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# Analysis of Multiple Tsetse Fly Populations in Uganda Reveals Limited Diversity and Species-Specific Gut Microbiota

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**The invertebrate microbiome contributes to multiple aspects of host physiology, including nutrient supplementation and immune maturation processes. We identified and compared gut microbial abundance and diversity in natural tsetse flies from Uganda using five genetically distinct populations of *Glossina fuscipes fuscipes* and multiple tsetse species (*Glossina morsitans morsitans*, *G. f. fuscipes*, and *Glossina pallidipes*) that occur in sympatry in one location. We used multiple approaches, including deep sequencing of the V4 hypervariable region of the 16S rRNA gene, 16S rRNA gene clone libraries, and bacterium-specific quantitative PCR (qPCR), to investigate the levels and patterns of gut microbial diversity from a total of 151 individuals. Our results show extremely limited diversity in field flies of different tsetse species. The obligate endosymbiont *Wigglesworthia* dominated all samples (>99%), but we also observed wide prevalence of low-density *Sodalis* (tsetse's commensal endosymbiont) infections (<0.05%). There were also several individuals (22%) with high *Sodalis* density, which also carried coinfections with *Serratia*. Albeit in low density, we noted differences in microbiota composition among the genetically distinct *G. f. fuscipes* flies and between different sympatric species. Interestingly, *Wigglesworthia* density varied in different species ($10^4$ to $10^6$ normalized genomes), with *G. f. fuscipes* having the highest levels. We describe the factors that may be responsible for the reduced diversity of tsetse's gut microbiota compared to those of other insects. Additionally, we discuss the implications of *Wigglesworthia* and *Sodalis* density variations as they relate to trypanosome transmission dynamics and vector competence variations associated with different tsetse species.**

The collection of microbes associated with a eukaryotic organism is termed the microbiota. The human gastrointestinal tract alone houses an estimated $10^{14}$ microbes that belong to over 500 distinct microbial taxa (1). The gut microbiota can contribute to various aspects of host physiology, ranging from facilitating the digestion of complex substrates and providing colonization resistance against pathogenic organisms to priming the development and maturation of host immune responses during development (2). While the complexity of the mammalian microbiome hinders the analysis of multifaceted interactions, the insect gut includes a less diverse community of organisms (3, 4). Because of their reduced microbiota complexity, insects are good experimental models to pinpoint the relative contribution(s) of individual microbes to host physiological processes. These microbes provide nutrients to their insect hosts and, moreover, influence their hosts' immunity, including their vector competence (ability to transmit disease-causing pathogens) (5, 6).

The majority of insects acquire pathogens during the feeding process that subsequently undergo development and/or establish infections in the host gut in close proximity to resident microbiota. In the laboratory, removal of the gut microbiota through supplementation of the host diet with antibiotics renders mosquitoes highly susceptible to infection with *Plasmodium* (7). In natural mosquito populations, analysis of the microbiota has shown a higher abundance of *Enterobacteriaceae* in *Plasmodium*-infected individuals than in uninfected flies (8), although a direct correlation between the composition of the microbiota and pathogen transmission remains to be determined. In a different study, an *Enterobacter* strain identified in the guts of natural populations of *Anopheline* mosquitoes interfered with *Plasmodium* transmission in laboratory experiments through the synthesis of reactive oxygen species (ROS) (9). In several other studies in mosquitoes, newly established infections with symbiotic *Wolbachia* increased host resistance to viruses, such as dengue virus (8, 10, 11) and West Nile virus (12), as well as to parasites, such as *Plasmodium* (13, 14). The introduction of novel *Wolbachia* infections into naive hosts is thought to prime host immune responses, which in turn restricts the ability of the host to transmit pathogens (15, 16). Thus, harnessing host-microbiota interactions has emerged as a novel vector control method to decrease pathogen infections. In particular, several paratransgenic systems have been developed that take advantage of modified microbiota cultures expressing antipathogen products, with the aim of repopulating insect guts to increase host resistance to pathogens (17, 18). The success of these methods requires identification of the full spectrum of organisms in the microbiota and a good understanding of the host-microbe-pathogen interactions.

This study focuses on tsetse flies (Diptera: Glossinidiae), which

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are vectors of African trypanosomes, the causative agents of human African trypanosomiasis (HAT) and animal African trypanosomiasis (AAT) across sub-Saharan Africa. Several aspects of tsetse's physiology are unique among Diptera. Both sexes only feed on vertebrate blood and can transmit disease. Also, tsetse reproductive physiology differs from that of other dipteran vectors in that the mother carries an intrauterine larva and produces a single, fully mature larval progeny per gonotrophic cycle. The progeny receive nutrients in the form of milk secreted by differentiated accessory glands (milk gland). Two endosymbiotic organisms associated with tsetse's digestive tract have been described to date: the obligate mutualist *Wigglesworthia glossinidia* (herein referred to as *Wigglesworthia*) (19) and the facultative commensal species *Sodalis glossinidius* (herein referred to as *Sodalis*) (20). The mutualist *Wigglesworthia* resides intracellularly in bacteriocytes, which collectively form an organ called the bacteriome that is attached to the epithelial cells of tsetse's anterior midgut (19). In contrast, *Sodalis* is more widely distributed throughout the fly tissues and compartments, including the gut epithelia and lumen (21), hemolymph, milk gland organ, and salivary glands (22). Both endosymbionts are also found extracellularly in the lumen of female milk glands and are vertically transmitted to intrauterine larvae in milk secretions during larval nourishment (23). While all individuals in different tsetse species carry *Wigglesworthia*, the prevalence of *Sodalis* in natural populations varies (24, 25). Previous studies have found a positive correlation between the presence of *Sodalis* and trypanosome infections in natural tsetse populations (reviewed in reference 26), and various *Sodalis* genotypes have been found in different tsetse species (27, 28). These observations, together with the fact that *Sodalis* can be cultured *in vitro* (29) and genetically modified (30), make this bacterium a good candidate for paratransgenic applications to reduce tsetse's trypanosome transmission ability (17, 31).

Although 16S rRNA gene clone library data suggested that the gut microbiota of *Glossina morsitans morsitans* wild-type (WT) colony flies includes only the two symbiotic microbes (21), analysis of natural populations using direct cultivation methods and clone libraries showed broader diversity (reviewed in reference 26). *Glossina fuscipes fuscipes* flies from western Kenya harbor low-frequency infections with 23 bacterial species, 69.5% of which correspond to the phylum *Firmicutes* (32). In *Glossina palpalis palpalis* in Angola, bacteria from three families (*Enterobacteriaceae*, *Moraxellaceae*, and *Enterococcaceae*) and one species of *Serratia* (33) were identified using culture-dependent methods, while similar studies in Cameroon reported 16 genera from *G. p. palpalis* (34, 35). While these studies have identified a number of different microorganisms residing in the guts of individual tsetse flies of different species, the relative abundances of these microbes, the number of taxa shared across individual flies, and the bacterial species that make up the core microbiome remain to be investigated.

The *palpalis* and *morsitans* subgenera of tsetses differ in their ability to transmit pathogenic trypanosomes, with *palpalis* group individuals generally displaying greater parasite resistance than their *morsitans* counterparts (36). Both *G. m. morsitans* and *Glossina pallidipes* belong to the *morsitans* subgenus, while *G. f. fuscipes* is a member of the *palpalis* subgenus. It remains to be seen whether differences in microbiota composition associated with tsetse species could contribute to varying parasite transmission dynamics. Here, we used deep sequencing of the 16S rRNA V4 hypervariable region and 16S rRNA gene clone libraries to analyze the diversity of gut-associated microbes in individuals from five natural populations of *G. f. fuscipes* across Uganda. We also sampled from one sympatric location in Uganda with multiple tsetse species (*G. f. fuscipes*, *G. m. morsitans*, and *G. pallidipes*) to compare the resident gut microbiota between different species. We used quantitative PCR (qPCR) to validate the deep-sequencing data and to compare the relative densities of the two endosymbionts, *Sodalis* and *Wigglesworthia*. We discuss our findings of the microbiota present in natural populations in light of the various vector competencies, host preferences, and ecological niches the different tsetse species display.

