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Faculdade de Ciências Agrárias e Veterinárias - Campus de Jaboticabal
Departamento de Patologia, Reprodução e Saúde Única

UNRAVELLING THE RELATIONSHIP BETWEEN *BARTONELLA* SPP. AND ARTHROPOD-VECTORS BY "OMICS" APPROACHES

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APRESENTAÇÃO

Este Relatório Científico refere-se ao Projeto de Pesquisa "UNRAVELLING THE RELATIONSHIP BETWEEN *Bartonella* spp. AND ARTHROPOD-VECTORS BY 'OMICS' APPROACHES", desenvolvido junto ao Departamento de Patologia, Reprodução e Saúde Única da FCAV/UNESP, Jaboticabal-SP. O relatório contempla as atividades desenvolvidas pela bolsista Dra. Michelli Inácio Gonçalves Funnicelli, sob a supervisão do Prof. Dr. Marcos Rogério André, incluindo as atividades de pesquisa realizadas durante o período de vigência da bolsa, a produção científica diretamente relacionada ao projeto, bem como outras produções e atividades científicas desenvolvidas no mesmo período.

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ORIGINAL ARTICLE

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Bacterial composition of *Polygenis* (*Polygenis*) *bohlsi bohlsi* (Wagner, 1901) (Siphonaptera: Rhopalopsyllidae) associated with *Thrichomys fosteri* (Rodentia: Echimyidae) in the Brazilian Pantanal wetland

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Abstract

Fleas (Insecta: Siphonaptera) are important vectors of zoonotic bacterial pathogens. However, the bacterial communities hosted by fleas remain poorly understood, especially regarding the interactions between nonpathogenic bacteria and pathogens, as well as the factors shaping these bacterial communities. This study aimed to explore the bacterial composition and diversity between female and male *Polygenis* (*Polygenis*) *bohlsi bohlsi* fleas collected from free-living *Thrichomys fosteri* (Rodentia: Echimyidae) in the Nhecolândia region of central-western Brazil. Here, bacterial profiling of fleas was performed using a Next-Generation Sequencing approach targeting the V3–V4 hypervariable region of the 16S rRNA gene to assess community structure. Bacterial diversity in *P. (P.) bohlsi bohlsi* fleas varied by sex, as indicated by significant differences in alpha diversity, while beta diversity showed a trend toward separation between sexes.

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Pseudomonadota was the predominant phylum in the flea microbiome. Ten bacterial genera showed differentially abundance between male and female fleas, including *Bartonella* spp., which was detected exclusively in males, and *Wolbachia* spp., which was more abundant in females than in males. Phylogenetic analysis positioned *Bartonella gltA* sequences detected in two fleas within the same clade as *Bartonella harrusi*. While the *Wolbachia wsp* and 16S rRNA sequences detected in *P. (P.) bohlsi bohlsi* grouped within the supergroups S (associated with pseudoscorpions), *gatB* sequences grouped with V (cat flea-related). These findings suggest sex-associated differences in the microbiome of *P. (P.) bohlsi bohlsi* fleas and highlight the need for further investigation into the potential role of *Wolbachia* spp. in shaping flea-associated microbial communities and their interactions with *Bartonella* spp.

KEYWORDS
endosymbionts, microbiota, pathogens, rodents, Siphonaptera

INTRODUCTION

Fleas (Insecta: Siphonaptera) are hematophagous ectoparasites that play a key role in the transmission of zoonotic bacteria, including *Bartonella* spp., *Rickettsia* spp. and *Yersinia pestis* (Leulmi et al., 2014; Silaghi et al., 2016; Vogel & Schaub, 2025). Their ability to parasitize a wide range of hosts, including wildlife, domestic animals and humans, highlights their epidemiological importance due to their potential to transmit zoonotic pathogens (Bitam et al., 2010).

The bacterial microbiota of hematophagous arthropods has emerged as an important component of vector biology. Resident microbial communities contribute to several aspects of host physiology, including nutrition, immune response, development and reproductive fitness, and may also influence interactions between vectors and the pathogens they transmit (Wang et al., 2023). Consequently, characterizing vector-associated microbiota has become an important approach for understanding factors that may affect pathogen maintenance and transmission. In the specific case of fleas, investigating these bacterial communities is crucial to understanding how they shape host physiology and interactions with flea-borne pathogens. Studies on several flea species have characterized their bacterial communities and investigated the ecological factors influencing microbiome composition (Carcauzon et al., 2025; Jones et al., 2015; Lawrence et al., 2015; Manvell et al., 2022; Matthee et al., 2023; Moore et al., 2023); however, it remains poorly characterized in most Neotropical flea species.

Rodent-associated fleas deserve particular attention because rodents (order Rodentia) host a wide range of zoonotic pathogens and are commonly associated with flea infestations, making them central to the maintenance and transmission of arthropod-borne pathogens due to their broad ecological distribution and high flea species diversity (Dahmana et al., 2020; Gutiérrez et al., 2015; Meerburg et al., 2009). Among these fleas, the genus *Polygenis* represents the most species-rich lineage within the family Rhopalopsyllidae and exhibits a broad geographic distribution across the Americas. Although most species parasitize rodents, they have also been reported in larger mammals, indicating low host specificity and a high potential for bridging pathogen transmission across different hosts (Pesenato et al., 2025). Species of this genus have been associated with the maintenance of sylvatic plague cycles involving *Y. pestis* (Holdenried, 1952; Reis Da Silva Fernandes et al., 2021) and of other zoonotic bacteria, including *Rickettsia* spp. (Melis et al., 2020) and *Bartonella* spp. (De Sousa et al., 2018).

In this context, this study aims to characterize the bacterial community associated with the poorly characterized Neotropical flea species *Polygenis (Polygenis) bohlsi bohlsi* collected from wild rodent hosts in the Brazilian Pantanal, the largest wetland in South America, and to provide baseline data for future investigations addressing the relationship between flea microbiomes and pathogen transmission dynamics.

MATERIALS AND METHODS

Ethics statement

Procedures and management protocols involving wild rodents were approved by the 'Institute Chico Mendes for Conservation of Biodiversity' (SISBIO number 64,204) and by the 'Ethics Committees on Animal Use of the School of Agricultural and Veterinary Sciences' (FCAV/ UNESP) (protocol number #11,794).

Flea sampling

Fleas were collected from wild rodents within a single habitat type during a consistent timeframe (December 2018 and February 2019), corresponding to the rainy summer season. Sampling was conducted in the Nhecolândia region (−19.050399; −56.675623), in the Pantanal wetland, Mato Grosso do Sul state, central-western Brazil. Wild rodents were captured using live traps (Tomahawk and Sherman) baited with a mixture of oats and peanut butter, arranged in transects with capture stations distributed every 10 m. Captured animals were

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chemically immobilized (ketamine hydrochloride/acepromazine combination, 9:1; 0.1 mL/100 g, intramuscularly) and handled according to the protocol previously described (Do Amaral, Cardozo, Varani, Gonçalves, et al., 2022). After the animals died, fleas were carefully removed from the hosts using entomological forceps and individually stored in sterile microcentrifuge tubes containing absolute ethanol until laboratory processing.

Morphological characterization

Flea specimens were morphologically identified to the species level using standard taxonomic keys based on external characteristics such as body size, shape, combs (ctenidia), setae patterns and genital structures (Linardi & Guimarães, 2000).

Sample processing and DNA extraction

Prior to DNA extraction, fleas were individually cleaned using a sequential surface washing process: first with 1% sodium hypochlorite for 2 min, followed by 70% ethanol for 2 min, and then rinsed with ultrapure water (Invitrogen®, Carlsbad, California, USA), repeating each step twice. Incubation was performed under agitation using the Fisherbrand Bead Mill 4 (speed = 1). The surface sterilization protocol was adapted from previously published methods (Binetruy et al., 2019; Jones et al., 2015; Kumar et al., 2022). DNA from each flea sample was extracted individually using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol. During the extraction process, two types of controls were included: homogenization control (HC) and extraction control (EC), with two replicates of each ($n = 2$ for HC and $n = 2$ for EC). The HC consisted of a microtube containing only a lysis buffer, which was kept open during the manual maceration of the fleas, simulating reagent exposure during sample processing. The EC, albeit also containing a lysis buffer, remained closed throughout the procedure and served as a negative reagent control (Lejal et al., 2020). No biological material was added to either control. The extracted DNA was eluted in 75 μL of AE buffer, of which 50 μL were preserved for NGS sequencing. The remaining volume (25 μL) was used for quantification and molecular characterization of the fleas, as well as for the detection of the genera Bartonella and Wolbachia. Noteworthy, although the sampled fleas had been superficially decontaminated, they might still have contained residual host blood and, consequently, host-derived microorganisms. As a result, the bacterial community described here likely represents a composite of flea-associated symbionts and transient taxa originating from the rodent host.

The extracted DNA was quantified by spectrophotometry (NanoDrop™; Thermo Fisher Scientific Inc., USA). Conventional polymerase chain reaction (cPCR) was performed for the flea endogenous gene (*cox1*) (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3′) (~710 bp) (Folmer et al., 1994) to confirm successful DNA extraction.

Molecular identity of fleas

For the molecular characterization of fleas, DNA from three specimens was selected and subjected to cPCR targeting the insect cytochrome c oxidase subunit 1 mitochondrial gene (*cox1*) (Folmer et al., 1994). For each reaction, negative [UltraPure DNase/RNase-Free Distilled Water (Invitrogen™ Life Technologies, USA)] and positive (*Lutzomyia longipalpis*) (Lee et al., 2024) controls were included. Sequencing of the amplified products was performed using the dideoxy chain termination method of Sanger et al. (1977) using an ABI PRISM 3700 DNA Analyser (Applied Biosystems®, USA). The sequences obtained were submitted to an assembly using the Phred–Phrap software (v. 23) (Ewing et al., 1998; Ewing & Green, 1998; Gordon, 2003). The consensus sequences were subjected to BLASTn (Altschul et al., 1990) searches against the NCBI Core Nucleotide database to identify the most closely related sequences available in GenBank.

Quantitative real-time PCR (qPCR) assay and molecular characterization of *Bartonella* spp.

