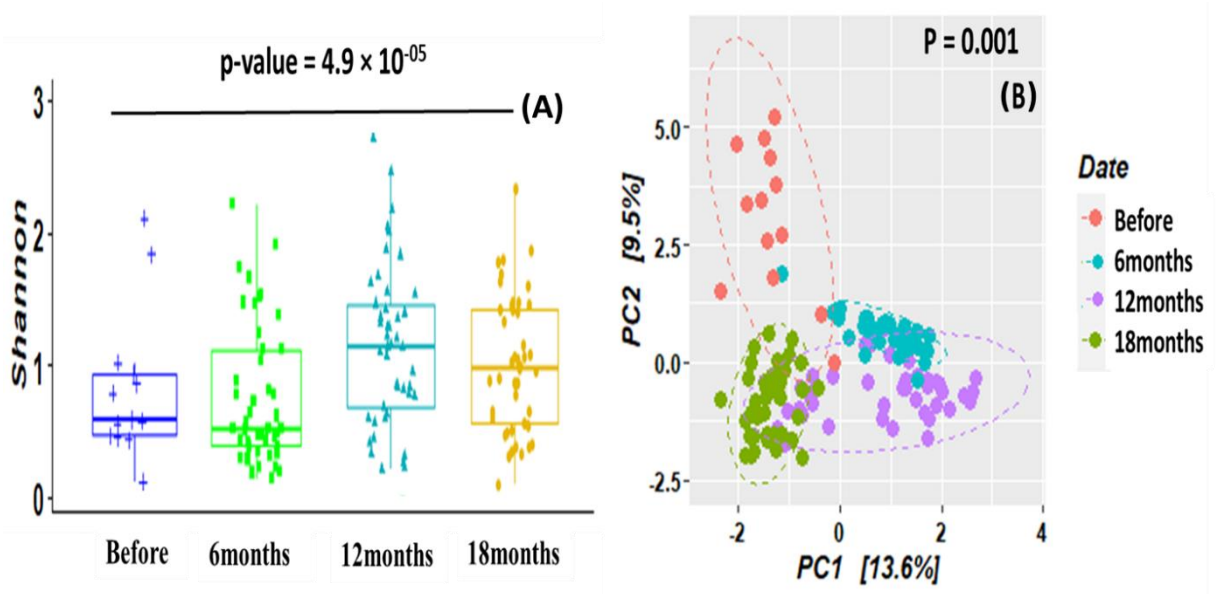

Figure 35. Bacteria diversity in tsetse fly (A) and distribution of tsetse fly samples according to their bacterial composition based on principal coordinates analysis using Bray-Curtis index (B) according to sampling periods (BF: before)

III.1.5.4. Differential abundance testing between bacterial composition of tsetse flies collected before and eighteen months after vector control implementation

Looking at the difference in the diversity of the tsetse flies collected before and after eighteen months of tiny targets implementation, differential abundance testing showed numerous OTUs that contributed to these differences with high fold change (>5) at 0.01 threshold (Figure 36). Twenty-seven OTUs among which 12 classified at the genus level were differentially abundant. *Novimethylophilus* OTU097 went from 0% before to 0.01% eighteen months later (log2 Fold Change = -26.29, p<0.001), *Simkania* OTU071 from 0% to 0.08% (log2 Fold Change = -24.28, p<0.001) and *Cupriavidus* OTU082 went from 0% to 0.01% (log2 Fold Change = -18.78, p<0.001). In addition, other bacteria genera shown a decrease in abundance such as *Cupriavidus* OTU83 which went from 0.22% to 0% (log2 Fold Change = 26.22,

p<0.001); *Methylophilus* OTU022 went from 1.97% to 0% (log2 Fold Change = 12.06, p<0.001).

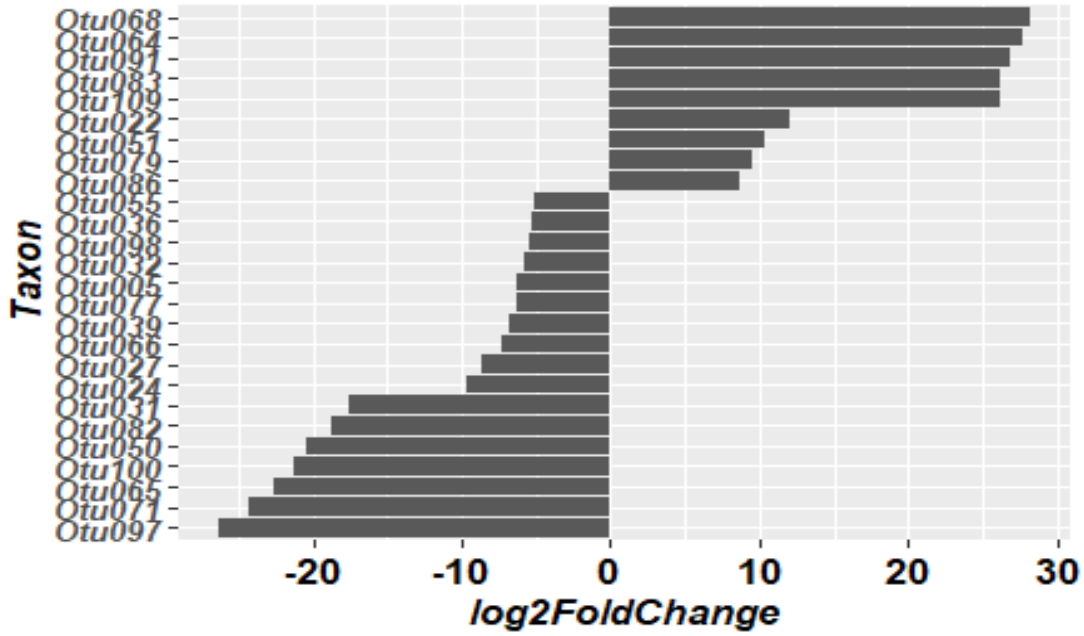

Figure 36. Operational Taxonomic Units displaying a significant difference in abundance between tsetse flies collected before and after 18 months of tiny targets implementation.

III.1.7. Genetic diversity of some bacteria of interest

The study of bacterial composition showed several taxa associated with the flies infection with trypanosomes, but also with the maturation of this latter. However, some of these bacterial genera were represented by several operational taxonomic units (OTUs) or phylotypes. The phylogenetic trees analyses showed that these OTUs presented a certain degree of genetic polymorphism within the taxon.

Three different OTUs were found belonging to the genus *Pseudomonas* (OTU 4, 38 and 45) with one OTU having sequences similarities between *pseudomonas* and *Escherichia* (OTU 33) according to SILVA database. Two of these OTUs showed differential abundances between infected and uninfected flies (OTU33 and OTU45). The phylogenetic tree shows that these two OTUs were highly similar in their 16S rRNA sequences and could belong to the same species, strain or genotype. For OTU4, some of the sequences were close to the species *Pseudomonas aeruginosa* while others are close to the species *Pseudomonas alcaliphila* or *P. protegens* identified in several insect (Figure 37).

Concerning the genus *Serratia*, two different OTUs were found (OTU3 and OTU55). The OTU55 showed differential abundance between infected and uninfected flies but also between infected flies and those carrying mature infections. Phylogenetic analyses showed that theses 2 OTUs were clearly diverse in their 16S sequences with the main phylotype (the most abundant, OTU3) similar to the *Serratia marcescens* species previously described in several tsetse fly species (Figure 38).

Regarding *Wigglesworthia* genus, two different UTOs were found (OTU1 and OTU18). The OTU18 showed a significant difference in abundance between infected and uninfected flies. Phylogenetic analyses presented this OTU forming a cluster well separated from the main phylotype. The 16S DNA sequences of this predominant OTU (OTU1) showed a clear relationship with the sequences of *Wigglesworthia glossinidia* previously described in *Glossina palpalis*, *Glossina pallidipes* and *Glossina austeni* (Figure 39).