## MATERIALS AND METHODS

**Biological materials.** Flies from the species *G. f. fuscipes*, *G. m. morsitans*, and *G. pallidipes* were collected in the Murchison Falls State Park (MF) area in western Uganda. For *G. f. fuscipes*, samples were also obtained from Busime (BU), Kaberamaido (KB), Dokolo (DK), and Otubio (OT), using biconical traps baited with cow urine and acetone (Fig. 1). The age of all flies analyzed was estimated based on wing fray score (37), and only those with a score of ≥2 (estimated to be 3 to 4 weeks old) were included for further analysis, to ensure that flies had acquired multiple blood meals. The location of the traps, methods used for sex and wing fray analysis (used as a proxy for age), surface sterilization process, tissues available for each sample, and gut dissection process for excluding bacteriomes are described in Table S1 and Materials and Methods in the supplemental material. Following the sterilization process, the dissected guts, with and without the bacteriome, and the corresponding carcasses were stored in individual tubes in 100% ethanol until DNA extraction. In total, we analyzed DNA from 129 whole guts (49 male and 80 females), 18 guts without bacteriomes, and 4 carcasses of females.

**Genomic DNA extraction and PCR assay for symbiont and trypanosome detection and quantification.** Total DNA was extracted with the MasterPure complete DNA purification kit (Epicentre, Madison, WI, USA), using sterile water as the template during each DNA extraction batch to detect possible contamination. The quality of the prepared DNA was first assessed by PCR amplification of a 500-bp fragment of the tsetse β-tubulin gene, using primers and protocols detailed in Table 1. The PCR cycling conditions for the tsetse β-tubulin gene were as follows: initial denaturation at 94°C for 2 min; 34 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. For *Sodalis* detection, the three genes encoding flagellin (*fliC*), outer membrane protein (*ompC*), and hemolysin (*hemA*) (42, 43) were used, and samples were considered positive only when two independent reactions were positive for at least two of the genes tested (see Table S1 in the supplemental material). The PCR cycling conditions for *fliC* were as follows: initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The conditions for *ompC* were as follows: initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The conditions for *hemA* were as follows: initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. For trypanosome infection, internal transcribed spacer 1 (ITS-1; the spacer between 5.8S and 25S rRNA [41]) was used, which can also differentiate certain parasite species based on amplicon size. The ITS-1 cycling conditions were as follows: initial denaturation at 94°C for 3 min; 4 cycles of 94°C for 40 s, 58°C for 50 s, and 72°C for 1 min; 8 cycles of 94°C for 40 s, 56°C for 50 s, and 72°C for 1 min; 23 cycles of 94°C for 40 s, 54°C for 50 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. *Sodalis* and *Wigglesworthia* densities were determined by qPCR using *fliC*- and *thiC*-specific primers, respectively (39, 40). *Wigglesworthia* cells are polyploid (39), and thus, in our analysis, we measured the genome copy number normalized to the tsetse host β-tubulin gene (38) level as described pre-

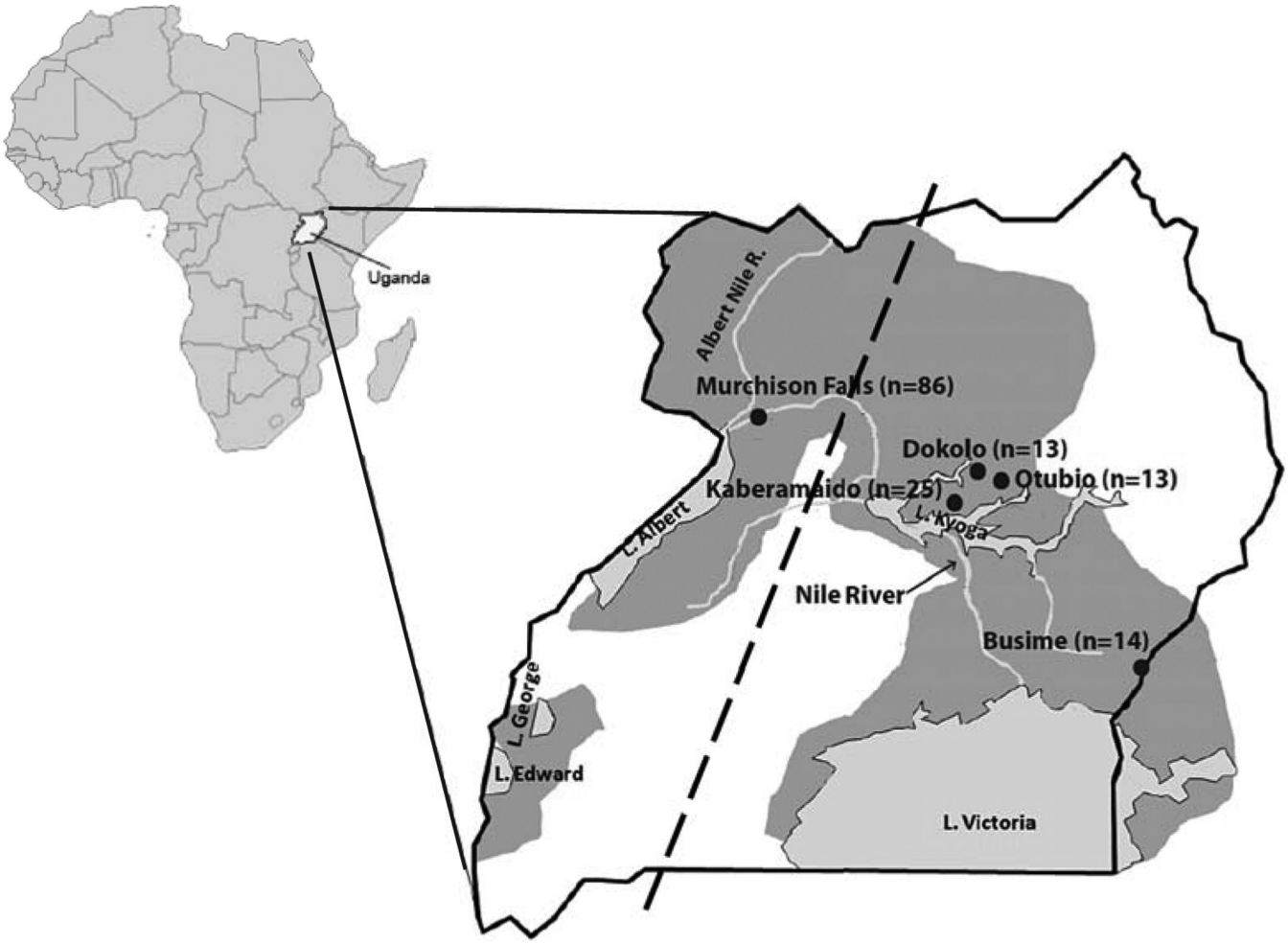


**FIG 1** Map of Uganda showing the locations of the tsetse sampling sites analyzed in this study. The position of Uganda on the African continent is also shown. The dotted line demarcates the separation of *Trypanosoma brucei gambiense* areas of endemicity in the northwest from the *Trypanosoma brucei rhodesiense* areas of endemicity in the southeast of Uganda. The area shaded in dark gray represents the range of *Glossina fuscipes fuscipes*. Light gray areas identify major bodies of water. Sampling site names are reported, and the number of tsetse flies from each site is shown. For Murchison Falls, the number of individuals assayed included samples from three different *Glossina* species (*Glossina morsitans morsitans*, $n = 10$; *Glossina pallidipes*, $n = 53$; *G. f. fuscipes*, $n = 11$). For all other sites, only *G. f. fuscipes* individuals were sampled. Maps were downloaded from "Uganda" article in South African History Online (www.sahistory.org.za).

viously (45). The quantitative PCR conditions were as follows: initial denaturation at 95°C for 8 min; 40 cycles of 95°C for 15 s, 55°C for 30 s, 95°C for 1 min, and 55°C for 1 min; and a melt curve of 55°C to 95°C in increments of 0.5°C for 30 s. A similar method was applied to *Sodalis* quantification. Student's *t* test was employed to evaluate statistical differences in symbiont densities from the qPCR data. The procedures, all PCR primers, and the amplification conditions used are described in Table 1. Information on reaction efficiencies for quantitative PCR analysis, including standard curves and melt peaks, is included in Fig. S6 in the supplemental material.

