Diversity of bacterial symbionts associated with the tropical plant bug *Monalonion velezangeli* (Hemiptera: Miridae) revealed by high-throughput 16S-rRNA sequencing.

Lucio Navarro-Escalante[*&]
Department of Entomology, National Coffee Research Center - CENICAFE, Manizales, Colombia. Email: lucio.navarro@cafedecolombia.com
ORCID Number: 0000-0001-7315-5669

Pablo Benavides
Department of Entomology, National Coffee Research Center - CENICAFE, Manizales, Colombia. Email: pablo.benavides@cafedecolombia.com
ORCID Number: 0000-0003-2227-4232

Flor E. Acevedo*
Department of Entomology, Pennsylvania State University, University Park, PA, USA.
Email: fea5007@psu.edu
ORCID Number: 0000-0002-0946-9951

*Corresponding authors.
[&]Current position: Department of Molecular Biosciences, The University of Texas at Austin, Austin TX, USA. Email: lucio.navarro@austin.utexas.edu

Abstract: Insects and microbes have developed complex symbiotic relationships that evolutionarily and ecologically play beneficial roles for both, the symbiont and the host. In most Hemiptera insects, bacterial symbionts offer mainly nutritional, defensive, and reproductive roles in addition to promoting the adaptive radiation of several hemipteran phytophagous lineages. The tropical plant bug *Monalonion velezangeli* (Hemiptera: Miridae) is a polyphagous herbivore considered an important insect pest for several economically relevant tropical crops, but information about the composition of its bacterial microbiota was missing. In this study, we describe the diversity and structure of the bacterial microbiota in the nymph and adult life stages of *M*. *velezangeli* using Illumina high-throughput sequencing of 16S ribosomal RNA gene amplicons (meta-barcoding). We found that both insect life stages share a similar microbiota in terms of bacterial diversity and community structure. The intracellular symbiont *Wolbachia* dominated the overall microbiome composition (~92%) in these life stages. Members of the core microbiota include *Wolbachia, Romboutsia*, *Ignavibacterium*, *Clostridium*, *Allobaculum*, *Paracoccus*, *Methylobacterium*, *Faecalibacterium*, *Collinsella*, *Rothia*, *Sphingomonas* and 4 other undetermined bacterial genera. Based on PCR screening and DNA sequencing of the *wsp* gene, *Wolbachia* infection was confirmed in almost 80% of samples, and represented by two different isolates or strains within the supergroup B. This data offer opportunities for studying the contribution of symbiotic bacteria in the biological performance of this insect pest, and provides a base to explore other insect control methods.

Key Words: bacterial microbiota, endosymbiont, plant bug, *Wolbachia*, *Monalonion*, *Coffea*.

Introduction:

Most insects harbor diverse microbiota inside their body that collectively perform important biological roles for the insect-host in processes such as nutrition, reproduction, immunity, and development. These symbiotic interactions involve microbes adapted to live inside specialized host cells (intracellular symbionts), or outside cells (extracellular symbionts). The vast majority of insect-associated microbes reside in the gut lumen, and some are adapted to live within specialized structures in the insect posterior midgut. Insect gut-associated microorganisms have been proposed as key players in the adaptive radiation of herbivorous insects by allowing them to metabolize or assimilate recalcitrant plant compounds, or to exploit low-nutrient plant contents by providing additional nutritious molecules (Motta et al. 2022; Ge et al. 2023; Janson et al. 2008; Sudakaran et al. 2017). In some cases the outcomes of this symbiotic interplay in plant-feeding insects has also extended to the control of host-plant defense responses for the benefit of the insect (Chung et al. 2013; Acevedo et al. 2017; Schausberger 2018; Li et al. 2019). Moreover, insect-associated microbial symbionts have been shown to confer resistance to chemical insecticides in various pest insects (Kikuchi et al. 2012; Blanton and Peterson 2020; Sato et al. 2021).

Equally important, intracellular symbionts (e.g. *Wolbachia*) that frequently reside within the reproductive tissues of most insects are well known as manipulators of insect reproduction. *Wolbachia* are maternally inherited bacterial symbionts that infect at least 65% of insect species (Hilgenboecker et al. 2008), and are capable of altering host reproduction and fitness in order to achieve high frequency of infection in the host populations (Stouthamer et al. 1999). This manipulation can involve cytoplasmic incompatibility (CI) (Sinkins 2004; Dylan Shropshire et al. 2020), parthenogenesis (Werren 1997; Vavre et al. 2004; Zhou et al. 2021), male-killing (Hurst et al. 1999; Fukui et al. 2015), and feminization (O'Neill et al. 1997; Hiroki et al. 2002; Narita et al. 2007). Additionally, several lines of evidence show that *Wolbachia* can affect behavioral patterns in their hosts by altering mating, feeding, locomotion, or aggressive behavior in addition to learning and memory capacity (reviewed by (Jie Bi 2020)).

Several Hemiptera plant-feeding insect species in the suborders Sternorrhyncha, Auchenorrhyncha and Heteroptera display a variety of insect-microbial symbiosis. Phytophagous Sternorrhyncha and Auchenorrhyncha species have piercing and sucking mouthparts for stylet-sheath feeding (phloem and xylem sap-suckers) as in aphids and leafhoppers. Phytophagous Heteroptera species have macerate-and-flush feeding mouthparts (sucking of extra-orally digested plant tissues) as seen in stink bugs and plant bugs. In consequence, several of these insect species are agricultural pests of economic importance. Most members of Sternorrhyncha and Auchenorrhyncha harbor intracellular obligate symbionts within specialized cells or bacteriocytes that provide essential amino-acids and vitamins to the insect (Moran and Telang 1998). However, most phytophagous Heteroptera members lack intracellular symbionts, but instead have developed relationships with extracellular symbionts in special midgut compartments such as midgut crypts and caeca. These extracellular symbionts are mainly found in stink bugs, flat bugs and seed bugs, within the infraorder Pentatomomorpha. Nonetheless, special symbiont-harboring midgut compartments seems to be absent in most

phytophagous species in the infraorder Cimicomorpha which includes true plant bugs such as *Monalonion velezangeli*.

The plant bug *M. velezangeli* (Hemiptera: Miridae: Bryocorinae) is a neotropical polyphagous insect native to Central and South America. This insect feeds on 21 plant species in 14 families (Giraldo J. and Benavides M. 2012; Rodas et al. 2014; Ocampo Flórez et al. 2018). It is considered as a strict phytophagous insect based on the lack of reports of other feeding habits, and the fact that all known members of the mirid subfamily Bryocorinae are herbivorous as well (Jung and Lee 2012; Namyatova and Cassis 2016). This plant bug is a notorious agricultural pest of cacao (*Theobroma cacao*, Malvaceae), avocado (*Persea americana*, Lauraceae), guava (*Psidium guava*, Myrtaceae), and tea (*Camellia sinensis*, Theaceae) (Jaimes et al. 2015; Ramírez-Gil et al. 2019). *Monalonion velezangeli* is also an emerging pest for coffee crops in Colombia especially in the southern coffee-producing regions of the country (Ramirez C. et al. 2008). The immature (nymph) and adult stages of this plant bug feed on terminal shoot tips, young leaves or fruits causing cell-death at the feeding sites as the main direct damage. Severe plant damage is mainly caused by nymphal stages when they inject enzyme-rich saliva into the plant tissues for extra-oral digestion of the cell contents. Current recommendations for pest management vary according to host crops. Common methods include cultural practices (e.g. manual collection of insects in the field or flaming), biological control with fungal entomopathogens, and insecticides. Despite its significance as an agricultural pest, several aspects of the biology of *M. velezangeli* remain poorly studied including the composition of its microbiota.

Diversity and functional characterization of symbiotic microbiota in Miridae plant bugs have been poorly studied except for the strictly phytophagous cotton fleahopper *Pseudatomoscelis seriatus* and the omnivorous *Adelphocoris suturalis* to the best of our knowledge (Fu et al. 2021; Xue et al. 2021; Luo et al. 2021). Knowledge of *M. velezangeli* associated microbiota is fundamental not only to better understand its biology, but also could provide new opportunities for the development of insect management methods. For example, symbiont-mediated RNA interference (smRNAi) is emerging as a potential approach for control of pest insects in agriculture (Dyson et al. 2022), and as an efficient tool for insect gene functional analysis (Lariviere et al. 2022). It is necessary to characterize the taxonomic composition of microbes within the insect body before any study on the role of the microbiota in insect biology or exploration of symbiont-based methods for pest control can be carried out. In this study, we analyzed for the first time the diversity and structure of the symbiotic microbiota within *M. velezangeli* nymph and adult life stages using high-throughput DNA amplicon sequencing of bacterial 16S rRNA gene (DNA meta-barcoding). Here we discovered a diverse microbiota across all life stages that is dominated by few bacterial genera, highlighting the presence of the endosymbiont *Wolbachia*.

Methods:

Insect collection, DNA isolation and 16S rRNA sequencing:

Samples of immature and adult individuals of *M*. *velezangeli* feeding on leaves of multiple coffee plants (*Coffea arabica* var. Castillo) were collected from a coffee plantation in the Department of Huila (Segovianas, Coordinates: 2.3784, -75.88291), Colombia. Insects were externally sterilized by washing three times with 75% ethanol and immediately conserved in 96% ethanol for DNA isolation. Three independent samples of immature (pools of 5 nymphal stages, one per instar) and three independent samples of adults (pools of one female and one male) of *M*. *velezangeli* were used for microbiota analysis. Total DNA was isolated from whole-body insects using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) including a lysozyme treatment according to the manufacturer protocol. DNA integrity was checked on agarose gel and quantified on Nanodrop (Invitrogen, Waltham, MA, USA). PCR amplification of the hyper-variable region V3V4 of the bacterial 16S rRNA gene was performed using primers 341F (5′-CCT AYG GGR BGC ASC AG- 3′) and 806R (5′- GAC TAC NNG GGT ATC TAA T- 3′) (Caporaso et al. 2011; Klindworth et al. 2012). Illumina sequencing libraries were generated with NEBNext® Ultra™ DNA Library Prep Kit (New England BioLabs, Ipswich, MA, USA). The 16S rRNA amplicon Illumina 250PE libraries were sequenced using NovaSeq platform (Illumina, San Diego, CA, USA) at Novogene Corporation Inc. (Sacramento, CA, USA).