DNA extracted from fleas (Section 2.4) was subjected to a qPCR assay for *Bartonella* spp. based on the 16S–23S rRNA intergenic region (Breitschwerdt & Maggi, 2019; Oteo et al., 2017) (Table SM1). The number of DNA copies/μL was quantified using a G-block containing the target sequence of *Bartonella henselae*, from which serial dilutions were prepared with concentrations ranging from 2.0×10^7 to 2.0×10^0 copies/μL. The estimated number of copies was calculated using the formula: (Xg/μL of DNA ÷ [plasmid size (bp) × 660]) × 6.022×10^{23}. The qPCRs were performed in duplicates. Amplification efficiency was obtained from the slope of the standard curve for each assay, using the equation $E = 10^{-1/\text{slope}}$, as described by Bustin et al. (2009). The UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen™ Life Technologies, USA) was used as a negative control in all reactions. All samples were also subjected to cPCR assays for the 16S–23S rRNA intergenic region (ITS; variable 453–704 bp) (Maggi & Breitschwerdt, 2005).

Samples testing positive in the qPCR assay were additionally analysed by cPCR targeting the *gltA* gene (~400 bp) (Norman et al., 1995) (Table SM1). UltraPure DNase/RNase-Free Distilled Water (Invitrogen™ Life Technologies) was used as a negative control for all reactions. DNA from *Bartonella henselae* (Dias et al., 2023) was used as a positive control for all reactions. The products obtained from the cPCR assays showing a single band were purified using the ExoSAP-IT™ PCR Product Cleanup (Applied Biosystems®, USA), following the manufacturer's instructions, whereas those from cPCR assays with nonspecific bands were purified using the Wizard® PCR and Gel Clean-Up System (Promega®, USA). The sequencing procedure and sequence analysis were conducted as described in Section 2.5.

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Molecular characterization of *Wolbachia* spp.

Extracted DNA samples from fleas (Section 2.4) were subjected to cPCR assay targeting the *wsp* gene. Initially, the primer set 81F and 691R (590–632 bp) was used to amplify the universal *wsp* gene (Table SM2) (Baldo et al., 2006). Subsequently, a semi-nested PCR (snPCR) was performed using the group-B-specific primer set 81F and 522R (~440 bp) (Table SM2). As the negative control, UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen™, Carlsbad, CA, USA) was used for all reactions. A DNA sample of *Wolbachia*-infected *Ctenocephalides felis* was used as a positive control. For the snPCR assays, 1 μL of the amplified product from the first PCR was used as the template DNA for the second reaction. For amplification of the *gatB* gene (Table SM2) (Baldo et al., 2006), DNA samples from fleas were amplified by cPCR using the primer pair *gat*B_F1/gatB_R1 and the same reaction mixture described for *wsp*.

The products obtained from the cPCR assays showing a single band were purified using the ExoSAP-IT™ PCR Product Cleanup (Applied Biosystems®, USA), following the manufacturer's instructions, whereas those from cPCR assays with nonspecific bands were purified using the Wizard® PCR and Gel Clean-Up System (Promega®, USA). The sequencing procedure and sequence analysis were conducted as described in Section 2.5.

Amplicon library construction and high-throughput sequencing

The flea-extracted DNA samples (Section 2.4.) were subjected to a cPCR targeting the V3–V4 region of the prokaryotes 16S rRNA gene (341F: 5'-CCTAYGGGRBGCASCAG-3′ and 806R: 5'-GGACTACNNGGGTATCTAAT-3′) (~470 pb) (Yu et al., 2005). Samples that showed a positive band were subsequently subjected to library preparation targeting the V3-V4 region of the prokaryotes 16S rRNA gene (Yu et al., 2005) followed by Illumina deep sequencing according to the manufacturer's instructions. Sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina, USA) configured to generate 2 × 250 bp paired-end reads.

Amplicon processing and data analysis

Quality control of the demultiplexed FASTQ files was performed using FastQC (v0.11.9) (Andrews, 2010). Dynamic trimming of the 3′ end of sequences was conducted with fastp (v0.23.2) (Chen et al., 2018), removing bases with an average quality <25 in a window size of five bases (−-cut_right_window_size 5, −-cut_right_mean_quality 25), and discarding reads shorter than 150 bp. Remaining reads were merged using FLASH (v1.2.11) (Magoč & Salzberg, 2011) with a minimum overlap of 12 bp, a maximum overlap of 250 bp, and a maximum mismatch density of 0.2. The primer sequences were identified and removed with search_pcr (−minamp 300, −maxamp 600) from the USEARCH (v.11.0.667) (Edgar, 2010).

Preprocessed reads were analysed using the DADA2 package (v1.14.1) (Callahan et al., 2016) in R (R Core Team, 2023) for the inference and quantification of Amplicon Sequence Variants (ASVs). Merged reads were filtered using the 'filterAndTrim' function with parameters truncLen = 440, maxEE = 2 and maxN = 0. Error rates were estimated using the 'learnErrors' function with a LOESS model, and sequence redundancy was removed via the 'derepFastq' function. Error correction and chimaera removal were carried out using the 'removeBimeraDenovo' function.

Taxonomic assignment of ASVs was performed with the 'assignTaxonomy' function in the R package DADA2, using the Greengenes2 database (v2.0) (McDonald et al., 2024) and a Naive Bayesian classifier (Wang et al., 2007). Following classification, filtering steps were applied to remove spurious or contaminant sequences, including ASVs unclassified at the kingdom level, and those assigned to mitochondria or plastids. Subsequently, the ASV counts were transformed into a 'phyloseq' object (R package 'phyloseq') (v. 1.46.0) (McMurdie & Holmes, 2013) for downstream analyses. Potential exogenous contaminants sequences were further identified and removed using the R package 'decontam' (v. 1.22.0) (Davis et al., 2018) with reference negative controls (HC and EC).

The ASV phylogenetic relatedness was performed from multiple alignment from ASVs using the 'AlignSeqs' function from the R package 'DECIPHER' (v.2.30.0) (Wright, 2016). The phylogenetic tree was constructed by performing the Neighbour-Joining algorithm using the 'NJ' function and fitting a GTR + G + I (Generalized time-reversible with Gamma rate variation) using the fitGTR function, both from the R package 'phangorn' (v.2.12.1) (Schliep, 2011).

Sample reads were normalized by sequencing depth using the rarefaction rarefy_even_depth function from the R package 'phylsoeq' (v. 1.46.0) (McMurdie & Holmes, 2013). Rarefaction was used only for diversity analyses requiring standardized sequencing depth. The rarefaction threshold was set to the first quartile (25%) of the sequence count distribution across samples, and only libraries with counts above this threshold were rarefied.

Statistical analysis

Alpha diversity was calculated as observed richness, Shannon diversity and dominance core abundance using the 'phyloseq' package (v.1.54.2) and as Faith's phylogenetic diversity (Faith, 1992) using the picante package (v.1.8.2) (Kembel et al., 2010). Prior to modelling, core abundance dominance was transformed using the formula $(y \times (N-1)+0.5)/N$, where y represents the original value and N is the total sample size to avoid boundary values (Smithson & Verkuilen, 2006). The effect of flea sex on alpha diversity was analysed using generalized linear mixed-effects models (GLMMs) using the glmmTMB package (v.1.1.14) (Brooks et al., 2017; McGillycuddy et al., 2025). Observed features were modelled using a negative binomial GLMM; while Shannon diversity and the transformed core abundance dominance were modelled using Gaussian GLMMs and Beta GLMMs, respectively. Faith's phylogenetic diversity was modelled

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using Gamma GLMMs (with a log link). The flea sex was included as a fixed effect, and individual host identity was specified as a random effect. The fit with model assumptions (e.g., assessments of dispersion, zero inflation and outliers) was verified using the DHARMa package (v.0.4.7) (Hartig, 2024) (Figure SM2). Estimated marginal means (EMMs) were calculated using the 'emmeans' package (v. 2.0.3) (Lenth & Piaskowski, 2026), considering the fixed effects of the model while accounting for the random-effects structure.

Beta diversity was calculated based on the Weighted UniFrac metric (Lozupone et al., 2011) using the phyloseq package (v.1.54.2) (McMurdie & Holmes, 2013). To evaluate the influence of flea sex, a permutational multivariate analysis of variance (PERMANOVA) was performed with 9999 permutations using the 'adonis2' function from the 'vegan' package (v.2.6.8) (Oksanen et al., 2025). Permutations were constrained within individual hosts (using the strata argument) to account for the host effect. Group dispersion homogeneity was assessed using the 'betadisper' function from the 'vegan' package (v. 2.6.8) (Oksanen et al., 2025). The resulting distance matrix was also used for principal coordinate analysis (PCoA).

Differences in the abundance of bacterial genera between male and female fleas were assessed using the Wald test implemented in the R package 'DESeq2' (Love et al., 2014). Genera detected in fewer than three samples were excluded prior to analysis, and a pseudo-count of 1 was added to the count matrix. Differential abundance was defined as a false discorevy rate (FDR)-adjusted p-value ≤0.05.

BLASTn and phylogenetic analyses

The consensus sequences were subjected to BLASTn (Altschul et al., 1990) searches against the NCBI Core Nucleotide database to identify the most closely related sequences available in GenBank.

Partial 16S rRNA gene sequences were extracted to determine the relationships of *Bartonella* and *Wolbachia*-associated ASVs from the microbiota dataset with representative species from each genus in the microbiome data. The most abundant ASV of each genus (defined as the variant with the highest read count) was considered the representative sequence for each genus in the dataset. The sequences obtained in this study for the partial 16S rRNA and *gltA* gene of *Bartonella* spp., 16S rRNA, *wsp* and *gatB* gene of *Wolbachia* spp., along with representative species' sequences for each gene from GenBank, were aligned using the MAFFT (v. 7.511) software (Katoh et al., 2019). Phylogenetic inference was performed using the Maximum Likelihood (ML) method in the W-IQ-TREE server[1] (Trifinopoulos et al., 2016) (v. 1.6.11). The best-fit substitution model for each dataset was selected automatically by ModelFinder, implemented in W-IQ-TREE, under the Bayesian Information Criterion (BIC). Branch support was assessed using ultrafast bootstrap with 1000 replicates. The resulting tree was visualized and edited using TreeGraph 2 (v.2.15.0–887 beta) (Stöver & Müller, 2010) and iTOL (v.7) (Letunic & Bork, 2024).