**16S rRNA gene clone library.** The 16S rRNA gene clone library was constructed using DNA from the guts of 7 individual tsetse flies (4 *G. pallidipes*, 2 *G. m. morsitans*, and 1 *G. f. fuscipes*) that had relatively low *Wigglesworthia* densities. A eubacterium-specific primer set (27F-1429R) (Table 1) (44) was used to generate 1,402-bp PCR amplification products, which were digested with the restriction enzymes TaqI and HaeIII (New England BioLabs) and cloned into the pGEM-T vector (Promega) and transformed into *Escherichia coli* DH5α cells. The PCR conditions for eubacterial 16S rRNA were as follows: an initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 30 s, 49°C for 30 s, and 72°C for 90 s; and a final extension at 72°C for 10 min. DNA prepared from the individual clones was PCR amplified using T7 and Sp6 primers, the amplification product was digested with the restriction enzymes TaqI and HaeIII, and the profiles generated were visualized on a 2.0% Metaphor agarose gel stained with ethidium bromide (see the example shown in Fig. S7 in the supplemental material) (21). All clone inserts that displayed restriction profiles different from that generated by the *Wigglesworthia*-specific sequence were subjected to Sanger sequencing at the Yale DNA Analysis Facility on Science Hill. The resulting DNA sequences were compared to the NCBI BLAST database and the Ribosomal Database Project II (RDP) (http://rdp.cme.msu.edu/) using CLC Genomics Workbench (version 6). It is possible that these enzymes may generate a similar restriction profile from bacteria other than *Wigglesworthia*, in which case they would have been excluded from our sequence analysis. However, our previous efforts with sequencing the 16S rRNA gene clones without prior selection had resulted in the identification of only *Wigglesworthia* and *Sodalis*, given the dominance of these organisms in the gut.

**Illumina library preparation.** Library preparation was carried out using Illumina fusion primers (46). A description of the Illumina sequencing and barcoded fusion primers used for this project can be found at (ftp://ftp.metagenomics.anl.gov/data/misc/EMP/SupplementaryFile1_barcoded_primers_515F_806R.txt). Each sample was assigned a unique

**TABLE 1** List of PCR primers used in this study

| Primer | Target | Sequence | Product size (bp) | Reference or source |
|---|---|---|---|---|
| qPCR-β-Tubulin-F | Tsetse β-tubulin gene | 5′-CCATTCCCACGTCTTCACTT-3′ | 151 | 38 |
| qPCR-β-Tubulin-R | | 5′-GACCATGACGTGGATCACAG-3′ | | |
| qPCR-Wig-thiC-F | *Wigglesworthia thiC* (thiamine biosynthesis gene) | 5′-AAGTTATGATAGAAGGACCAGGAC-3′ | 130 | 39 |
| qPCR-Wig-thiC-R | | 5′-CCCGGAGCAATATCAGTAGTTAG-3′ | | |
| qPCR-α-Tubulin-F | *Trypanosoma* α-tubulin gene | 5′-GAGAAGGCCTACCACGAGC-3′ | 128 | This study |
| qPCR-α-Tubulin-R | | 5′-CCACGGTACATGAGGCAGC-3′ | | |
| qPCR-Sod-fliC-F | *Sodalis fliC* (flagellin) | 5′-GAAGGTGTGATTTCTACGGGCAAAG-3′ | 169 | 40 |
| qPCR-Sod-fliC-R | | 5′-GCGACTGGCTCAGGTTGGC-3′ | | |
| KIN-1-F | *Trypanosoma* (internal transcribed spacer-1) | 5′-GCGTTCAAAGATTGGGCAAT-3′ | | 41 |
| KIN-2-R | | 5′-CGCCCGAAAGTTCACC-3′ | | |
| Sod-fliC-F | *Sodalis fliC* (flagellin) | 5′-GCAGTTTCAGGATACCC-3′ | 508 | 42 |
| Sod-fliC-R | | 5′-GGCGGAAAATGGTATAG-3′ | | |
| Sod-opmC-F | *Sodalis ompC* (outer membrane protein C) | 5′-CGGCATGCGCTATATGTCTA-3′ | 900 | 42 |
| Sod-opmC-R | | 5′-GTCACGGGTGAAGTCGTTTT-3′ | | |
| Sod-hemA-F | *Sodalis hemA* (hemolysin A) | 5′-ATGGGAAACAAACCATTAGCCA-3′ | 650 | 43 |
| Sod-hemA-R | | 5′-TCAAGTGACAAACAGATAAATC-3′ | | |
| 16S Eubacterial-F | 16S rRNA | 5′-AGAGTTTGATCCTGGCTCAG-3′ | 1,465 | 44 |
| 16S Eubacterial-R | | 5′-GGTTACCTTGTTACGACTT-3′ | | |
| β-Tubulin-F | Tsetse β-tubulin gene | 5′-ACGTATTCATTTCCCTTTGG-3′ | 650 | 38 |
| β-Tubulin-R | | 5′-AATGGCTGTGGTGTTGGACAAC-3′ | | |

12-bp Golay barcode located on the 806R primer. Each PCR was carried out in a 30-μl volume containing 1 μl of DNA, 0.2 μl of Phusion *Taq* (catalog number M0530L; New England BioLabs), 6 μl of 5× reaction buffer, 0.6 μl of 10 mM deoxynucleoside triphosphates (dNTPs), 0.75 μl of 10 μM forward and reverse primers (Table 1), and 20.7 μl of distilled water. The cycling conditions were as follows: 1 min of initial denaturation at 98°C; 35 cycles of 98°C for 10 s, 54°C for 15 s, and 72°C for 15 s; and a final elongation step at 72°C for 2 min. PCRs were performed in triplicate, pooled, and analyzed on a 2% agarose gel to confirm the amplification of the expected 383-bp fragment. The PCR fragments were cleaned with Agencourt AMpure XP beads (catalog number A63880; Beckman Coulter) using the manufacturer's protocol. The PCR products were quantified using the Qubit double-stranded DNA (dsDNA) high-sensitivity assay (catalog number Q32851; Life Technologies, Guilford, CT). Positive reaction mixtures were pooled at an equal molar concentration. For this project, negative extractions for the Illumina Epicenter DNA extraction kit and the PCR reagents were not included in the sequencing pool. In total, we pooled 16S rRNA gene sequences from 151 individual tsetse flies (see Table S1 in the supplemental material). The pooled sample was sent to Yale Center for Genome Analysis for analysis in a MiSeq 500-cycle sequencing run.

**Next-generation-sequencing data analyses.** The 16S rRNA gene sequence data set generated from the MiSeq run was demultiplexed, and forward and reverse reads were paired using SeqPrep. To improve the sequencing accuracy for low-diversity samples, phiX DNA was added as part of the standard procedure prior to sequencing as described in the Illumina protocol (http://res.illumina.com/documents/products/technotes/technote_low_diversity_rta.pdf). To remove the phiX spike-in contamination from the sequencing run, paired reads were mapped to the phiX genome using Bowtie2 (47). A header list of reads not mapping to the phiX genome was generated using SamTools (48), and the resulting reads were separated from the data file using the QIIME version 1.7.0 software package (49) filter_faster.py script. A barcode file was created using a custom script, parse_bc_reads_labels.py, and sequences were entered into the QIIME pipeline using the split_libraries_fastq.py command. Sequences were clustered using Uclust (50) at 97% sequence similarity against the Greengenes ribosomal database (http://greengenes.secondgenome.com/downloads). A custom reference file was created to include the corresponding V4 16S rRNA regions for *Wigglesworthia* and *Sodalis*, which were absent from the Greengenes database, and for other organisms either previously described in tsetse flies or described in the 16S clone library in this study. To remove possible chimeras and PCR errors, all operational taxonomic units (OTUs) that did not align to the Greengenes database were removed from the analysis. Representative sequences were picked and assigned to a taxon using RDP Classifier 2.2 against the Greengenes 13_5 database. The fly samples were analyzed separately based on species, geographical location, bacteriome presence, and *Sodalis* densities. To remove low-abundance OTUs, which may be the result of spurious hits, OTUs below 0.005% were eliminated from further analysis (51).