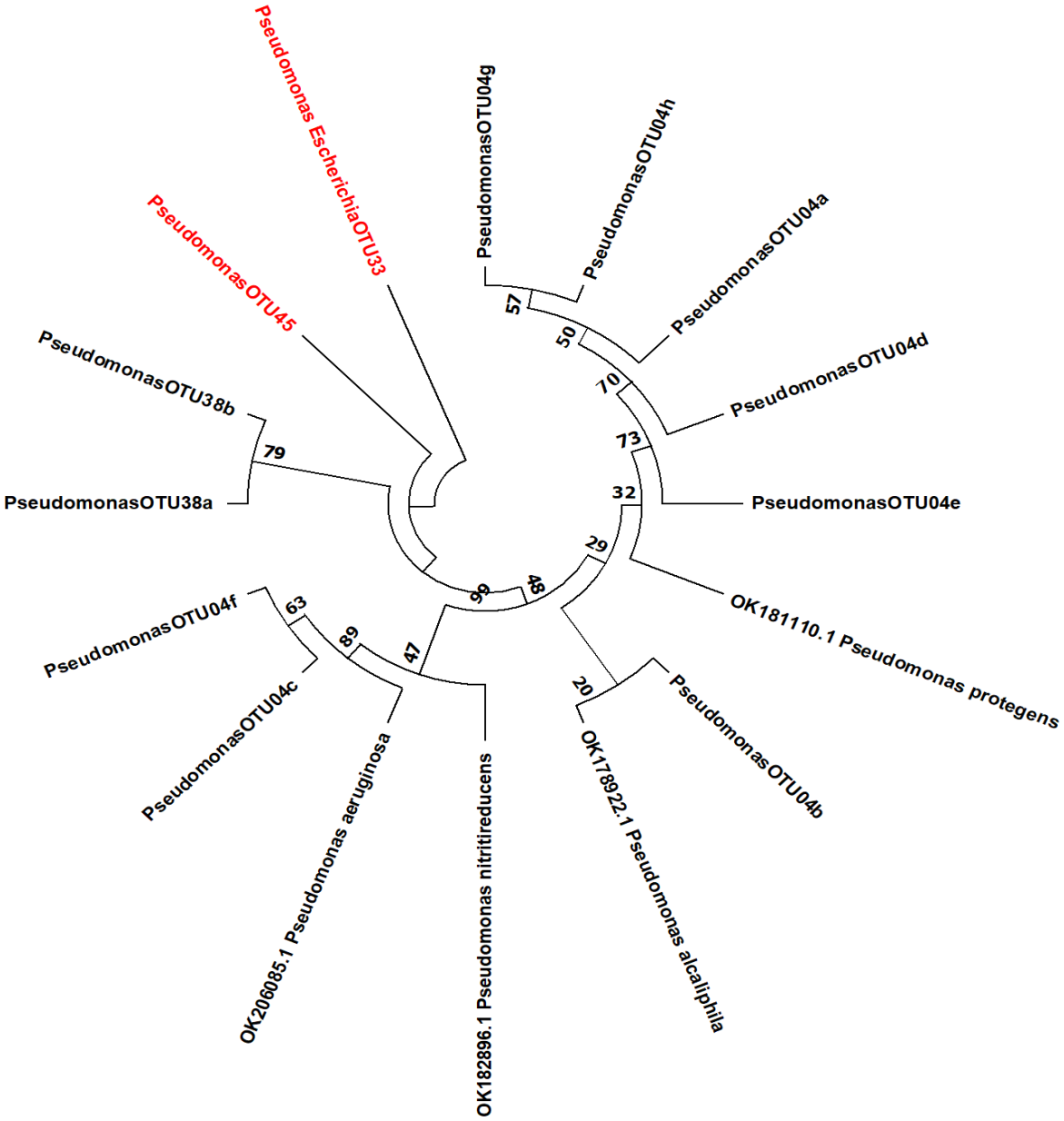

Figure 37. Phylogenetic tree of Pseudomonas OTUs

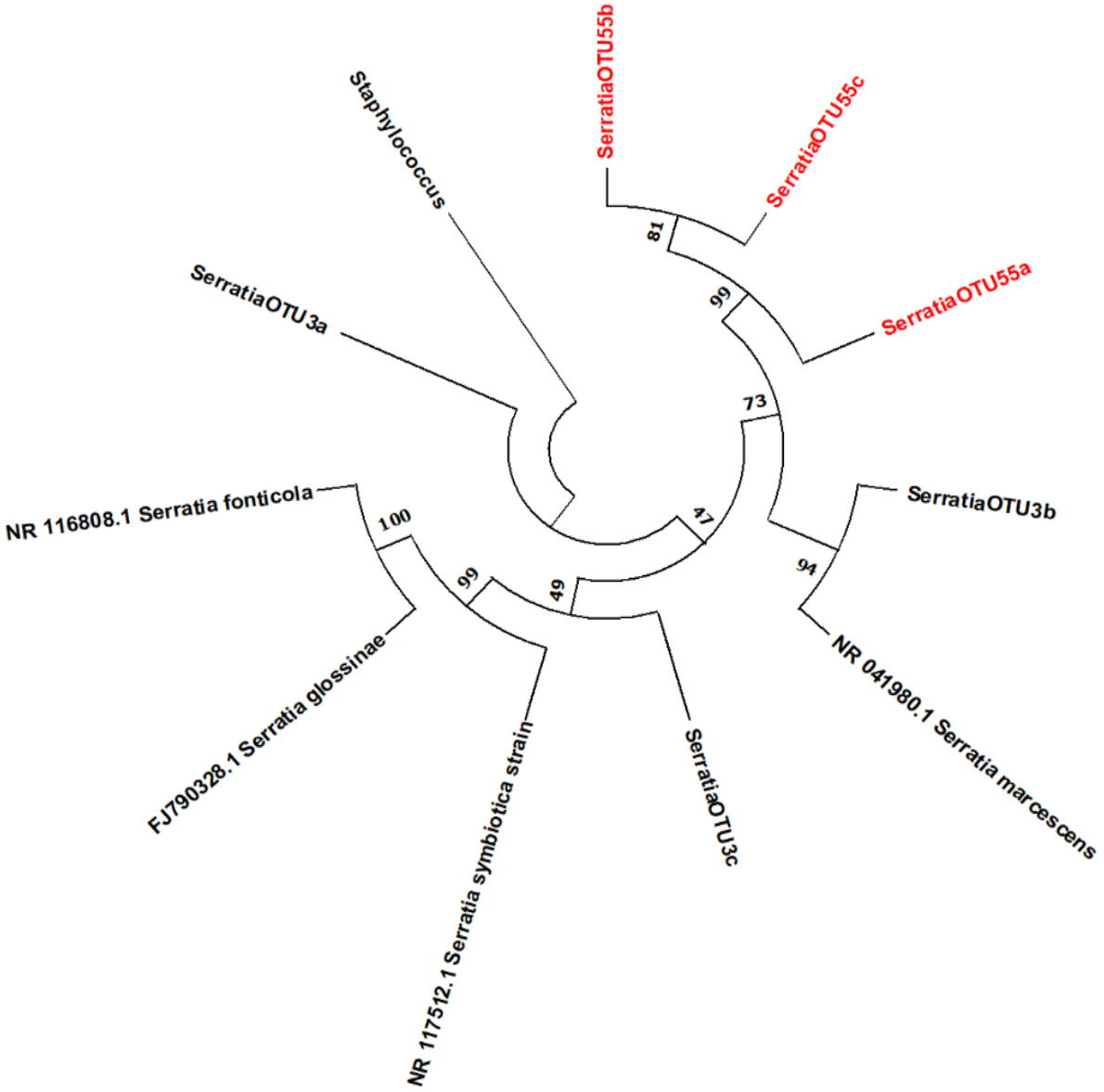

Figure 38 : Phylogenetic tree of *Serratia* OTUs

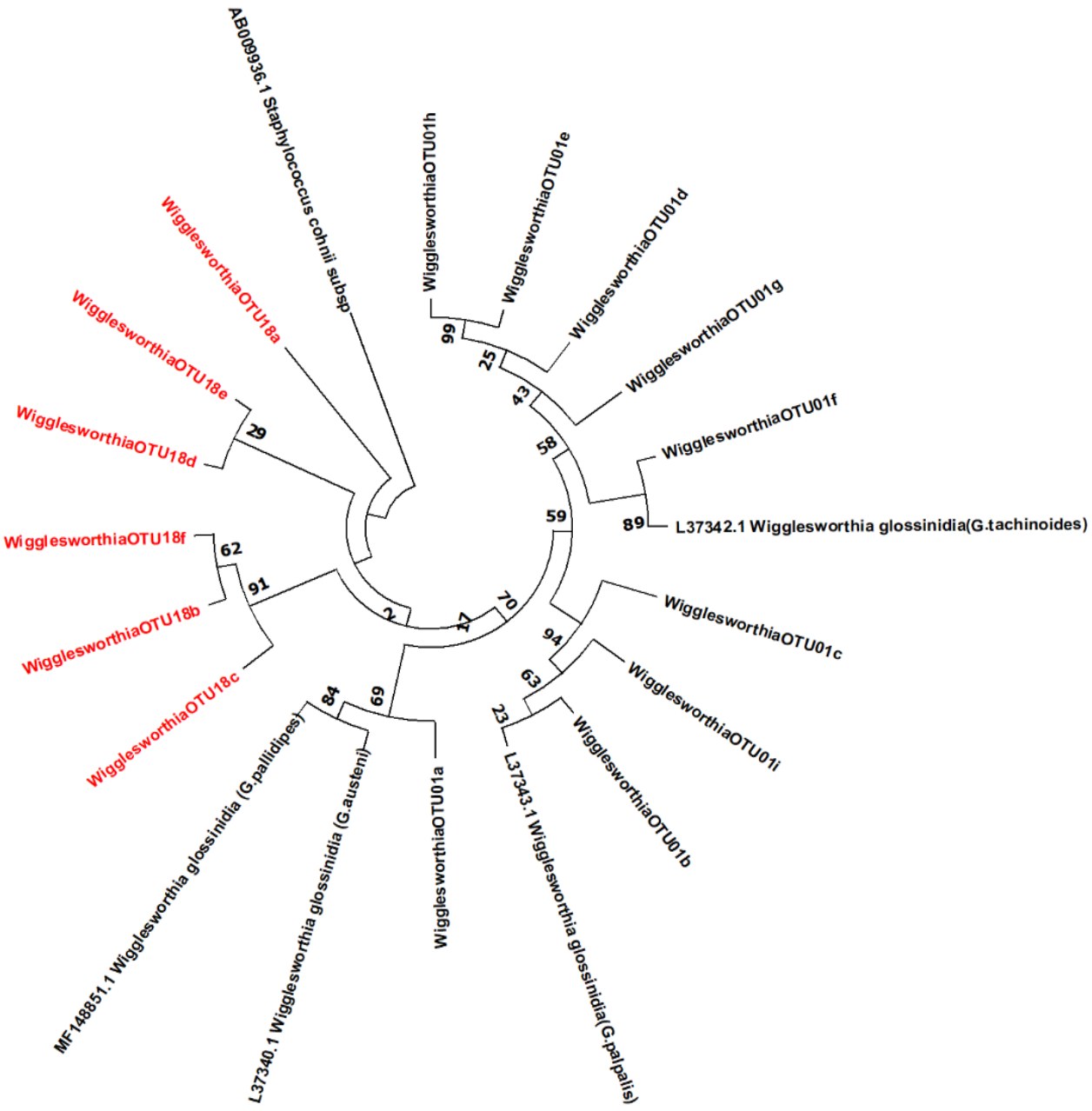

Figure 39 : Phylogenetic tree of *Serratia* OTUs

III.2. Discussion

The description of microbiome harboured by arthropod vectors presents increasing interest, owing to their role in modulating vector fitness or competence and their potential use in vector control. Although most studies on tsetse fly vector of both human and animal trypanosomiasis have mainly described the microbial diversity in tsetse fly guts, few have either looked at the microbiome in the whole fly or have established strong associations between the microbiome composition and the maturation of trypanosomes in the flies. In the present study, we conducted a high throughput sequencing of the V3-V4 region of the bacterial 16S rRNA gene in the tsetse fly *Glossina palpalis palpalis*, the main vector of human sleeping sickness in the forest area of Cameroon. We included few samples of three additional tsetse species in order to make a complete inventory of the microbial communities they harbour. Bacterial taxa associated with the infection of tsetse with trypanosomes, or the maturation of these later, were also investigated.

