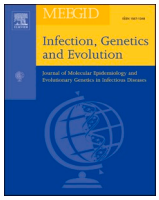
Infection, Genetics and Evolution 125 (2024) 105683

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid



Spatial and temporal diversity of *Simulium damnosum s.l.* gut microbiota and association with *Onchocerca volvulus* infection in Cameroon

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A R T I C L E I N F O

Keywords:
Onchocerca volvulus
Simulium damnosum s.l.
Microbiota
Vector control

A B S T R A C T

Arthropod microbiota plays an important role in host physiology, and there is growing interest in using vector symbionts to modify vector competence and control parasite transmission. This study aims to characterise the blackfly *Simulium damnosum* s.l. gut microbiota and to explore possible associations with various bio-ecological determinants of the *Onchocerca volvulus* establishment and the transmission in blackfly. Adult female blackflies were caught in three Cameroonian health districts belonging to different bioecological zones endemic for onchocerciasis. Flies were dissected and qPCR screened for *Onchocerca volvulus* infection. The diversity of the blackflies gut microbiota was assessed by high-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S ribosomal RNA. Subsequent metataxo-genomic, multivariate, and association analysis were used to investigate the variables that influence the microbiota diversity.

Transmission index rates ranging from 20.7 to 6.0 % and 6.2to 2.0 % for infection and infectivity rates, respectively, indicate ongoing transmission of onchocerciasis across all surveyed health districts. The identified bacterial taxa were clustered into four phyla, five classes, and 23 genera. The *S. damnosum* s.l. gut microbiota was dominated by *Wolbachia* and by *Rosenbergiella* in *Wolbachia*-free *Simulium*. Significant differences were observed in the diversity of *S. damnosum* s.l. microbiota concerning parity status ($P = 0.007$), health district of origin ($P = 0.001$), and the presence of the *Onchocerca volvulus*. *Simulium* from the Bafia health district also showed increased bacterial diversity between two consecutive years ($P = 0.001$). Four bacterial taxa, including *Serratia*, were associated with the absence of the *O. volvulus* infection.

These results indicate that *S. damnosum* s.l. from different onchocerciasis foci in Cameroon, exhibit distinguishable gut microbial compositions which are dynamic over time. Some bacterial species are associated with the *O. volvulus* infection and could be further investigated as biological target/tool for vector modified-based onchocerciasis control.

1. Introduction

Onchocerciasis or river blindness is a debilitating parasitic disease due to *Onchocerca volvulus*, prevailing in tropical climates and currently endemic in 34 countries, including 31 in sub-Saharan Africa (World Health Organization, 2019). The disease is transmitted by a

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https://doi.org/10.1016/j.meegid.2024.105683
Received 8 June 2024; Received in revised form 19 October 2024; Accepted 19 October 2024
Available online 21 October 2024

hematophagous arthropod insect of the genus *Simulium*, (Adler and Crosskey, 2012). In Africa, transmission patterns and vector species distributions are geographically differentiated. Indeed, 90 % of transmission is associated with the bites of the *S. damnosum* complex, mainly found in West and Central Africa, while the remaining 10 % is due to *S. neavei* in East Africa (Crosskey, 1990). The clinical picture of onchocerciasis includes mild skin lesions or the hyperreactive form (Hyperactive Onchocerciasis) and visual impairment leading to irreversible blindness. An estimated 500,000 blindness cases have been attributable to onchocerciasis, making the latter the third most important cause of preventable blindness in the tropics and the second leading cause of infectious blindness (Narita and Taylor, 1993; Resnikoff and Keys, 2012). Onchocerciasis have been associated with epilepsy, nodding syndrome, as well as excess mortality and Nagana syndrome (Kipp et al., 1996; Colebunders et al., 2017b, 2017a; Chesnais et al., 2018). Historically, the disease burden and vector biting nuisance in African villages close to vector breeding sites led to the desertion of vast areas of fertile land, leading to significant socio-economic repercussions (Resnikoff and Keys, 2012).

The approval of ivermectin for the treatment of human onchocerciasis has been pivotal in controlling the morbidity related to onchocerciasis. This very potent microfilaricidal and easy to administer anthelminthic drug has enabled the scale-up of the control efforts in all the endemic countries. However, the elimination of onchocerciasis using ivermectin solely remains a challenge in Africa, as only four countries have reported the interruption of transmission in some foci since decades of annual non-interrupted preventive chemotherapy (Traore et al., 2012; Tekle et al., 2016; Miri et al., 2022). Moreover, vast endemic areas remain untreated due to the risk of potentially fatal serious adverse events related to the co-infection with loiasis, another filarial parasite endemic in the forest zone in Central and West Africa (Zouré et al., 2011; Emukah et al., 2018).

Vector control strategies with the aim of reducing vector population density therefore appears as a suitable alternative to ivermectin in areas where mass treatment is not feasible and can be considered as complementary strategy where the disease is persistent. However, the primarily vector control approach based on larvicides spraying on blackflies' breeding sites remains limited because of ecological pollution, reduced efficacy consecutive to resistance, and difficulties of implementation, especially in forest areas where river networks are complex and access breeding sites difficult (Gebrezgabiher et al., 2019). Innovative vector control approaches have recently been developed to curb the transmission of other vector-borne diseases based on the modification or alteration of vector competence. Indeed, the vector ability to acquire, mature, and transmit pathogens to vertebrates is known to be influenced by several factors, including the microbiome. Studies have demonstrated that the gut microbiota can both positively and negatively affect vector competence and thus potentiate or alter the ability of adult vectors to mature and transmit a pathogen (Azambuja et al., 2005; Engel and Moran, 2013). These effects generally appear as the result of complex interactions of the vector biological function, triggered by an intermediate metabolite released by a single bacterial species (Werren et al., 2008; Chouaia et al., 2012; Bando et al., 2013) or the combined action of sub-groups of bacteria in the microbiota (Crotti et al., 2010). These bacterial species can then be explored as effective tools or targets for new generations of vector control strategies (Favia et al., 2008; McMeniman et al., 2009; Gupta et al., 2012; Wilke and Marrelli, 2015).

Blackfly gut microbiota revealed a heterogenic composition dominated by the genus *Wolbachia* in the Mbam valley in Cameroon (Efon Ekangouo et al., 2021). However, the impact of the bioecological diversity and the biotic and abiotic contacts of *S. damnosum* s.l. with the environment on the composition of the gut microbiota remains unknown. This study aims to update the composition of the *S. damnosum* s.l. gut microbiome and to assess the effects of spatio-temporal and biological determinants on its diversity across various onchocerciasis-endemic foci in Cameroon.

2. Material and methods

2.1. Study sites

Seven first-line villages (located in less than 5 km from the blackflies river breeding sites) were selected for sampling of female adult *Simulium* (Fig. 1). The villages were located in three health districts of Cameroon, with distinguishable bioecological features and endemicity to onchocerciasis: Bafia health district (Centre Region), located in the forest-savannah transition zone, Ndom and Yabassi health districts (Littoral Region) located in the forest area. Community-directed treatment with ivermectin have been implemented in the three health districts since 2000 with therapeutic coverage fluctuating around 80 % since 2014 (Kamga et al., 2016). Despite decades of treatment, these health districts remain the primary hotspots for onchocerciasis in the country with community-based prevalence ranging from 10 to 60 % of microfilarodermia prevalence (Kamga et al., 2016; Sumo et al., 2024).

2.2. Blackfly collection, dissection and identification

The sample collection was conducted between April 2019 and July 2020 in Bafia and in 2020 in Ndom and Yabassi health districts. The sample size estimation was based on the null hypothesis that beta-diversity does not differ between infected and non-infected *Simulium* groups, and determined as described for the microbiome studies: $n = 2\left(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta}\right)^2 / \Delta^2$, where $\Delta$is the effect size, $Z_{1-\alpha}, Z_{1-\beta}$ are the upper tail normal quantiles associated with the desired type I and type II errors; α and β, respectively (Casals-Pascual et al., 2020; Ferdous et al., 2022). As we measure dissimilarity metric for beta diversity (bray-curtis index) to test our hypothesis, the minimum number of required blackflies to see a moderate effect size (0.60) with a significance of 5 % and statistical power of 80 % is 100 flies (50 per group).