**Alpha diversity.** For α diversity (species richness) calculations, data sets were rarefied to 15,000 sequences per sample. Alpha diversity was calculated for each location in Uganda using the metric "Observed Species" with 10 iterations at each sequencing depth to count the unique OTUs found. The number of OTUs observed at each sampling depth was averaged to generate the rarefaction curves. To compare α diversity between different species of tsetse flies (i.e., *G. f. fuscipes* versus *G. m. morsitans*) or *G. f. fuscipes* flies from different locations (i.e., DK versus KB), we used a nonparametric two-group *t* test (Monte Carlo permutations) for location comparisons.

**Beta diversity.** Beta diversity (microbiome community variation) was calculated for microbiota comparison between *G. f. fuscipes* flies from different locations and between multiple tsetse species in MF using Bray-Curtis dissimilarity (52) and Unifrac metrics (53). Bray-Curtis is based on shared OTU counts between individuals, while Unifrac measures the distance between shared and unshared branch lengths. We used jackknifed principal coordinate analysis (PCoA) to visualize differences between the microbial communities, using 10 jackknife replicates.

## RESULTS

Previous 16S rRNA gene clone library results indicated that the tsetse fly gut microbiota only contained the endosymbionts *Wigglesworthia* and *Sodalis* (21). To further investigate tsetse gut microbiota diversity, we utilized a deep-sequencing approach on the Illumina MiSeq platform. A total of 16,669,849 reads were generated for 151 barcoded individuals. After quality filtering and read merging, a total of 7,290,315 reads (average of 47,000 se-

**TABLE 2** Summary of 16S rRNA deep-sequencing results

| Sampling site or source | Tissue | Tsetse species (no. of flies) | Avg no. of 16S rRNA sequences per sample |
|---|---|---|---|
| Otubio | Gut | *G. f. fuscipes* (13) | 49,423 |
| Kaberamaido | Gut | *G. f. fuscipes* (25) | 49,692 |
| Dokolo | Gut | *G. f. fuscipes* (13) | 59,132 |
| Busime | Gut | *G. f. fuscipes* (14) | 25,369 |
| Busime | Bacteriome-removed gut | *G. f. fuscipes* (4) | 33,419 |
| Murchison Falls | Gut | *G. m. morsitans* (6), *G. pallidipes* (42), *G. f. fuscipes* (11) | 57,806, 49,893, 61,986 |
| Murchison Falls | Bacteriome-removed gut | *G. m. morsitans* (3), *G. pallidipes* (11) | 33,191, 39,182 |
| Murchison Falls | Carcass | *G. m. morsitans* (1), *G. pallidipes* (3) | 19,141, 37,574 |
| Laboratory colony | Gut | *G. m. morsitans* (3) | 58,966 |
| Laboratory colony | Infected gut | *G. m. morsitans* (2) | 42,427 |

quences per sample) (Table 2) were obtained for alignment and taxonomic assignment with the QIIME software pipeline (49).

**Microbiota diversity across multiple *G. f. fuscipes* populations.** To understand the diversity of the gut microbiota in natural populations, we obtained data from whole guts of 76 (40 male and 36 female) *G. f. fuscipes* flies from five sampling sites from southern (BU, $n = 14$), northern (OT, $n = 13$; DK, $n = 13$; and KB, $n = 25$), and western (MF, $n = 11$) Uganda (Table 2, Fig. 1) (54–56). Analysis was also completed on four *G. f. fuscipes* guts from BU after removing the *Wigglesworthia*-harboring bacteriome organ during dissection.

The 16S rRNA deep-sequencing results from *G. f. fuscipes* field samples showed that *Wigglesworthia* represented the majority of the microbiota (BU, 99.97%; DK, 99.92%; KB, 99.87%; and OT, 99.91%) (Fig. 2A). The overwhelming presence of *Wigglesworthia* in field samples was also confirmed in a small set of similarly analyzed flies from the *G. m. morsitans* Yale strain (see Fig. S1 in the supplemental material). *Sodalis* was detected in all field sam-

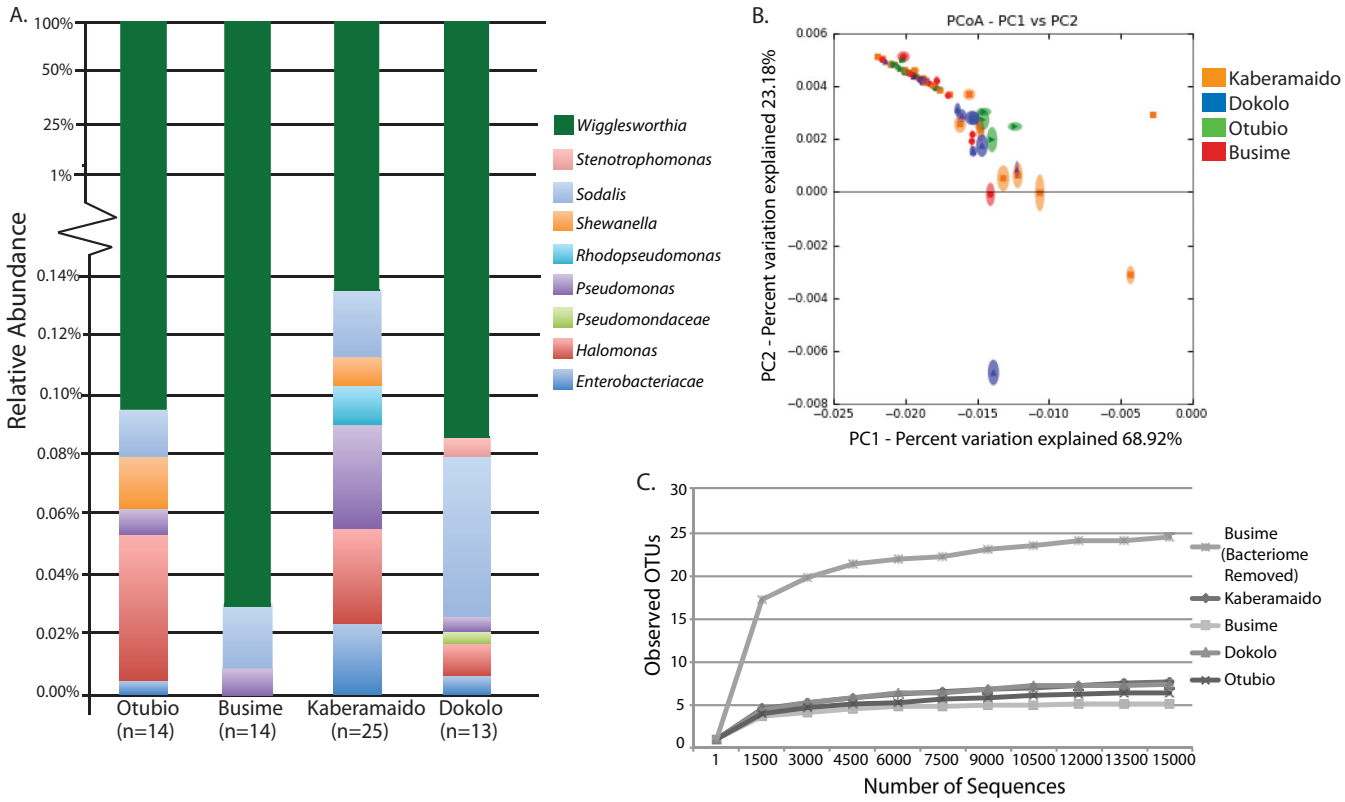


**FIG 2** 16S rRNA analysis of *G. f. fuscipes* flies from different populations in Uganda. (A) Microbiota taxon summary for *G. f. fuscipes* flies from four locations. Individual taxonomy summaries were averaged for each location to represent the core microbiome for that particular sampling site. (B) We performed jackknifed principal coordinate analysis (PCoA) using the Bray-Curtis dissimilarity distance metric to uncover bacterial composition differences between locations. The Bray-Curtis dissimilarity metric is based on shared OTU counts between samples. Only principal coordinate 1 (PC1) versus PC2 is shown here; the rest of the plots, as well as uniweighted Unifrac plots, can be seen in Fig. S2 in the supplemental material. (C) Rarefaction curves for the four distinct locations and the bacteriome-removed individuals from Busime. We used the metric "Observed Species" to determine species richness in each location and Monte Carlo permutations to calculate the *P* value (Bonferroni corrected) to determine whether species richness differences existed between each location. Data can be seen in Table S3 in the supplemental material.