[1]https://www.hiv.lanl.gov/content/sequence/IQTREE/iqtree.html.

RESULTS

Specimens' collection and morphological identification

A total of 21 *Polygenis (Polygenis) bohlsi bohlsi* (Wagner, 1901) flea specimens (17 females and 4 males) were collected from 14 *Thrichomys fosteri* individuals (Wagner, 1845) (Rodentia: Echimyidae), with hosts harbouring between one and three fleas each (mean infestation intensity: 1.2 fleas/host). Of the 21 specimens collected, one female (54A) was slide-mounted and deposited in the Siphonaptera Collection of the Museum of Zoology at the Universidade Estadual de Campinas (ZUEC-SIP-10), whereas two additional female specimens had been used in previous laboratory tests and were therefore not included in the present molecular analyses. The remaining 18 specimens were processed for molecular analyses.

Morphologically, males of this subspecies can be distinguished from the other subspecies [*Polygenis (Polygenis) bohlsi jordani* (Lima, 1937)] by having the ventral arm of sternite IX longer than the vertical arm, and the apex of the ventral arm distinctly rounded (whereas in *P. (P.) bohlsi jordani*, it is tapered), with a dense group of downward-pointing setae contrasting with the surrounding setae (in the other subspecies, the group of setae is less dense). Females can be distinguished by the shape of the posterior margin of sternite VII. In *P. (P.) bohlsi bohlsi* is only slightly projected, while in *P. (P.) bohlsi jordani* it is strongly pronounced.

Screening and characterization of *Bartonella* spp.

The qPCR analysis performed on DNA extracted from 16 *P. (P.) bohlsi bohlsi* fleas revealed the presence of *Bartonella* spp. in 18.7% of the samples (2/16), based on amplification of the 16S–23S rRNA intergenic spacer (ITS) region. The absolute quantification of *Bartonella* DNA ranged from 9.44×10^{-1} to 1.30×10^{3} copies/μL. The reactions showed an average efficiency of 96.6%, with a slope of −3.407, R^2 of 0.996 and a y-intercept of 38.083. Both qPCR-positive samples were also detected by cPCR targeting the *gltA* gene. The BLASTn analysis of the obtained *gltA* sequences revealed 99.73% nucleotide identity with an uncultured *Bartonella* (KY304482.1) sequence previously detected in fleas collected from wild rodents in the Pantanal region, Mato Grosso do Sul (De Sousa et al., 2018) (Table SM3). In the PCR targeting the 16S–23S rRNA ITS region of *Bartonella* spp., two samples produced bands of the same size as the positive control. However, only one sample (POSS13) displayed a single distinct band and generated a readable sequence, which showed 100% identity to *Bartonella harrusi* (CP101114.1) (Do Amaral, Cardozo, Varani, Furquim, et al., 2022) (Table SM3).

The phylogenetic reconstruction based on partial 16S rRNA gene sequences lacked sufficient resolution to confidently infer the relationship between the representative sequence [ASV0124] and *Bartonella* strains. The BLASTn searches using the Core nucleotide BLAST database showed that the partial 16S rRNA sequence of ASV0124 had 100% coverage and 99.09% similarity to *Bartonella harrusi* strain

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117A (CP101114.1), matching the ITS findings. To achieve higher phylogenetic resolution, additional analyses based on the *gltA* gene positioned the genotypes detected in *P. (P.) bohlsi bohlsi* within a clade alongside other *Bartonella* sequences previously identified in *P.* (*P.*) *bohlsi bohlsi* fleas and marsupials from the Pantanal region, Mato Grosso do Sul (Figure 1; Table SM3).

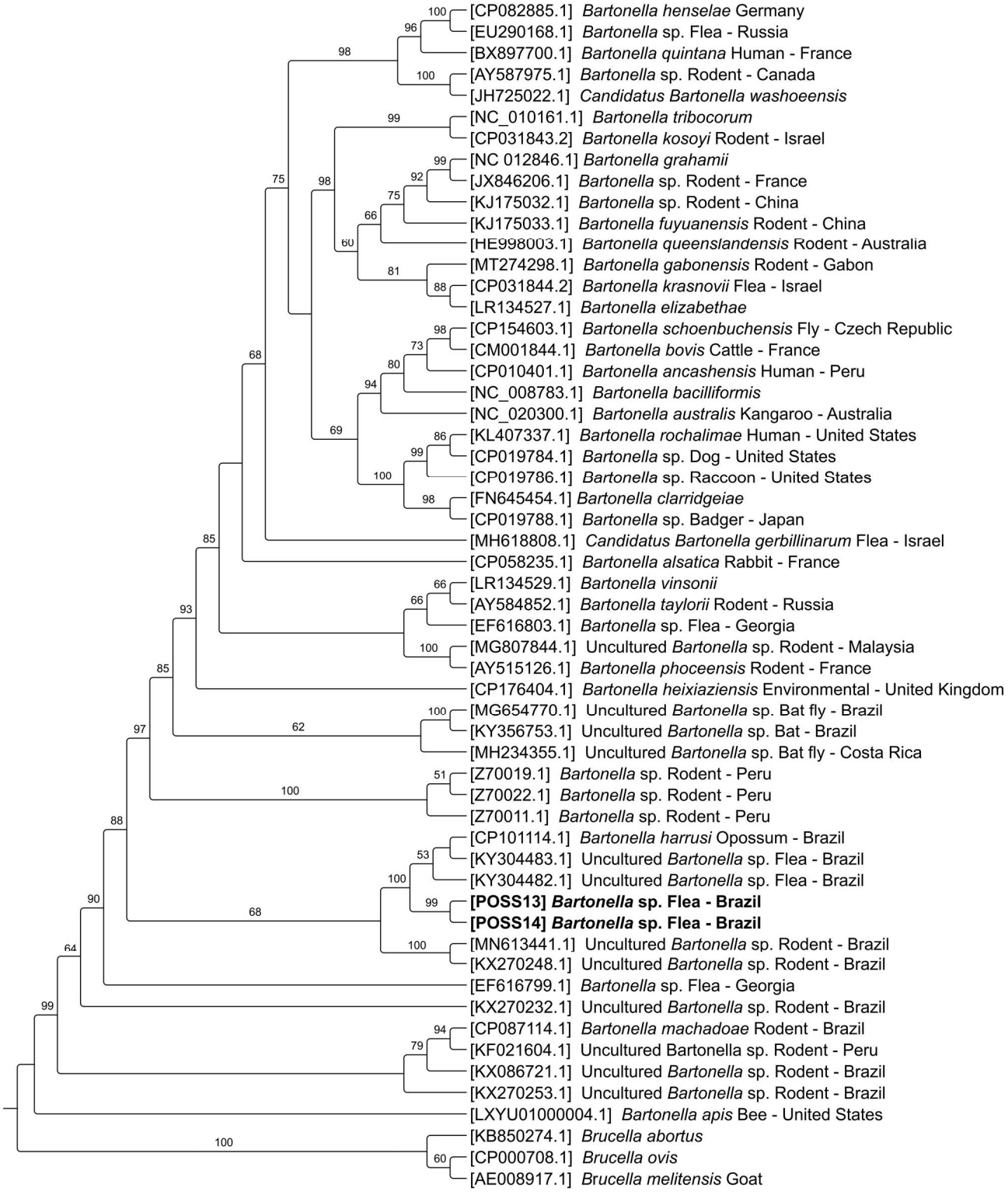


FIGURE 1 Phylogenetic analysis of the *gltA* gene using Maximum Likelihood of *Bartonella* spp. detected in *Polygenis (Polygenis) bohlsi bohlsi* parasitizing free-living *Thrichomys fosteri* trapped at the Pantanal region, Brazil. The phylogeny was inferred from a 352 bp alignment of the partial *gltA* gene, implemented with the evolutionary model TIM + F + I + G4 [selected by Bayesian Information Criterion (BIC)]. Outgroups included *Brucella abortus* (KB850274.1), *Brucella melitensis* (AE008917.1) and *Brucella ovis* (CP000708.1). Sequences generated in this study are highlighted in bold. Bootstrap support values (≥50%) are shown at the nodes. The list of sample names and accession numbers is provided in Table SM4.

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Screening and characterization of *Wolbachia* spp.

The similarity analysis using BLASTn searches showed that *Wolbachia* representative 16S rRNA sequence ASV0002 had 100% coverage and 98.86% similarity with *Wolbachia* endosymbiont of *Tunga penetrans* (Linnaeus, 1758) (Siphonaptera: Tungidae) (MH337864.1). The phylogenetic analysis clustered the ASV0002 sequence into the *Wolbachia* supergroup S, which has also been detected in pseudoscorpions of the species *Atemnus politus* (Simon, 1878) (Figure 2).

Among the 16 fleas, amplification of the *wsp* gene was achieved in 10 female specimens (62.5%), while 14 specimens (87.5%), including 12 females and 2 males, were positive in the PCR targeting the *Wolbachia gatB* gene. The BLASTn similarity analysis showed that the *wsp* sequences, with the exception of sample POSS05, which shared 86.93% similarity with the *Wolbachia* endosymbiont of *Basilia* bat flies (Paracyclopodia) [CP061738.1], shared a mean identity of 87.55% with the *Wolbachia* endosymbiont of *Cimex hemipterius* tropical bed bug (KT751167.1) (Table SM5). For the *gatB* gene, the sequences matched those of *Wolbachia* endosymbionts of *Cimex lectularius* (AP013028.1) and *Cystococcus pomiformis* (MW511353.1), with mean similarities of approximately 91.39% (Table SM5).

Phylogenetic analysis based on the *wsp* gene revealed that the sequences of *P. (P.) bohlsi bohlsi* clustered with the S supergroup, which is related to the *Wolbachia* endosymbiont of pseudoscorpion (Lefoulon et al., 2020). Furthermore, the phylogenetic positioning indicates evolutionary proximity to clades F (including both filarial and arthropod symbionts) and V (cat flea *Wolbachia*) (Figure 3a). In the phylogeny based on the *gatB* gene, the sequences of *P. (P.) bohlsi bohlsi* clustered together with the *Wolbachia* lineage of the cat flea (wCfeJ), within supergroup V (Sharma & Som, 2023), since there are no available sequences belonging to supergroup S for this gene (Figure 3b).