In each selected village, three catch points were set up between 0.5 and 1 km from the nearest blackfly breeding sites. Blackflies were caught using the human landing catch technique (Tambwe et al., 2023) and identified under binocular stereomicroscope by experienced entomologists using validated identification keys (Philippon, 1977). Only flies belonging to the *S. damnosum* complex were retained for further analysis. Female *Simulium damnosum* s.l. were surface sterilized as previously described (Efon Ekangouo et al., 2021) and individually dissected in a drop of saline. The parity rates of dissected flies was determined as described by Walsh et al. (Walsh et al., 1978), and enabled to differentiate nulliparous flies (females that have never laid eggs) from parous flies (females that have completed at least one gonotrophic cycles). All *Simulium* infected with *O. volvulus* and parous flies were systematically retained for sequencing, and equivalent number of nulliparous *Simulium* were randomly selected from each health district.

2.3. Onchocerca volvulus detection in blackflies

The presence of *O. volvulus* larval stages was detected in gut (L1 or L2 stages) and head (infective larval stage or L3) of blackflies using the real-time PCR. Briefly, the DNA was purified from different parts (abdomen/thorax and head) of each blackfly and purified using the QIAamp DNA Mini kit (Qiagen Inc., Les Ulis, France). A species-specific *singleplex* qPCR was used to target a 128 bp sequence encoding the ND5 subunit of the *Onchocerca volvulus*. This recently developed biomarker has been demonstrated to be more sensitive and species-specific than the O-150 marker, which targets a non-coding repetitive DNA sequence for the molecular diagnostic of *O. volvulus* (Doherty et al., 2023). Reactions were carried out using StepOnePlus PCR system (Applied Biosystems,

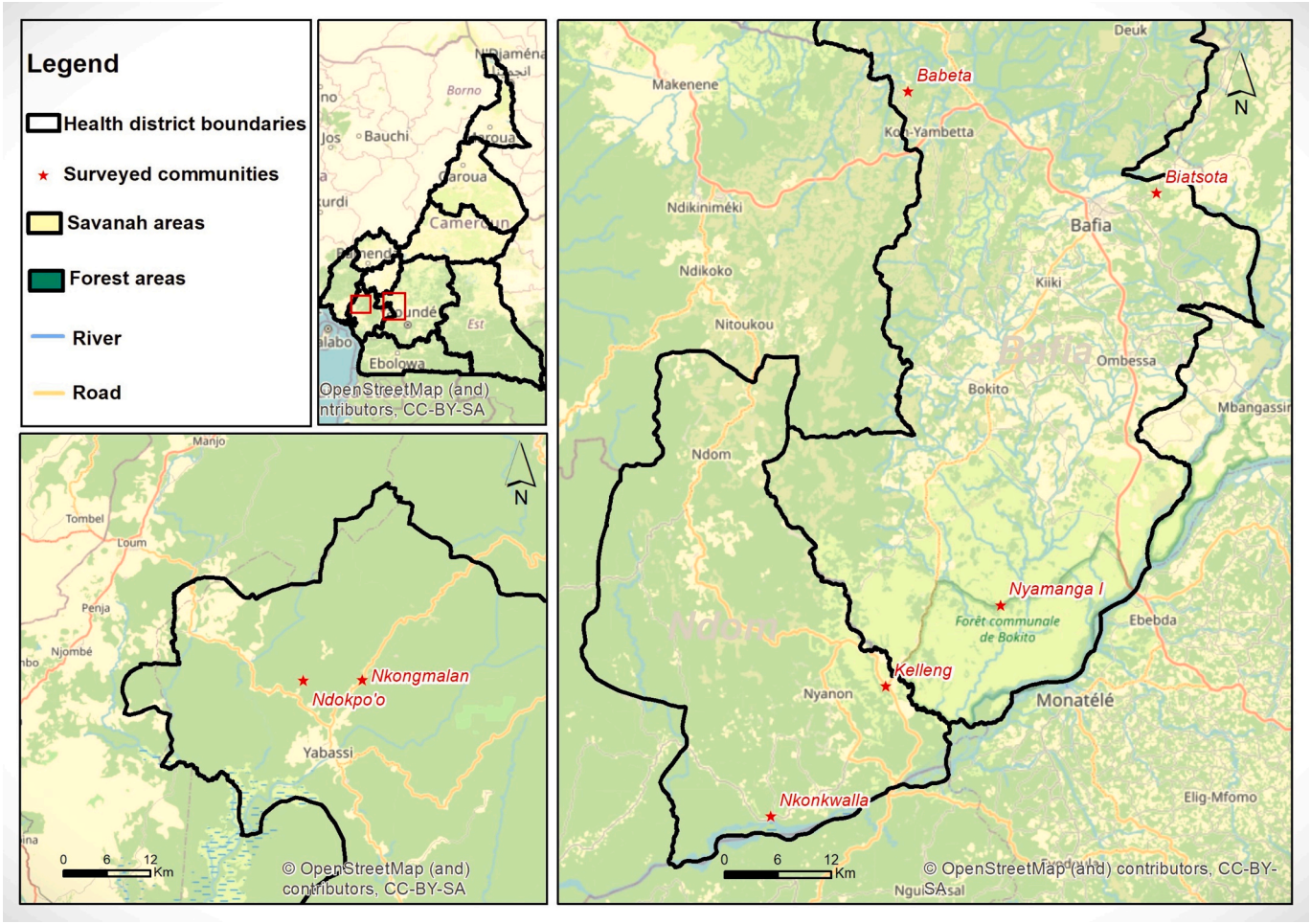

Fig. 1. Geographical map of communities where blackflies were sampled.

Foster City, CA). The final solution of 20 µL, contained 2 µL of template DNA (DNA samples, plasmid with inserted ND5 target sequence as positive control and nuclease free water as negative control) and 18 µL of PCR Master Mix made up of 12 µL of molecular biology-grade water, 2 µL of 10× Taq polymerase Buffer, 2.4 µL of $MgSO_4$ (4.5 mM), 0.1 µL of dNTP (40 mM), 0.1 µL of HotStar polymerase at 5 U/µL, 0.6 µL of each primer (10 mM) (OvOo ND5 forward: GCTATTGGTAGGGGTTTGCAT and OvOo ND5 reverse: CCACGATAATCCTGTTGACCA), and 0.2 µL of *O. volvulus* ND5 probe (Taqman probe: FAM-TAAGAGGTTAAGATGG-BHQ1). Cycling conditions involved initial denaturation at 95 °C for 15 min, followed by 45 cycles at 95 °C for 10 s and 61 °C for 30 s.

2.4. Illumina MiSeq sample preparation, and high-throughput sequencing

PCR amplicon libraries were generated for each gut DNA sample. The hypervariable region V3-V4 of the 16S ribosomal RNA was targeted to enable the accurate detection of the bacterial communities. Library was generated using V3F (5'-GGCCTACGGGAGGCAGCAG-3′) and V4R (5'-CCGGACTACHVGGTWTCTAAT-3′) primers previously designed for a similar study (Jacob et al., 2017). PCR was performed using the HotStar Taq Plus Master Mix Kit (Qiagen Inc., Texas, USA) with an initial denaturation at 94 °C for 3 min followed by 32 cycles of amplification of which each cycle consisted of a denaturation step at 94 °C for 30 s, an annealing step at 53 °C for 40 s, and an extension step at 72 °C for 1 min, followed by the final extension at 72 °C for 5 min, and a final hold at 4 °C. PCR products were run on 2 % agarose gel and fragment of approximately 380 bp were excised, barcoded, pooled and purified for sequencing. High Throughput Sequencing was performed using the Illumina MiSeq platform (www.mrdnalab.com, Shallowater, TX, USA) incorporating the paired-end kits from Illumina specific to the V3-V4 region of the 16S rRNA gene.