ples at much lower relative abundances (BU, 0.021%; DK, 0.052%; KB, 0.022%; and OT, 0.015%), except for two flies, one each from OT and DK (see Table S2, flies OT 05 and DK 32). OT 05 did not have any *Sodalis*, while DK 32 harbored a higher relative abundance of this bacterium (0.26%). *Pseudomonas* was detected in tsetses from all four sampling sites (BU, 0.009%; DK, 0.005%; OT, 0.009%; and KB, 0.034%). However, its frequency varied in the field; for example, it was detected in only 2 of 14 individuals in BU and in 9 of 14 individuals in OT (see Table S2). In addition, we found similarities and differences in the microbiota composition among the three *G. f. fuscipes* populations from northern Uganda (Fig. 1, DK, KB, and OT). While flies from all three sites harbored low levels of both *Halomonas* (DK, 0.004%; KB, 0.031%; and OT, 0.048%) and *Enterobacteriaceae* (DK, 0.006%; KB, 0.023%; and OT, 0.004%), each location also had a few unique taxonomic groups. For instance, *Stenotrophomonas* was only found in DK (0.007%, 8/13 samples), while *Rhodopseudomonas* was only found in KB (0.013%, 18/25 samples) (see Table S2).

The 16S rRNA deep-sequencing data from the four *G. f. fuscipes* individuals from BU that had their bacteriomes removed during dissection showed, as expected, a lower *Wigglesworthia* abundance (BU 124, 14.46%; BU 92, 44.28%; BU 99, 68.56%; and BU 77, 91.31%) (see Table S2 in the supplemental material) than was found in whole-gut samples analyzed from the same population (>99%). However, even with the bacteriome removed, we still noted a high *Wigglesworthia* abundance in these samples. This could be due to either incomplete bacteriome organ removal or bacteriome rupture during the field dissection process. *Sodalis*, which was identified in almost all of the whole-gut samples at low relative abundance, was still detected at low levels in these four bacteriome-removed samples (BU 124, 0.07%; BU 92, 0.1%; BU 99, 0.05%; and BU 77, 0.3%) (see Fig. S3). Although previously unidentified organisms were discovered in this analysis, such as *Delftia*, *Erwinia*, and *Trabulsiella*, the majority of the microbiota were still dominated by two to three organisms, with only a few appearing at a depth of >1% (see Table S2). Some of the dominant microbes included bacteria from diverse families, such as *Pseudomonadaceae* (BU 124, 77%), *Ochrobactrum* (BU 92, 35%), *Proteus* (BU 99, 31%), and *Enterobacteriaceae* (BU 77, 5%). Interestingly, *Ochrobactrum* and *Proteus* were not detected in the whole-gut analysis but were found in high abundance in the bacteriome-removed individuals, BU 92 and BU 99, respectively (see Fig. S3).

We measured the α diversity (species richness) in the *G. f. fuscipes* gut samples from four sampling sites (BU, DK, OT, and KB, $n = 66$) and in four bacteriome-removed *G. f. fuscipes* guts from BU (Fig. 2C). The rarefaction curves leveled off after 5,000 sequences per sample, indicating that an adequate sequencing depth and OTU discovery were achieved. We observed significant differences in α diversity between the four geographical locations (see Table S3 in the supplemental material). Flies from the southern sampling site (BU) had the lowest species richness (5.11 ± 0.325 [mean ± standard deviation]), which was significantly different ($P = 0.006$) from the species richness present in the three northern sampling sites (OT, 6.4 ± 0.99; DK, 7.46 ± 1.16; and KB, 7.612 ± 1.57). There were no differences, however, in α diversity between *G. f. fuscipes* individuals from the three northern sites, OT, DK, and KB ($P > 0.05$). In contrast, the bacteriome-removed *G. f. fuscipes* samples from BU showed significantly higher complexity than those found in all other sampling sites ($P = 0.01$, Fig. 2C).

**Microbiota differences between sympatric tsetse species.** The gut microbiota composition data from the three tsetse species captured in the same site in Uganda (MF) also indicated that *Wigglesworthia* was the dominant taxon (*G. pallidipes*, 99.99%; *G. m. morsitans*, 99.98%; and *G. f. fuscipes*, 99.98%) and that all flies also harbored *Sodalis* infections in low abundance (*G. pallidipes*, 0.01%; *G. m. morsitans*, 0.02%; and *G. f. fuscipes*, 0.02%). In *G. f. fuscipes* flies only, low-density *Halomonas* was detected in most individuals analyzed (0.01% in 9/11 individuals) (see Table S2 in the supplemental material).

In addition to 16S rRNA deep sequencing, a random subset of samples (2 *G. m. morsitans*, 1 *G. f. fuscipes*, and 4 *G. pallidipes*) with relatively low *Wigglesworthia* densities were analyzed for their microbiota identification by using a modified full-length 16S rRNA gene clone library approach. Given that our deep-sequencing results described above and our prior results from *G. m. morsitans* colony fly guts (21) both showed overwhelming *Wigglesworthia* dominance, we applied a restriction assay to eliminate PCR amplification products corresponding to *Wigglesworthia*-specific 16S rRNA prior to cloning and sequencing, as described in Materials and Methods. Despite this enrichment, only 77 of the 141 clones analyzed were identified as non-*Wigglesworthia* sequences based on BLAST analysis of the full 16S rRNA gene sequence against the NCBI database (see Table S4 in the supplemental material). Among the genera identified were *Pseudomonas*, *Stenotrophomonas*, *Rhodopseudomonas*, *Thermoanaerobacterium*, *Sodalis*, and several uncultured bacteria, which were also present in the deep-sequencing data. In the *G. f. fuscipes* sample designated Gff22, both *Delftia acidovarans* and *Delftia lacustris* were detected, suggesting the presence of multiple species within one host.

The microbiota composition differences between the three sympatric species were also analyzed for α diversity (see Fig. S1 in the supplemental material) and β diversity (see Fig. S4). We found significant differences in α diversity between *G. pallidipes* and *G. f. fuscipes* ($P > 0.003$) (see Table S3). We also found significant differences in β diversity among all three species, as shown by the weighted Unifrac plot data (see Fig. S4). However, the β diversity differences are likely due to the *Wigglesworthia* 16S rRNA species-specific sequence variations rather than microbiome community variations.

**Microbiota composition in bacteriome-removed gut samples in MF.** The taxa identified in the bacteriome-removed gut samples from *G. pallidipes* and *G. m. morsitans* in MF are shown in Fig. S5 in the supplemental material. The two tsetse species shared similar bacterial taxa, including *Acinetobacter*, *Halomonas*, and *Cloacibacterium*, but there were also species-specific differences. For instance, *Lysobacter*, *Limnohabitans*, and *Comamonas* were uniquely associated with *G. m. morsitans*. Although our data suggest that the *G. m. morsitans* microbiota (26.9 ± 7.47 OTUs, $n = 2$) is more complex than the *G. pallidipes* microbiota (14.42 ± 1.97 OTUs, $n = 5$), this difference was not statistically significant ($P = 0.056$) (see Fig. S4 and Table S3 in the supplemental material). When we compared the bacterial diversities in *G. m. morsitans* and *G. pallidipes* bacteriome-removed samples with those of similarly analyzed *G. f. fuscipes* samples from BU, we found that some of the abundant organisms, such as *Delftia* and *Proteus*, that were detected in BU individuals were not present in flies from MF.