Bacterial microbiome profiling of fleas

A total of 18 flea specimens from 14 individual rodent hosts were processed for molecular analysis. Three hosts (1, 4 and 12) each contributed one male and one female flea, whereas host 8 contributed two female fleas. Of the 18 processed specimens, two failed PCR amplification. High-throughput sequencing of the noncuticular (internal) microbiome was performed on the remaining 16 flea specimens (12 females and 4 males) (Table SM4), together with four ECs. A total of 1,452,170 paired-end reads were generated, with a mean of 72,608.50 (±40,861.13) reads per library. The controls accounted for 21,244 reads in total, with a mean of 5311 (±3153.70). After quality filtering and processing, libraries retained a mean of 32,445.05 (±19,326.96) reads per library, identifying 1983 bacterial ASVs. Following decontamination, a total of 1809 ASVs remained. The rarefaction curve based on observed ASVs approached a saturation plateau, indicating that the sequencing depth was sufficient to capture the bacterial diversity associated with the flea samples (Figure SM1).

Alpha diversity analyses revealed significant sex-associated differences in microbial community structure (Figure 4a). Observed richness was significantly higher in males than in females (negative binomial GLMM, log link: estimate = 0.96 ± 0.28, $z = 3.44$, $p < 0.001$), with values approximately 2.61-fold greater (e0.96 = 2.61). A similar pattern was observed for Faith's phylogenetic diversity (Gamma GLMM, log link: estimate = 0.99 ± 0.41, $z = 2.41$, $p = 0.016$), corresponding to an estimated 2.71-fold increase. In contrast, dominance core abundance differed significantly between sexes and was higher in females (beta GLMM, logit link: estimate = −3.39 ± 0.63, $z = -5.42$, $p < 0.001$), with males showing markedly lower odds than females (odds ratio = 0.034). No significant difference between males and females was detected for the Shannon diversity index (Gaussian GLMM, identity link: estimate = 0.60 ± 0.34, $z = 1.77$, $p = 0.077$).

Beta-diversity analysis revealed a pattern of microbial community structuring according to sex. Weighted UniFrac-based PCoA demonstrated a separation between male and female fleas, particularly along PCoA1, which accounted for 44.03% of the total observed variation, while PCoA2 explained an additional 26.61%, totaling 70.64% of the explained variation (Figure 4b). Despite the apparent visual separation between female and male fleas in the ordination space, PERMANOVA ($R^2 = 0.41$; $F = 9.92$; $p = 0.125$) did not detect significant differences in overall community composition between sexes. However, permutational analysis of multivariate dispersions (PERMDISP: $F = 3.73$; $p = 0.048$) indicated significant differences in within-group variability, with males showing greater dispersion relative to the group centroid, indicating higher heterogeneity in microbial community composition.

The bacterial flea microbiome was partitioned into 28 phyla, 63 classes, 141 orders, 264 families and 496 genera. Among the ASVs identified, 82.4% achieved taxonomic classification at the genus level. At the phylum level, the five most abundant phyla accounted for an average of 98% of the total relative abundance. These included Pseudomonadota (80.84%), Bacillota (10.46%), Bacteroidota (2.62%), Actinomycetota (2.53%) and Fusobacteriota (1.53%).

The main genera associated with female fleas included *Wolbachia* (mean relative abundance of reads = 82.28 ± 23.83%; prevalence = 12/12), followed by *Staphylococcus* (8.38 ± 20.76%), unclassified Pasteurellaceae (3.45% ± 6.68), *Haoranjiania* (2.51 ± 3.62%) and *Rothia* (1.04 ± 3.58%). On the other hand, the microbiome of male fleas was more variable. The most abundant genus was *Bartonella* (36.86 ± 44.29%; prevalence = 3/4), which was not detected in females. Other genera found in males included *Pseudoleptotrichia* (5.44 ± 8.89%), unclassified Neisseriaceae (4.74 ± 8.45%), *Pseudomonas* (2.91 ± 5.36%) and *Wolbachia* (2.85 ± 3.02%; prevalence = 2/4). The high abundance of *Bartonella* in males is largely due to its dominance in two individuals, while the bacterial profiles of the other two males were dominated by different taxa, indicating a heterogeneous microbiome in this group. Meanwhile, the strong predominance of *Wolbachia* in females, along with its reduced abundance and lower prevalence in males, highlights a sex-associated difference of flea microbiome structure (Figure 5a).

Out of the total ASVs, 72.36% (1309 ASVs) were exclusive to male fleas, 22.50% (407 ASVs) to females and only 5.14%

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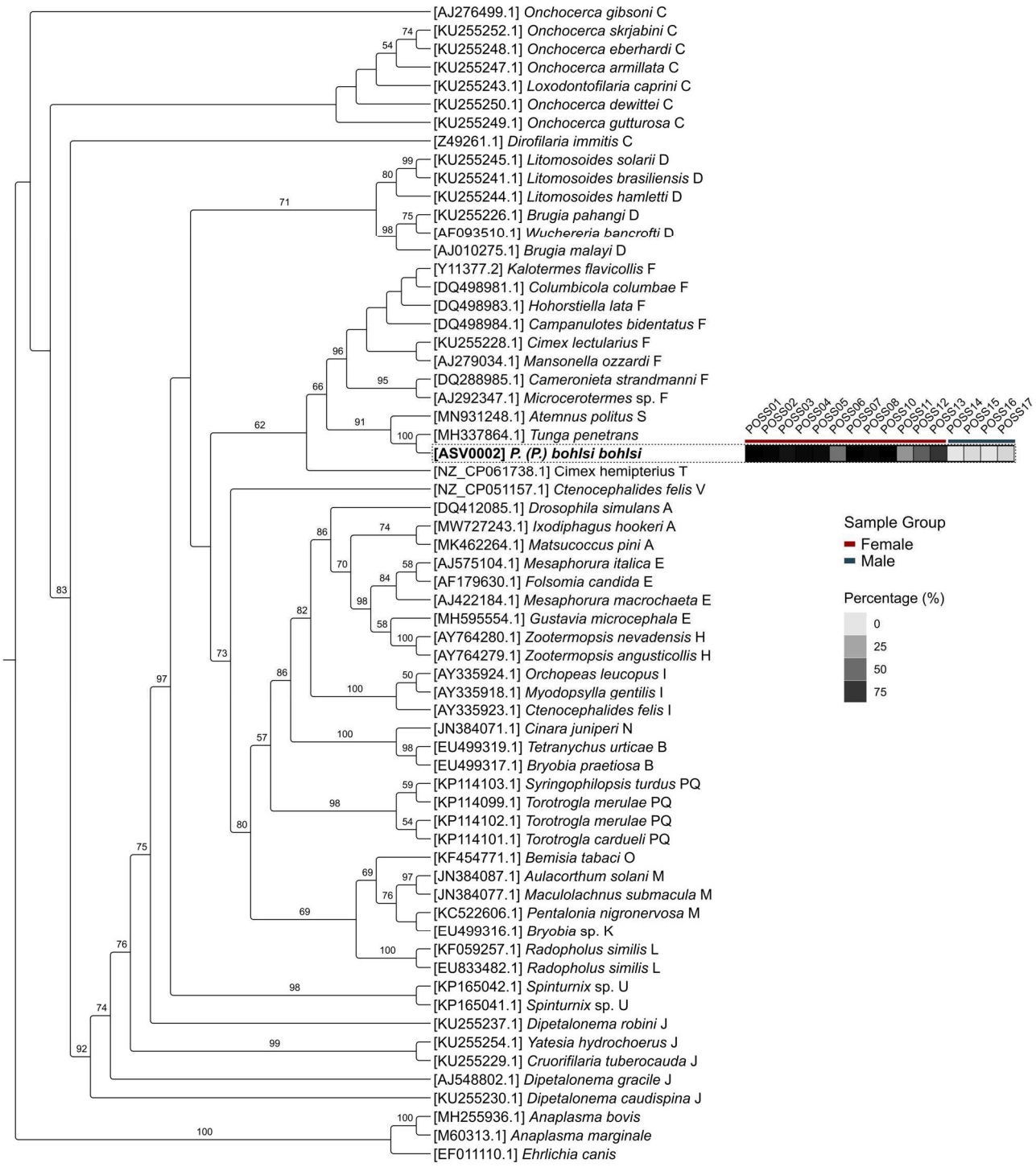


FIGURE 2 Phylogenetic analysis of the 16S rRNA gene of *Wolbachia* spp. detected in *Polygenis (Polygenis) bohlsi bohlsi* parasitizing free-living *Thrichomys fosteri* trapped at the Pantanal region, Brazil, using the Maximum Likelihood method. The tree was inferred from a 446 bp alignment of the partial 16S rRNA gene, using the K2P + R2 evolutionary model [selected by Bayesian Information Criterion (BIC)]. Outgroups included *Anaplasma marginale* (M60313.1), *Anaplasma bovis* (MH255936.1), and *Ehrlichia canis* (EF011110.1). The representative sequence from this study is shown in bold. The heatmap indicates the relative abundance (%) of *Wolbachia* in each sample. Bootstrap support values (≥50%) are shown at the nodes.

(93 ASVs) were shared between both sexes. At the genus level, 65.52% (325 genera) were found exclusively in males, 18.95% (94 genera) in females and 15.52% (77 genera) were common to both genders (Figure 5b). A total of 118 ASVs were assigned to *Wolbachia*, 40 to *Bartonella*, whereas *Rickettsia* was represented by a single ASV.