Processing of 16S rRNA sequence data and taxonomic classification:
Demultiplexed raw 16S rRNA sequences were processed using QIIME2 v.2020.8 (Bolyen et al. 2019) as follows. Paired-end read sequences were quality-filtered, denoised and clustered using DADA2 (Callahan et al. 2016) (*dada2 denoise-paired*) to produce Amplicon Sequence Variants (ASV). The ASVs were taxonomically classified using the plugin *feature-classifier classify-sklearn* with the GreenGenes database (version 13_8) using default confidence threshold (≥ 0.7). The ASVs that could not be identified to genus with Greengenes were blasted against the NCBI Microbial Genome sequences (Bacteria and Archaea) to identify best hits and also compared with the EzBioCloud Database (version 2021.07.07) for assignation of genus using 97% identity threshold on both searches. The original GreenGenes identification taxon level was maintained when NCBI-BLAST and EzBiocloud resulted in contradictory genus best-hits at >=97% identify respectively. Contaminant sequences identified as chloroplast or mitochondria were removed from processed data tables and excluded from further analyses.

Diversity analysis and taxon abundance comparisons:
The ASV tables for raw abundance and taxonomy classification were exported from QIIME2 and processed through the MicrobiomeAnalyst tool (Dhariwal et al. 2017; Chong et al. 2020) using the Marker Data Profiling (MDP) pipeline as follows. The ASV abundances were brought to the total sum scaling for data normalization and further analysis of diversity. Alpha-diversity was estimated using the number of observed taxa (Observed), Chao1, ACE, Fisher and Shannon (H') indexes. Statistical differences between groups (Nymph vs Adult) were assessed with Mann-Whitney *U* test. Beta-diversity was assessed using Bray-Curtis distance between groups and their ordination visualized with Principal Coordinate Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS). Statistical differences in community structure between groups was tested with the permutational multivariate analysis of variance (PERMANOVA, one-way) and the analysis of similarities (ANOSIM, one-way). Both were based on Bray-Curtis distance as implemented on Past v.4.08 (Hammer-Muntz et al. 2001). Differences in dispersion

within each group was tested using PERMDISP (Anderson and Walsh 2013). Bacteria taxon abundance bar-plots were built with the MicrobiomeAnalyst tool and the heatmap plots using Matrix2png (Pavlidis and Noble 2003). Statistical differences for taxon abundances between groups were tested with the Mann-Whitney *U* test.

Molecular screening of *Wolbachia* endosymbiont:

Abdomen samples were separately dissected from nymphs or adults under sterile conditions in a stereoscope from the *M. velazangeli* individuals collected in this study and individually used for DNA isolation with DNeasy Kit (Qiagen) as described above. Detection and classification of *Wolbachia* was performed following the *wsp* gene (*Wolbachia* surface protein) PCR-based method established by Zhou et al. (Zhou et al. 1998) as follows. PCR screening was done with the *wsp*-specific primers wsp81F (5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′) and wsp691R (5′-AAA AAT TAA ACG CTA CTC CA-3′) in 20 µL reactions containing 1x Green GoTaq® reaction buffer (Promega, USA), 250 µM dNTPs, 0.5 µM of each primer, 0.5 u of GoTaq® polymerase (Promega, Madison, WI, USA) and 1 µL of DNA template. PCR cycling involved one initial step of denaturation at 95°C for 2 min, and then followed by 35 cycles of three steps including 95°C for 40 sec, 55°C for 30 sec and 72°C for 40 sec. The cycle ends with a final extension of 72°C for 5 min. DNA template integrity was additionally tested by PCR with universal primers for arthropod 28SrRNA gene sequences (28sF3633: 5′-TAC CGT GAG GGA AAG TTG AAA-3′, and 28sR4076: 5′-AGA CTC CTT GGT CCG TGT TT-3′) using the same PCR reaction conditions and cycling described above. Total DNA from a naturally *Wolbachia*-infested fruit fly (*Drosophila melanogaster*) strain was used as positive control in the PCR screening experiments. PCR amplicons were visualized with agarose gel electrophoresis.

Sanger DNA sequencing and phylogenetic analysis of *Wolbachia wsp* amplicons:

A group of 10 randomly selected *wsp* PCR amplicons (wsp81F/691R primers) derived from the *M. velezangeli* DNA samples were further purified using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer protocol. Purified amplicons were directly submitted to ABI automated bidirectional sequencing with wsp81F and wsp691R primers. DNA sequence chromatograms were processed in Chromas v2.6.6 (https://technelysium.com.au/wp/chromas/) for quality and primer-sequence trimming. Bidirectional sequences for each sample were clustered into single DNA consensus sequences (isolates) using GeneStudio v.2.2.0 (https://sourceforge.net/projects/genestudio/). The DNA consensus sequences were compared to available sequence data at GeneBank nt database using BLASTn search algorithm and were deposited at GeneBank under accession numbers OR129441-OR129450.

A phylogenetic analysis of the *M. velezangeli*-derived *wsp* sequences was performed using the web-based Phylogeny.fr platform (Dereeper et al. 2008) along with *wsp* sequences from insect-derived *Wolbachia* isolates at the GeneBank database as representatives of major *Wolbachia* subgroups found in insects according to Zhou et al. (Zhou et al. 1998). Sequences were aligned using ClustalW (v2.1) (Thompson et al. 1994). After alignment, positions with gaps were removed from the alignment. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT) (Guindon and Gascuel 2003). The default substitution model was selected assuming an estimated proportion of

invariant sites (of 0.003) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma=0.398). Reliability for internal branches was assessed using the aLRT test (SH-Like) (Anisimova and Gascuel 2006).

Results:

Microbial 16S rRNA sequence data:

A total of 491,802 denoised non-chimeric merged sequences for the 16S rRNA V3V4 variable region were produced among all samples (nymph and adult) after removing putative contaminant sequences. Sequence clustering produced 123 ASVs, with an average number of ASVs for adult and nymph samples of 57 and 79 respectively. The number of Illumina reads and ASV sequences for each sample are detailed in Table 1. Rarefaction curves showed that all samples reached richness saturation (Fig. 1A) indicating that sequencing effort was enough to capture total diversity (Good's coverage > 99.99% for all samples, Table 1).

Diversity of bacterial community:

The bacterial diversity associated with the nymph and adult life stages of *M. velezangeli* was analyzed through five alpha-diversity indices (Fig. 1B and Table 2). No statistical differences for species richness (Observed species), abundance (Chao1 and ACE) and abundance distribution indices (Fisher and Shannon) were detected between nymph and adult stages (Observed species: $U = 0$, $p = 0.1$; Chao1: $U = 0$, $p = 0.1$; ACE: $U = 0$, $p = 0.1$; Fisher: $U = 0$, $p = 0.1$; Shannon: $U = 3$, $p = 0.7$).

Differences in microbial community structure (beta-diversity) between nymph and adult was assessed with PERMANOVA and ANOSIM analyses, and their ordinal distances (Bray-Curtis dissimilarity) visualized with PCoA and NMSD plotting (Fig. 1C,D). PERMANOVA tests whether distance in community structure differs between groups (e.g. nymph vs adult) (Anderson 2001) whereas ANOSIM tests whether distances between groups are greater than within groups (Clarke 1993). Both analyses indicated no significant differences in microbial community structure between nymphs and adults (PERMANOVA: F-value: 0.4774; R-squared: 0.1135; p-value = 0.5016; ANOSIM: R: -0.1111; p-value = 0.7019). We assessed the differences in dispersion (variance) within groups with PERMDISP (Anderson and Walsh 2013) considering that PERMANOVA and ANOSIM are sensitive to variance within groups. This analysis showed that there is homogeneity of multivariate dispersions between nymph and adult samples (PERMDISP: F-value: 0.1958; p-value: 0.681).

Removal of *Wolbachia*-associated sequences is a regular practice for microbiome analysis in insects when they are detected in bacterial 16S rRNA libraries (Chandler et al. 2014; Rudman et al. 2019). We compared the overall microbial community structure in our samples when *Wolbachia* sequences are kept or removed from the data. The removal of *Wolbachia* reads did not alter the similarities in alpha diversity indices between nymphs and adults (Supplementary Table S1) (Observed species: $U = 0$, $p = 0.1$; Chao1: $U = 0$, $p = 0.1$; ACE: $U = 0$, $p = 0.1$; Fisher: $U = 0$, $p = 0.1$; Shannon: $U = 3$, $p = 0.1$). Similarity in microbial community structure between life

stages also remained unchanged (PERMANOVA: F-value: 1.698; R-squared: 0.5142; p-value = 0.2028; ANOSIM: R: 0.2593; p-value = 0.2992; PERMDISP: F-value: 0.0993; p-value = 0.7684).