During two entomological surveys, 3215 tsetse flies were collected belonging to four tsetse species: *G. palpalis*, *G. pallicera*, *G. nigrofusca*, and *G. caliginea*, and a fifth species (with two individuals) that could not be identified morphologically using the key available. These results are in agreement with those reported in previous studies, indicating that ecological conditions of these biotopes remain favourable to the development of these taxa (Simo *et al*., 2008; Farikou *et al*., 2010; Grébaut *et al*., 2016). *G. palpalis palpalis*, which is the vector of sleeping sickness in Campo, was the predominant species captured, whatever the sampling period or the sampling point (93%). This result is consistent with previous studies in the same area (Simo *et al*., 2008; Grébaut *et al*., 2016). The predominance of this species is due to the fact it is more anthropophilic and persists in human-degraded areas compared to other species that are more zoophilic (Simo *et al*., 2008). The deforestation and creation of farmlands like the ongoing 70,000 hectares palm grove near the Campo natural game reserve or wood exploitations in Campo, and other projects like the autonomous deep sea port at Kribi (around 35 km from Campo) or the hydroelectric dam at Memvele increase the local population, and their settlement made the Campo environment more anthropized and thus, suitable for colonization by *G. palpalis palpalis* (Simo *et al*., 2014). It is important to note that records of the years 1985 (Eouzan *et al*., 1974) or 2001 (Mbida Mbida, Unpublished results) show *G. palpalis* relative abundance not exceeding 65%. Moreover, the present study has shown high apparent densities for this species in the two seasons, i.e., 3.87 and 2.59 flies/trap/day. These densities are 2 to 3 times greater than the ones observed in 2012 in the same area *(*Grébaut *et al*., 2016). This observation indicates the high potential risk of contact between human and

tsetse flies, and thus an increased disease transmission risk. Also, this result confirms that the deep ongoing forest degradation and implantation of agricultural-related activities are favourable to the development of the anthropophilic *G. palpalis palpalis*, which is unfortunately the responsible of sleeping sickness transmission in the area.

Molecular identification of trypanosomes revealed a high prevalence of the animal trypanosome species *T. congolense*, *T. brucei brucei*, *T. vivax* and *T. simiae,* and the presence of the human parasite, *T. brucei gambiense* in *G. palpalis palpalis* captured in Campo. These trypanosome species were already reported in tsetse captured in Campo by several authors (Simo *et al*., 2015; Grébaut *et al*., 2016; Tsagmo Ngoune *et al*., 2017; Ngambia *et al*., 2021), or in animals of the same area or from other trypanosomiases foci in Cameroon (Njiokou *et al*., 2004; Simo *et al*., 2006; Mamoudou *et al*., 2006; Tanenbe *et al*., 2010; Nimpaye *et al*., 2011; Tchouomene-labou et al., 2013). These results confirm the current circulation of trypanosomes in the Campo forest area of Cameroon. However, a significant difference was observed in trypanosome infection rates in flies between December 2018 and July 2019. Indeed, *T. congolense* infection rate was low in July compared to December, whereas *T. brucei* and *T. vivax* rates where ~5 times higher in July. These results suggest that trypanosome circulation is dynamic over the time and may be linked to vertebrate hosts present and their movements from villages to the deep bush. In fact, a study conducted in Campo on the dynamics of potential wild tsetse hosts shown that although some wild animal species occur in all biotopes in all seasons, other move across biotopes (from farmlands to forest near villages) according to the season (Massussi *et al*., 2009). Also, the fact that 22.6% of infected flies in December 2018 were carrying a mature infection with *T. congolense* and none from July 2019 was found with mature infection, suggest that these later flies were recently infected and didn't have enough time to develop their parasites before being captured, meaning that flies' infection in the nature may follow cycles of hosts availability in their biotopes. In our study, the occurrence of mature trypanosome infections was certainly underestimated, since we didn't dissect the fly salivary glands that are the localization of mature *T. brucei* infections. Among the four trypanosome species identified, *T. congolense* predominated. *T. congolense* is one of the trypanosome species widely distributed in forest areas like Campo, where it presents a high infectivity to domestic animals (Bengaly *et al*., 2002; Nimpaye *et al*., 2011; Simo *et al*., 2013), but also, it has been reported in wild animals species of the area (Herder *et al*., 2002; Njiokou *et al*., 2004). Concerning *T. simiae*, it was detected with a low infection rate in *G. palpalis palpalis*. This result corroborate the low prevalence of this parasite, previously reported in domestic and wild animals in Campo (Njiokou *et al*., 2004; Nimpaye *et al*., 2011). This parasite is a potential

constraint for pigs rearing implemented in the area due to its high pathogenicity for pigs (Moloo *et al*., 1992); the low observed prevalence may be indicative of the fact that infected animals die before having enough time for infecting the neighbouring tsetse flies. The mixed trypanosome infections identified in this study reflect the presence of such infections in vertebrate hosts of this area since during a blood meal, tsetse flies can ingest one or more trypanosomes species from an animal. These results are already reported in previous studies (Tchouomene-Labou *et al*., 2013; Simo *et al*., 2015; Grébaut *et al*., 2016).

Two blood meals out of 85 were identified in *G. palpalis palpalis* as originating from human, while the others (belonging to 4 profiles) being non identified; this result suggests that apart of humans, the tsetse flies feed on other vertebrates which in this case are wild animals not having their cytochrome b sequences in the Genbank database used for the identification.

T. brucei gambiense, responsible for human sleeping sickness, was found in the villages Mabiogo, Nazareth, Mvass and military surveillance posts on the border between Cameroon and Equatorial Guinea. These villages are those were sleeping sickness cases were detected these later years. In Mvass, the implementation of a new farmland where young palm trees are produced for the palm grove, with more than 1000 employees represents a great risk of disease transmission. Moreover, these villages bordered by mangrove with high tsetse densities are mainly located at the border between Cameroon and Equatorial Guinea, where human activities increase the risk of transborder transmission of the sleeping sickness. Also, many soldiers guarding the border have been found infected and treated for sleeping sickness; their regular movement from Campo to other areas in the northern Cameroon infested by tsetse vectors like *G. palpalis*, *G. fuscipes* and *G. tachinoides*, or to other countries like the Central African Republic for security issues clearly extend the risk of expanding the disease at a larger scale.

During the tsetse control initiated in Campo using tiny targets (January 2020 to January 2022), the mean daily catches of *G. palpalis palpalis* declined by 73.69% at the twelfth month of vector control. This reduction was low than the expected reduction 80% to 90% during the six first months as observed in DCR (Tirados *et al*., 2020), Chad (Rayaisse *et al*., 2020), Côte d'Ivoire (Berté *et al*., 2019) and Uganda (Tirados *et al*., 2015). The first factor that could explain the difference between the pattern of tsetse densities in Cameroon and in other countries during the vector control could be the great difference in tsetse habitats between countries. Indeed, Campo is a dense forest area with high land coverage with vegetation, swampy areas, marshes, and mangroves, representing probably one of the most challenging environment for vector control, as large areas infested with tsetse flies are not easily accessible. For example, although tiny targets were easily installed in most of the mangrove along the Ntem River, most of the

small tributaries coming from the mainland and crossing high surfaces of marshes and swamps that seem to be tsetse breeding hotspots were not accessible because of the vegetation congestion on Riverbeds. Streams forming those tributaries on the mainland, on which villagers carry out activities were previously shown to harbour high tsetse fly densities (Grébaut et al., 2016) and we hypothesize that despite the presence of tiny targets in accessible areas, there is a constant flies supply from downstream. Secondly, although more that 90% of tiny targets installed were still in good physical condition (upright position) after six months (that was quite good compared to the 50 % fallen expected (LSTM, 2016), many were already hidden with vegetation after 3 months or others became less effective because of particles film deposited on them and preventing flies to get in contact with the insecticide. This certainly favour slight increase in densities during the three months other months before monitoring, but this hypothesis couldn't be assessed as in many studies were monitoring is done every three months (Tirados *et al*., 2015, 2020; Mahamat *et al*., 2017).

Our study also showed a partial gradual recovery (almost 20%) of the tsetse population density, during the second year of vector control. This result could be explained by environmental mutations or changes that occurred in Campo biotope during the intervention with the implementation of a new palm grove (3 500 hectares set on 70 000 hectares expected), through a huge deforestation near the Campo natural game reserve. Indeed, the increase of the tsetse densities in the second year was in parallel with villagers complains of elephants invading the villages, destroying crops, and these elephants are known locally to move along with swarms of tsetse flies. As deforestation was done without preliminary orientation of animals to the natural game reserve side, the animals that were on the villages side probably came close to villages to avoid the noise, and unfortunately constitute permanent tsetse attractants.

In the control area as expected, the tsetse densities stayed high over the two years of monitoring, with a slight non-significant increase in the second year, also following elephants coming near villages since the starting of deforestation for the palm grove. This result confirm that tiny targets were the factors responsible of reducing the tsetse densities in the intervention area.