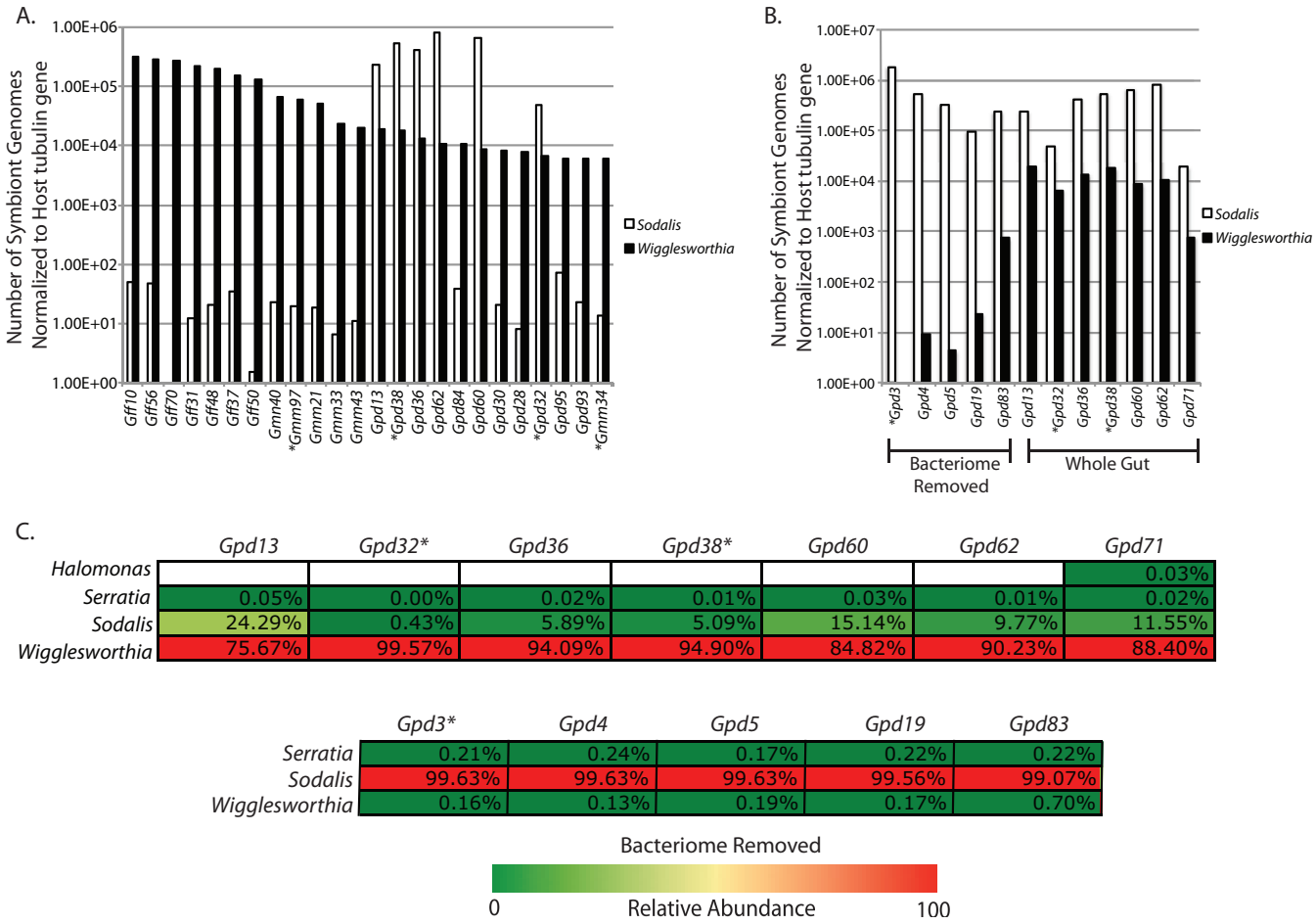


**FIG 3** Quantitative PCR and 16S rRNA analysis from Murchison Falls. (A) Symbiont genome copy numbers for *Wigglesworthia* and *Sodalis* normalized to tsetse host tubulin gene based on quantitative real-time PCR analysis of individuals from Murchison Falls. Both *Wigglesworthia* and *Sodalis* densities differed in the individuals, with *G. f. fuscipes* flies having the highest densities of *Wigglesworthia*. An asterisk by a sample designation indicates the fly was PCR positive for trypanosomes (using the ITS-1 primers). Seven (2 *G. m. morsitans* and 5 *G. pallidipes*) of the total of 73 samples from Murchison Falls tested positive for the presence of trypanosomes. Values below 1.00E+02 were extrapolated from the standard curve. In sample designations, species are indicated as follows: *Gff*, *Glossina fuscipes fuscipes*; *Gmm*, *Glossina morsitans morsitans*; *Gpd*, *Glossina pallidipes*. (B) Symbiont genome copy numbers for *Wigglesworthia* and *Sodalis* normalized to tsetse host tubulin gene based on quantitative real-time PCR analysis are shown for seven whole-gut samples and for five bacteriome-removed samples. (C) 16S rRNA deep-sequencing results corresponding to the same seven whole-gut samples and five bacteriome-removed samples for which results are shown in panel B. The number inside each box corresponds to the relative abundance of the taxon in that individual. Three of the 12 *G. pallidipes* flies with a high density of *Sodalis* were found to be positive for trypanosomes; these 3 samples are indicated by asterisks.

***Wigglesworthia* and *Sodalis* symbiont densities in natural populations.** To measure *Wigglesworthia* and *Sodalis* densities, we performed qPCR analysis on a subset of the three species analyzed from MF (Fig. 3A). Although *Wigglesworthia* densities varied between individuals in all species, *G. f. fuscipes* guts on average had a 10-fold-higher density of *Wigglesworthia* than guts from *G. m. morsitans* and *G. pallidipes* (Fig. 3A). Deep-sequencing data also indicated the presence of *Sodalis* in all samples at low levels (see Table S3 in the supplemental material). To confirm the deep-sequencing results, we performed standard PCR amplifications with three *Sodalis glossinidius*-specific genes, encoding hemolysin A (*hemA*), outer membrane protein (*ompC*), and flagellin (*fliC*). These results showed that the prevalence of *Sodalis* varied across the three species (*G. m. morsitans*, 33%; *G. pallidipes*, 53%; and *G. f. fuscipes*, 13%) (see Table S1). As the sensitivity of standard PCR amplification methods can be limiting, we used qPCR to quantify *Sodalis* densities (see Table S1), which confirmed the presence of low-level *Sodalis* infections (below $10^2$ normalized genome copies) in all individuals (Fig. 3A). However, based on qPCR results, 7 of the 33 *G. pallidipes* gut samples tested and 5 of the 14 *G. pallidipes* bacteriome-removed guts carried high levels of *Sodalis* infection, ranging from $4.8 \times 10^4$ to $1.8 \times 10^6$ normalized genome copies (Fig. 3B; see also Table S1).

Comparison of the deep-sequencing data from individuals with a high relative abundance of *Sodalis* showed the presence of *Serratia* at low abundance in all 12 samples (Fig. 3C). Interestingly, *Serratia* was absent from the 9 bacteriome-removed individuals in MF that also had low-density *Sodalis* infections (see Fig. S5 in the supplemental material). However, *Serratia* was present in other field and colony samples, including the 4 bacteriome-removed *G. f. fuscipes* samples from BU (see Fig. S3) and the *G. m. morsitans* WT laboratory strain (see Fig. S1). Given that *Sodalis* infection has been suggested to influence the parasite transmission ability of the tsetse host (26), we also tested the 12 indi-