Taxonomic composition of bacterial community:

From the 123 ASV, 107 (87%) were taxonomically assigned to at least the Phylum level. Taxonomic distribution of ASVs included 10 bacteria phyla, 18 classes, 22 orders, 33 families and 36 genera. Distribution of relative abundances for phylum, order and genus levels are shown in Figure 2 and fully detailed for all taxonomic levels in Supplementary Tables S2 to S6. Overall, the Phylum Proteobacteria (92.6%) and Firmicutes (5.2%) represented almost the full microbiota detected in this study (Supplementary Table S2, Fig.2A). The orders Rickettsiales (Phylum Proteobacteria: Class Alphaproteobacteria) and Clostridiales (Phylum Firmicutes: Class Clostridia) with abundances 91.9% and 4.8% respectively dominated the bacterial community. To a lesser extent, other 20 orders were present at or below 1% overall abundance (Supplementary table S4, Figure 2B).

From the total 123 ASVs, 95 (77.2%) were assigned to the genus level where 66 ASVs (53.7%) were annotated using GreenGenes (≥ 0.7 confidence level) and 29 ASVs (23.6%) annotated using BLASTn and BioCloud (≥ 97% identity to top-hit for both algorithms). The remaining 28 ASVs (22.8%) were considered as undetermined at genus level (Not Assigned). At the genus level, *Wolbachia* (Rickettsiaceae) dominated the overall abundance (91.9%) across nymph and adult samples followed by *Romboutsia* (1.8%), *Ignavibacterium* (0.8%), *Clostridium* (0.70%), *Mycoplasma* (0.5%), *Allobaculum* (0.4%), *Blautia* (0.4%), *Eubacterium*_g23 (0.3%), *Sporobacter* (0.3%), *Paracoccus* (0.3), *Methylobacterium* (0.2%), *Dorea* (0.2%), *Sediminibacterium* (0.1%), *Faecalibacterium* (0.1%), and *Ruminococcus* (0.1%) as the top 15 taxa. Other 34 genera were present at abundances below 0.1% across all life stages (Supplementary Table S6, Figure 2C). The relative abundances for bacteria taxa in all taxonomic levels (Phylum to Genus) were similar between both insect life stages (Mann-Whitney *U* test, p-values > 0.05, Supplementary Tables S2 to S6). Similarly, no statistical differences were found at bacterial ASV level between both life stages (Mann-Whitney *U* test, p-values > 0.05).

Removal of *Wolbachia* sequences from this analysis did not alter the similarities in the overall relative abundances at ASV or genus levels between life stages (Mann-Whitney *U* test, p-values > 0.05) as estimated above despite changes in the proportions of total reads counts and relative taxon abundances across the individual samples. Additionally, apart from *Wolbachia*, the list of the top ten most abundant genus remained unchanged; and in all cases the microbiota was dominated by *Romboutsia* with few changes in the order of the remaining genera (Fig. 3B). However, the exclusion of *Wolbachia* resulted in Firmicutes (69.3%) as the overall dominant Phylum, followed by Proteobacteria (10.4%), Chlorobi (6.2%) and other seven Phylum to a lesser extent (Fig. 3A).

Core microbiota:

According to the data collected in this study, the core microbiota of *M. velezangeli* is composed of 21 bacterial ASVs (17% of all ASVs) that were consistently shared between the nymph and adult life stages (ASVs present in all samples in this study) (Fig. 2D). These core ASVs were

identified by analyzing 42 ASVs present in all samples of both life stage. The remaining 21 ASVs were present at either nymph or adult. Other 81 ASVs (65.9% of all ASVs) were not consistently detected in all samples of each life stage and may represent transient or non-resident microbes within the microbiome of *M*. *velezangeli*.

The bacterial genus assignments for the core 21 ASVs based on 16S GreenGenes database are shown in Table 3. These core bacterial genera, listed in decreasing order of abundance, included: *Wolbachia*, *Romboutsia*, *Ignavibacterium, Clostridium*, *Allobaculum, Paracoccus*, undetermined Anaerolineaceae, *Methylobacterium, Faecalibacterium,* undetermined Lachnospiraceae, *Collinsella*, *Rothia,* undetermined Peptostreptococcaceae, *Sphingomonas* and undetermined Coriobacteriaceae.

***Wolbachia* PCR detection and profiling**:

Taking into account the large proportion of *Wolbachia*-associated ASVs found in our samples, we decided to further investigate the presence of *Wolbachia* endosymbiont in *M. velezangeli* by PCR screening of the *wsp* gene. About 79% of the insect individuals tested (22 out of 28) from the Segovianas locality resulted positive for *Wolbachia* infection according to the amplification of a ~600 bp DNA band (Figure 4). A PCR test for DNA template integrity showed that all 28 (100%) *M. velezangeli* DNA samples were PCR quality grade based on the successful amplification of a DNA band for the arthropod 28S rRNA gene target. This indicates that the no *wsp* amplification in 21% (6/28) of the samples could be explained by the absence of *Wolbachia* infection and not because of a low DNA template quality.

DNA Sanger sequencing was performed for 10 randomly selected *wsp* DNA amplicons derived from *M. velezangeli* samples. All DNA sequences had clearly defined single-pick chromatograms, which suggested the presence of single *Wolbachia*-strain infections in each sequenced sample. A multiple sequence alignment showed that these isolates can be grouped in two distinct *wsp* sequence haplotypes that share 78% similarity between them (Supplementary Figure S1). Haplotype 1 (hereafter wMvel1) was represented by 80% (8/10) of the sequence isolates in this study, whereas haplotype 2 (hereafter wMvel2) was represented by the remaining 20% (2/10) isolates. A BLASTn search against the GeneBank database showed that wMvel1 *wsp* sequence was 99.46% identical (top hit) to a *Wolbachia wsp* isolate from the butterfly *Acraea equitorialis* (GenBank accession: AJ271195) whereas wMvel2 *wsp* was 98.91% identical (top hit) to a *Wolbachia wsp* isolate from the planthopper *Perkinsiella saccharicida* (GenBank accession: GU190768) (Hughes et al. 2011). Phylogenetic analysis clustered all wMvel *wsp* sequences within the *Wolbachia wsp* B supergroup clade (Figure 5) and assigned the distinct wMvel *wsp* haplotypes within two distant subclades respectively along with their corresponding *wsp* BLASTn top-hits.

Discussion:

We used 16S rRNA amplicon high-throughput sequencing to investigate for the first time the diversity of the symbiotic bacteria community associated with the tropical plant bug *M*. *velezangeli*. Here, we found a relatively diverse core microbiota dominated by genera *Wolbachia*, *Romboutsia*, *Ignavibacterium* and *Clostridium*. Although this plant bug is a

polyphagous herbivore considered a pest for various tropical crops in America, in this study we focused the bacteria screening on a population feeding on coffee plants in Colombia. We found that overall bacteria diversity (Alpha diversity, Fig. 1B) was similar between the nymph and the adult life stages. Based on the most abundant taxa (ASVs with overall abundance >0.01%), the bacterial community composition (Beta diversity) is conserved between these two developmental stages. However, there is a degree of variability regarding the presence of bacteria with low abundance within and between life stages. The immature forms of *M. velezangeli* go through 5 nymphal instars that differ among them mainly in body size (Giraldo J. et al. 2010). The microbial composition we present in this work for the nymph is based on pooled individuals from all instars. Hence, whether the overall bacterial community diversity and structure experience any changes along nymphal development needs to be addressed in future analyses. The bacterial 16S rRNA gene sequence has been used historically as a gold standard genetic marker to infer bacteria taxonomic identity and community diversity in high-throughput microbiome studies especially with the use of the partial sequencing of some of its nine hypervariable sequence regions (V1 to V9) (Van de Peer et al. 1996). In our study, we used the sequences of the combined V3-V4 variable regions, a 16S sequence section commonly utilized in microbiome analysis; however, it must be noticed that the used of partial sequences of this gene marker can result in overestimation of microbial diversity due to bacterial intragenomic heterogeneity (Sun et al. 2013), and does not offer enough accuracy for bacteria identification at the species or strain level (Johnson et al. 2019a). Being aware of this bias, we mainly describe the microbial taxonomic diversity in this study at genus level as the deepest taxonomic rank.

We found that the intracellular symbiont *Wolbachia* dominated the full microbiota associated with *M. velezangeli* which represent about 92% of the bacterial load within the body of nymph and adult stages. The observed high abundance of *Wolbachia* in our samples may indicate a proportionally elevated titer of this endosymbiont in the analyzed insects as well. Presence of *Wolbachia* was also confirmed by PCR screening in *M. velezangeli* samples. Additionally, DNA sequence analysis of wMvel *wsp* isolates indicates that they belong to *Wolbachia* B supergroup. Insect-infecting *Wolbachia* strains with major biological effects have been mostly associated with host reproductive disturbances such as CI, parthenogenesis, male-killing and feminization (Serbus et al. 2008; Werren et al. 2008; Kaur et al. 2021). Furthermore, recent studies suggest that *Wolbachia* infections may also influence other behavioral and physiological processes including nutrition, defense and insecticide-resistance (Hosokawa et al. 2010; Nikoh et al. 2014; Zug and Hammerstein 2015; Zhang et al. 2020; Soh and Veera 2022). In other mirid species the presence of *Wolbachia* has been associated with reproductive alterations and nutritional roles. For example in the predatory mirid bug *Macrolophus pygmaeus* this parasitic bacteria induces strong CI (Machtelinckx et al. 2009). *Wolbachia* infection in the hematophagous bed bugs *Cimex lectularius* and *Cimex hemipterus* (Hemiptera: Miridae) creates an obligate mutualism that is essential for normal insect growth and reproduction via provision of B vitamins (Hosokawa et al. 2010; Laidoudi et al. 2020). *Wolbachia* infections in insects have been mainly associated with host reproductive tissues, but it is also commonly found in several insect somatic organs or tissues including brain, salivary glands, gut, malpighian tubules, muscles, fat bodies and also as habitant of bacteriocytes (Casper-Lindley et al. 2011; Pietri et al. 2016; Diouf et al. 2018; Hosokawa et al. 2010). The presence of this parasitic endosymbiont in *M.*

velezangeli raises new questions about the possible biological implications for this plant bug. The detection of two distinct wMvel *wsp* haplotypes in our analysis suggest that multiple *Wolbachia* strains are present in the insect population tested here. However, insect individuals seem to be infected by single *Wolbachia* strains. Additionally, the prevalence of infection is not 100% across all insect individuals, which seems to indicate that an obligate mutualism is not the proper characterization of the *M. velezangeli* - *Wolbachia* relationship.