The tiny targets had a great impact in reducing trypanosomes circulation in tsetse flies captured in Campo during the vector control. Indeed, overall trypanosome infection rates decreased by 95% in the treated area during the first 18 months of vector control. The impact of vector control on trypanosome circulation was higher than what recently reported in Côte d'Ivoire (Kaba *et al*., 2021), where tsetse infection rates to trypanosomes were generally lower following the deployment of targets but not significantly, despite a great reduction in tsetse

densities. Furthermore, in this study, no *T. brucei gambiense* infections were detected amongst all flies captured during the two years of vector control; this result indicates that the circulation of the human parasite has regressed in this area. More importantly, no mature infections were detected in flies captured during the vector control, indicating that the infections detected in these flies were recent, and also that flies infected are likely to get in contact with tiny targets and die before maturing and transmitting their trypanosomes. However, the reduction in trypanosome infections rate was also observed in the control area. This finding indicates that the control area was not far enough from the treatment area and that same parasite populations circulate in the whole Campo focus through animal movements.

Using whole tsetse fly bodies, a total of 85 OTUs, belonging to 32 bacterial genera, were identified in 4 phyla. They were largely dominated by the phylum *Proteobacteria*, with a mean relative abundance of 95.04%. This observation is consistent with previous studies that showed the predominance of this phylum (~90%) in the guts of tsetse flies (Geiger et al., 2011; Jacob et al., 2017; Ngambia Freitas et al., 2021). Such predominance is due to the high relative abundance of the primary symbiont *Wigglesworthia* that represented 47.29% of the total microbiome and other taxa like *Serratia* (16.58%), *Pantoae_Klebsiella_Enterobacter_Kluyvera* (16.30%), and *Pseudomonas* (6.16%). This result is not surprising, since *Proteobacteria* species are known to easily adapt and develop in different biotopes. The high abundance of the primary mutualist symbiont of tsetse flies, *Wigglesworthia*, corroborates the result reported in previous studies (Yabu & Takayanagi, 1988). However, the relatively lower abundance of *Wigglesworthia* found in the present study (47% of the tsetse gut bacteriome) compared to previous studies (90–99%) is probably due to the fact that whole fly bodies were investigated here, rather than the midgut only. *Wigglesworthia* may be predominant in the midgut, whereas the other taxa may be more abundant in other tissues; this result suggest that the abundances of other bacteria taxa were largely underestimated in the previous studies, where midguts only were used for the analysis. Despite that *Wigglesworthia* was the predominant bacteria in most of the flies, some *Glossina palpalis palpalis* individuals were dominated by *Pantoae_Klebsiella_Enterobacter_Kluyvera* or *Serratia*, and some *G. tachinoides* were dominated by *Burkholderia*. Members of the genus *Burkholderia* are widespread in soil rhizospheres and plant surfaces, and some species are known to be associated with insects feeding on plants (Kikuchi *et al*., 2005, 2007; Compant *et al*., 2008). A previous study reported that tsetse flies may ingest bacteria present on the epidermis of a variety of vertebrates (Simo *et al*., 2008) or in plants' nectar when they feed (Colman *et al*., 2012; Solano *et al*., 2015). Although infection by *Burkholderia* is non-essential

for growth and reproduction of the mosquito (Boissière *et al*., 2012) for example, association studies revealed mutualistic relationships with insect, where the symbiont presence increases the insect fitness or protects the insect from entomopathogenic fungi (Santos *et al*., 2004; Kikuchi *et al*., 2007). On the other hand, *Pantoea* has been shown to cross-colonise several mosquito species and is readily transformed and cultured, and therefore, has been proposed for paratransgenic applications (Djadid *et al*., 2011). Further investigations on these predominant bacteria dynamics throughout the tsetse fly life cycle are required to better define the nature of the microbe–fly association. *Serratia* was detected in more than 90% of the flies in our study, with an overall abundance of 16.58%; this was not expected, since this bacterium has been previously reported in only around 50% of flies by Jacob *et al.* (2017) and with only 0.0012% abundance by Tsagmo-Ngoune *et al.* (2019). This observation strengthens the hypothesis that fly tissues other than those in the midgut are key localizations for bacteria development, and the potential importance of these bacteria deserves to be further investigated.

Bacterial taxa richness and evenness were different between tsetse fly species. The microbiota composition of *G. palpalis palpalis* and *G. caliginea* were significantly more diverse and evenly distributed compared to that of *G. pallicera* and *G. tachinoides*. This result may be due to the differences in the environmental conditions of these different tsetse species and in the food supply from which some of these microbes originate, as suggested previously (Geiger *et al*., 2013; Aksoy *et al*., 2014). Moreover, differences in the gut physiological conditions and/or the fly's innate immune system may impair the proliferation of some bacterial taxa in different fly species, modulating the composition of microbial communities, as suggested in studies on mosquitoes (Muturi *et al*., 2017). This observation is strengthened by the significant difference detected in the alpha and beta diversities when comparing non-teneral and teneral *G. palpalis palpalis*, indicating that blood meals have a significant impact on the tsetse microbiome. Indeed, an increase in the relative abundance of *Pantoae_Klebsiella_Enterobacter_Kluyvera*, *Serratia*, *Pseudomonas*, *Staphyloccocus*, *Methylophilus*, and other bacteria from teneral to non-teneral flies was concomitant with a decrease in *Wigglesworthia* from 70% in teneral flies, to 37% in non-teneral flies. A similar result was observed in blood-fed ticks compared to unfed ticks (Heise *et al*., 2010). However, more work is needed to obtain a complete and accurate picture of the bacteria associated with blood meals of the tsetse fly and to understand how and why these bacteria establish in their hosts.

Although no significant difference was observed when comparing bacteria richness and evenness between flies harbouring trypanosomes in their midguts and non-infected flies, the

abundances of some bacteria taxa were nevertheless different in the two groups. This result is consistent with previous results obtained by Jacob *et al*. (2017) and Tsagmo-Ngoune et al. (2019) on the same tsetse species in Cameroon. However, alpha and beta diversities of the tsetse microbiome were significantly different between flies harbouring mature trypanosome infections and those with only non-mature infection in the midgut. Indeed, a significant drop was observed in the alpha diversity of flies with mature trypanosome infection, and this observation was strengthened by hierarchical clustering of these flies, at least at low levels, distinguishing them from those with non-mature trypanosome infections. Differential abundance testing showed some bacteria phylotypes from the genera *Dechloromonas*, *Ralstonia*, *Serratia*, *Pseudomonas*, *Enteroccocus*, *Wigglesworthia*, *Methylophilus*, *Escherichia*, *Enhydrobacter*, and *Staphyloccocus* associated with the infection status of flies. The roles of these bacteria genera in tsetse fly biology remain unknown and poorly documented in other insects. *Enterobacter*, *Escherichia coli*, *Serratia marcescens*, and *Enterococcus* spp. are able to produce toxic molecules with potential antiparasitic activity (such as prodigiosin (Coburn & Gilmore, 2003)) that were shown to be toxic to *Plasmodium falciparum* and to *Trypanosoma cruzi* (Azambuja *et al*., 2004). However, *Serratia odorifera* was shown to enhance the susceptibility of *Aedes aegypti* to the chikungunya virus (Apte-Deshpande *et al*., 2014), as well as its susceptibility to the dengue-2 virus (Apte-Deshpande *et al*., 2012). In addition, the genus *Bacillus* spp is thought to be essential for *Culex pipiens* reproduction (Fouda *et al*., 2001), and some species were shown to play a role in the digestion of polysaccharides and aromatic compounds such as chitin and lignocellulose in termites (König, 2006). *Pseudomonas aeruginosa* are known to play an important role in mosquito *Culex quinquefasciatus* and *Culex tarsalis* larvae adaptation to hypereutrophic aquatic habitats by providing phosphorus enhancing growth rates (Peck & Walton, 2006). Therefore, based on the importance of these bacteria taxa in other insects, further investigations into their potential roles in tsetse fly biology, or their interaction with the trypanosomes that these flies transmit, are required. Moreover, the identification of *Wigglesworthia* OTU 18 and *Serratia* OTU 55 associated with the tsetse fly infection with trypanosomes, rather than the predominant OTUs (OTU 01 and OTU 03, respectively, for the two genera), further strengthen the opinion that tsetse vector competence might be linked to given bacterial genotypes or their abundance, as previously suggested by Geiger *et al*. (2007), rather than simply the presence/absence of the bacteria taxa.

The endosymbionts *Sodalis glossinidius* and *Wolbachia*, commonly reported in tsetse flies (Doudoumis *et al*., 2012; Aksoy *et al*., 2014; Jacob *et al*., 2017), could not be described in

this study, since they were eliminated by the abundance threshold of 0.01% set for the analyses. *Sodalis* is known to be involved in modulating the ability of the tsetse fly to acquire trypanosomes (Farikou *et al*., 2010; Hamidou *et al*., 2014) and *Wolbachia*, to induce a variety of reproductive phenotypes, such as cytoplasmic incompatibility, parthenogenesis, and feminization, into the host population(Stouthamer *et al*., 1999; Blagrove *et al*., 2012). As these symbionts exhibit a wide tissue tropism and can be found intra or extra-cellularly in various tissues, including the midgut, fat body, milk gland, salivary glands and hemocoel (Chen *et al*., 1999; Balmand *et al*., 2013), it is probable that they have a generally low relative abundance, which is a constraint to their identification and thus, a limitation to the NGS method used.