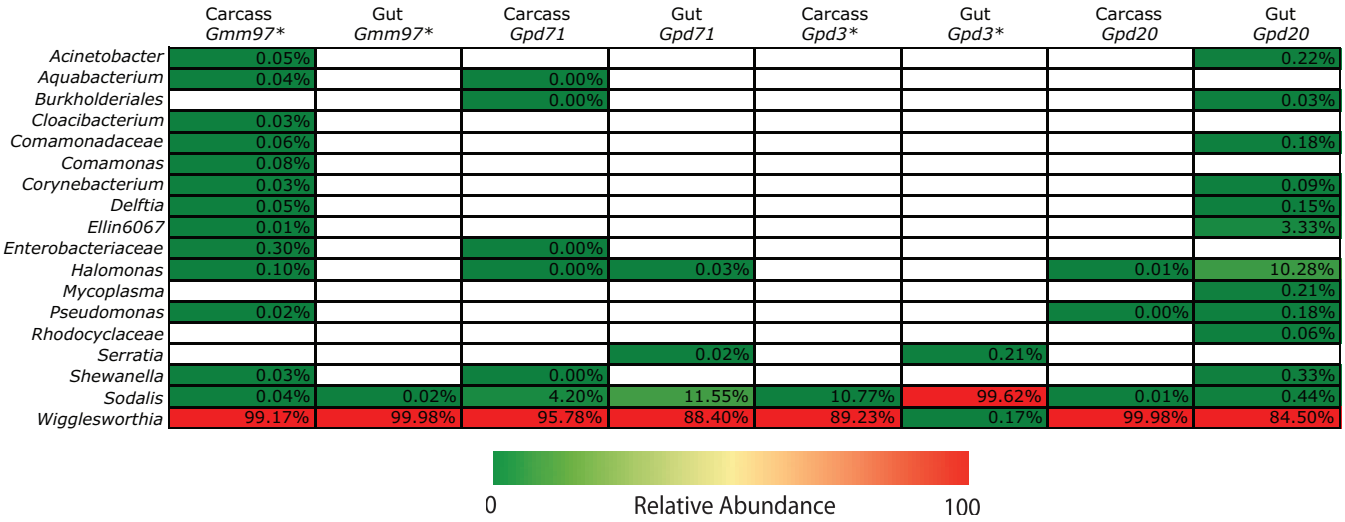


**FIG 4** Relative abundances of microbial taxa in tsetse guts and corresponding carcass tissues. Four female flies had their guts and corresponding carcasses analyzed by 16S rRNA deep-sequencing method based on quantitative real-time PCR analysis for the symbiont *Sodalis*. The number inside each box corresponds to the relative abundance of the taxon in that individual. An asterisk by a sample designation indicates the fly was PCR positive for trypanosomes (using the ITS-1 primers).

viduals with high-density *Sodalis* infections for the presence of trypanosomes using the ITS-1 primers (41). We found three of the 12 individuals to be positive for trypanosome presence (see Table S1).

**Vertical transmission of *Sodalis* to intrauterine progeny.** Based on the above-described normalized qPCR analysis, *Sodalis* gut infection levels were high for the *G. pallidipes* samples designated Gpd3 ($1.8 \times 10^6$) and Gpd71 ($1.9 \times 10^4$), medium for Gpd20 ($1.2 \times 10^2$), and low for Gmm97 (19.58) (Fig. 3B). In the laboratory line of *G. m. morsitans*, *Sodalis* is maternally transmitted to the tsetses' intrauterine larval progeny in milk secretions produced by the milk gland organ. The milk gland expands throughout the abdominal cavity of the fly (carcass) as bifurcating tubules intertwining with fat body tissue (23, 57). We selected the corresponding carcasses from these four females for deep-sequencing analysis to investigate whether they carried the maternally transmitted *Sodalis* (Fig. 4A). As expected, *Wigglesworthia*, which is also transmitted to progeny through mother's milk secretions, was present in high abundance in all four carcasses sampled (see Table S2 in the supplemental material). Beyond *Wigglesworthia*, two samples (Gpd3 and Gpd71) had relatively high *Sodalis* abundances (10% and 5%, respectively), while the abundance of *Sodalis* in the other two carcass samples was much lower (>0.1%). The latter two samples had a number of other organisms present at very low levels, some of which were also present in their corresponding guts, suggesting possible crossover from the gut contents during the dissection process.

## DISCUSSION

Our 16S rRNA deep-sequencing analysis, 16S rRNA gene clone libraries, and qPCR data indicate both spatial and host species-specific differences for the tsetse gut microbiota. Collectively, these results show that tsetse's gut microbiota is overwhelmingly dominated by its obligate endosymbiont *Wigglesworthia* (>99%). Even when the *Wigglesworthia*-harboring bacteriome organ was excluded by dissection at the time of collection, our analysis still detected only a limited microbial community in the gut. However, in addition to *Wigglesworthia*, almost all flies carried low-density infections with tsetse's commensal endosymbiont *Sodalis*, but a small subset of individuals housed high-density infections with *Sodalis* and coassociation with *Serratia*. Our qPCR results showed various *Wigglesworthia* densities in natural populations, with *G. f. fuscipes*, a species in the *palpalis* subgenus known to be highly resistant to trypanosome infections, harboring the highest density levels.

This study represents the first attempt at using a high-throughput approach to characterize microbial diversity in the tsetse fly gut. Our findings are suggestive of the difficulty associated with identifying the tsetse fly gut microbiota using a 16S rRNA deep-sequencing approach. The extreme overabundance of *Wigglesworthia* in tsetse's gut was unexpected, and this phenomenon prevented us from identifying many other bacterial taxa that may be residing in this niche. The exogenous microbes that we identified in this study represented less than 1% of the cumulative population. Some of these may have arisen from contamination from environmental sources, such as during field-based dissection processes, through extraction kit contamination, and/or because of technical limitations (e.g., index misalignment and carryover [58, 59]) of the MiSeq platform. Because the strict vertical transmission of *Wigglesworthia* generates a tractable host-symbiont evolutionary history (60), we were able to analyze the data from the three different tsetse species for the small sequence variations exhibited in their respective *Wigglesworthia* V4 regions. By way of this analysis, we detected small amounts (~0.1%) of *Wigglesworthia* carryover between the different tsetse species sampled. This outcome may have arisen from low-level contamination between samples, although pinpointing the source of this complication is difficult. However, using a combination of standard PCR and qPCR methods that target regions other than the 16S rRNA gene, we were able to confirm that *Sodalis* exhibits a wide prevalence in the samples we analyzed. Uniquely in this study, we show that the

density of *Sodalis* varies in the hosts it infects, with many individuals carrying it at low levels.

Based on mitochondrial and nuclear DNA markers, the populations of the major disease vector species *G. f. fuscipes* in Uganda are genetically differentiated and form three spatially distinct clusters in northern, southern, and western regions of the country (reviewed in reference 56). Furthermore, the *G. f. fuscipes* genetic clusters are associated with different disease belts, with the southern BU population historically transmitting *Trypanosoma brucei rhodesiense* (causative agent of the acute form of HAT) and the western MF population historically transmitting *Trypanosoma brucei gambiense* (causative agent of the chronic form of HAT) (61). Since the composition of the microbiota has been shown to influence pathogen transmission traits of several insect hosts, we analyzed the microbiota diversity of the individuals from the genetically distinct Ugandan *G. f. fuscipes* sampling sites. The deep-sequencing data showed that the gut microbiota of all *G. f. fuscipes* populations displayed low complexity, being dominated to near saturation by *Wigglesworthia* (Fig. 2A). However, there were variations in the microbiota composition beyond *Wigglesworthia* and *Sodalis* in *G. f. fuscipes* samples obtained from different genetic clusters (Fig. 2A). Based on our rarefaction curves (Fig. 2C), we had obtained enough sequencing reads to saturation levels to be able to identify all the microbial taxa present in the individuals analyzed. Some of the low-abundance organisms we identified by the deep-sequencing method had either been previously reported from tsetses based on culture-dependent methods, e.g., members of the genera *Serratia*, *Staphylococcus*, *Acinetobacter*, and *Pseudomonas* (32, 35), or represented novel microbes, some of which we also observed in the 16S rRNA gene clone library results (see Table S4 in the supplemental material). The α diversity analysis (Fig. 2C) confirmed microbiota complexity differences between the genetically distinct populations, with the composition in flies from the southern site (BU) being less diverse than in flies from the three northern sites (KB, OT, and DK).

Given that the high *Wigglesworthia* densities present in the gut could inhibit the ability to detect low-density microbes, we investigated several randomly selected individuals from BU and MF from which the bacteriome organ was removed at the time of field collection (see Fig. S3 and S5 in the supplemental material). This approach enabled the identification of a more diverse microbiota, yet individuals still harbored only 1 or 2 taxa that were represented at above 1% (see Table S2). However, some of these taxa are not present in the whole-gut deep-sequencing data (Fig. 2A). This is interesting, since the rarefaction analysis indicated that we had exceeded the necessary sequencing depth to detect all potential taxa. Thus, multiple methodologies are needed to capture the diversity of the core gut microbial community.

The difference in the bacterial diversities present in *G. f. fuscipes* flies from the southern and northern Uganda sampling sites could reflect various abiotic factors, such as humidity and wetlands, that play a role in environmental exposure in these two different habitats. Beyond abiotic factors, it remains to be seen if this difference could reflect host genetic factors resulting from the various levels of historical exposure of *G. f. fuscipes* flies from different localities to trypanosome parasites. The tsetses from the northern sites (DK, KB, and OT) represent areas previously unexposed to HAT parasites, while the southern populations are from sites where *T. brucei rhodesiense*-caused disease is historically endemic (reviewed in reference 56).