The extremely high abundance of ASV sequences identified as *Wolbachia* in our samples (~92% overall abundance) could introduce a potential confounding effect in the estimation of relative abundances for the actual gut-associated bacterial taxa. This possible issue was recently analyzed by Wilches et al. (Wilches et al. 2021) using the spotted-wing drosophila (*Drosophila suzukii*) as a case of study when high-throughput sequencing is applied to investigate the microbiome in *Wolbachia*-infected insect samples. The authors detected large discrepancies in the measures of alpha and beta diversity as well as in the relative abundances of several bacteria taxa in the microbiome between *Wolbachia*-infected fly samples (mean abundance of 98.8% for *Wolbachia* sequences) and non-infected. This and other work has shown that in some cases removing the *Wolbachia*-associated reads from the analyses could also have major impacts in the interpretation of the study results which may be especially relevant when comparing infected samples versus non-infected (Wilches et al. 2021; Henry and Ayroles 2021). We addressed the impact of removing *Wolbachia* reads in microbiota diversity and structure in *M*. *velezangeli*. Here, the exclusion of *Wolbachia*-associated sequences did not affect the similarity in microbiota composition between the life stages.

Apart from *Wolbachia* (Proteobacteria) the remaining top 10 most abundant bacterial genera detected in *M*. *velezangeli* include members of Phylum Firmicutes (5.2% overall abundance) such as *Romboutsia*, *Clostridium*, *Allobaculum*, Blautia, *Eubacterium*_g23, Sporobacter, *Dorea* and *Faecalibacterium,* as well as the Proteobacteria genera *Paracoccus*, *Methylobacterium* and the Chlorobi genus *Ignavibacterium*. Members of these Firmicutes genera have been previously found in the alimentary canals of other arthropods (Grech-Mora et al. 1996; Husseneder et al. 2017; Li et al. 2020; Shukla and Beran 2020; Fang et al. 2020; Mejía-Alvarado et al. 2021). In our study, *Romboutsia* (1.75%) (Firmicutes: Peptostreptococcaceae) was the second most abundant bacterial genus across all samples. Members of this genus have been mainly found in the gut microbiota from several vertebrate animals and insects (Gerritsen et al. 2014, 2017; Ricaboni et al. 2016; Johnson et al. 2019B; Shukla and Beran 2020). There is no information about the functional roles of the *Romboutsia* members as gut symbionts; however, they seem to be well adapted to live within animal guts (Gerritsen et al. 2017, 2019). Similarly, members of *Paracoccus*, *Methylobacterium* and *Ignavibacterium* are regular habitants of arthropod guts (Zhang et al. 2016, 2018; Sajnaga et al. 2022). We infer that most abundant bacteria genera found in this study, except *Wolbachia,* are likely residents of the *M. velezangeli* gut lumen and may be involved in important biological processes for this plant bug. Several of these symbionts (*Romboutsia*, *Ignavibacterium, Clostridium*, *Paracoccus*, *Allobaculum*, *Methylobacterium*, *Faecalibacterium*, *Collinsella*, *Rothia* and *Sphingomonas*) were found to be consistently present in all our samples of nymph and adult stages and we consider them as members of the insect gut-associated core microbiota. Most of these genera, except for *Paracoccus, Methylobacterium*

and *Sphingomonas*, are primarily anaerobic bacteria taxa. Compared with the microbiota associated with the cotton fleahopper *P*. *seriatus* (Hemiptera: Miridae) (Fu et al. 2021) and *A*. *suturalis* (Hemiptera: Miridae) (Xue et al. 2021) the composition at the genus level within *M*. *velezangeli* is clearly different. In *P*. *seriatus*, the gut microbiome is dominated by bacteria *Diaphorobacter*, *Lactococcus*, *Pseudomonas*, *Pantoea* and *Izhakiella*; whereas in *A*. *suturalis*, the gut microbiome is dominated by *Erwinia*, *Acinetobacter*, *Staphylococcus*, and *Lactococcus*. These differences in microbiota composition could be associated with environmental differences due to host-plant species, feeding habits and geographical origins.

Several bacteria isolates found in *M. velezangeli*'s microbiota that belong to genera *Paracoccus, Methylobacterium* and *Sphingomonas* are potential culturable strains and may also represent candidate symbionts for paratransgenic approaches such as symbiont-mediated RNAi (Dyson et al. 2022). The use of bacteria within paratransgenesis applications requires a culturable symbiont genetically manipulable and especially amenable under aerobic culturable conditions (Ratcliffe et al. 2022). Conditions like these make easier bacterial engineering and experimentation processes. Future attempts for selection of culturable bacterial isolates from the *M. velezangeli* gut will reveal what microbes have these characteristics.

Conclusions:

The tropical plant bug *M*. *velezangeli* harbors a diverse microbiota, and in some cases it can be dominated by the intracellular symbiont *Wolbachia.* The *M. velezangeli* microbiota also contain potential gut-associated members of the genera *Romboutsia*, *Ignavibacterium, Clostridium*, *Paracoccus*, *Allobaculum*, *Methylobacterium*, *Faecalibacterium*, *Collinsella*, *Rothia* and *Sphingomonas*. The persistent detection of these bacteria genera in nymphal and adult life stages indicates they seem to be part of the core microbiome and likely play important biological roles in the normal development of *M*. *velezangeli*. Additionally, our observations suggest that multiple *Wolbachia* strains are present in *M. velezangeli* populations, but insect individuals seem to harbor single-strain infections. The findings reported by this study offer new avenues to improve our understanding of the microbiome contribution in the biology of Miridae plant bugs such as the tropical insect pest *M*. *velezangeli*.

Statements and Declarations:

Acknowledgements: The authors thank the National Federation of Coffee Growers of Colombia (FNC) and the personnel of the Extension Service of FNC at the Department of Huila (Colombia) for their support and logistic arrangements for insect sample collection. The authors also thank the coffee farmers at the Municipality of La Argentina (Huila, Colombia) who kindly allowed the insect sampling in their coffee plantations.

Data Availability: The datasets generated or analyzed during the current study are included in this article and its supplementary information files. The raw sequence data is accessible at the NCBI Sequence Read Archive (SRA) database under the bioproject number PRJNA875474 (http://www.ncbi.nlm.nih.gov/bioproject/875474).

Funding: This work was supported by the National Coffee Research Center at the National Federation of Coffee Growers of Colombia [Funder ID 100019597, project number ENT101006]; and Sistema General de Regalías del Departamento de Huila, Colombia [convenio 0244 del 2014].

Competing Interests: The authors have no relevant financial or non-financial interests to disclose.

Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Lucio Navarro-Escalante and Flor E. Acevedo. Pablo Benavides contributed to data interpretation. The first draft of the manuscript was written by Lucio Navarro-Escalante and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

References:

Acevedo FE, Peiffer M, Tan C-W, et al (2017) Fall armyworm-associated gut bacteria modulate plant defense responses. Mol Plant Microbe Interact 30:127–137

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecol 26:32–46

Anderson MJ, Walsh DCI (2013) PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? Ecol Monogr 83:557–574

Anisimova M, Gascuel O (2006) Approximate likelihood-ratio test for branches: A fast, Accurate, and powerful alternative. Syst Biol 55:539–552

Blanton AG, Peterson BF (2020) Symbiont-mediated insecticide detoxification as an emerging problem in insect pests. Front Microbiol 0.: https://doi.org/10.3389/fmicb.2020.547108

Bolyen E, Rideout JR, Dillon MR, et al (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852–857

Callahan BJ, McMurdie PJ, Rosen MJ, et al (2016) DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583

Caporaso JG, Lauber CL, Walters WA, et al (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A 108 Suppl 1:4516–4522

Casper-Lindley C, Kimura S, Saxton DS, et al (2011) Rapid fluorescence-based screening for *Wolbachia* endosymbionts in *Drosophila* germ line and somatic tissues. Appl Environ Microbiol 77:4788

Chandler JA, James PM, Jospin G, Lang JM (2014) The bacterial communities of *Drosophila suzukii* collected from undamaged cherries. PeerJ 2.: https://doi.org/10.7717/peerj.474

Chong J, Liu P, Zhou G, Xia J (2020) Using MicrobiomeAnalyst for comprehensive statistical,

functional, and meta-analysis of microbiome data. Nat Protoc 15.: https://doi.org/10.1038/s41596-019-0264-1

Chung SH, Rosa C, Scully ED, et al (2013) Herbivore exploits orally secreted bacteria to suppress plant defenses. Proc Natl Acad Sci U S A 110:15728–15733

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Austral Ecol. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x

Dereeper A, Guignon V, Blanc G, et al (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res 36:W465–W469

Dhariwal A, Chong J, Habib S, et al (2017) MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res 45.: https://doi.org/10.1093/nar/gkx295

Diouf M, Miambi E, Mora P, et al (2018) Variations in the relative abundance of *Wolbachia* in the gut of *Nasutitermes arborum* across life stages and castes. FEMS Microbiol Lett 365.: https://doi.org/10.1093/femsle/fny046

Dylan Shropshire J, Leigh B, Bordenstein SR (2020) Symbiont-mediated cytoplasmic incompatibility: What have we learned in 50 years? https://doi.org/10.7554/eLife.61989