The differential abundance analysis revealed that, in addition to *Wolbachia*, an unclassified genus within the family Pasteurellaceae

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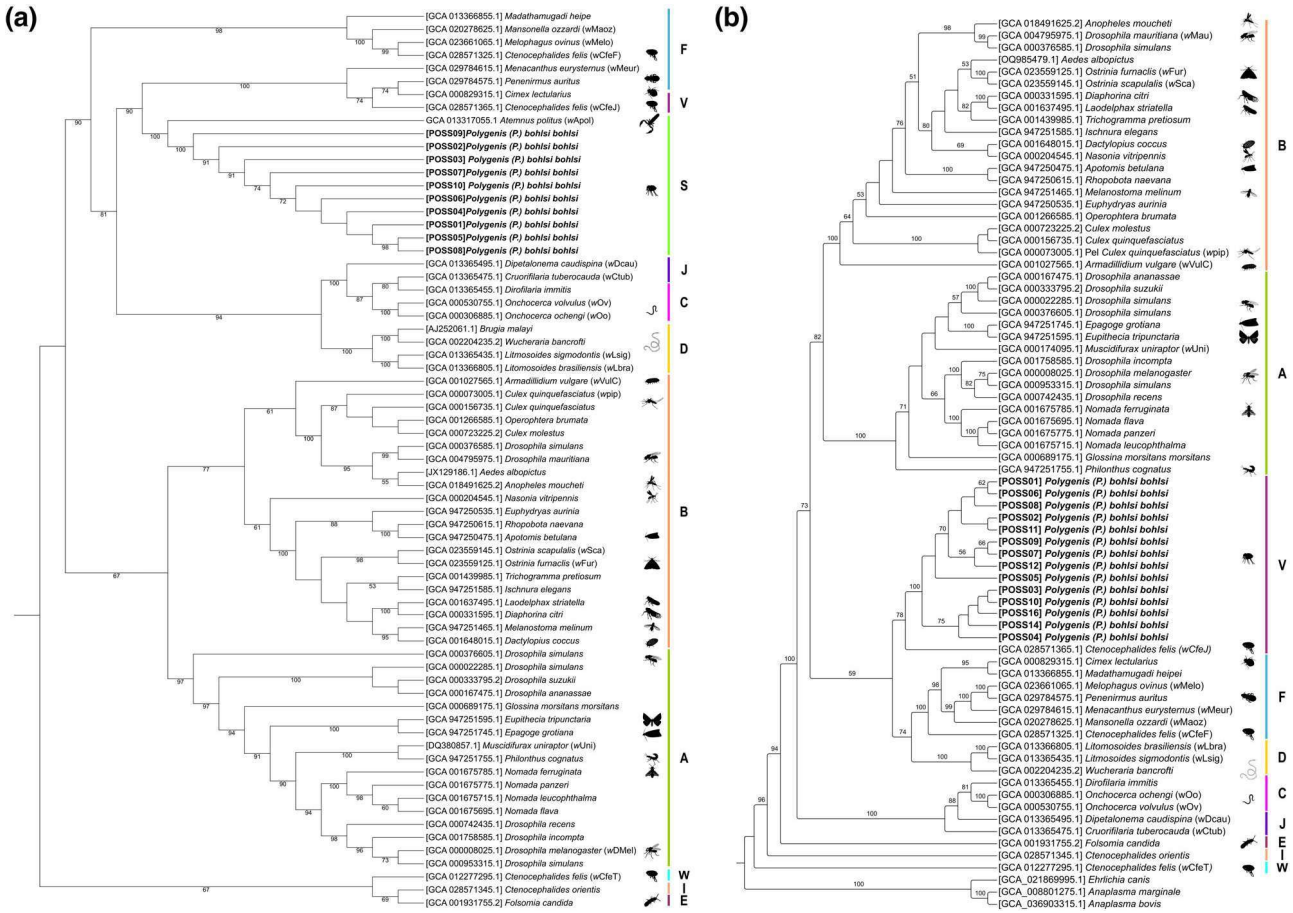


FIGURE 3 Maximum Likelihood (ML) phylogenetic tree of *Wolbachia* spp. detected in *Polygenis (Polygenis) bohlsi bohlsi* parasitizing free-living *Thrichomys fosteri* trapped at the Pantanal region, Brazil. (a) Phylogeny inferred using the ML method based on the *wsp* gene. The tree was inferred from a 616 bp alignment of the partial *wsp* gene, using the TVM + F + I + G4 evolutionary model [selected by Bayesian Information Criterion (BIC)], and the tree is midpoint rooted. (b) Phylogeny inferred using the ML method based on the *gatB* gene. The tree was inferred from an 800 bp alignment of the partial *gatB* gene, using the GTR + F + I + G4 evolutionary model (selected by BIC). Outgroups included *Anaplasma marginale* (GCA_ 008801275.1), *Anaplasma bovis* (GCA_036903315.), and *Ehrlichia canis* (GCA_021869995.1). Sequences generated in this study are highlighted in bold. Bootstrap support values (≥50%) are shown at the nodes. Supergroups are indicated by coloured bars. The list of sample names and accession numbers is available in Table SM4.

(Pseudomonadota) was significantly enriched in female fleas (log2FC < −5) (Figure 6). In contrast, male fleas showed eight differentially abundant genera (log2FC > 3). Notably, the genera *Bartonella* (Proteobacteria), *Escherichia* (Proteobacteria), *Pseudoleptotrichia* (Fusobacteriota), an unclassified genus within the family Lachnospiraceae (Bacillota), *Acinetobacter* (Proteobacteria) and *Prevotella* (Bacteroidota) exhibited the highest log2FC values (log2FC > 3) (Figure 6).

DISCUSSION

Rodents represent 87.9% of the hosts in which *P. (P.) bohlsi bohlsi* specimens have been reported, with members of the family Cricetidae being the most frequently parasitized (Linardi & Guimarães, 2000). In addition to rodents, marsupials have also been reported as hosts (9.5%), followed by carnivores (1.3%), xenarthrans (0.6%) and birds (0.6%). This flea species has been recorded in several localities across the states of Goiás (Mello & Brasil, 1979), Minas Gerais (Botelho & Linardi, 1992), Mato Grosso do Sul (De Sousa et al., 2018) and Rio Grande do Sul (Linardi et al., 2005), indicating a wide distribution across central regions of Brazil. Previously, *Bartonella* spp. was detected in *P. (P.) bohlsi bohlsi* collected from *T. fosteri* rodents in the Brazilian Pantanal (De Sousa et al., 2018).

Regarding the flea microbiome, our findings are consistent with previous studies (Carcauzon et al., 2025; Guo et al., 2024; Jones et al., 2015), which have also reported the presence of bacterial phyla such as Pseudomonadota, Bacillota and Bacteroidota. In particular, our results reinforce that the microbiome of female fleas is strongly dominated by the endosymbiont *Wolbachia*, as previously described (Carcauzon et al., 2025; Lawrence et al., 2015; Wu et al., 2023). This dominance suggests that *Wolbachia* may play a central role in shaping the microbial community structure of *P. (P.) bohlsi bohlsi*, potentially influencing the flea's reproductive biology and its capacity to harbour or transmit pathogens.

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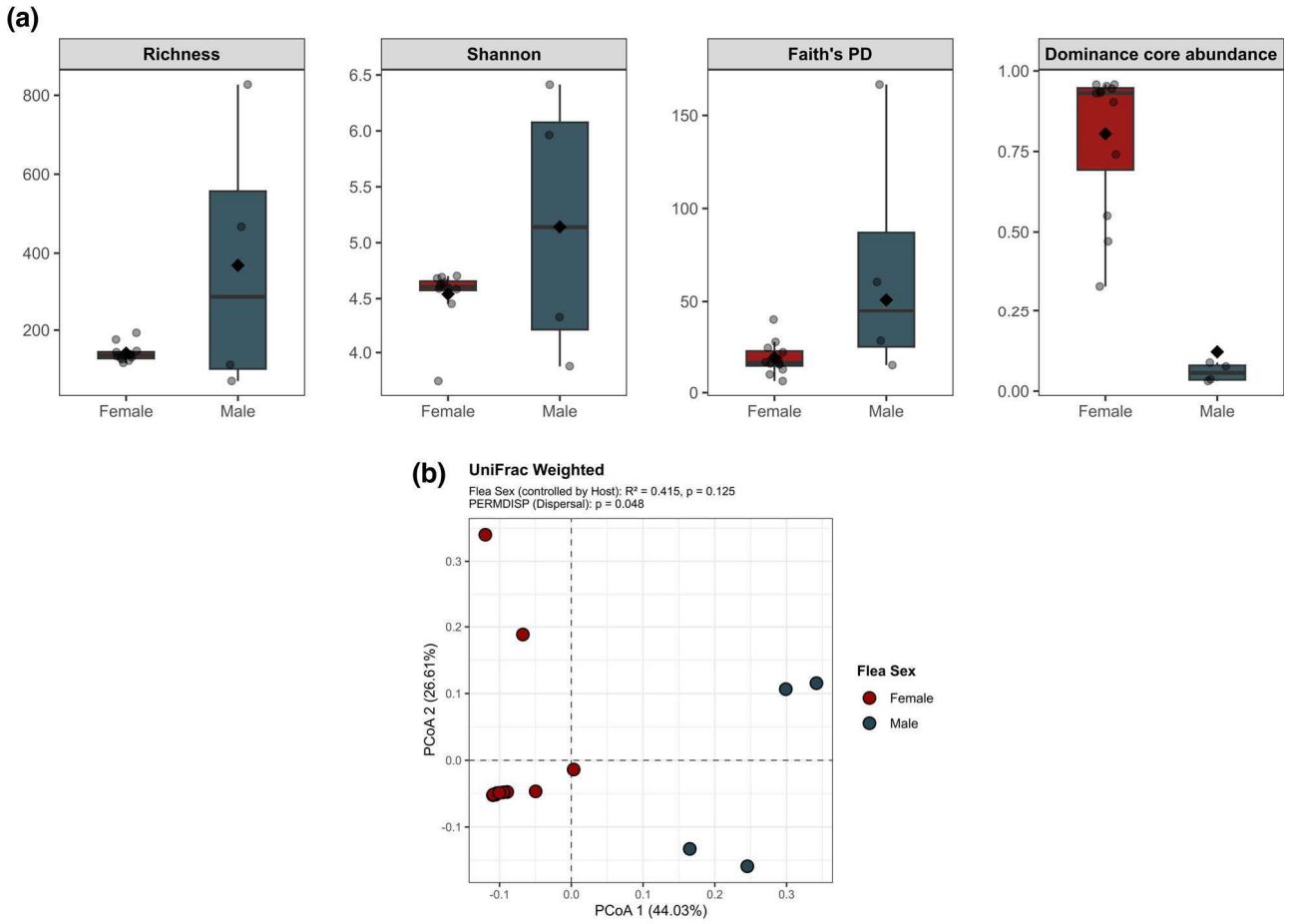


FIGURE 4 Analysis of the bacterial diversity in *Polygenis (Polygenis) bohlsi bohlsi* flea populations parasitizing free-living *Thrichomys fosteri* trapped at the Pantanal region, Brazil. (a) Boxplots of alpha diversity indices (observed richness, Shannon diversity index, Faith's phylogenetic diversity [Faith's PD], and dominance core abundance). Diamonds within the boxplots represent estimated marginal means obtained from the GLMMs. (b) Principal coordinate analysis (PCoA) based on weighted UniFrac distances. The two-dimensional PCoA explained 70.64% of the variance in bacterial community composition. Statistical significance of differences between male and female flea microbiomes was assessed using PERMANOVA ($F = 9.92$, $R^2 = 0.41$, $p = 0.125$).