2.5. Metabarcoding analysis and taxonomic assignment

The raw sequence data generated by high-throughput sequencing were demultiplexed and quality control was performed using FastQC program. The paired-ends reads were quality-based filtered with a threshold value of 20 using the CutAdapt V3.5 program (Martin, 2011). In addition to CutAdapt, VSearch V2.21.1 program (Rognes et al., 2016) was used to merge the reads (forward and reverse), trim the read extensions (barcodes and primers), perform the sample-level demultiplexing of reads. Output reads were pooled into a single FASTA file and subsequently dereplicated and clustered using Swarm V2.2.2 program (Mahé et al., 2015). VSearch was also employed to eliminate chimeric reads. The STAMPA pipeline (https://github.com/frederic-mahe/stampa) allowed taxonomic assignment of representative OTUs to a reference database (previously generated from SILVA SSU Database (Quast et al., 2012) using CutAdapt program) in order to generate the OTU table.

2.6. Filtering of contaminants and rarefaction analysis

The generated The OTU table was pre-processed using the open-source decontam R package (Davis et al., 2018) that implements a statistical classification procedure that identifies contaminants taxa from controls samples (blanks). The prevalence-based contaminant

identification method was used with a threshold of 20 % (Davis et al., 2018). Contaminants empirically associated with sample processing (Salter et al., 2014) as well as sequences associated to chloroplasts and OTU with less than 10 reads across the dataset were also discarded.

2.7. Statistical analyses

The statistical analysis was performed using R version 4.3.2. The entomological indexes included parity, infectivity and infection rates. Infection and infectivity rates were calculated as the proportion of parous blackflies harbouring *O. volvulus* L1 and/or L2 in their gut, and infective larval stage (L3) in their heads (salivary gland), respectively. The parity rate was computed as the proportion of dissected adult female blackflies that were classed as parous (individuals that completed at least a one reproductive cycle at the time of the capture). Chi-square tests were performed to compare the entomological indices between covariables. *MicrobiomeAnalyst* v2.0 (Lu et al., 2023) was used to determine the taxonomic distribution and diversity metrics. Total sum normalization (TSS) combined with square root transformation (Hellinger transformation) was used to normalize data count. This method converted raw feature counts to relative abundance. Alpha diversity describing the bacterial species diversity at the scale of a focus (ecosystem) was assessed by estimating the Shannon index (an estimator of taxa diversity, combining richness and evenness) using non transformed or normalized data (Bertrand et al., 2011; Lemos et al., 2011). Beta diversity which refers to the change in the bacterial diversity across the different foci (ecosystem) was determined by calculating the Bray-Curtis dissimilarity index (Izsak and Price, 2001). Principal component analyses were used to visualise the distribution of samples between the variables (health districts, infection status, parity status and year of sampling). Differential analysis of the abundance of taxa with regards to the different variables (health districts, infection status and parity) was performed by applying a generalised linear model using the EdgeR package of the R software (Chen et al., 2022). The search of taxonomic groups potentially associated with specific biological features (biomarkers), was performed using linear discriminant analysis of effect size (LEfSe) (Segata et al., 2011). The significance level was set at $\alpha = 0.05$.

3. Results

3.1. Frequency of Onchocerca volvulus infection in Simulium damnosum s.l.

A total of 3376 blackflies caught were dissected. The parity rate was at 12.3 %, unevenly distributed between health districts, with a significantly higher proportion of parous blackflies in Bafia health district ($P < 0.0001$). The same trend was observed with a significantly higher infectivity rate in Bafia (6.2 %) than in Yabassi (2.9 %) and Ndom (2.0 %). In terms of distribution of infection rate, the highest proportion of black flies harbouring early-stage parasite larvae (L1,L2) was found in the Yabassi health district ($P < 0.0002$) (Table 1).

3.2. Composition of the Simulium damnosum s.l. gut microbiota

One hundred and fifty-eight (158) total DNA samples purified from blackfly abdomen/thorax were selected for microbiome characterization. The origin of *Simulium* and their bioecological features are summarized in Table 2.

MiSeq sequencing of the V3—V4 region of 16S rRNA generated a total of 12,945,809 reads with a mean of 77,058 reads per sample (varying between a minimum of 6207 and a maximum of 256,294 reads). Taxonomic assignment showed that the sequences clustered into four phyla, five classes with an overall abundance equal to, or greater than, 1 %. The majority of Operational Taxonomic Unit (OTU) belonged to the phylum *Proteobacteria* (97.5 %) consisting of *Alphaproteobacteria* (86.4 %) and *Gammaproteobacteria* (11.1 %). Other observed phyla included *Firmicutes* (0.5 %), *Bacteroidota* (1.7 %) and *Actinobacteriota* (0.3 %). The *Anaplasmataceae* (78.6 %), *Erwiniaceae* (9.4 %), and *Acetobacteraceae* (8.1 %) were the mainly represented families. The microbial composition of blackfly guts revealed 23 genera, with *Wolbachia* (78.6 %) by far the most abundant, followed by *Rosenbergiella* (7.4 %) and *Asaia* (4.6 %) (Fig. 2). *Vogesella* (0.1 %) and *Tanticharoenia* (0.2 %) were the least represented genera among all the samples processed.

The bacterial composition of the blackfly guts showed a high heterogeneity with regards to the geographic origin (Fig. 3a). Indeed, the gut microbiota of blackflies from Yabassi health district was dominated by the genera *Rosenbergiella* (52.9 %), *Asaia* (20.8 %), and *Pantaea* (10.7 %), whereas those from Bafia health district showed an absolute predominance of *Wolbachia* which represented 96.7 % of all bacterial genera identified. The microbiota of the blackflies collected in Ndom health district presented an intermediate composition dominated by both *Wolbachia* (58.0 %) and *Saccharibacter* (18.8 %) (Fig. 3). The single factor analysis of the significance of the difference in genera abundance with the regard to the geographic origin, showed that *Asaia* ($P < 0.0001$), *Wolbachia* ($P < 0.0001$) and *Rosenbergiella* ($P < 0.0001$) were mainly represented in Ndom, Bafia and Yabassi health districts, respectively (Fig. 3b). While *Wolbachia* was present in all samples collected in the Bafia and Ndom health districts, it was represented in only 50 % of samples from Yabassi. This representativeness was reversed with the genus *Rosenbergiella*, which was present in all *Simuliidae* from Yabassi and in 50 % of individuals from Bafia.

The composition of blackfly gut microbiota according to the biological features (*O. volvulus* infection and parity statuses) showed a

Table 2
Sample selected to characterise the gut microbiota.

Health district	*O. volvulus* infected Blackflies	*O. volvulus* uninfected Blackflies		Total
		Pare	Nulliparous	
Bafia	32	29	18	79
Ndom	3	9	14	26
Yabassi	29	11	14	54
Total	**64**	**49**	**46**	**158**

Table 1
Parity, infection and infectivity rates of dissected blackflies according to health districts and their constituting communities.