The MF area of Uganda hosts a sympatric population comprised of three *Glossina* species, *morsitans*, *pallidipes*, and *fuscipes*. Analysis of individuals from different species captured in the same traps in this sympatric population allowed us to investigate host species-specific microbiota differences, which might be epidemiologically relevant, as these taxa display various vector competence levels (36) and host feeding preferences (62). In general, species in the subgenus *palpalis* group, such as *G. f. fuscipes*, are more resistant to parasite transmission than species in the subgenus *morsitans* group, such as *G. m. morsitans* and *G. pallidipes* (36). The species in the *palpalis* group also have strong human host preferences, while the species in the *morsitans* complex typically have broader host specificity (63). Given that genetic data from Uganda suggest that tsetse individuals do not move for long distances (64, 65), we assumed that flies captured in the same locations were likely to have been exposed to similar microenvironments. Thus, we reasoned that microbiota composition differences observed in individuals of the different species in MF may arise from biotic differences associated with blood-feeding preferences or genetic differences.

Beyond the obligate *Wigglesworthia*, which was found in every individual, our results, including for the bacteriome-removed individuals, indicate the presence of only a few low-abundance organisms (see Fig. S3 and S5 in the supplemental material). Interestingly, some of these are species-specific taxa present in multiple individuals analyzed (see Fig. S3). The association between *Halomonas* and *G. f. fuscipes* was found both in MF and in the other Ugandan sites (Fig. 2A), while *Halomonas* was absent from *G. pallidipes* and *G. m. morsitans* flies. Bacterial species differences were also noted in the 16S rRNA gene clone library analysis, along with the presence of multiple species of the same genus in one individual (see Table S4).

The abundance of *Wigglesworthia* was confirmed by qPCR analysis of guts from colony and field flies (Fig. 3A; see also Fig. S1 in the supplemental material). These data also indicated variation in density levels between individuals of the different species, with *G. f. fuscipes* having higher relative densities of *Wigglesworthia* ($>10^5$) than *G. pallidipes* and *G. m. morsitans* ($<10^5$) (Fig. 3A). In laboratory studies, *Wigglesworthia* density has been shown to influence host traits for resistance to trypanosome infections through the induction of a host immune protein, peptidoglycan recognition protein LB (PGRP-LB) (66). The same host factor, PGRP-LB, was also shown to exhibit antibacterial activity *in vitro* (66, 67). Thus, the various *Wigglesworthia* densities found in natural populations could impact the vector competence of these individuals and species and the ability of other microbes to survive in their guts.

Previous experiments using standard PCR methods, which typically can amplify products present above $10^3$ copies (32), failed to detect the presence of *Sodalis* in *G. f. fuscipes* flies from several field sites in western Kenya (32) and in Uganda (68). However, the results of our qPCR-based experiments presented here indicate the presence of low-density *Sodalis* ($<10^2$ normalized genome copies) in almost all individuals, with the exception of 12 *G. pallidipes* individuals (22% of the number sampled) in which significantly higher densities were observed ($>10^4$ normalized genome copies) (Fig. 3A). Understanding how *Sodalis* outcompetes other gut microbes in a small percentage of individuals is of interest. It also remains to be seen whether the *Sodalis* species we identified show strain variation among the tsetse species and individ-

uals we analyzed, as we cannot distinguish different *Sodalis* strains based on the sequence information present in the 16S V4 region. As free-living *Sodalis* has been identified from the environment (69) and *Sodalis*-allied organisms have been reported from diverse insects (70–72), the comparison of genomic contents of the different *Sodalis* species, their routes of acquisition, and their association with different insect hosts will be of interest for future investigations.

Evaluation of the qPCR and 16S rRNA data showed that all 12 individuals with high *Sodalis* densities also had *Serratia* (Fig. 3B and C). *Serratia* has been reported in guts from *G. f. fuscipes* flies from western Kenya (32), as well as in *Glossina palpalis gambiensis* flies in West Africa (33). In laboratory studies, challenge with *Serratia marcescens* has been shown to cause high mortality in tsetses (73). In *Anopheles gambiae* mosquito midguts, multiple strains of *Serratia* have been identified, with some inhibiting *Plasmodium* infections (74). In the case of aphids, *Serratia* has been identified as a newly acquired symbiotic partner (75). We found that 3 of the 12 *G. pallidipes* individuals we analyzed also tested positive for trypanosome infection. Thus, understanding the coassociation of *Sodalis* and *Serratia* and their functional role in tsetse physiology could provide critical knowledge about trypanosome transmission dynamics in tsetses.

It remains to be seen why the tsetse gut microbiota is overwhelmingly dominated by *Wigglesworthia*, as this level of dominance by a single organism (>99%) is highly unusual and is unprecedented in other insect microbiota. A 16S rRNA-based study in the lone star tick, *Amblyomma americanum*, which also has symbiotic microbes and a restricted blood diet, identified at least 9 taxa, representing ~70% of the gut population (76). A study of natural populations of *A. gambiae* in Kenya also found the gut microbiota to be more complex than in tsetses, with 147.64 OTUs (±88.49, at a 0.04% occurrence threshold) and with refraction curves showing large variability in bacterial complexity among samples (varying from 13 to 340 OTUs) (77). The core microbial community identified in the mosquito was comprised of *Asaia*, *Burkholderia*, *Ralstonia*, and *Escherichia/Shigella* but also had several genera which overlapped with tsetses, including *Acinetobacter*, *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptococcus*, and *Serratia*. Interestingly, *Asaia* sp., which was present in all mosquito guts sampled, was absent from our analysis of tsetses, as well as from ticks (76). The differing diets and the various larval habitats can account for the microbial differences noted between tsetses and other vector species. While tsetses and mosquitoes both blood feed as adults, the mosquito diet is broader and includes sugar feeding in the adult stages. Mosquitoes also have an aquatic larval stage, which exposes them to a broad range of microbes, unlike a tsetse larva, which develops in the mother's uterus and depends entirely on the maternal blood diet for microbial exposure.

It is possible that tsetses' strict vertebrate blood diet may restrict their exposure to a broad spectrum of microbes and serve as a resource-poor diet for many microbes. In many insects, a large microbiota diversity may also serve to provide hosts with greater nutritional flexibility and protection from pathogens (78). It is possible that given the young age of the tsetse-*Wigglesworthia* symbiosis, estimated to be ~50 million years old (60), *Wigglesworthia* has not yet undergone extensive functional erosion and can provide its tsetse host with complete dietary supplementation necessary for survival on the single-vertebrate blood diet. Beyond dietary provisioning, *Wigglesworthia* has also been shown to enhance host fecundity (79) and immunity (40), further contributing to host fitness. It has been found that as mutualists undergo reductive genome evolution as a function of symbiotic age, they lose functional complexity that is present in their related free-living ancestors (80). As a host-symbiont association ages, essential host physiological functions may no longer be supplemented by the mutualistic associations that have undergone extensive loss of function. It is possible in this case that new microbial partners will be taken aboard to fulfill host fitness needs.

**Conclusions.** The invertebrate microbiome plays an important role in host nutritional physiology and can also influence the transmission of vector-borne pathogens. To date, efforts to characterize the core gut microbiota from field tsetse flies have relied on culture-dependent methods and 16S rRNA gene clone libraries. Using 16S rRNA deep sequencing on the Illumina MiSeq platform, we observed that the gut microbiota of tsetse flies from multiple species and geographic locations is taxonomically low in diversity, being dominated by the obligate symbiont *Wigglesworthia*. Our study suggests the presence of species-specific microbiota compositions in cooccurring tsetse samples and differences between microbiota of *G. f. fuscipes* flies from different geographical regions across Uganda. Interestingly, we found that the majority of flies analyzed harbored low-density infections of the commensal organism *Sodalis*, which was previously thought to be absent in flies from these field sites when they were screened using traditional PCR methods. We also found a correlation between a high density of *Sodalis* and low-density *Serratia* infections in a subset of our samples. Future studies will aim at understanding the epidemiological relevance of these field observations for trypanosome transmission biology.

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