Although *Wolbachia* is a widespread endosymbiont among arthropods, including fleas, accumulating evidence suggests that distinct *Wolbachia* strains may play distinct roles in flea host biology. Genomic analyses of *C. felis felis* suggest that the *Wolbachia* strain *w*CfeT may function as a nutritional endosymbiont. In contrast, the coinfecting strain *w*CfeJ harbours a putative toxin-antidote (TA) operon analogous to the *w*Pip *cinAB* operon (a known CI inducer in flies), highlighting the coexistence of distinct symbiotic roles within the same host species (Driscoll et al., 2020). In *Synosternus cleopatrae*, *Wolbachia* did not affect flea survival or reproduction, and no detectable fitness benefits or reproductive manipulation were observed when infections were evaluated without distinguishing among strains (Flatau et al., 2021). However, a subsequent study revealed that *S. cleopatrae* harbours two coinfecting *Wolbachia* strains (*w*Sc1 and *w*Sc2) with distinct effects. While *w*Sc1 was associated with a direct fitness advantage to its hosts (responsible for the continuously high incidence observed in female fleas), *w*Sc2 was negatively associated with the number of male offspring and the total number of offspring per female, supporting male-killing activity (Flatau et al., 2025). Together, these findings indicate that the ecological and evolutionary consequences of *Wolbachia* infection in fleas are likely strain-specific and may range from mutualistic to reproductive-parasitic interactions.

The phylogeny of *Wolbachia* lineages is essential for understanding their diversity and the evolutionary relationships established with their hosts. The phylogenetic analyses based on the 16S rRNA and *wsp* genes revealed that the *Wolbachia* genotype detected in *P. (P.) bohlsi bohlsi* clustered within supergroup S, along with previously described genotypes detected in pseudoscorpions *Atemnus politus* (*w*Apol) and *Cordylochernes scorpioides* (*w*Csco) (Lefoulon et al., 2020). The resulting topology of the S and F clades was consistent with the clustering reported by Sharma and Som (2023). In contrast, the phylogeny inferred with the *gatB* gene grouped the *P. (P.) bohlsi bohlsi*-associated *Wolbachia* genotype within the *w*CfeJ lineage, belonging to supergroup V (Sharma & Som, 2023). Although such a phylogenetic positioning differed from that observed for the 16S rRNA and *wsp* genes, it is important to consider the limitations of these two markers. The 16S rRNA fragment sequenced here is relatively short and might not provide the phylogenetic resolution necessary to reliably

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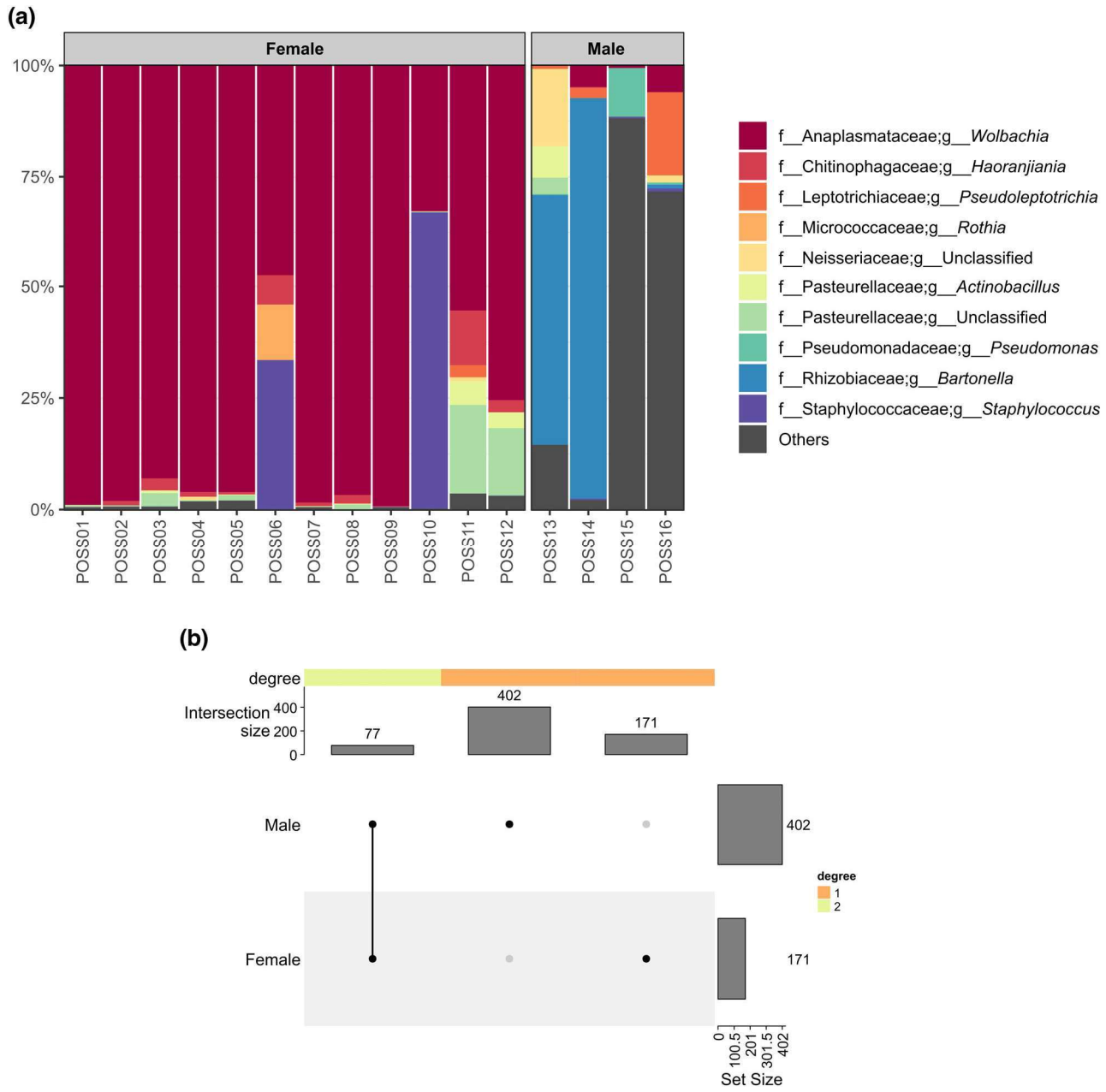


FIGURE 5 Distribution of bacterial genera in *Polygenis (Polygenis) bohlsi bohlsi* flea populations parasitizing free-living *Thrichomys fosteri* trapped at the Pantanal region, Brazil. (a) Mean relative abundance of the top 10 most abundant bacterial genera, grouped by sex (female and male). Genera with lower abundance were grouped under the 'Others' category. (b) UpSet plot showing the distribution of shared and unique bacterial genera among male and female *P.* (*P.*) *bohlsi bohlsi* flea samples.

distinguish among recently diverged supergroups. Likewise, the *wsp* gene, despite being widely used, is known to undergo extensive intragenic recombination and horizontal transfer between supergroups, which can lead to misleading phylogenetic inferences (Baldo & Werren, 2007). Thus, although 16S rRNA and *wsp* appear to show concordant topologies, this agreement does not necessarily reflect the true evolutionary history of the lineage, but rather the limited resolution of the 16S rRNA fragment and the highly variable nature of the *wsp* gene. On the other hand, the phylogenetic inference based on *gatB*, a conserved MLST marker with lower recombination rates, provides more robust support for the *P. (P.) bohlsi bohlsi-associated Wolbachia* positioning together the *w*CfeJ lineage. Such a discrepancy between the molecular markers may also reflect the lack of availability of *gatB* sequences from representatives of supergroup S, which limits broader comparative analyses. This finding is intriguing, as supergroups S and V are relatively uncommon and poorly characterized compared to the more frequently reported supergroups A and B in arthropods. Their presence in fleas suggests either an underestimated diversity of *Wolbachia* lineages in Siphonaptera or possible horizontal transfer events. Further studies are needed to clarify the evolutionary

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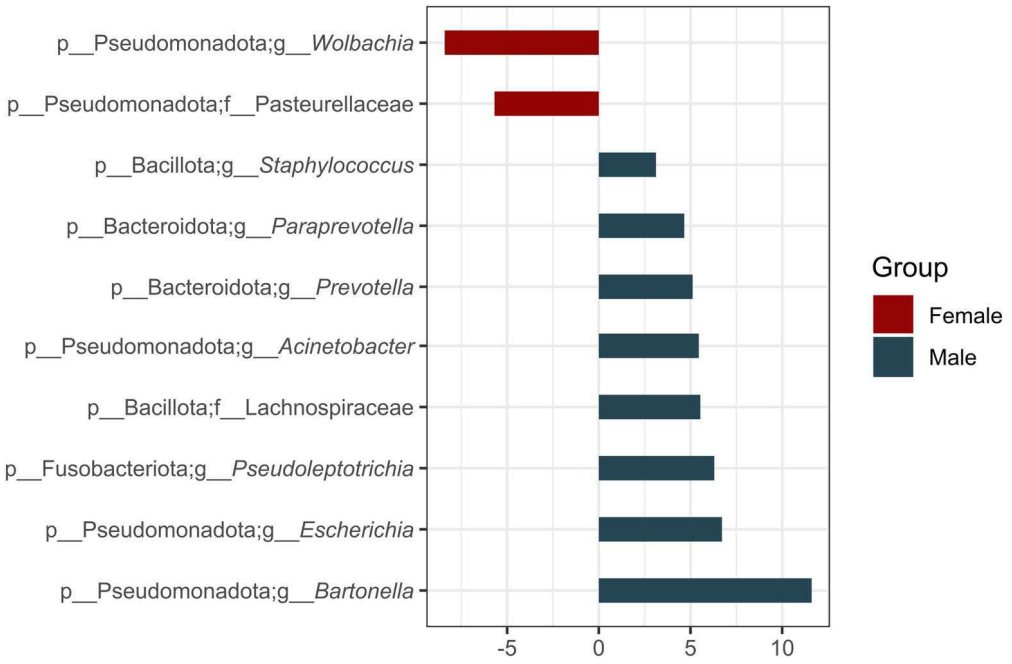