Dyson P, Figueiredo M, Andongma AA, Whitten MMA (2022) Symbiont-mediated RNA interference (SMR): Using symbiotic bacteria as vectors for delivering RNAi to insects. Methods Mol Biol 2360:295–306

Fang J-X, Zhang S-F, Liu F, et al (2020) Differences in gut bacterial communities of *Ips typographus* (Coleoptera: Curculionidae) Induced by enantiomer-specific α-pinene. Environ Entomol 49:1198–1205

Fukui T, Kawamoto M, Shoji K, et al (2015) The endosymbiotic bacterium *Wolbachia* selectively kills male hosts by targeting the masculinizing gene. PLoS Pathog 11:e1005048

Fu Z, Antwi JB, Sword GA, et al (2021) Geographic variation of bacterial communities associated with cotton fleahopper, *Pseudatomoscelis seriatus*. swen 46:17–32

Gerritsen J, Fuentes S, Grievink W, et al (2014) Characterization of *Romboutsia ilealis* gen. nov., sp. nov., isolated from the gastro-intestinal tract of a rat, and proposal for the reclassification of five closely related members of the genus *Clostridium* into the genera *Romboutsia* gen. nov., *Intestinibacter* gen. nov., *Terrisporobacter* gen. nov. and *Asaccharospora* gen. nov. Int J Syst Evol Microbiol 64:1600–1616

Gerritsen J, Hornung B, Renckens B, et al (2017) Genomic and functional analysis of *Romboutsia ilealis* CRIBT reveals adaptation to the small intestine. PeerJ 5.: https://doi.org/10.7717/peerj.3698

Gerritsen J, Hornung B, Ritari J, et al (2019) A comparative and functional genomics analysis of the genus *Romboutsia* provides insight into adaptation to an intestinal lifestyle. bioRxiv 845511

Ge S-X, Li T-F, Ren L-L, Zong S-X (2023) Host-plant adaptation in xylophagous insect-microbiome systems: Contributionsof longicorns and gut symbionts revealed by parallel

metatranscriptome. iScience 26:106680

Giraldo J. M, Benavides M. P (2012) Conozca los hospedantes sitios de alimentación y oviposición de la chinche de la chamusquina del café. Cenicafe. Avance Tecnico #418. URI: http://hdl.handle.net/10778/332

Giraldo J. M, Benavides M. P, Villegas G. C (2010) Aspectos morfológicos y biológicos de *Monalonion velezangeli* Carvalho and Costa (Hemiptera : Miridae) en café. Cenicafe 6:195–205

Grech-Mora I, Fardeau M-L, Patel BKC, et al (1996) Isolation and characterization of *Sporobacter termitidis* gen. Nov., sp. Nov., from the digestive tract of the wood-feeding termite *Nasutitermes lujae*. Int J Syst Evol Microbiol 46:512–518

Guindon S, Gascuel O (2003) A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. Syst Biol 52:696–704

Hammer-Muntz O, Harper D, Ryan P (2001) PAST: paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4:1–9

Henry LP, Ayroles JF (2021) Meta-analysis suggests the microbiome responds to evolve and resequence experiments in *Drosophila melanogaster*. BMC Microbiol 21:1–14

Hilgenboecker K, Hammerstein P, Schlattmann P, et al (2008) How many species are infected with *Wolbachia*? A statistical analysis of current data. FEMS Microbiol Lett 281:215–220

Hiroki M, Kato Y, Kamito T, Miura K (2002) Feminization of genetic males by a symbiotic bacterium in a butterfly, *Eurema hecabe* (Lepidoptera: Pieridae). Naturwissenschaften 89.: https://doi.org/10.1007/s00114-002-0303-5

Hosokawa T, Koga R, Kikuchi Y, et al (2010) *Wolbachia* as a bacteriocyte-associated nutritional mutualist. Proc Natl Acad Sci U S A 107:769

Hughes GL, Allsopp PG, Brumbley SM, et al (2011) Variable infection frequency and high diversity of multiple strains of Wolbachia pipientis in Perkinsiella Planthoppers. Appl Environ Microbiol 77:2165–2168

Hurst GDD, Jiggins FM, von der Schulenburg JHG, et al (1999) Male-killing *Wolbachia* in two species of insect. Proceedings of the Royal Society B: Biological Sciences 266:735

Husseneder C, Park J-S, Howells A, et al (2017) Bacteria associated with *Piezodorus guildinii* (Hemiptera: Pentatomidae), with special reference to those transmitted by feeding. Environ Entomol 46:159–166

Jaimes LFT, Valenzuela JRC, Londoño GAC, et al (2015) Relación entre la presencia y el daño de *Monalonion velezangeli* Carvalho & Costa y algunos factores climáticos en cultivos de aguacate cv. Hass. CTA 16:79–85

Janson EM, Stireman JO 3rd, Singer MS, Abbot P (2008) Phytophagous insect-microbe mutualisms and adaptive evolutionary diversification. Evolution 62:997–1012

Jie Bi YW (2020) The effect of the endosymbiont *Wolbachia* on the behavior of insect hosts. Insect Sci 27:846

599 Johnson JS, Spakowicz DJ, Hong B-Y, et al (2019a) Evaluation of 16S rRNA gene sequencing
600 for species and strain-level microbiome analysis. Nat Commun 10:1–11

601 Johnson TA, Sylte MJ, Looft T (2019b) In-feed bacitracin methylene disalicylate modulates the
602 turkey microbiota and metabolome in a dose-dependent manner. Sci Rep 9.:
603 https://doi.org/10.1038/s41598-019-44338-5

604 Jung S, Lee S (2012) Molecular phylogeny of the plant bugs (Heteroptera: Miridae) and the
605 evolution of feeding habits. Cladistics 28:50–79

606 Kaur R, Shropshire JD, Cross KL, et al (2021) Living in the endosymbiotic world of *Wolbachia*: A
607 centennial review. Cell Host Microbe 29:879–893

608 Kikuchi Y, Hayatsu M, Hosokawa T, et al (2012) Symbiont-mediated insecticide resistance. Proc
609 Natl Acad Sci U S A 109.: https://doi.org/10.1073/pnas.1200231109

610 Klindworth A, Pruesse E, Schweer T, et al (2012) Evaluation of general 16S ribosomal RNA
611 gene PCR primers for classical and next-generation sequencing-based diversity studies.
612 Nucleic Acids Res 41:e1–e1

613 Laidoudi Y, Levasseur A, Medkour H, et al (2020) An earliest endosymbiont, *Wolbachia*
614 *massiliensis* sp. nov., strain PL13 from the bed Bbg (*Cimex hemipterus*), type strain of a
615 new supergroup T. Int J Mol Sci 21:8064

616 Lariviere PJ, Leonard SP, Horak RD, et al (2022) Honey bee functional genomics using
617 symbiont-mediated RNAi. bioRxiv 2022.04.22.489157

618 Li Q, Fan J, Sun J, et al (2019) Anti-plant defense response strategies mediated by the
619 secondary symbiont *Hamiltonella defensa* in the wheat aphid *Sitobion miscanthi*. Front
620 Microbiol 0.: https://doi.org/10.3389/fmicb.2019.02419

621 Li R, Rongrong LI, Min LI, et al (2020) Composition and function of the microbiotas in the
622 different parts of the midgut of *Pyrrhocoris sibiricus* (Hemiptera: Pyrrhocoridae) revealed
623 using high-throughput sequencing of 16S rRNA. Eur J Entomol 117:352–371

624 Luo J, Cheng Y, Guo L, et al (2021) Variation of gut microbiota caused by an imbalance diet is
625 detrimental to bugs' survival. Sci Total Environ 771:144880

626 Machtelinckx T, Van Leeuwen T, Vanholme B, et al (2009) *Wolbachia* induces strong
627 cytoplasmic incompatibility in the predatory bug *Macrolophus pygmaeus*. Insect Mol Biol
628 18:373–381

629 Mejía-Alvarado FS, Ghneim-Herrera T, Góngora CE, et al (2021) Structure and dynamics of the
630 gut bacterial community across the developmental stages of the coffee berry borer,
631 *Hypothenemus hampei*. Front Microbiol.: https://doi.org/10.3389/fmicb.2021.639868

632 Moran NA, Telang A (1998) Bacteriocyte-associated symbionts of insects: A variety of insect
633 groups harbor ancient prokaryotic endosymbionts. Bioscience 48:295–304

634 Motta EVS, Gage A, Smith TE, et al (2022) Host-microbiome metabolism of a plant toxin in
635 bees. Elife 11.: https://doi.org/10.7554/eLife.82595

636 Namyatova AA, Cassis G (2016) Systematic revision and phylogeny of the plant bug tribe

637 Monaloniini (Insecta: Heteroptera: Miridae: Bryocorinae) of the world. Zool J Linn Soc
638 176:36–136

639 Narita S, Kageyama D, Nomura M, Fukatsu T (2007) Unexpected mechanism of symbiont-
640 induced reversal of insect sex: feminizing *Wolbachia* continuously acts on the butterfly
641 *Eurema hecabe* during larval development. Appl Environ Microbiol 73:4332–4341

642 Nikoh N, Hosokawa T, Moriyama M, et al (2014) Evolutionary origin of insect-*Wolbachia*
643 nutritional mutualism. Proc Natl Acad Sci U S A 111.:
644 https://doi.org/10.1073/pnas.1409284111

645 Ocampo Flórez V, Durán Prieto J, Albornoz M, Forero D (2018) New plant associations for
646 *Monalonion velezangeli* (Hemiptera: Miridae) in green urban areas of Bogotá (Colombia).
647 Acta biolColomb 23:205–208