Health district	Village	N blackflies dissected	N parous	Parity rate (%)	Infection rate (%)	Infectivity rate (%)
Bafia	Biatsota	598	120	20.1	18.3	8.3
	Nyamongo	512	73	14.3	9.6	4.1
	Bayomen	200	34	17.0	8.8	2.9
	Total	***1310***	***227***	***17.3***	***14.1***	***6.2***
Yabassi	Nkongmalan	554	68	12.3	13.2	1.5
	Ndokpo'o	523	72	13.8	27.8	4.2
	Total	***1077***	***140***	***13.0***	***20.7***	***2.9***
Ndom	Kelleng	490	33	6.7	6.1	0.0
	Nkonkwalla	499	17	3.4	5.9	5.9
	Total	***989***	***50***	***5.1***	***6.0***	***2.0***

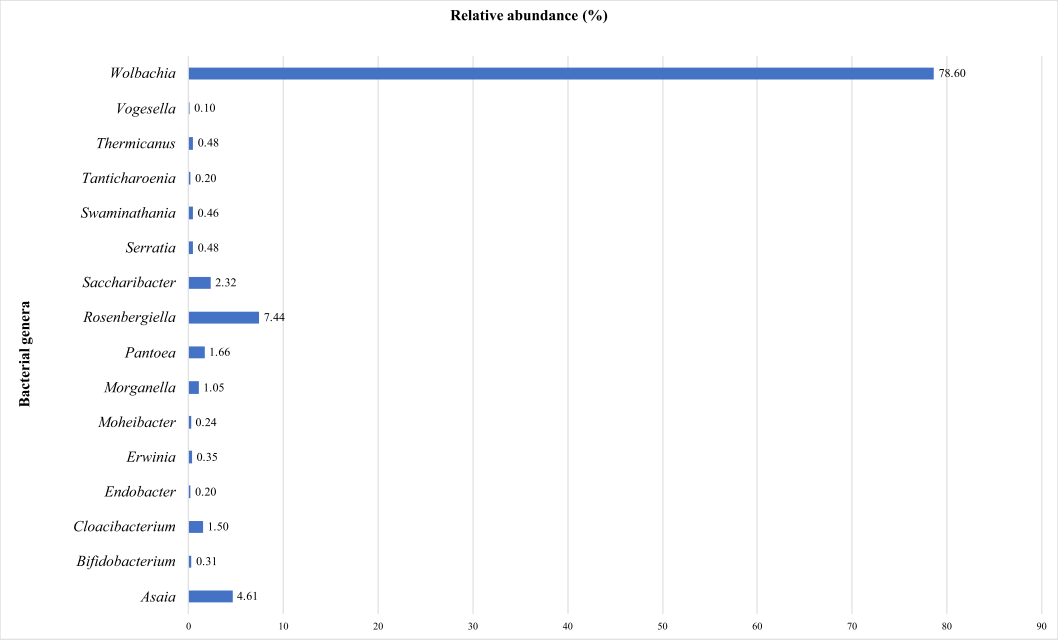

Fig. 2. Relative abundance of the top bacterial genera in the gut microbiota of *Simulium*.

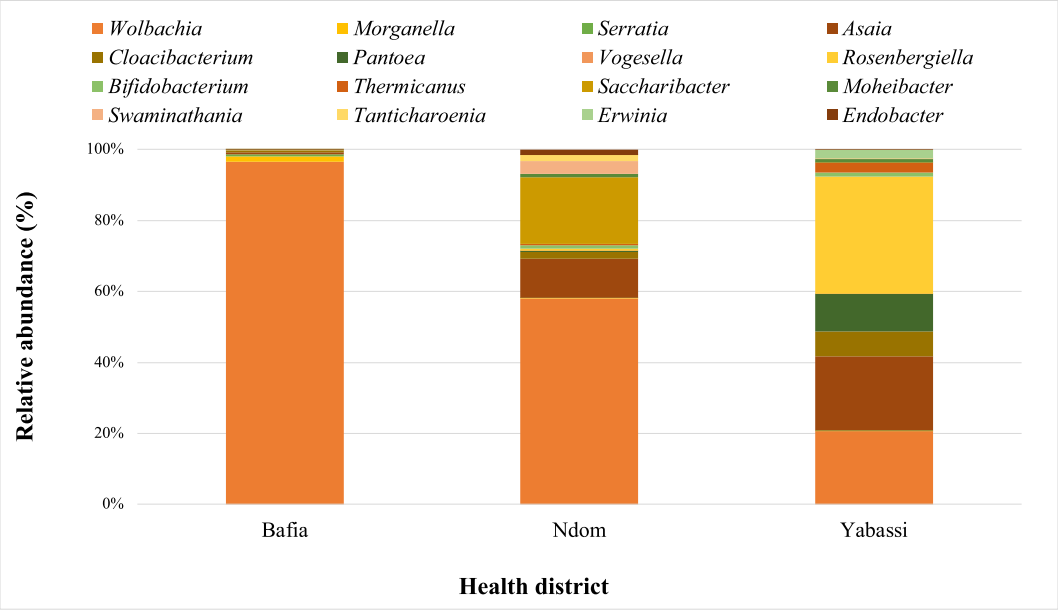

Fig. 3. Relative abundances of the most predominant taxa of the *Simulium* gut microbiota according to the health district of origin.

homogeneity in the distribution of many taxa in the different groups (Fig. 4). Overall, *Wolbachia* showed the highest proportion in the majority of blackflies processed, regardless of the biological features considered. A significant increase was found in the abundance of *Wolbachia* and *Asaia* in parous blackfly compared with nulliparous, and the parity status of flies reduced the abundance of *Erwinia*, *Rosenbergiella* and *Pantoea*. The presence of the larval stages L1 and/or L2 significantly reduced the abundance of eight genera, including *Erwinia*, *Morganella*, *Pantoea* and *Rosenbergiella*, and was significantly associated with an increase in *Asaia*, *Bifidobacterium* and *Cloacibacterium*. The presence of L3 larvae was associated with a reduction in the abundance of *Serratia* ($P = 0.03$) and *Erwinia* ($P = 0.002$), and a significant increase in *Wolbachia* abundance ($P = 0.03$).

3.3. Alpha diversity

The bacterial diversity, assessed using the index of bacterial richness (Shannon H), revealed a significantly more evenness distribution of abundances in the Ndom health district (H index: 2.17) than in Bafia and Yabassi health districts (H Indexes: 1.34 and 1.92 respectively) (Fig. 5a). Overall, the diversity was reduced among parous flies than nulliparous ones (H Index: 1.54 versus 1.88; $P = 0.008$) (Fig. 5b). Moreover, the presence of L1 and/or L2 larval stages reduced the bacterial richness (H-Index: 1.66 against 1.71; $P = 0.03$) in infected blackflies compared to their uninfected counterparts (Fig. 5c). The comparison of two populations collected in two consecutive years in the Bafia health district (Fig. 5d) showed no significant changes of the alpha diversity in the