FIGURE 6 Differentially abundant (DA) bacterial genera between male and female *Polygenis (Polygenis) bohlsi bohlsi* parasitizing free-living *Thrichomys fosteri* trapped in the Pantanal region of Brazil, identified using DESeq2. The x-axis indicates the log2 fold change, and the genera shown were significantly different (FDR <0.05).

and the functional implications of these supergroups in fleas and whether they might influence pathogen dynamics, as interactions between *Wolbachia* and vector-borne pathogens have been reported in other arthropod vectors (O'Neill et al., 2019).

Another taxon frequently associated with co-infections involving *Bartonella* spp. and *Wolbachia* spp. in fleas is *Rickettsia* spp. In our study, *Rickettsia* spp. was represented by a single ASV, which was detected in only one sample (POSS14) with low abundance. Although co-infections between *Bartonella* and *Rickettsia* have been documented in other flea species (Beliavskaia et al., 2023; Manvell et al., 2022), few have been reported in fleas of the genus *Polygenis*. López Berrizbeitia et al. (2024) detected co-infection with *Bartonella quintana* and *Rickettsia felis* in one specimen of *Polygenis (Polygenis) acodontis* (Jordan & Rothschild, 1923) collected from *Akodon sylvanus* (Thomas, 1921) and one specimen of *Akodon spegazzinii* (Thomas, 1897) in northwestern Argentina. Likewise, Schott et al. (2020) identified *Rickettsia* sp. (strain Taim), closely related to *Rickettsia parkeri*, in *Polygenis* spp. collected from wild rodents in southern Brazil.

Considering the low detection of Rickettsia observed in our study, it is important to note that sample processing can directly affect the recovery of certain taxa. Lawrence et al. (2015) demonstrated that, although the overall microbial community structure of fleas remained largely consistent across protocols, surface sterilization with bleach resulted in the depletion of specific bacterial genera, including *Rickettsia*. Furthermore, sequencing depth can also influence the detection of low-abundance taxa; therefore, both chemical treatment and sequencing depth represent important methodological limitations that might have contributed to the low prevalence of *Rickettsia* observed in our dataset. Beyond methodological considerations, biological factors might also have limited the detection of rare taxa. The overwhelming dominance of *Wolbachia* in arthropod-associated microbiomes can account for a substantial proportion of sequencing reads, thereby reducing the effective sequencing depth available for less abundant bacterial taxa. Consequently, rare microorganisms may be underrepresented or remain undetected in amplicon-based surveys. This phenomenon has been documented in several arthropod systems, where highly abundant endosymbionts can mask the occurrence and diversity of co-existing bacterial taxa (Audsley et al., 2018; Henry et al., 2024; Ourry et al., 2021; Wilches et al., 2021).

The phylogenetic analysis of the representative 16S rRNA *Bartonella* sequence identified in the bacteriome of *P.* (*P.*) *bohlsi bohlsi* revealed clustering within the *Bartonella harrusi* clade. This finding is noteworthy, as *B. harrusi* was recently detected by Do Amaral, Cardozo, Varani, Furquim, et al. (2022) in the marsupial *Thylamys macrurus* (Olfers, 1818) from the Pantanal region of Mato Grosso do Sul, suggesting that this bacterial species may circulate among diverse mammalian hosts in this biome. In contrast, de Sousa et al. (2018) reported that *Bartonella* sequences based on *nuoG* + *ftsZ* genes obtained from *P.* (*P.*) *bohlsi bohlsi* fleas collected from *T. fosteri* in the same region clustered with the *Bartonella alsatica* clade. These divergent findings may reflect temporal shifts, host-related factors, or geographic variation in the circulation of *Bartonella* species in Pantanal ecosystems. Nevertheless, the broad host range previously reported for *P. (P.) bohlsi bohlsi* (Linardi & Guimarães, 2000) indicates that this flea exhibits low host specificity, parasitizing a wide range of vertebrate taxa. Such ecological flexibility may facilitate the transmission and maintenance of diverse *Bartonella* spp. in natural environments, especially in biodiverse regions like the Pantanal.

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The microbiome of male and female *Polygenis (Polygenis) bohlsi bohlsi* fleas differed in alpha diversity, with males exhibiting higher ASV richness and greater phylogenetic diversity. Although the number of males analysed was limited ($n = 4$) and this group exhibited greater dispersion, the magnitude of the estimated effect suggests that sex may be associated with important variations in bacterial community structure. However, the low representation of males makes these results highly sensitive to individual variation and random colonization events, reducing the statistical power to detect consistent patterns in the population. In female fleas, the higher core abundance dominance values suggest a community more strongly dominated by a few abundant taxa. This pattern is likely associated with the high abundance of *Wolbachia*, which was predominant in the female microbiome. As *Wolbachia* is an intracellular endosymbiont commonly associated with arthropod reproduction and maternally transmitted, it tends to reach high densities in female reproductive tissues, particularly the ovaries, facilitating its vertical transmission across generations (Frydman et al., 2006). This transmission strategy may contribute to its high representation within the female microbiome. Additionally, phylogenetic analyses indicated that the *Wolbachia* detected in this study is closely related to the *w*CfeJ strain described from *Ctenocephalides felis felis*, whose genome contains candidate genes associated with reproductive parasitism mechanisms, such as a *cinAB*-like TA operon associated with cytoplasmic incompatibility in other *Wolbachia* lineages (Driscoll et al., 2020). Furthermore, studies across distinct arthropods suggest that the presence and abundance of *Wolbachia* can shape the structure of host-associated microbial communities (Brinker et al., 2026; Duan et al., 2020; Ourry et al., 2021). In addition, previous studies have highlighted the difficulty of disentangling the effects of vector sex and *Wolbachia* infection on microbiome composition, as both factors may be closely associated in some arthropod systems (Dittmer & Bouchon, 2018). Given the predominance of *Wolbachia* in female fleas, it remains unclear whether the differences in alpha diversity observed in the present study primarily reflect vector sex, variation in Wolbachia abundance, or an interaction between these factors. Because these relationships remain poorly investigated in fleas, future studies examining the effects of both vector sex and *Wolbachia* infection status will be important for disentangling their relative contributions to microbiome structure.

The occurrence of *Bartonella*-positive male fleas and *Bartonella*-negative female fleas on the same vertebrate hosts (two of three wild rodents) suggests that host sharing alone may not fully explain patterns of *Bartonella* acquisition. In this context, the absence of *Bartonella* detection in females may be associated with factors beyond host exposure, as the abundance of vector-borne bacteria can be influenced by endosymbiotic bacteria within the vector (e.g., *Wolbachia*) as well as by interactions with other microorganisms co-occurring in both the vector and the vertebrate host bloodstream. Furthermore, seasonal variation may also contribute to differences in *Bartonella* occurrence. Cohen et al. (2015) reported a decrease in the relative abundance of both *Wolbachia* and *Bartonella* phylotypes in female fleas from spring to summer, suggesting that seasonal shifts in microbial community composition may influence *Bartonella* persistence and detection. Although seasonal effects were not evaluated in the present study, since sampling was performed only during the summer, such variation could represent an additional factor contributing to the observed sex-associated pattern. Therefore, the microbial composition differences observed between males and females may reflect not only patterns of exposure but also ecological interactions among microorganisms that could influence pathogen acquisition and maintenance within the host-vector system. Although one could argue that the sampled fleas might have harboured bacterial loads below the sequencing detection threshold, as observed in a previous study (Moore et al., 2023), herein we also performed molecular screening for *Bartonella* using a qPCR assay. Future investigations combining molecular analyses of both host blood and vector-associated microbiota are needed to determine host infection status at the time of flea collection in order to clarify how host infection status and microbial interactions within the vector jointly shape the maintenance and transmission dynamics of vector-borne pathogens, including *Bartonella*.

In addition to *Wolbachia* and *Bartonella*, other bacterial taxa exhibited differential abundance between male and female fleas. Noteworthy, an unclassified genus belonging to the family Pasteurellaceae was detected in both flea sexes, albeit significantly more abundant in females. The detection of this taxon in both male and female fleas indicates its widespread distribution among the specimens analysed. Similarly, while investigating the microbiome of different flea species associated with rodents, Jones et al. (2015) identified species-specific associations between fleas and Pasteurellaceae lineages, highlighting the recurrent occurrence of this bacterial family across different flea species. Taken together, these findings indicate that members of the family Pasteurellaceae are frequent components of the microbiota associated with rodent-associated fleas. The genus *Prevotella*, which showed the highest relative abundance in male fleas sampled herein, has been reported in *Rhadinopsylla dahurica vicina* fleas, in the intestines of the plateau pika (*Ochotona curzoniae*), and in environmental samples such as plants and soil (Li et al., 2016; Li et al., 2018). Similarly, *Staphylococcus* has been detected in both fleas and the blood of rodents (Cohen et al., 2015). Altogether, these observations suggest that the occurrence of these taxa in fleas may be influenced, at least in part, by their association with vertebrate hosts. The genus *Acinetobacter*, identified in our study, has also been detected in the microbiome of other flea species (Guo et al., 2024; Lawrence et al., 2015; Seidi et al., 2025; Wu et al., 2023), indicating that its occurrence may be relatively common among these ectoparasites. An unclassified member of the family Lachnospiraceae was also identified among the differentially abundant taxa. Members of this family are common gut commensals in mammals and insects and have been suggested to contribute to host and gut community health (Vera-Ponce de Leon et al., 2022). Additionally, Lachnospiraceae has been previously reported in *C. felis felis*, representing one of the most diverse bacterial groups detected within the flea microbiome (Moore et al., 2023).