648 O'Neill SL, Hoffmann AA, Werren JH (1997) Influential Passengers: Inherited Microorganisms
649 and Arthropod Reproduction. Oxford University Press, USA

650 Pavlidis P, Noble WS (2003) Matrix2png: a utility for visualizing matrix data. Bioinformatics 19.:
651 https://doi.org/10.1093/bioinformatics/19.2.295

652 Pietri JE, DeBruhl H, Sullivan W (2016) The rich somatic life of *Wolbachia*. Microbiologyopen
653 5:923

654 Ramirez C. HJ, Gil P. ZN, Benavides M. P, Bustillo P. AE (2008) *Monalonion velezangeli*: La
655 chinche de la chamusquina del café. Cenicafe

656 Ramírez-Gil JG, López JH, Henao-Rojas JC (2019) Causes of Hass avocado fruit rejection in
657 preharvest, harvest, and packinghouse: economic losses and associated variables.
658 Agronomy 10:8

659 Ratcliffe NA, Furtado Pacheco JP, Dyson P, et al (2022) Overview of paratransgenesis as a
660 strategy to control pathogen transmission by insect vectors. Parasit Vectors 15:112

661 Ricaboni D, Mailhe M, Khelaifia S, et al (2016) *Romboutsia timonensis*, a new species isolated
662 from human gut. New Microbes and New Infections 12:6–7

663 Rodas CA, Serna R, Hurley BP, et al (2014) Three new and important insect pests recorded for
664 the first time in Colombian plantations. Southern Forests 76:245–252

665 Rudman SM, Greenblum S, Hughes RC, et al (2019) Microbiome composition shapes rapid
666 genomic adaptation of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 116.:
667 https://doi.org/10.1073/pnas.1907787116

668 Sajnaga E, Skowronek M, Kalwasińska A, et al (2022) Comparative Nanopore sequencing-
669 based evaluation of the midgut microbiota of the summer chafer (l.) associated with
670 possible resistance to entomopathogenic nematodes. Int J Environ Res Public Health 19.:
671 https://doi.org/10.3390/ijerph19063480

672 Sato Y, Jang S, Takeshita K, et al (2021) Insecticide resistance by a host-symbiont reciprocal
673 detoxification. Nat Commun 12:1–8

674 Schausberger P (2018) Herbivore-associated bacteria as potential mediators and modifiers of

induced plant defense against spider mites and thrips. Front Plant Sci 0.: https://doi.org/10.3389/fpls.2018.01107

Serbus LR, Casper-Lindley C, Landmann F, Sullivan W (2008) The genetics and cell biology of *Wolbachia*-host interactions. Annu Rev Genet 42:683–707

Shukla SP, Beran F (2020) Gut microbiota degrades toxic isothiocyanates in a flea beetle pest. Molecular Ecology 29:4692–4705

Sinkins SP (2004) *Wolbachia* and cytoplasmic incompatibility in mosquitoes. Insect Biochem Mol Biol 34:723–729

Soh LS, Veera SG (2022) Bacterial symbionts influence host susceptibility to fenitrothion and imidacloprid in the obligate hematophagous bed bug, *Cimex hemipterus*. Sci Rep 12.: https://doi.org/10.1038/s41598-022-09015-0

Stouthamer R, Breeuwer JA, Hurst GD (1999) *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu Rev Microbiol 53:71–102

Sudakaran S, Kost C, Kaltenpoth M (2017) Symbiont acquisition and replacement as a source of ecological innovation. Trends Microbiol 25:375–390

Sun D-L, Jiang X, Wu QL, Zhou N-Y (2013) Intragenomic heterogeneity of 16S rRNA genes causes overestimation of prokaryotic diversity. Appl Environ Microbiol. https://doi.org/10.1128/AEM.01282-13

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Van de Peer Y, Chapelle S, De Wachter R (1996) A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Res 24:3381–3391

Vavre F, de Jong JH, Stouthamer R (2004) Cytogenetic mechanism and genetic consequences of thelytoky in the wasp *Trichogramma cacoeciae*. Heredity 93:592–596

Werren JH (1997) Biology of *Wolbachia*. Annu Rev Entomol 42:587–609

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6:741–751

Wilches D, Coghlin P, Floate K (2021) Next generation sequencing, insect microbiomes, and the confounding effect of *Wolbachia*: a case study using *Drosophila suzukii Matsumura* (Diptera: Drosophilidae). Canadian Journal of Zoology 99.: https://doi.org/10.1139/cjz-2020-0260

Xue H, Zhu X, Wang L, et al (2021) Gut bacterial diversity in different life cycle stages of *Adelphocoris suturalis* (Hemiptera: Miridae). Front Microbiol 0.: https://doi.org/10.3389/fmicb.2021.670383

Zhang D, Wang Y, He K, et al (2020) *Wolbachia* limits pathogen infections through induction of host innate immune responses. PLoS One 15:e0226736

Zhang L, Yun Y, Hu G, Peng Y (2018) Insights into the bacterial symbiont diversity in spiders. Ecol Evol 8:4899–4906

Zhang S, Gan L, Qin Q, et al (2016) *Paracoccus acridae* sp. nov., isolated from the insect *Acrida cinerea* living in deserted cropland. Int J Syst Evol Microbiol 66:3492–3497

Zhou J-C, Zhao Q, Liu S-M, et al (2021) Effects of thelytokous parthenogenesis-inducing *Wolbachia* on the fitness of *Trichogramma dendrolimi* Matsumura (Hymenoptera: Trichogrammatidae) in superparasitised and single-parasitised hosts. Front Ecol Evol 0.: https://doi.org/10.3389/fevo.2021.730664

Zhou W, Rousset F, O'Neil S (1998) Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences. Proc Biol Sci 265:509–515

Zug R, Hammerstein P (2015) *Wolbachia* and the insect immune system: what reactive oxygen species can tell us about the mechanisms of *Wolbachia*–host interactions. Front Microbiol 0.: https://doi.org/10.3389/fmicb.2015.01201

Tables:

Table 1. Overview of Illumina 16SrRNA-amplicon sequencing of the bacterial microbiota in *Monalonion velezangeli*.

Sample	Raw PE reads	Clean PE reads	Raw merged sequences	Clean merged sequences	Total ASVs	Good's coverage
Adult 1	173,233	96,265	74,667	69,782	63	100%
Adult 2	162,775	109,344	90,923	90,168	62	100%
Adult 3	167,579	109,789	91,180	90,009	45	100%
Nymph 1	170,239	110,169	87,856	86,212	82	99.99%
Nymph 2	172,490	85,101	60,756	58,026	85	100%
Nymph 3	169,525	115,394	97,798	97,605	71	100%

Table 2. Alpha diversity indices for 16SrRNA-based microbiota in *Monalonion velezangeli*.

Sample	Observed	Chao1 (±se)	ACE (±se)	Fisher	Shannon (H')
Adult1	63	63 (±0.0)	63 (±1.69)	6.82	0.81
Adult2	62	62 (±0.0)	62 (±2.90)	6.50	0.41
Adult3	45	45 (±0.0)	45 (±1.91)	4.55	0.37
Nymph1	82	83 (±2.33)	82.5 (±3.25)	8.94	0.46
Nymph2	85	85 (±0.0)	85 (±2.97)	9.78	1.12
Nymph3	71	71 (±0.0)	71 (±2.66)	7.49	0.39

Table 3. Bacterial genus annotations for ASVs considered as members of the core microbiota in *Monalonion velezangeli*.

ID	Overall abundance	Genus (Family) rank annotation[#]
ASV01	91.7%	*Wolbachia* (Rickettsiaceae)
ASV02	0.97%	*Romboutsia* (Peptostreptococcaceae)*
ASV03	0.79%	*Romboutsia* (Peptostreptococcaceae)*
ASV04	0.73%	*Ignavibacterium* (Ignavibacteriaceae)*
ASV05	0.47%	*Clostridium* (Clostridiaceae)*
ASV06	0.26%	*Paracoccus* (Rhodobacteraceae)
ASV07	0.25%	Undetermined (Anaerolineaceae)*
ASV08	0.22%	*Allobaculum* (Erysipelotrichaceae)
ASV09	0.19%	*Methylobacterium* (Methylobacteriaceae)
ASV10	0.14%	*Sediminibacterium* (Chitinophagaceae)
ASV11	0.13%	*Allobaculum* (Erysipelotrichaceae)
ASV12	0.12%	*Faecalibacterium* (Ruminococcaceae)
ASV13	0.11%	*Clostridium* (Clostridiaceae)
ASV14	0.09%	Undetermined (Lachnospiraceae)
ASV15	0.09%	*Collinsella* (Coriobacteriaceae)
ASV16	0.09%	*Rothia* (Micrococcaceae)
ASV17	0.06%	*Clostridium* (Clostridiaceae)
ASV18	0.06%	Undetermined (Peptostreptococcaceae)
ASV19	0.06%	*Allobaculum* (Erysipelotrichaceae)
ASV20	0.05%	*Sphingomonas* (Sphingomonadaceae)
ASV21	0.04%	Undetermined (Coriobacteriaceae)

The taxonomic classification was determined using the 16S GreenGenes (GG) database with a confidence level of ≥0.7. For ASVs where GG failed to assign a Genus taxon, the Genus identification was performed using the BLASTn and BioCloud search algorithms with a concomitant ≥97% sequence identity for their top hits (taxa denoted with asterisk [*]). Further details can be found in the Methods section.