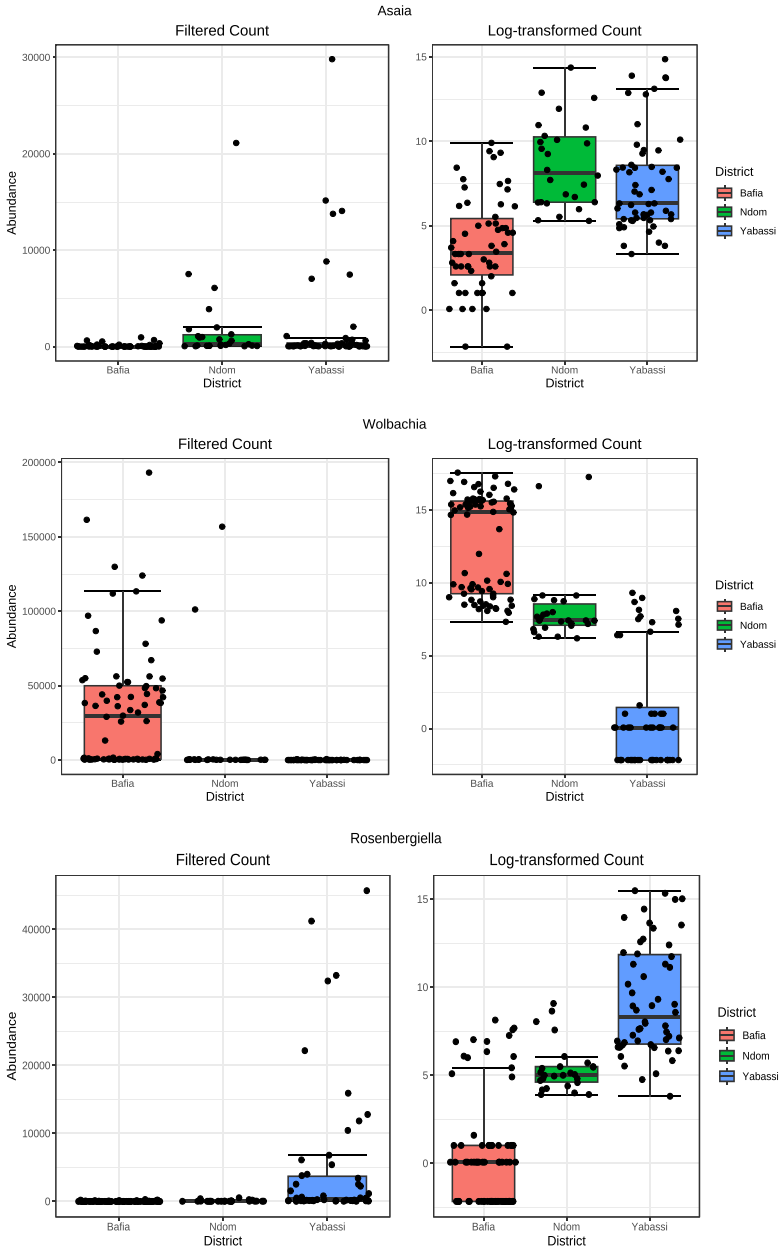

Fig. 3. (*continued*).

composition of the gut microbiota of blackflies over time.

Considering the diversity of the microbiota of Wolbachia-free *Simulium* (Wolbachia excluded from the microbiota), we observed an overall increase in the alpha diversity of the microbiota of *Simulium* initially heavily infected with Wolbachia. Indeed, while alpha diversity remained significantly pronounced in *Simulium* originating from Ndom health district compared with Yabassi health district (H index: 2.2 vs 1.8; $P = 0.02$), it increased significantly in *Simulium* originating from Bafia health district compared with Yabassi health district (H index: 2.1 vs 1.8; $P = 0.01$) and remained unchanged between Ndom health district and Bafia health district ($P = 0.7$) (Fig. S1). Excluding *Wolbachia* also unsurprisingly increased the diversity of parous and infected *Simulium*, reducing the effect of infection (Infected vs. non-infected, $P = 0.4$) and parity (Parous vs. Nulliparous $P = 0.3$) on *Simulium* diversity (Fig. S1).

3.4. Beta diversity

Figure 6 shows the Principal Component Analysis (PCA) plot based on Bray-Curtis distance matrix, an index of dissimilarity describing gut microbial composition of different blackfly groups based on their health district of origin (Fig. 6a), blood feed uptake (Fig. 6b), infection with L1 and L2 larvae stage of *O. volvulus* (Fig. 6c), and year of flies' capture (Fig. 6d). The two-dimensional PCA plot shows 51.9 % of the total variance between the blackfly samples collected in 2019 and those collected in 2020 in the Bafia health district with composition profiles that markedly differed between the two groups. The same differential clustering of bacterial gut was observed between health districts and regarding the blood uptake status of the flies. However, the profiles overlapped between the different subgroups with regard to the infection status for the larval stages L1 and/or L2. Analyses of Bray-Curtis dissimilarity index definitively showed the dissimilarity of the blackfly microbiota compositions between parous and nulliparous groups (analysis based on the Bray-Curtis distance, $P = 0.007$) and between the simulium collected in 2019 and those of 2020 in Bafia health district ($P = 0.001$). The dissimilarity was also significant according to geographical origins ($P = 0.001$) and was confirmed by the organization

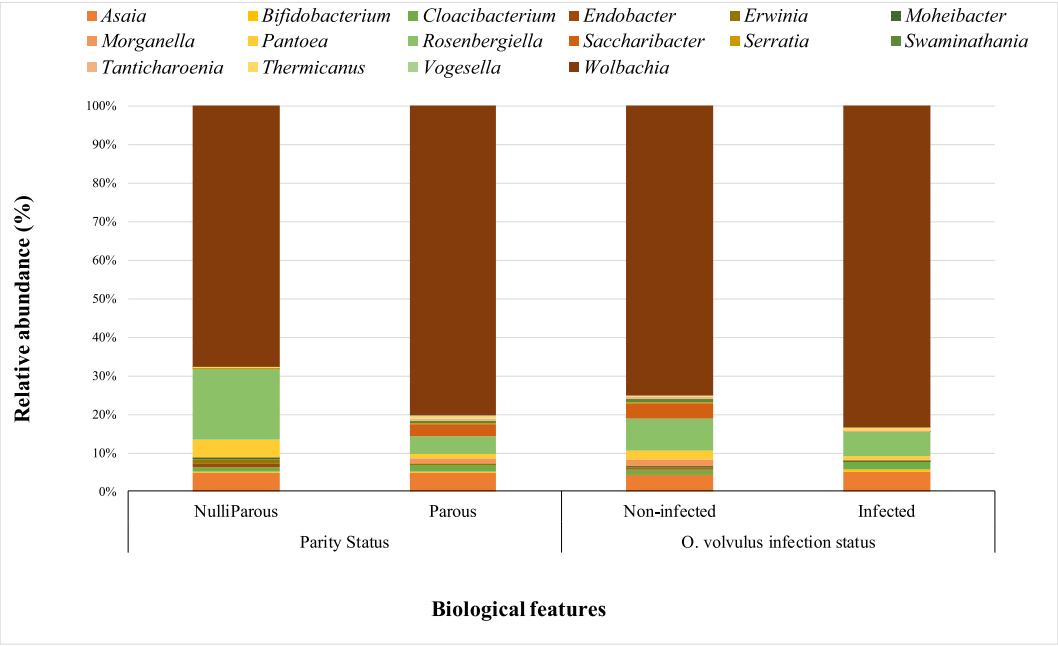

Fig. 4. Distribution of the three most predominant taxa of the *Simulium* gut microbiota according to the health district of origin.