Bacteria belonging to the phylum *Proteobacteria* were still predominant in the samples analysed throughout the vector control, with a mean relative abundance of 96.69%, the primary symbiont *Wigglesworthia* representing 71,76% of the total microbiome. Although the microbiome composition at the phylum level was almost similar to that of the samples before vector control, the few variation found was mainly due to differences in relative abundances of some bacterial taxa present.The main variation observed in the tsetse bacteria composition between sampling periods during the vector control was the overall increase in bacterial alpha diversity between six and twelve months of control, along with a decrease of the relative abundance of *Wigglesworthia* from 82.21% to 62.06%. The relative abundance of many bacteria taxa increased significantly, especially *Pelomonas* (4.64% to 8.36%) *Klebsiella* (0.56% to 3.18%) and *Curvibacter* (0.64 % to 4.66%) and many others. Analyses of the beta diversity showed significant difference between tsetse flies collected before and during the vector control; the number of OTUs identified increased from 88 in flies before the vector control to 96 and 98 in flies collected twelve and heighten months later respectively. These results suggest a change in the tsetse fly population itself over the vector control, i.e., most of the flies caught during the vector control are not likely to belong to the original population. This suggestion is reinforced by the perfect clustering of the flies from each capture period, and by the fact that most of the genera differentially abundant are unique to sample sets. Such increase in bacterial taxa richness was recently reported by Juma *et al*. (2020) after exposition of *Aedes albopictus* and *Culex pipiens* to malathion and permethrin. Adaptive microbes may facilitate the ability of hosts to explore the fitness landscape to match local environmental stressors (Henry *et al*., 2021); like for instance, microbes with large effective population sizes or rapid generation time may evolve novel functions faster (Koonin & Wolf, 2012; Lakhani *et al*., 2019), and provide their hosts with adaptive abilities in a changing local environment. In the studied tsetse flies' population, we didn't establish any clear link between this increase in microbiome

diversity and particular bacteria taxa that could be involved in flies' ability to escape insecticide pressure. A more probable explanation of microbiome richness increase is that tsetse flies with different microbiome composition are easily reinvading the surface area covered by vector control from surrounding areas like Campo national game reserve that borders the surface under vector control. This hypothesis is strengthened by the greater bacterial diversity observed in males compared to females, because male flies disperse more than females as shown by tsetse population genetics studies (Koné et al., 2011; Tito et al., 2011). Nonetheless, *Curvibacter* and *Acinetobacter* showed a regular significant increase in relative abundance over the 18 months of vector control and deserve further investigations to determine their potential role if any, in maintaining the tsetse population under control.

Some bacterial operational taxonomic units or phylotypes were found associated with fly's infection with trypanosomes (OTU 18, OTU 45, OUT 55, etc …). Phylogenetic analyses showed that for each bacterial genus, the OTUs associated to trypanosome infections were closely related to each other, and clearly separated from the other OTUs of the genus. As the analyses could allow to assign OTUs up to the genus level, the different OTUs of a genus could either correspond to species, or strains of the same species; specific analyses like qPCR with specific primers are required for confirmation. Our results corroborate previous research by Geiger *et al*. (2007) showing the presence of several genetically distinct genotypes of *Sodalis Glossinidius*, in *G. palpalis gambiensis*, with some specific associated to trypanosome infections. These results strengthen the hypothesis that tsetse vector competence might be linked to a given genotype of bacteria rather than the simple presence or abundance of the whole taxon.

CONCLUSION AND PERSPECTIVES

The description of the microbiome hosted by arthropod vectors is of growing interest because of its potential use in vector control. Our study aimed at making a complete inventory of the bacterial communities hosted by the flies tested by high-throughput sequencing of the V3-V4 region of the 16S rRNA bacteria gene, to identify the taxa potentially associated with the presence and/or maturation of trypanosomes and the potential flies' fitness during vector control with "Tiny Targets".

The results of this study have provided an updated spatial distribution of the tsetse fly species in the Campo sleeping sickness and animal trypanosomoses focus of southern Cameroon. We showed high level of trypanosome circulation in Campo, including the presence of the human parasite, *Trypanosoma Brucei gambiense*, despite the efforts made by WHO and the national control programme in cleaning the human reservoir of the parasite, therefore, showing that trypanosomiases remain a serious threat in Campo. However, this study has demonstrated the positive impact of the deltamethrin impregnated screens called tiny targets on reducing the tsetse vector population density in Campo that was associated to a good reduction of human and animal trypanosomes circulation. The impact of this vector control tool is hindered by a major local development agricultural project, and a more integrated action involving these actors is required to mitigate the effects of the plantation in restoring the tsetse population density. Tiny targets have proven a good additional tool that can help in achieving the elimination of sleeping sickness in Cameroon. They were easy to set-up, with the engagement of local trained people and therefore can further be transferred to them to ensure sustainability. The impact of tiny targets may be improved in Campo by increasing the targets coverage through accessing difficult areas that are considered hotspots of tsetse breeding, and by maintaining the targets deployed every 3 months through clearing around and changing the ones recovered with particles.

The present study also provides some updates on the composition and diversity of the tsetse fly bacterial communities in Cameroon. Using the whole fly body, we revealed a higher bacterial diversity than previously observed in the midgut only, which indicates that various localizations other than the midguts should be considered in further investigations of the tsetse fly microbiome. Moreover, the significant difference in microbiome diversity between flies harbouring mature and non-mature trypanosome infections suggests either a change in microbiome diversity and composition for trypanosome infection maturation, or that trypanosome maturation is possible only with the particular abundances of some microbial taxa or strains. Therefore, the strong association between some identified bacteria genera and

trypanosome infection status deserves additional investigations for the development of novel tools that can help to control disease transmission, by blocking the trypanosome development in tsetse flies.

Our results also showed an increase of bacteria diversity and composition during the vector control which might be in response of the tiny target implementation and therefore contributing to tsetse flies' fitness. This effect was certainly poorly estimated, as it was observed in a short term, so further studies are needed to better understand this variation in the microbiome during the vector control. However, these initial findings lay the groundwork for future research that will elucidate the role of the tsetse microbiota in conferring tsetse fitness.

In view of the results obtained, a certain amount of information needs to be provided in order to better understand the role of the microbiome in the biology of the tsetse fly.

- Carry out metataxogenomic analyses of the different parts of the tsetse fly to better understand the distribution of bacteria in different tsetse fly tissues.
- Use specific tools like qPCR with specific primers to confirm the species/strains of bacteria that were found associated with trypanosome infections and perform bioassays to determine their mechanisms of action.
- Study the variation of bacteria diversity and composition of tsetse during 5 to 10 years of vector control to better understand their role in fly's fitness.
- Select and study the best candidate bacteria to understand the mechanism of action of interaction with trypanosome in tsetse flies.

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APPENDIXES

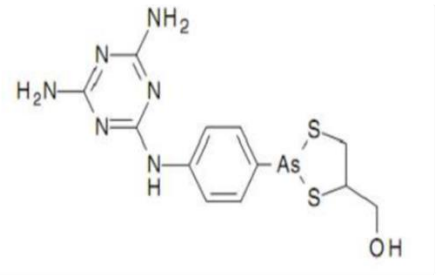
Appendix 1: List of published articles from the thesis

Bouaka Tsakeng, C.U., Melachio Tanekou, T.T., Feudjio Soffack, S., Tirados, I., Noutchih, C., Njiokou, F., Bigoga, J.D., Wondji, C.S. (2022) Assessing the Tsetse Fly Microbiome Composition and the Potential Association of Some Bacteria Taxa with Trypanosome Establishment. *Microorganisms*, 10, 1141. https://doi.org/10.3390/microorganisms 10061141

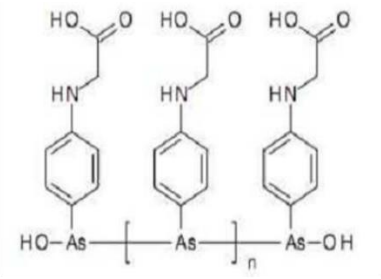
Melachio Tanekou, T.T., **Bouaka Tsakeng, C.U.**, Tirados, I., Torr, S.J., Njiokou, F., Acho, A. Wondji, S. (2022) Environmental mutations in the Campo focus challenge elimination of sleeping sickness transmission in Cameroon. *Medical and Veterinary Entomology*,1–9. https://doi.org/10.1111/mve.1257

Melachio Tanekou, T. T., **Bouaka Tsakeng, C. U.,** Tirados, I., Acho, A., Bigoga, J., Wondji, C. S., & Njiokou, F. (2023). Impact of a small-scale tsetse fly control operation with deltamethrin impregnated "Tiny Targets" on tsetse density and trypanosomes' circulation in the Campo sleeping sickness focus of South Cameroon. PLoS Neglected Tropical Diseases, 17(11), 1–14. https://doi.org/10.1371/journal.pntd.0011802

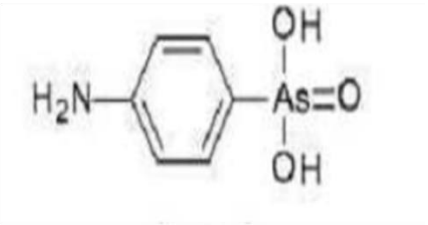
Appendix 2: Chemical structure of the different trypanocides