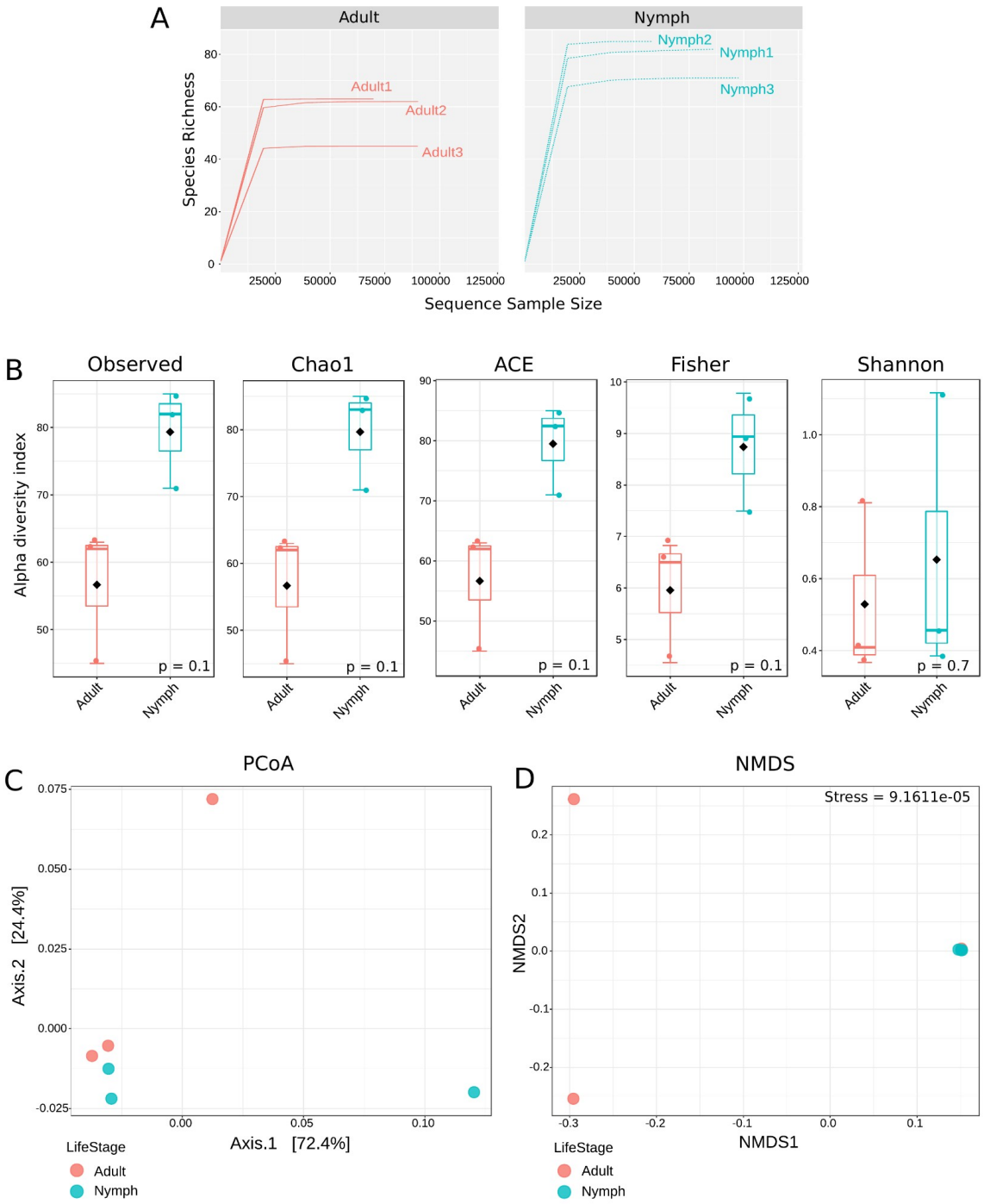


Figure 1. Diversity and community structure of the bacterial microbiota in adult and nymph life stages of *Monalonion velezangeli*. (A) Rarefaction curves. (B) Alpha diversity indices and their corresponding p-value of the Mann-Whitney U test. (C) Principal coordinate analysis (PCoA) plot based on Bray-Curtis dissimilarity of bacterial communities in nymphs and adults. (D) Non-metric multidimensional scaling (NMDS) ordination analysis plot based on Bray-Curtis dissimilarity of bacterial communities in nymphs and adults. Stress value represents the goodness-of-fit for the NMDS analysis.

22

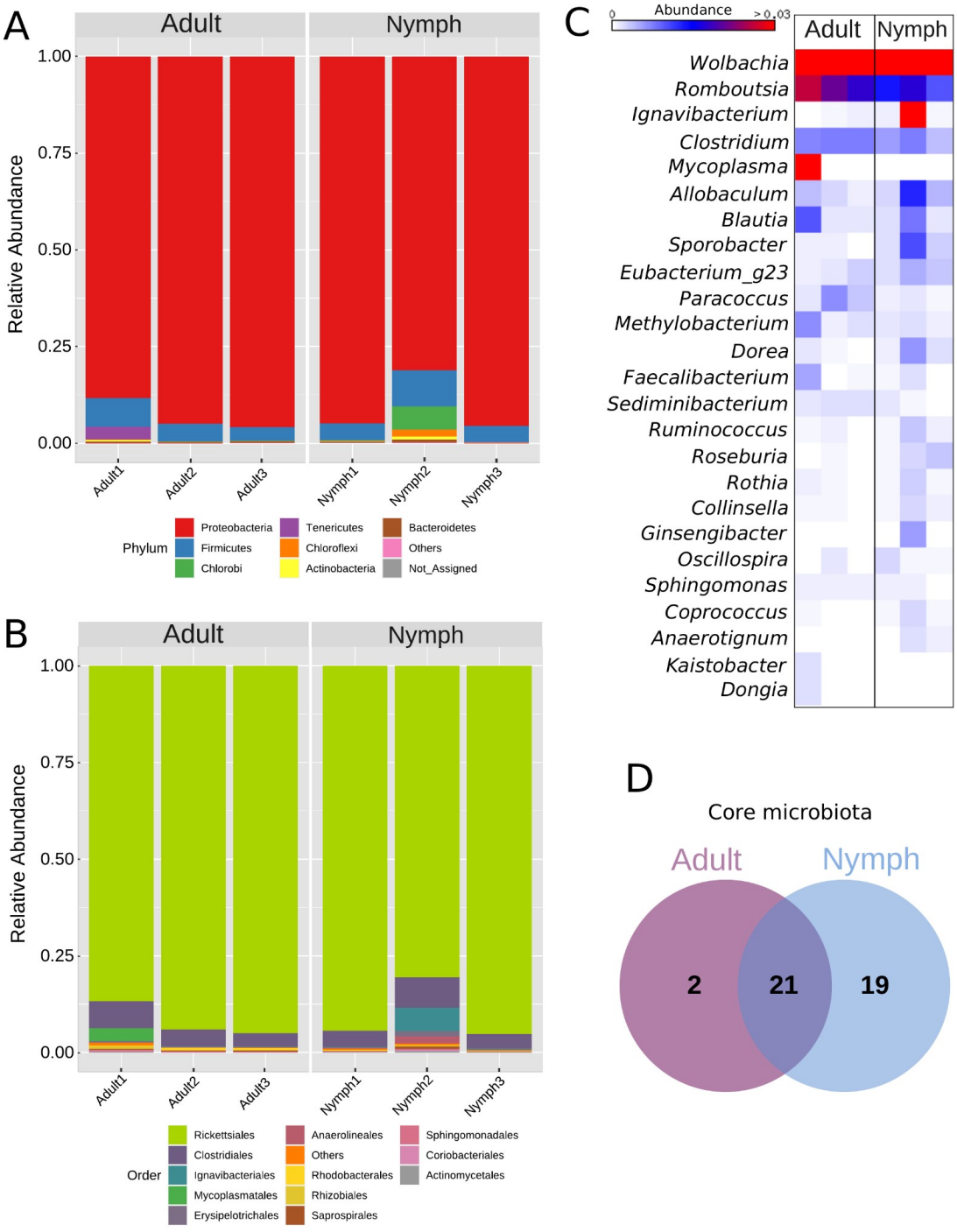


Figure 2. Taxonomic composition of the bacterial microbiota in nymphs and adults of *Monalonion velezangeli*. (A) Relative abundance at Phylum level. (B) Relative abundance at Order level. (C) Heatmap for relative abundances at genus level. (D) Number of ASV sequences consistently detected on either adults of nymphs and number of shared ASVs (circle intersection) as members of the core microbiota.