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Paraprevotella is an anaerobic member of the family Prevotellaceae and is commonly associated with the gastrointestinal microbiota of mammals (Wang et al., 2022). *Pseudoleptotrichia goodfellowii* (formerly *Leptotrichia goodfellowii*), belonging to the family Leptotrichiaceae, has been reported as an opportunistic pathogen associated with bacteremia and endocarditis in humans (Lieberman et al., 2021) and has also been identified in the gut of the swine-associated *Haematopinus suis* louse (Deng et al., 2024). The detection of these taxa in fleas broadens their known host associations, although their occurrence and ecological significance in these ectoparasites remain poorly understood. *Escherichia* was also identified among the differentially abundant taxa, although members of this genus have been reported as potential contaminants in low-biomass microbiome studies (Eisenhofer et al., 2019; Gin et al., 2026). Nevertheless, laboratory procedural controls were included to monitor environmental contamination, supporting a cautious interpretation of this finding. Although the ecological significance of these patterns remains unclear, the differential abundance of these bacterial taxa suggests that sex may contribute to variation in the flea microbiome. Additional studies will be necessary to determine the functional roles of these taxa and to elucidate the mechanisms underlying these patterns.

The contrasting bacterial profiles observed between female and male fleas may have epidemiological implications. The strong predominance of *Wolbachia* in females and the detection of *Bartonella* mainly in males raise the possibility that endosymbiont-pathogen interactions contribute to shaping flea vector competence. In other arthropods, *Wolbachia* has been associated with modulation of pathogen colonization through immune activation, resource competition, or alteration of host physiology (Pan et al., 2018; Pavanelo et al., 2023; Perlmutter et al., 2025). Although causality cannot be inferred from the present study, our findings raise the possibility that sex-specific microbiome composition may be relevant to the maintenance and transmission dynamics of flea-borne bacteria. From a public health perspective, microbiome surveys in fleas are relevant because these ectoparasites participate in the transmission cycles of several zoonotic agents. Identifying bacterial taxa associated with pathogen presence may help clarify ecological and biological factors underlying vector competence. In the Pantanal biome, where rodents, marsupials, domestic animals, wildlife and humans coexist in close ecological interfaces, understanding flea-associated microbial interactions may improve our comprehension of pathogen circulation and emergence risks.

In summary, our study provides novel insights into the microbiome composition and pathogen diversity associated with *P.* (*P.*) *bohlsi bohlsi*. The identification of *Bartonella* and a rare *Wolbachia* lineage suggests a complex microbial ecology with potential implications for pathogen transmission. Although *Bartonella* spp. was detected exclusively in male fleas in the present dataset, this pattern should be interpreted with caution, as the low number of male specimens analysed in this study limits the inference of sex-biased patterns of infection. Taken together, these findings underscore the ecological significance of *P.* (*P.*) *bohlsi bohlsi* as a host for diverse bacterial taxa and emphasize the need for further investigations into its vectorial capacity and the evolutionary relationships between fleas, their microbiomes and the pathogens they harbour.

AUTHOR CONTRIBUTIONS

Michelli Inácio Gonçalves Funnicelli: Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; software; formal analysis; data curation. **Renan Bressianini do Amaral:** Investigation; methodology; writing – review and editing. **Victória Valente Califre de Mello:** Investigation; methodology; writing – review and editing. **Ricardo Bassini-Silva:** Investigation; writing – review and editing; methodology. **Lizeth Fernanda Banguero Micolta:** Investigation; methodology; writing – review and editing. **Marie Buysse:** Investigation; methodology; writing – review and editing; software. **William Oliveira de Assis:** Methodology; writing – review and editing. **Alanderson Rodrigues da Silva:** Methodology; writing – review and editing. **Heitor Miraglia Herrera:** Investigation; methodology; writing – review and editing. **Rosangela Zacarias Machado:** Conceptualization; writing – review and editing. **Darci Moraes Barros-Battesti:** Methodology; investigation; writing – review and editing. **Daniel Guariz Pinheiro:** Investigation; methodology; validation; writing – review and editing; software; formal analysis; data curation. **Olivier Duron:** Investigation; methodology; writing – review and editing; data curation. **Marcos Rogério André:** Conceptualization; investigation; funding acquisition; writing – review and editing; project administration; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The Supporting Information for this study is publicly available in Zenodo at: https://doi.org/10.5281/zenodo.18496541. A female

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specimen (54A) of *Polygenis Polygenis bohlsi bohlsi*, collected from *Thrichomys fosteri*, was slide-mounted and deposited in the Siphonaptera Collection of the Museum of Zoology at the Universidade Estadual de Campinas (ZUEC), under the accession number ZUEC-SIP-10 (https://www.ncbi.nlm.nih.gov/). Mitochondrial cytochrome *c* oxidase subunit I (*cox1*) gene sequences from three female flea specimens (1A [POSS01], 14A [POSS07] and 120A [POSS12]) were deposited in GenBank under accession numbers PV658968, PV658969 and PV658970, respectively. Partial sequences of *Bartonella* 16S–23S ribosomal RNA ITS amplified from flea sample POSS13 [1B (male)] were submitted to GenBank and are available under the accession number PX633061, and citrate synthase (*gltA*) gene from fleas' samples POSS1 [1B (male)] and POSS14 [4A (male)] are available under the accession numbers PX580481 and PX580482, respectively. Partial sequences of the *Wolbachia* Outer surface protein (*wsp*) gene amplified from flea samples were submitted to GenBank and are available under accession numbers PX580483–PX580492, and partial Glutamyl-tRNA(Gln) amidotransferase subunit B (*gatB*) gene sequences from fleas are available under accession numbers PX580493–PX580506. The raw microbiome sequences targeting the hypervariable V3–V4 region generated for this study were deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA1370812.

ETHICS STATEMENT

Procedures and management protocols involving wild rodents were approved by the 'Institute Chico Mendes for Conservation of Biodiversity' (SISBIO number 64,204) and by the 'Ethics Committees on Animal Use of the School of Agricultural and Veterinary Sciences' (FCAV/ UNESP) (protocol number #11,794). Consent for publication: All authors consent to publication.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table SM1. Molecular assays used for the detection and characterization of *Bartonella* spp. in this study, including information on target genes, primer/probe sequences, thermal cycling conditions, amplicon sizes and references.

Table SM2. Molecular assays used for the characterization of *Wolbachia* spp. in this study, including information on target genes, primer sequences, thermal cycling conditions, amplicon sizes and references.

Table SM3. Flea specimens collected from wild rodents in the Pantanal region (Mato Grosso do Sul state, central-western Brazil) that

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tested positive in cPCR assays for *Bartonella* spp. based on the 16S–23S rRNA intergenic spacer (ITS) region and the citrate synthase gene (*gltA*). The table presents BLASTn results for sequenced amplicons.

Table SM4. Identification of the samples obtained in the present study and their respective accession numbers for the sequences generated and deposited in the GenBank database.

Table SM5. Flea specimens collected from wild rodents in the Pantanal region (Mato Grosso do Sul state, central-western Brazil) that tested positive in cPCR assays for *Wolbachia* spp. based on the Wolbachia surface protein (*wsp*) and the Glutamyl-tRNA(Gln) amidotransferase subunit B (*gatB*). The table presents BLASTn results for sequenced amplicons.

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1. ORIENTAÇÕES

Nada a declarar

2. DISCIPLINAS MINISTRADAS COM A RESPECTIVA CARGA HORÁRIA E/OU A REALIZAÇÃO DE ATIVIDADES DE EXTENSÃO.

Atuação em corregência na disciplina Tópicos Especiais: Bioinformática Aplicada a Estudo de Microbiomas: Diversidade Taxonômica por Marcadores Moleculares (códigos TEBM1 e TEBAM1), oferecida aos Programas de Pós-Graduação em Ciências Veterinárias e em Microbiologia Agropecuária da Faculdade de Ciências Agrárias e Veterinárias – Câmpus de Jaboticabal, durante o 2º semestre de 2026, com carga horária de 20 horas. [Anexo 01]

3. COMENTÁRIOS DO SUPERVISOR

A pós-doutoranda desenvolveu as atividades concernentes ao seu estágio de Pós-doutoramento de maneira exímia, sempre muito prestativa, alinhada aos objetivos norteadores do nosso grupo de pesquisa. Além de conduzir seus experimentos, colaborou e continua colaborando com outros projetos desenvolvidos no *Vector-Borne Bioagents Laboratory*. Realizou período de pós-doutoramento na University of Montpellier, participou de congresso internacional e ministrou aulas em disciplina de Pós-Graduação, demonstrando maturidade científica e profissional.

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ATESTADO

ATESTAMOS que a Profa. Dra. Michelli Inácio Gonçalves Funnicelli, RG 47.953.369-6 - SSP, ministrou/ministra aulas aos discentes dos Programas de Pós-graduação desta Unidade Universitária, conforme abaixo descrito:

Programa: Microbiologia Agropecuária

Disciplina	Ano	Dt.Início	Dt.Término	Curso	Discentes	Hr/Aula
Tópicos Especiais: Bioinformática Aplicada a Estudo de Microbiomas: Diversidade Taxonômica por Marcadores Moleculares	2025	29/09/2025	07/10/2025	MD	9	20

Programa: Ciências Veterinárias

Disciplina	Ano	Dt.Início	Dt.Término	Curso	Discentes	Hr/Aula
Tópicos Especiais: Bioinformática Aplicada a Estudo de Microbiomas: Diversidade Taxonômica por Marcadores Moleculares	2025	29/09/2025	07/10/2025	MD	10	20

Jaboticabal, 29 de setembro de 2026

Márcia Luciana Natareli
Supervisora de Seção Técnica de Pós-Graduação