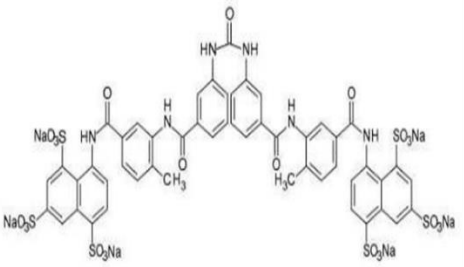
Melarsoprol



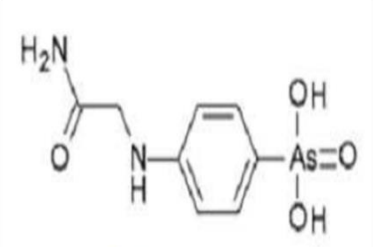
Arsenophenylglycine



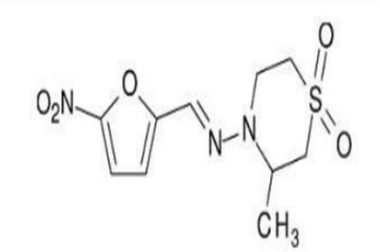
Atoxyl



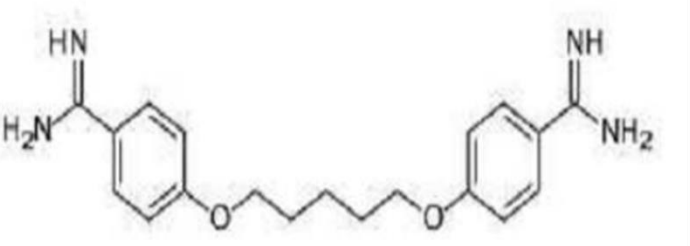
Suramine



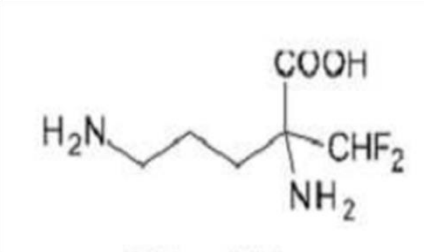
Tryparsamide



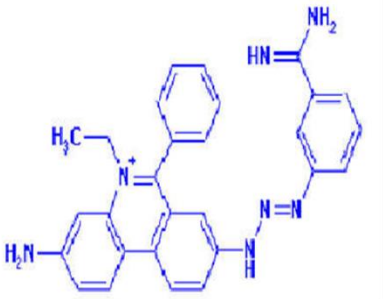
Nifurtimox



Pentamidine

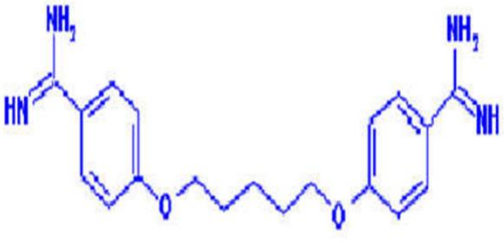


Eflornithine



Isométamidium

Homidium



Acéturate

Appendix 3: Tsetse DNA samples for the first bacteria sequencing

Samples	Villages	Tsetse_Sp	Sex	Teneral	Midgut_Inf	Mature_Inf	Tryp_Sp
T18n2	Mabiogo	Gpp	F	NT	MGInf	Hinf	TC/TBB
T18n4	Mabiogo	Gpp	F	NT	MGInf	Hinf	TC
T18n5	Mabiogo	Gpp	F	NT	MGInf	Hinf	TC
T19n14	Mabiogo	Gpp	M	NT	MGInf	Hinf	TC
T19n16	Mabiogo	Gpp	M	NT	MGInf	Hinf	TC
T19n32	Mabiogo	Gpp	F	NT	MGInf	Hinf	TC
T19n4	Mabiogo	Gpp	F	NT	MGInf	Hinf	TC/TBG/TV
T20n15	Mabiogo	Gpp	M	NT	MGInf	Hinf	TC
T20n17	Mabiogo	Gpp	F	NT	MGInf	Hinf	TC
T34n10	Nazareth	Gpp	F	NT	MGInf	Hinf	TC
T36n11	CampoB	Gpp	F	NT	MGInf	Hinf	TC
T36n13	CampoB	Gpp	M	NT	MGInf	Hinf	TC
T37n1	CampoB	Gpp	F	NT	MGInf	Hinf	TC/TS
T39n12	CampoB	Gpp	M	NT	MGInf	Hinf	TC
T40n2	CampoB	Gpp	F	NT	MGInf	Hinf	TC
T45n3	Ipono	Gpp	M	NT	MGInf	Hinf	TC
T45n7	Ipono	Gpp	F	NT	MGInf	Hinf	TC/TB
T47n1	Ipono	Gpp	F	NT	MGInf	Hinf	TC
T47n12	Ipono	Gpp	F	NT	MGInf	Hinf	TC
T47n13	Ipono	Gpp	F	NT	MGInf	Hinf	TC
T47n6	Ipono	Gpp	M	NT	MGInf	Hinf	TC/TB
T6n117	Afan_E	Gpp	F	NT	MGInf	Hinf	TC
T64n53	Itonde_AE	Gpp	M	NT	MGInf	Hinf	TC/TS
T72n2	Campo_Town	Gpp	F	NT	MGInf	Hinf	TC
T74n9	Ipono	Gpp	F	NT	MGInf	Hinf	TC
T75n6	Ipono	Gpp	M	NT	MGInf	Hinf	TC
T75n7	Ipono	Gpp	F	NT	MGInf	Hinf	TC
T86n7	Ntem_Bank	Gpp	F	NT	MGInf	Hinf	TC
T91n20	Ntem_Bank	Gpp	F	NT	MGInf	Hinf	TC
T98n26	Ntem_Bank	Gpp	M	NT	MGInf	Hinf	TC
T98n37	Ntem_Bank	Gpp	M	NT	MGInf	Hinf	TC
T98n44	Ntem_Bank	Gpp	M	NT	MGInf	Hinf	TC
T100n10	Ntem_Bank	Gpp	M	NT	MGInf	HNinf	TV
T19n23	Mabiogo	Gpp	M	NT	MGInf	HNinf	TC
T19n27	Mabiogo	Gpp	F	NT	MGInf	HNinf	TC
T26n14	Mabiogo	Gpp	F	NT	MGInf	HNinf	TC
T26n15	Mabiogo	Gpp	F	NT	MGInf	HNinf	TC
T26n17	Mabiogo	Gpp	M	NT	MGInf	HNinf	TC
T26n23	Mabiogo	Gpp	F	NT	MGInf	HNinf	TC
T26n4	Mabiogo	Gpp	F	NT	MGInf	HNinf	TC
T36n2	CampoB	Gpp	F	NT	MGInf	HNinf	TC
T36n4	CampoB	Gpp	F	NT	MGInf	HNinf	TC
T36n9	CampoB	Gpp	F	NT	MGInf	HNinf	TC

T38n13	CampoB	Gpp	M	NT	MGInf	HNinf	TC
T46n11	Ipono	Gpp	M	NT	MGInf	HNinf	TBB
T47n17	Ipono	Gpp	F	NT	MGInf	HNinf	TC
T48n4	Mvass	Gpp	F	NT	MGInf	HNinf	TC
T49n14	Mvass	Gpp	M	NT	MGInf	HNinf	TC
T49n4	Mvass	Gpp	F	NT	MGInf	HNinf	TC
T50n9	Mvass	Gpp	F	NT	MGInf	HNinf	TC
T51n15	Mvass	Gpp	F	NT	MGInf	HNinf	TC
T52n22	Mvass	Gpp	F	NT	MGInf	HNinf	TC
T52n40	Mvass	Gpp	F	NT	MGInf	HNinf	TC
T52n57	Mvass	Gpp	M	NT	MGInf	HNinf	TC
T52n58	Mvass	Gpp	M	NT	MGInf	HNinf	TC
T53n2	Mvass	Gpp	F	NT	MGInf	HNinf	TC
T54n3	Mvass	Gpp	F	NT	MGInf	HNinf	TBB
T70n27	Doumassi	Gpp	F	NT	MGInf	HNinf	TC
T70n28	Doumassi	Gpp	F	NT	MGInf	HNinf	TC
T70n5	Doumassi	Gpp	F	NT	MGInf	HNinf	TC
T74n1	Ipono	Gpp	F	NT	MGInf	HNinf	TC
T74n11	Ipono	Gpp	F	NT	MGInf	HNinf	TC
T74n12	Ipono	Gpp	F	NT	MGInf	HNinf	TC
T74n14	Ipono	Gpp	F	NT	MGInf	HNinf	TC
T74n18	Ipono	Gpp	M	NT	MGInf	HNinf	TC
T86n1	Ntem_Bank	Gpp	F	NT	MGInf	HNinf	TC
T86n2	Ntem_Bank	Gpp	F	NT	MGInf	HNinf	TC
T89n3	Ntem_Bank	Gpp	F	NT	MGInf	HNinf	TC
T89n6	Ntem_Bank	Gpp	M	NT	MGInf	HNinf	TC
T90n4	Ntem_Bank	Gpp	M	NT	MGInf	HNinf	TC
T91n1	Ntem_Bank	Gpp	F	NT	MGInf	HNinf	TC
T21n3	Mabiogo	Gpal	F	NT	MGInf	HNinf	TC
T26n8	Mabiogo	Gpal	M	NT	MGInf	HNinf	TC
T29n17	Mabiogo	Gpal	M	NT	MGInf	HNinf	TC
T34n3	Nazareth	Gpal	F	NT	MGInf	HNinf	TC
T34n37	Nazareth	Gpal	M	NT	MGInf	HNinf	TC
T38n28	CampoB	Gcal	M	NT	MGInf	HNinf	TC
T70n24	Doumassi	Gcal	M	NT	MGInf	HNinf	TC
T70n25	Doumassi	Gcal	M	NT	MGInf	HNinf	TC
T100n12	Ntem_Bank	Gpp	M	NT	MGNInf	NA	Ninf
T100n46	Ntem_Bank	Gcal	F	NT	MGNInf	NA	Ninf
T100n47	Ntem_Bank	Gcal	F	NT	MGNInf	NA	Ninf
T11n4	Assok	Gpp	F	NT	MGNInf	NA	Ninf
T11n5	Assok	Gpp	F	NT	MGNInf	NA	Ninf
T138n13	Ipono	Gcal	F	NT	MGNInf	NA	Ninf
T17n4	Mabiogo	Gpal	M	NT	MGNInf	NA	Ninf
T18n1	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T18n31	Mabiogo	Gpal	F	NT	MGNInf	NA	Ninf
T19n10	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf

T19n12	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n15	Mabiogo	Gpp	M	NT	MGNInf	NA	Ninf
T19n2	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n20	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n22	Mabiogo	Gpp	M	NT	MGNInf	NA	Ninf
T19n24	Mabiogo	Gpp	M	NT	MGNInf	NA	Ninf
T19n25	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n26	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n28	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n29	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n30	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n34	Mabiogo	Gpp	M	NT	MGNInf	NA	Ninf
T19n35	Mabiogo	Gpp	M	NT	MGNInf	NA	Ninf
T19n38	Mabiogo	Gpp	M	NT	MGNInf	NA	Ninf
T19n5	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n6	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n7	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T19n9	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T20n10	Mabiogo	Gpp	M	NT	MGNInf	NA	Ninf
T20n13	Mabiogo	Gpal	F	NT	MGNInf	NA	Ninf
T22n4	Mabiogo	Gpp	F	NT	MGNInf	NA	Ninf
T26n20	Mabiogo	Gpal	F	NT	MGNInf	NA	Ninf
T26n25	Mabiogo	Gpal	F	NT	MGNInf	NA	Ninf
T26n27	Mabiogo	Gpal	F	NT	MGNInf	NA	Ninf
T26n29	Mabiogo	Gcal	F	NT	MGNInf	NA	Ninf
T26n6	Mabiogo	Gpal	F	NT	MGNInf	NA	Ninf
T26n7	Mabiogo	Gpal	M	NT	MGNInf	NA	Ninf
T26n9	Mabiogo	Gpal	F	NT	MGNInf	NA	Ninf
T27n10	Mabiogo	Gpp	M	NT	MGNInf	NA	Ninf
T27n12	Mabiogo	Gpp	M	NT	MGNInf	NA	Ninf
T28n5	Mabiogo	Gpal	F	NT	MGNInf	NA	Ninf
T29n16	Mabiogo	Gpal	M	NT	MGNInf	NA	Ninf
T30n14	Nazareth	Gpp	F	NT	MGNInf	NA	Ninf
T30n8	Nazareth	Gpp	F	NT	MGNInf	NA	Ninf
T31n1	Nazareth	Gpp	F	NT	MGNInf	NA	Ninf
T31n29	Nazareth	Gpal	M	NT	MGNInf	NA	Ninf
T31n30	Nazareth	Gpal	M	NT	MGNInf	NA	Ninf
T34n18	Nazareth	Gpp	F	NT	MGNInf	NA	Ninf
T34n20	Nazareth	Gpp	F	NT	MGNInf	NA	Ninf
T34n21	Nazareth	Gpp	F	NT	MGNInf	NA	Ninf
T34n38	Nazareth	Gpal	M	NT	MGNInf	NA	Ninf
T36n15	CampoB	Gpp	F	NT	MGNInf	NA	Ninf
T36n21	CampoB	Gcal	M	NT	MGNInf	NA	Ninf
T38n11	CampoB	Gcal	F	NT	MGNInf	NA	Ninf
T38n12	CampoB	Gcal	F	NT	MGNInf	NA	Ninf
T38n26	CampoB	Gpp	M	NT	MGNInf	NA	Ninf

T38n51	CampoB	Gcal	M	NT	MGNInf	NA	Ninf
T38n52	CampoB	Gpal	M	NT	MGNInf	NA	Ninf
T41n1	CampoB	Gpp	F	NT	MGNInf	NA	Ninf
T46n12	Ipono	Gcal	M	NT	MGNInf	NA	Ninf
T47n22	Ipono	Gcal	M	NT	MGNInf	NA	Ninf
T50n10	Mvass	Gpp	F	NT	MGNInf	NA	Ninf
T50n13	Mvass	Gpp	M	NT	MGNInf	NA	Ninf
T50n16	Mvass	Gcal	M	NT	MGNInf	NA	Ninf
T70n31	Doumassi	Gcal	F	NT	MGNInf	NA	Ninf
T70n32	Doumassi	Gcal	F	NT	MGNInf	NA	Ninf
T70n33	Doumassi	Gcal	F	NT	MGNInf	NA	Ninf
T74n15	Ipono	Gpp	F	NT	MGNInf	NA	Ninf
T76n2	Bokome_C	Gcal	M	NT	MGNInf	NA	Ninf
T78n3	Bokome_C	Gcal	F	NT	MGNInf	NA	Ninf
T98n36	Ntem_Bank	Gpp	M	NT	MGNInf	NA	Ninf
M10	RaoSB	Gtach	F	NT	MGInf	NA	TV/TB
M11	RaoSB	Gtach	F	NT	MGInf	NA	TC
M12	RaoSB	Gtach	F	NT	MGInf	NA	TB/TS
M15	RaoSB	Gtach	F	NT	MGInf	NA	TV
M4	RaoSB	Gtach	M	NT	MGInf	NA	TV
T100n11	Ntem_Bank	Gpp	M	NT	MGNInf	NA	Ninf
b3	Ntem_Bank	Gcal	F	NT	MGNInf	NA	Ninf
M1	RaoSB	Gtach	F	NT	MGNInf	NA	Ninf
M13	RaoSB	Gtach	F	NT	MGNInf	NA	Ninf
M16	CampSB	Gtach	M	NT	MGNInf	NA	Ninf
M17	CampSB	Gtach	M	NT	MGNInf	NA	Ninf
M18	CampSB	Gtach	F	NT	MGNInf	NA	Ninf
M19	RaoSB	Gtach	F	NT	MGNInf	NA	Ninf
M2	CampSB	Gtach	F	NT	MGNInf	NA	Ninf
M20	CampSB	Gtach	M	NT	MGNInf	NA	Ninf
M3	RaoSB	Gtach	F	NT	MGNInf	NA	Ninf
M5	RaoSB	Gtach	F	NT	MGNInf	NA	Ninf
M6	RaoSB	Gtach	M	NT	MGNInf	NA	Ninf
M7	RaoSB	Gtach	F	NT	MGNInf	NA	Ninf
M14	RaoSB	Gtach	M	T	NA	NA	NA
M8	RaoSB	Gtach	M	T	NA	NA	NA
M9	RaoSB	Gtach	M	T	NA	NA	NA
T100n2	Ntem_Bank	Gpp	F	T	NA	NA	NA
T140n10	Ipono	Gpp	F	T	NA	NA	NA
T15n2	Nkouandjap	Gpp	F	T	NA	NA	NA
T157n14	Ntem_Bank	Gpp	F	T	NA	NA	NA
T160n20	Ntem_Bank	Gpp	F	T	NA	NA	NA
T162n14	Ntem_Bank	Gpp	F	T	NA	NA	NA
T162n15	Ntem_Bank	Gpp	F	T	NA	NA	NA
T18n19	Mabiogo	Gpp	F	T	NA	NA	NA
T18n23	Mabiogo	Gpp	M	T	NA	NA	NA

T29n15	Mabiogo	Gpp	F	T	NA	NA	NA
T31n16	Nazareth	Gpp	M	T	NA	NA	NA
T52n43	Mvass	Gpp	F	T	NA	NA	Ninf
T6n215	Afan_E	Gpp	F	T	NA	NA	Ninf
T6n217	Afan_E	Gpp	F	T	NA	NA	Ninf
T6n219	Afan_E	Gpp	F	T	NA	NA	Ninf
T65n28	Mbala	Gpp	F	T	NA	NA	Ninf
T65n29	Mbala	Gpp	F	T	NA	NA	Ninf
T8n30	Akak	Gpp	F	T	NA	NA	NA
T8n31	Akak	Gpp	F	T	NA	NA	NA
T90n6	Ntem_Bank	Gpp	F	T	NA	NA	NA

F: female; M: male; MGinf: midgut infected; MGNinf: midgut non infected; T: teneral; NT: non teneral; NA: not available; TC: *T. congolense*; TB: *T. brucei* s.l; TV: *T. vivax*

Appendix 3: Tsetse DNA samples for second bacterial sequencing

Samples	Villages	Sex	Midgut_Inf	Tryp_Sp	Tissues	Date
T38n44	Campo beach	F	MGNInf	Ninf	WH	BF
T38n46	Campo beach	F	MGNInf	Ninf	WH	BF
T41n4	Campo beach	F	MGInf	TB/TS	WH	BF
T48n5	Mvass	F	MGNInf	Ninf	WH	BF
T50n13	Mvass	M	MGNInf	Ninf	WH	BF
T51n5	Mvass	F	MGNInf	Ninf	WH	BF
T52n1	Mvass	F	MGNInf	Ninf	WH	BF
T52n14	Mvass	M	MGNInf	Ninf	WH	BF
T52n21	Mvass	F	MGNInf	Ninf	WH	BF
T88n1	Ntem_Bank	F	MGNInf	Ninf	WH	BF
T88n2	Ntem_Bank	F	MGNInf	Ninf	WH	BF
T89n1	Ntem_Bank	F	MGNInf	Ninf	WH	BF
T98n11	Ntem_Bank	F	MGNInf	Ninf	WH	BF
T111Cn3	Mabiogo	F	MGNInf	Ninf	WH	Sixmonths
T113Cn1	Mabiogo	F	MGNInf	Ninf	WH	Sixmonths
T114Cn2	Mabiogo	F	MGNInf	Ninf	WH	Sixmonths
T114Cn3	Mabiogo	F	MGNInf	Ninf	WH	Sixmonths
T114Cn6	Mabiogo	M	MGNInf	Ninf	WH	Sixmonths
T116Cn1	Mabiogo	M	MGNInf	Ninf	WH	Sixmonths
T117Cn2	Mabiogo	F	MGInf	TB	WH	Sixmonths
T120Cn10	Mabiogo	M	MGNInf	Ninf	WH	Sixmonths
T120Cn2	Mabiogo	F	MGNInf	Ninf	WH	Sixmonths
T121Cn1	Mabiogo	F	MGInf	TC	WH	Sixmonths
T124Cn4	Nazareth	F	MGInf	TB	WH	Sixmonths
T125Cn1	Nazareth	M	MGInf	TB	WH	Sixmonths
T126Cn10	Nazareth	F	MGInf	TB	WH	Sixmonths
T126Cn11	Nazareth	F	MGNInf	Ninf	WH	Sixmonths