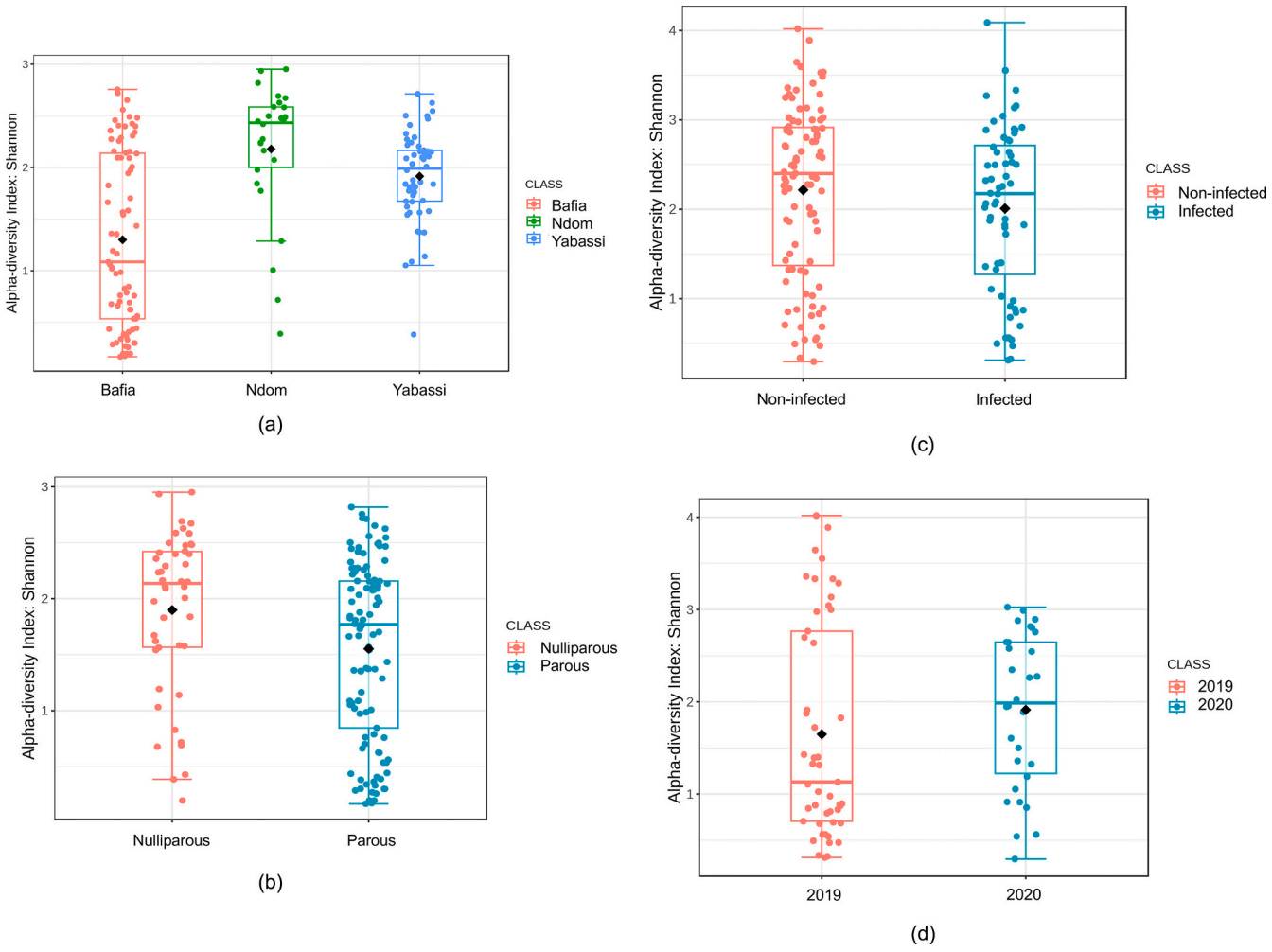

Fig. 5. Alpha diversity of the blackflies gut microbiota under diverse conditions with estimated Shannon index. (a): health district of origin: Bafia vs Ndom vs Yabassi ($P < 0.0001$); (b): infection status (Larvae L1/L2): Uninfected vs infected simulium (L1, L2) ($P = 0.03$); (c): Parity status: Nulliparous vs Parous ($P = 0.008$); (d): Year of capture (Bafia only): 2019 vs 2020 ($P = 0.17$).

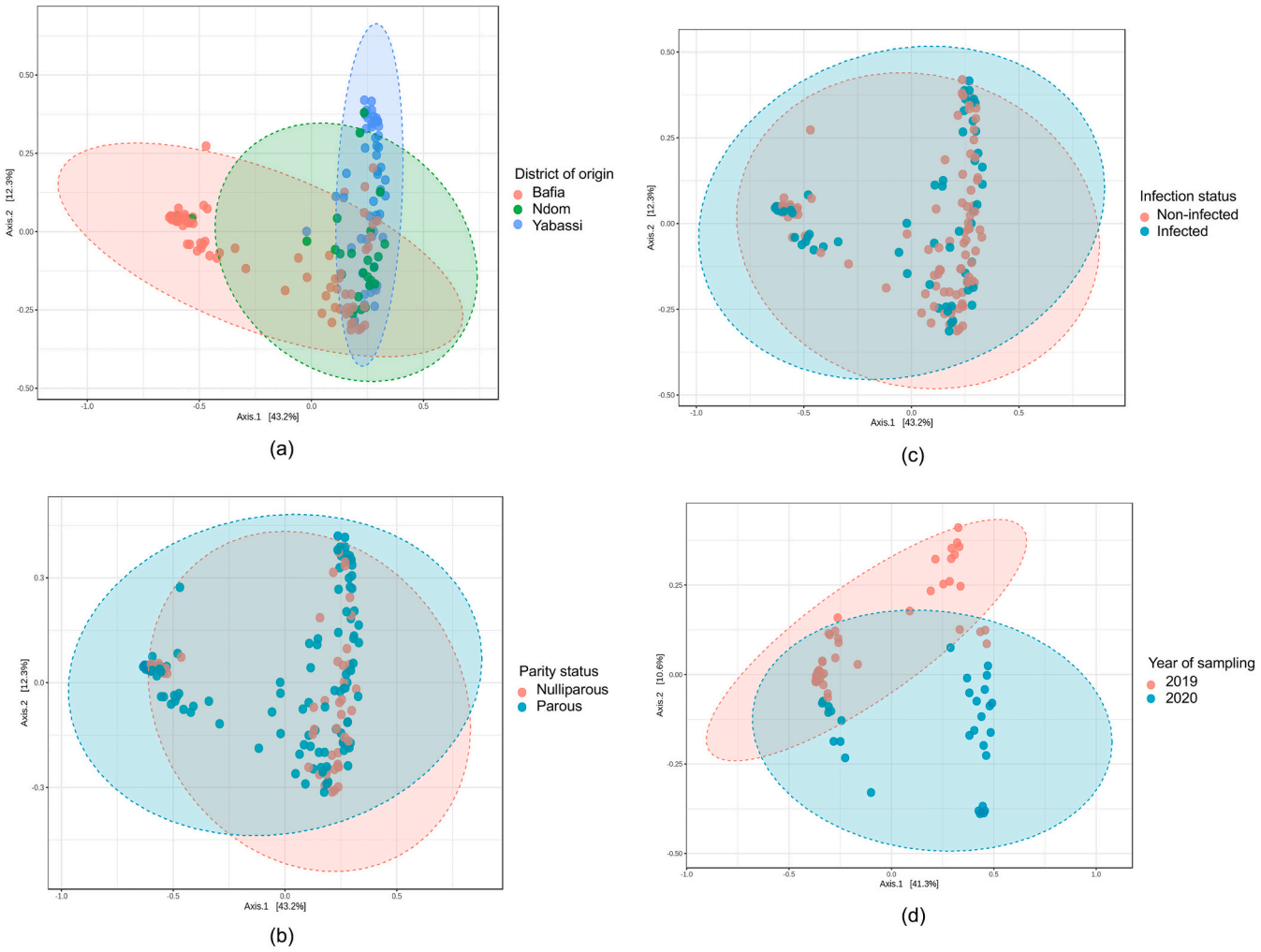

Fig. 6. Principal Component Analysis (PCA) plot based on Bray-Curtis distance matrix. (a): health district of origin, Bafia vs Ndom vs Yabassi ($P = 0.001$); (b): parity status, Nulliparous vs parous ($P = 0.007$); (c): infection status L1/L2, Uninfected (L1, L2) vs Infected (L1, L2) (P = 0,139); (d): collection year, 2019 vs 2020 (P = 0,001).

of the samples in the dendrogram (Fig. S2). Considering the beta diversity of the microbiota of *Simulium* without *Wolbachia* (*Wolbachia* excluded from the microbiota), the diversity of the microbiota of *Simulium* originating from Ndom health district shows more clearly that there is an overlap with the bacterial communities of *Simulium* originating from Yabassi and Bafia health districts (Fig. S2).