23

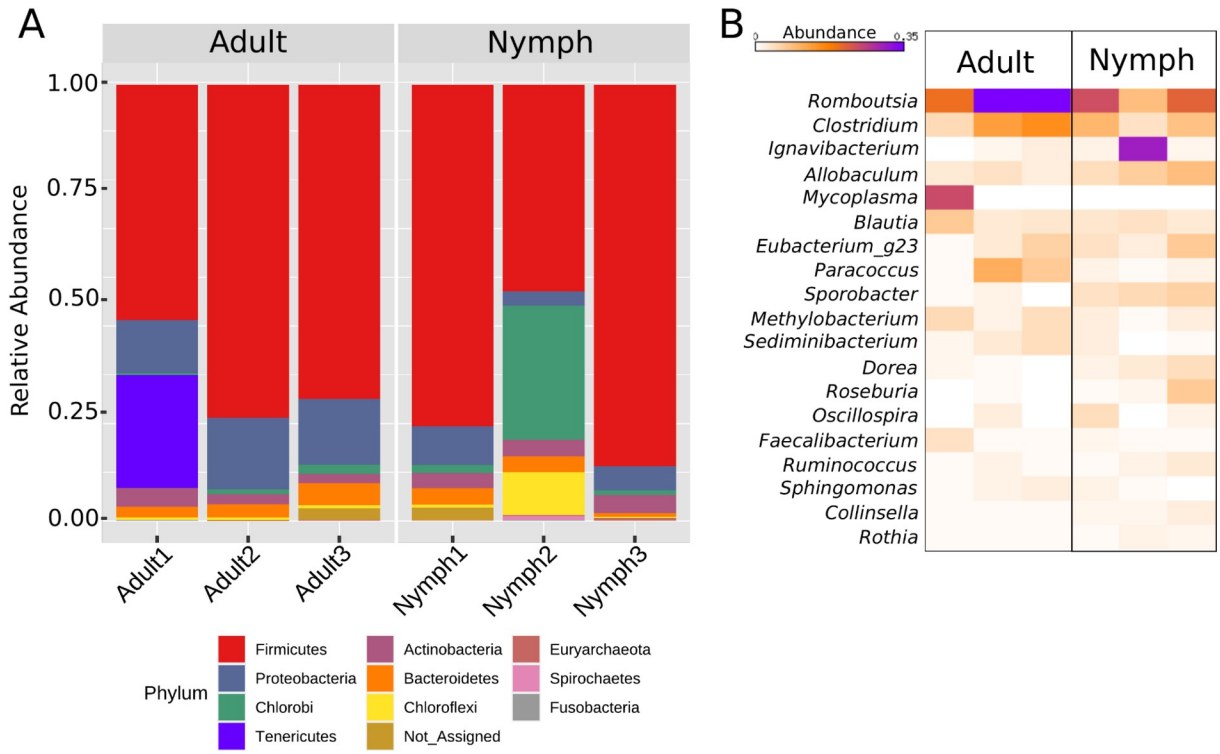
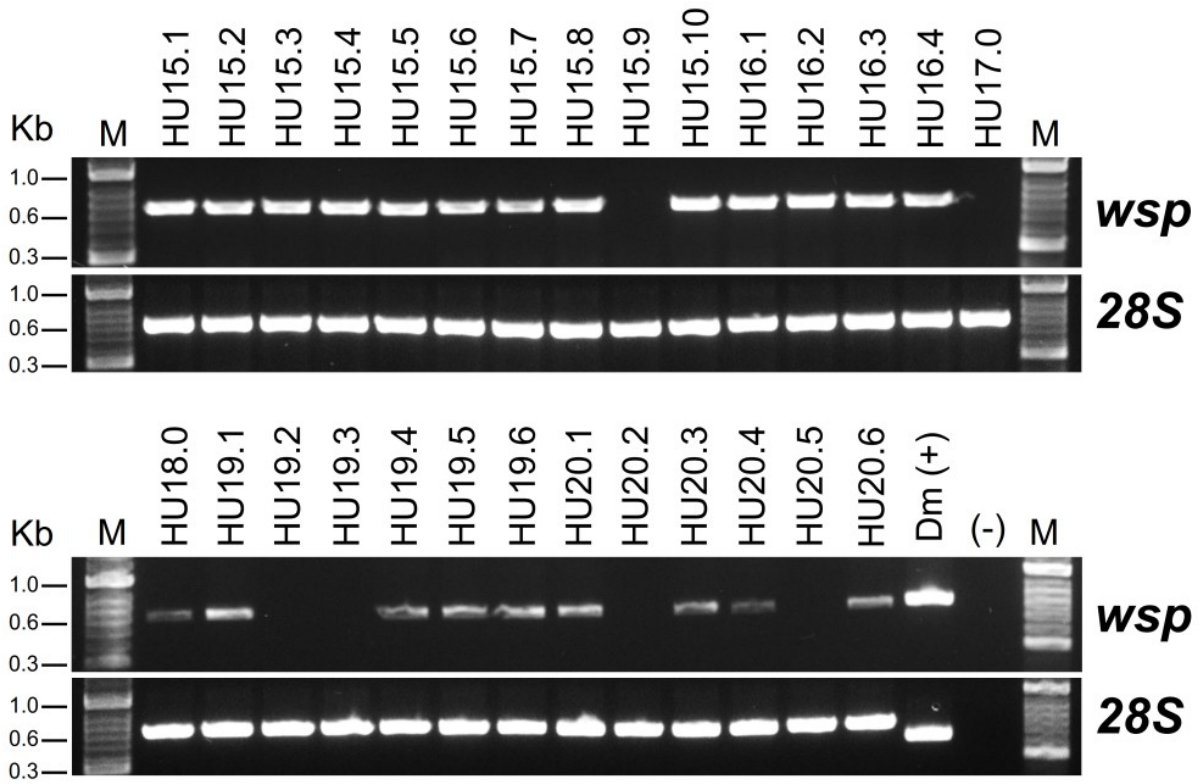


Figure 3. Relative abundance of the bacterial microbiota, with the exclusion of *Wolbachia*-associated sequences, for nymphs and adults of *Monalonion velezangeli*. (A) Relative abundances at Phylum level. (B) Heatmap of relative abundances at genus level.

24



822
823 **Figure 4.** Molecular screening for presence of *Wolbachia* endosymbiont in *Monalonion*
824 *velezangeli* samples. DNA samples from single insects (HU15.1 to HU20.6) were tested for
825 PCR amplification of the *Wolbachia wsp* gene using wsp81F and wsp691R primers. Quality of
826 DNA was tested by amplification of the 28S rRNA (28S) gene fragment (~700 bp). DNA from a
827 *Drosophila melanogaster* (Dm) population was used as positive control for *Wolbachia* infection,
828 and water (-) as negative control.
829
830
831

25

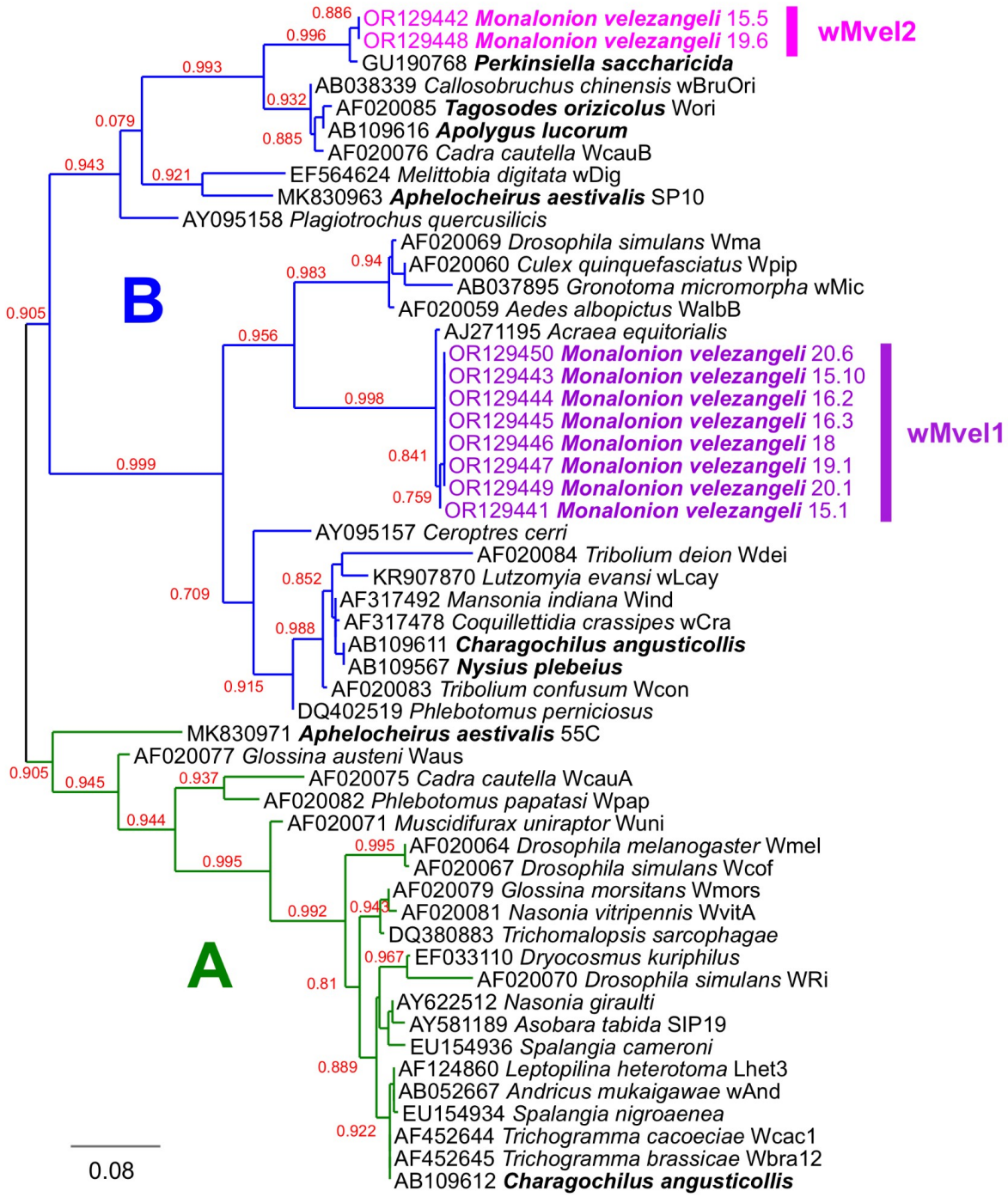


Figure 5. Maximum Likelihood phylogenetic tree of *Wolbachia wsp* sequences from *Monalonion velezangeli* and representative *Wolbachia* strains from other host insects at the GenBank database. *Wolbachia* supergroups A (green branch) and B (blue branch) clusters based on *wsp* sequences are shown. Sequence haplotypes clustering of the *M. velezangeli* wsp isolates, wMvel1 and wMvel2, are shown in purple and pink colors respectively. Hemiptera species are highlighted in bold letters. The aLRT branch supports are indicated as red numbers. Genbank accession numbers precede each sequence name.

26