T126Cn12	Nazareth	F	MGNInf	Ninf	WH	Sixmonths
T126Cn13	Nazareth	F	MGNInf	Ninf	WH	Sixmonths
T126Cn15	Nazareth	F	MGInf	TC	WH	Sixmonths
T126Cn19	Nazareth	F	MGNInf	Ninf	WH	Sixmonths
T126Cn21	Nazareth	F	MGInf	TB	WH	Sixmonths
T129Cn1	Nazareth	F	MGInf	TB	WH	Sixmonths
T129Cn25	Nazareth	M	MGInf	TC	WH	Sixmonths
T129Cn26	Nazareth	M	MGInf	TB	WH	Sixmonths
T130Cn7	Campo beach	F	MGNInf	Ninf	WH	Sixmonths
T130Cn8	Campo beach	M	MGNInf	Ninf	WH	Sixmonths
T131Cn1	Campo beach	M	MGNInf	Ninf	WH	Sixmonths
T131Cn2	Campo beach	F	MGNInf	Ninf	WH	Sixmonths
T131Cn3	Campo beach	M	MGInf	TS	WH	Sixmonths
T132Cn1	Campo beach	F	MGInf	TB	WH	Sixmonths
T133Cn4	Campo beach	F	MGInf	TC	WH	Sixmonths
T133Cn6	Campo beach	F	MGNInf	Ninf	WH	Sixmonths
T133Cn7	Campo beach	M	MGInf	TB	WH	Sixmonths
T133Cn8	Campo beach	M	MGNInf	Ninf	WH	Sixmonths
T134Cn1	Campo beach	F	MGNInf	Ninf	WH	Sixmonths
T135Cn1	Campo beach	M	MGNInf	Ninf	WH	Sixmonths
T136Cn1	Ipono	M	MGInf	TB	WH	Sixmonths
T142Cn2	Ipono	F	MGInf	TB	WH	Sixmonths
T142Cn6	Ipono	F	MGInf	TB	WH	Sixmonths
T147Cn14	Ipono	M	MGNInf	Ninf	WH	Sixmonths
T147Cn2	Ipono	F	MGNInf	Ninf	WH	Sixmonths
T147Cn7	Ipono	M	MGNInf	Ninf	WH	Sixmonths
T182Cn1	Mvass	M	MGInf	TB	WH	Sixmonths
T194Cn18	Itonde	F	MGInf	TB	WH	Sixmonths
T208Cn1	Campo ville	F	MGInf	TB	WH	Sixmonths
T211Cn2	Campo ville	F	MGInf	TB	WH	Sixmonths
T211Cn33	Campo ville	M	MGInf	TB	WH	Sixmonths
T126Dn52	Nazareth	M	MGNInf	Ninf	WH	Twmonths
T111Dn1	Mabiogo	F	MGNInf	Ninf	WH	Twmonths
T114Dn6	Mabiogo	F	MGNInf	Ninf	WH	Twmonths
T115Dn2	Mabiogo	F	MGNInf	Ninf	WH	Twmonths
T115Dn6	Mabiogo	F	MGNInf	Ninf	WH	Twmonths
T115Dn7	Mabiogo	M	MGNInf	Ninf	WH	Twmonths
T116Dn1	Mabiogo	F	MGNInf	Ninf	WH	Twmonths
T121Dn1	Mabiogo	F	MGNInf	Ninf	WH	Twmonths
T121Dn2	Mabiogo	F	MGNInf	Ninf	WH	Twmonths
T124Dn2	Nazareth	M	MGNInf	Ninf	WH	Twmonths
T126Dn1	Nazareth	F	MGNInf	Ninf	WH	Twmonths
T126Dn2	Nazareth	F	MGNInf	Ninf	WH	Twmonths
T126Dn3	Nazareth	F	MGNInf	Ninf	WH	Twmonths
T126Dn9	Nazareth	M	MGNInf	Ninf	WH	Twmonths
T126Dn10	Nazareth	M	MGNInf	Ninf	WH	Twmonths
T126Dn35	Nazareth	F	MGNInf	Ninf	WH	Twmonths

T126Dn36	Nazareth	F	MGNInf	Ninf	WH	Twmonths
T126Dn51	Nazareth	M	MGNInf	Ninf	WH	Twmonths
T127Dn1	Nazareth	F	MGNInf	Ninf	WH	Twmonths
T131Dn5	Campo beach	F	MGNInf	Ninf	WH	Twmonths
T132Dn1	Campo beach	M	MGNInf	Ninf	WH	Twmonths
T132Dn3	Campo beach	F	MGNInf	Ninf	WH	Twmonths
T140Dn1	Ipono	F	MGNInf	Ninf	WH	Twmonths
T140Dn3	Ipono	F	MGNInf	Ninf	WH	Twmonths
T140Dn4	Ipono	M	MGNInf	Ninf	WH	Twmonths
T142Dn3	Ipono	M	MGNInf	Ninf	WH	Twmonths
T142Dn4	Ipono	M	MGNInf	Ninf	WH	Twmonths
T147Dn1	Mvass	F	MGNInf	Ninf	WH	Twmonths
T147Dn2	Mvass	F	MGNInf	Ninf	WH	Twmonths
T151Dn6	Poste3	F	MGNInf	Ninf	WH	Twmonths
T151Dn7	Poste3	F	MGNInf	Ninf	WH	Twmonths
T152Dn18	Poste3	F	MGNInf	Ninf	WH	Twmonths
T152Dn19	Poste3	F	MGNInf	Ninf	WH	Twmonths
T152Dn20	Poste3	M	MGNInf	Ninf	WH	Twmonths
T153Dn4	Poste3	M	MGNInf	Ninf	WH	Twmonths
T154Dn1	Poste2	F	MGNInf	Ninf	WH	Twmonths
T154Dn2	Poste2	F	MGNInf	Ninf	WH	Twmonths
T154Dn5	Poste2	F	MGNInf	Ninf	WH	Twmonths
T154Dn36	Poste2	M	MGNInf	Ninf	WH	Twmonths
T154Dn37	Poste2	M	MGNInf	Ninf	WH	Twmonths
T154Dn38	Poste2	M	MGNInf	Ninf	WH	Twmonths
T154Dn50	Poste2	F	MGNInf	Ninf	WH	Twmonths
T154Dn58	Poste2	M	MGNInf	Ninf	WH	Twmonths
T154Dn65	Poste2	F	MGNInf	Ninf	WH	Twmonths
T158Dn1	Poste1	F	MGNInf	Ninf	WH	Twmonths
T145En8	Mvass	M	MGNInf	Ninf	WH	Hemonths
T148En2	Mvass	F	MGNInf	Ninf	WH	Hemonths
T120En1	Mabiogo	F	MGNInf	Ninf	WH	Hemonths
T121En7	Mabiogo	M	MGNInf	Ninf	WH	Hemonths
T114En13	Mabiogo	M	MGNInf	Ninf	WH	Hemonths
T130En5	Campo beach	F	MGNInf	Ninf	WH	Hemonths
T116En2	Mabiogo	F	MGNInf	Ninf	WH	Hemonths
T113En2	Mabiogo	F	MGNInf	Ninf	WH	Hemonths
T152En1	Poste3	F	MGNInf	Ninf	WH	Hemonths
T151En9	Poste3	M	MGNInf	Ninf	WH	Hemonths
T133En1	Campo beach	M	MGNInf	Ninf	WH	Hemonths
T134En2	Campo beach	F	MGNInf	Ninf	WH	Hemonths
T126En37	Nazareth	F	MGNInf	Ninf	WH	Hemonths
T112En4	Mabiogo	M	MGNInf	Ninf	WH	Hemonths
T146En1	Mvass	F	MGNInf	Ninf	WH	Hemonths
T151En7	Poste3	F	MGNInf	Ninf	WH	Hemonths
T114En12	Mabiogo	M	MGNInf	Ninf	WH	Hemonths
T131En2	Campo beach	M	MGNInf	Ninf	WH	Hemonths

T148En9	Mvass	F	MGNInf	Ninf	WH	Hemonths
T126En5	Nazareth	F	MGNInf	Ninf	WH	Hemonths
T148En14	Mvass	M	MGNInf	Ninf	WH	Hemonths
T113En1	Mabiogo	F	MGNInf	Ninf	WH	Hemonths
T192En12	Itonde	M	MGInf	TB	WH	Hemonths
T126En27	Nazareth	M	MGNInf	Ninf	WH	Hemonths
T151En8	Poste3	F	MGNInf	Ninf	WH	Hemonths
T146En5	Mvass	M	MGNInf	Ninf	WH	Hemonths
T148En3	Mvass	M	MGNInf	Ninf	WH	Hemonths
T157En1	Poste3	F	MGNInf	Ninf	WH	Hemonths
T111En1	Mabiogo	F	MGNInf	Ninf	WH	Hemonths
T126En25	Nazareth	M	MGNInf	Ninf	WH	Hemonths
T148En16	Mvass	M	MGNInf	Ninf	WH	Hemonths
T126En4	Nazareth	F	MGNInf	Ninf	WH	Hemonths
T157En9	Poste2	F	MGNInf	Ninf	WH	Hemonths
T129En1	Nazareth	F	MGInf	TC	WH	Hemonths
T126En26	Nazareth	M	MGNInf	Ninf	WH	Hemonths
T146En4	Mvass	M	MGNInf	Ninf	WH	Hemonths
T121En1	Mabiogo	F	MGNInf	Ninf	WH	Hemonths
T114En1	Mabiogo	F	MGNInf	Ninf	WH	Hemonths
T126En3	Nazareth	F	MGNInf	Ninf	WH	Hemonths
T123En1	Nazareth	F	MGNInf	Ninf	WH	Hemonths
T111En3	Mabiogo	F	MGNInf	Ninf	WH	Hemonths
T133En3	Campo beach	F	MGNInf	Ninf	WH	Hemonths
T114En3	Mabiogo	F	MGNInf	Ninf	WH	Hemonths
T134En1	Campo beach	F	MGNInf	Ninf	WH	Hemonths
T126En45	Nazareth	F	MGNInf	Ninf	WH	Hemonths

F: female; M: male; MGinf: midgut infected; MGNinf: midgut non infected; WH: whole fly; BF: before;