3.5. Metagenomic biomarker discovery

The Linear Discriminant Analysis Effect Size (LEfSe) on the filtered dataset was performed to find taxa differentially represented between different groups (Fig. 7). The LEfSe analysis with an LDA score of 3 showed that four genera contributed to greater dissimilarity (effect size) in non-infected blackflies, including *Serratia* ($P = 0.002$), *Saccharibacter* (P = 0.002), *Endobacter* ($P = 0.003$) and *Morganella* ($P = 0.01$) (Fig. 7b). No genus was associated with a significant dissimilarity effect in infected blackflies. According to the parity status, six genera were differentially associated with a dissimilarity effect. Indeed, *Serratia* ($P < 0.0001$), *Saccharibacter* (P = 0.001), *Endobacter* (P = 0.001), *Morganella* ($P = 0.004$), *Vogesella* (P = 0.01) and *Cloacibacterium* ($P = 0.02$) contributed the most to dissimilarity among parous flies, while only *Endobacter* significantly contributed to dissimilarity among nulliparous flies (Fig. 7c). According to the health district of origin of the blackflies processed, 15 genera were differentially associated with a dissimilarity effect (Fig. 7a). *Wolbachia* (P < 0.0001) and Vogesella (P < 0.0001) contributed the most to dissimilarity in Bafia health district, while *Rosenbergiella, Pantoea, Moheibacter* and *Erwinia* contributed significantly to dissimilarity in Yabassi health district. Ndom health district had the largest number of genera affecting dissimilarity, including *Asaia, Saccharibacter* and *endobacter*.

4. Discussion

The aim of this study was to characterise the gut microbiota of *Simulium damnosum s.l.*, and to assess its dynamics over time and the effects of some biological and ecological features on its diversity. The evaluation of the entomological indices revealed a relatively high parity rate (12.3 %), reflecting the ratio of blackflies that had completed at least one reproductive cycle among the overall sampled population. This ratio ranging from 5.1 % in Ndom health district to 20.1 % in Bafia health district indicates an important vector-host contact in the surveyed health districts and the high risk of *O. volvulus* transmission, since only females that have laid eggs at least once can harbour *O. volvulus* larvae and transmit the infective stage of the parasite during a subsequent blood meal. This suggests that transmission may be more effective in Bafia health district compared to Ndom health district, as confirmed by infection indices. Indeed, the highest infectivity rate, reflecting the proportion of blackflies capable to transmit the infective larval stage (L3), was obtained in Bafia health district. These results reinforce the evidence of very active transmission of onchocerciasis as previously

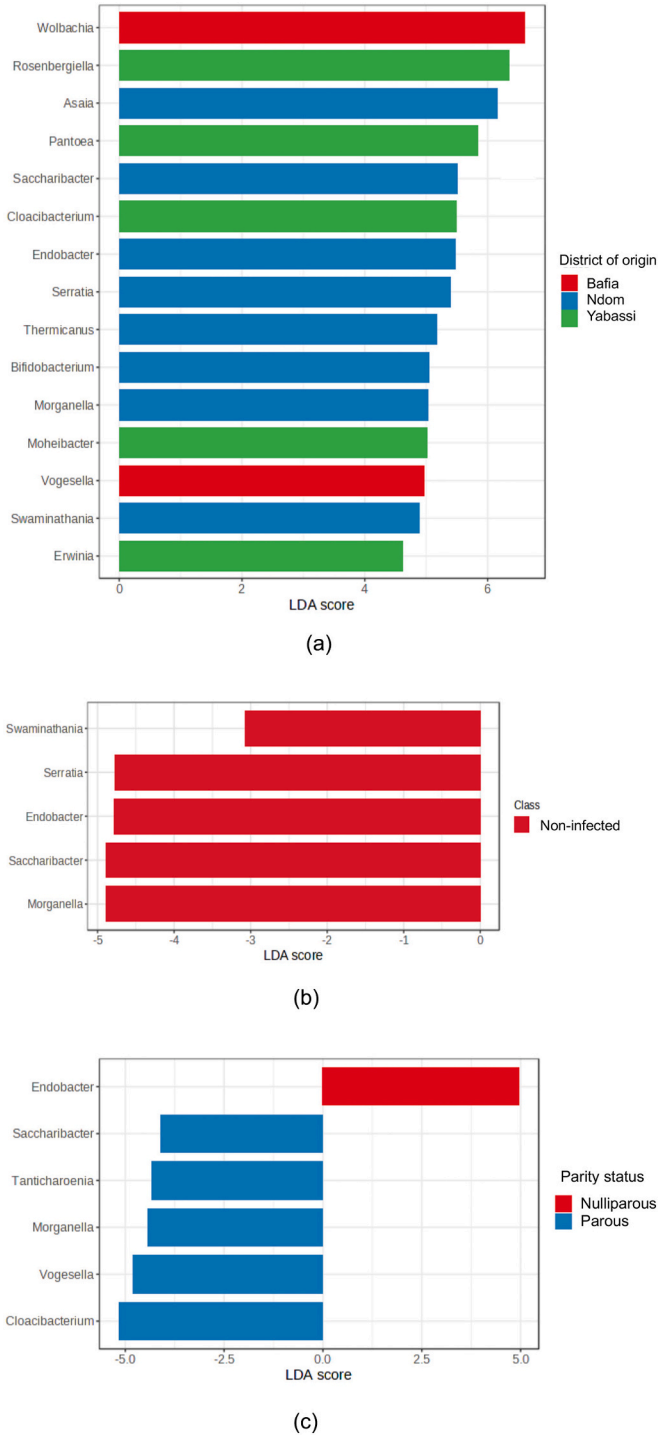

Fig. 7. The Linear Discriminant Analysis Effect Size (LEfSe) performed on relative abundance data of the bacterial community of blackflies according to the health district of origin (a), according to the infection status of the blackflies (b) and parity status (c). Only taxa enriched in each group with an LDA score > 3 are considered significant.

reported (Kamga et al., 2016; Nana-Djeunga et al., 2022; Domche et al., 2023; Efon-Ekangouo et al., 2023), and classification of the Bafia and Yabassi health districts as the meso-endemic zones for onchocerciasis despite more than two decades of mass ivermectin administration (Kamga et al., 2016; Tekle et al., 2016).

The characterization of *S. damnosum s.l.* gut microbial communities revealed *Proteobacteriae* as the predominant phylum, with a relative abundance of 97.5 %. The overwhelming predominance of this phylum which had also been reported in several arthropods of medical interest (Correa and Ballard, 2016; Caragata and Moreira, 2017; Jacob et al., 2017; Tsagmo Ngoune et al., 2019) can be explained by the importance of its role in energy metabolism and stress management in a variety of organisms and particularly in arthropods (Emerson et al., 2007; Heddi and Gross, 2012; Fiebig et al., 2015). *Proteobacteriae* is a heterogeneous phylum consisting of two bacterial classes essentially dominated by *Alphaproteobacteria* which accounted for 86.4 % of all taxa, with remarkable genomic plasticity associated with different modes of life (intracellular, facultative and free-living) (Le et al., 2014). Indeed, *Alphaproteobacteria* integrates environmental signals and consequently control the transcription of genes that ensure growth and survival under a range of stress conditions through a molecular system including the sigma factor σEcfG, its anti-σ factor NepR and the anti-anti-σ factor PhyR (Fiebig et al., 2015; Esposti and Romero, 2017). These biological features associated with the genetics of *Alphaproteobacteria* may therefore constitute a potential source of great interest in the adaptive repertoires of blackflies.

Wolbachia was the predominant genus identified, exhibiting a very high relative abundance of 78.6 % among all the taxa characterized across the blackfly populations analysed in this study. This intracellular endosymbiont, which is maternally inherited in arthropods, has already been described in more than 65 % of insect species and is also widespread in other invertebrates such as arachnids and crustaceans (Werren et al., 1995; Hilgenboecker et al., 2008; Ahantarig and Kittayapong, 2011). Wolbachia is known to play a major role in modulating the host's reproductive capacity to enhance its own spread (Duron and Hurst, 2013; Correa and Ballard, 2016). However, phylogenetic analyses indicate that the Wolbachia strain characterized from Simuliidae (wDam) does not derive from any of the known *Wolbachia* supergroups or lineages, appearing instead as an isolated lineage (Crainey et al., 2010, 2017). This may imply different or additional biological characteristics in Simuliidae compared with other clade groups. Furthermore, *Wolbachia* is also an important endosymbiont described in several filarial nematodes including *Onchocerca volvulus* (Foster et al., 2005; Coulibaly et al., 2009; Ahantarig and Kittayapong, 2011).

The differences in the representativeness and abundance of Wolbachia across various sampling zones (spatial variability) may be attributed to the heterogeneity of the studied *Simulium* population. Previous cytotaxonomic evaluations in the study area identified the presence of at least two cytospecies within the *S. damnosum* complex: *S. squamosum* and *S. mengense* (Traore-Lamizana et al., 2001). It is therefore possible that this symbiosis is not obligatory for other *Simulium* subspecies or some of these subspecies may exhibit varying levels of susceptibility or tolerance to *Wolbachia*, resulting in the potential absence or presence of this bacterium in the microbiota. This hypothesis should be explored further in future studies.

The primary gut microbiota of *S. damnosum s.l.* processed in this study comprises five genera, including *Rosenbergiella, Pantoea* and *Asaia*, which are known for their environmental origin. Indeed, these bacteria have mainly been described as originating from the nectar of numerous flowers such as *Amygdalus communis* (almond) and *Citrus paradisi* (grapefruit) (Halpern et al., 2013; Lenaerts et al., 2017) which are commonly found in the studied areas. These bacteria are probably ingested by female adults' blackflies during their sweet meal, which is a crucial energy resource for the flight. Furthermore, *Asaia* is an important endosymbiont of many vector-borne arthropods, notably *Anopheles coluzzii* and *An. stephensi* where its presence is associated with increased susceptibility to the insecticide deltamethrin, thus it importance for the development of a para-transgenic-based vector control approach for malaria elimination (Favia et al., 2007, 2008).

The alpha diversity of *Simulium* microbiota, when considering *Wolbachia*, was significantly higher in Ndom health district compared to Yabassi and Bafia health districts, and Bafia presented the lowest diversity of the three health districts. This could be explained by the

overabundance of the *Wolbachia* and *Rosenbergiella* genera in the microbiota of *Simulium* from Bafia and Yabassi health districts, respectively. These bacteria have a "crushing effect" on taxa that are poorly represented, thereby reducing diversity. The hypothesis of the "crushing" effect is reinforced by the reduced bacterial diversity as the consequence of the *O. volvulus* infection. Indeed, the increase in the Shannon index among infected flies compared to uninfected parous ones underlines the effect of the increased abundance of *Wolbachia* of parasitic origin in the microbiota of blackflies. This hypothesis is also reinforced by considering the evolution of the diversity of the *Simulium* microbiota when Wolbachia is excluded. Indeed, the relaxation of the crush effect allows the alpha and beta diversity of the *Simulium* to increase independently of the biological status (parity or infection status), the origin of the *Simulium* or the time of sampling.

The clustering of blackflies guts bacterial community profiles according to health district of origin, parous/nulliparous and infected/non-infected status was demonstrated by principal component analysis of the distances in the Bray-Curtis matrix. A significantly high dissimilarity in the gut microbial composition of blackfly populations originating from the Bafia health district compared with those from the Yabassi and Ndom health districts; similar dissimilarity was found among parous flies compared with nulliparous ones. This confirms the effect of environmental and biological features on the variability of the gut microbial composition of blackflies. The dissimilarity in the gut microbial composition of blackflies collected in the Bafia health district in two consecutive years (2019 and 2020), highlighted by the reduction in the alpha diversity index between the two years, demonstrated the temporal dynamics in microbial composition. This dynamic ecology of microbiota diversity is not intrinsically associated with simuliidae; this evidence has been demonstrated in the gut microbiota of mosquitoes (Novakova et al., 2017), humans (Turroni et al., 2017; Anderson et al., 2022), other mammals (Liu et al., 2019; Arfken et al., 2020) and results from a combined effect of complex changes in diet, stress and other bioecological conditions.

The study of bacterial genera (biomarkers) specifically associated with the infectious status of *S. damnosum s.l.* (excluding the *Wolbachia* effect) revealed a significant association between the presence of certain genera (*Serratia, Saccharibacter, Endobacter* and *Morganella*) and the absence of *O. volvulus* in the abdomen/thorax of blackflies, thus potentially reducing the competence of blackflies to host and mature the *Onchocerca volvulus* infection. Of these biomarkers, only the genus *Serratia* has been described in other arthropod vectors and its impact on parasite establishment appears to be vector-dependent. In mosquitoes, *Serratia odorifera* has been associated with the susceptibility of *Aedes aegypti* to chikungunya virus (Apte-Deshpande et al., 2014) and dengue virus (Apte-Deshpande et al., 2012). In addition, other studies have demonstrated the ability of *Serratia marcescens* to produce certain trypanolytic compounds that increase the resistance of *Rhodnius prolixus* to infection by *Trypanosoma cruzi* (Azambuja et al., 2004). However, further research is needed to decipher the host-bacteria interactions and to assess whether the biological role is mediated by a single bacterial species or by a synergy of a complex of bacterial genera. It will represent a step forward in the development of a transgenic vector control strategy for the elimination of onchocerciasis.

5. Conclusions

The present study was designed to determine the composition of the blackfly gut microbiota and to identify the effect of biological and environmental features on its variability. We found that the composition of the gut microbiota changes significantly depending on the area where blackflies were originated from. The diversity of the microbiota is dynamic over time and is influenced by the presence of *O. volvulus* larval stages, and the parity rate used as a proxy of blood feed. Four taxa, including *Serratia*, were associated with the absence of *O. volvulus* and are therefore suspected of negatively influencing parasite development. The role of these potential biomarkers on blackfly physiology needs to be further investigated in order to prompt the design of transmission-blocking approaches to control onchocerciasis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2024.105683.

List of abbreviations

OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction.

Ethics approval and consent to participate

Simulium samples were collected using the human landing technique, which requires volunteers. Consequently, ethical approval N°1011/CRERSH/C/2020 was granted by the Centre's Regional Ethics Committee for Human Health Research. Participation was voluntary and volunteers were free not to participate without fear of reprisal. The volunteers recruited lived at the sampling sites, so they were no more exposed to simulium bites than usual. In addition, volunteers were trained to capture the flies before being bitten. Finally, they were given preventive treatment with ivermectin.

Funding

This study was supported by the French National Research Institute for Sustainable Development (IRD) and a grant from the United States Agency for International Development (USAID) and UK aid from the British people (UK aid) through the Coalition for Operational Research on Neglected Tropical Diseases (COR-NTD) and administered by the African Research Network for Neglected Tropical Diseases (ARNTD) [grant number: Ref: SGPIII/0210/499]. Its contents are solely the responsibility of the author(s) and do not necessarily represent the views of IRD, USAID, UK aid, COR-NTD or ARNTD.

CRediT authorship contribution statement

Arnauld Efon-Ekangouo: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hugues C. Nana-Djeunga:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Philippe B. Nwane:** Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Narcisse Nzune-Toche:** Writing – review & editing, Methodology, Investigation. **Jeanne C. Sondi-Dissake:** Writing – review & editing, Methodology, Investigation. **Guilhem Sempere:** Writing – review & editing, Visualization, Validation, Software, Resources, Methodology, Formal analysis, Data curation. **André Domche:** Writing – review & editing, Methodology, Investigation. **Flobert Njiokou:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Joseph Kamgno:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Paul Moundipa-Fewou:** Writing – original draft, Validation, Supervision, Resources, Methodology, Conceptualization. **Anne Geiger:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interests.

Data availability

Data will be made available on request.

Acknowledgements

We are thankful to the and local authorities who hosted the research team in their villages and to the volunteers devoted their time and energy to collect the sample. This work has been technically supported by the South Green Bioinformatics platform and the CIRAD - UMR AGAP HPC Data Centre.

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