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

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

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Abstract: Insects and microbes have developed complex symbiotic relationships that evolutively and ecologically play beneficial roles for both, the symbiont and the host. In most Hemiptera insects, bacterial symbionts offer mainly nutritional, defense and reproductive roles and have promoted 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; however, 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* (~92%) dominated the overall microbiome composition in these life stages, and along with bacteria genera *Romboutsia*, *Ignavibacterium*, *Clostridium*, *Allobaculum*, *Paracoccus*, *Methylobacterium*, *Faecalibacterium*, *Collinsella*, *Rothia*, *Sphingomonas* and other 4 undetermined genera, were found as members of the core microbiota. Based on PCR screening and DNA sequencing of *wsp* gene, *Wolbachia* infection was confirmed in almost 80% of samples, represented by two different isolates or strains within the supergroup B. This data opens new questions and avenues to better understand the contribution of symbiotic bacteria in

the biological performance of this insect pest and provide bases 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) mostly in the gut lumen or within specialized structures in the posterior midgut. Insect gut-associated microorganisms (specially bacteria) 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. The outcomes of this symbiotic interplay in plant-feeding insects has also extended in some cases 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 conferring resistance to chemical insecticides in various pest insects (Kikuchi et al. 2012; Blanton and Peterson 2020; Sato et al. 2021).

Equally important, some 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, as well as learning and memory capacity (reviewed by (Jie Bi 2020)).

Several Hemiptera plant-feeding insect species in the suborders Sternorrhyncha (e.g. aphids, whiteflies, psyllids, scale insects, mealybugs); Auchenorrhyncha (e.g. planthoppers, leafhoppers and cicadas), and Heteroptera (e.g. stink bugs and plant bugs) harbor a variety of insect-microbial symbiosis. These insects have piercing and sucking mouthparts for stylet-sheath feeding (phloem and xylem sap-suckers) as in Sternorrhyncha and Auchenorrhyncha; or macerate-and-flush feeding (sucking of extraorally digested plant tissues) as seen in some Heteroptera. In consequence, several of these phytophagous species are agricultural pests of economic importance. Most members of Sternorrhyncha and Auchenorrhyncha harbor intracellular obligate symbionts within specialized cells (bacteriocytes) that provide essential amino-acids and vitamins to the insect; compounds commonly deprived from the poor nutritional plant-sap diet (Moran and Telang 1998). On the other hand, most phytophagous Heteroptera members lack intracellular symbionts but have developed relationships with extracellular

symbionts in special midgut compartments (e.g. midgut crypts and caeca), mainly within the infraorder Pentatomomorpha (e.g. stink bugs, flat bugs and seed bugs). However, special symbiont-harboring midgut compartments seems to be absent in most phytophagous species in the infraorder Cimicomorpha (e.g. true plant bugs and lace bugs).

The plant bug *Monalonion velezangeli* (Hemiptera: Miridae: Bryocorinae) is a neotropical polyphagous insect in the infraorder Cimicomorpha 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) and it is considered a notorious agricultural pest in cacao (*Theobroma cacao*, Malvaceae); avocado (*Persea americana*, Lauraceae) (Jaimes et al. 2015; Ramírez-Gil et al. 2019); guava (*Psidium guava*, Myrtaceae); and tea (*Camellia sinensis*, Theaceae). *Monalonion velezangeli* is also an emerging pest for coffee crops in Colombia, specially in the southern coffee-producing regions of the country (Ramirez C. et al. 2008). The immatures (nymph) and adults of *M. velezangeli* feed on terminal shoot tips, young leaves or fruits, causing cell-death at the feeding sites as the main direct damage. Severe plant damages are mainly caused by nymphal stages when they inject enzyme-rich saliva into the plant tissues for extraoral digestion of the cell contents. Current recommendations for pest management vary according to host crops; however, 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* (Fu et al. 2021) and the omnivorous *Adelphocoris suturalis* (Xue et al. 2021; Luo et al. 2021) to our knowledge. We consider *M. velezangeli* as a strict phytophagous insect based on the reports of host plant species identified for this plant bug (Giraldo J. and Benavides M. 2012; Rodas et al. 2014; Ocampo Flórez et al. 2018), 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). Knowledge about *M. velezangeli* associated microbiota is fundamental not only to better understand insect biology but also it could provide new opportunities for 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). However, 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, it is necessary to characterize the taxonomic composition of microbes within the insect body. 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 16SrRNA gene (DNA meta-barcoding). Here we discovered a diverse microbiota stable across life stages but dominated by few bacterial genera, including the presence of the endosymbiont *Wolbachia*.

Methods:

Insect collection, DNA isolation and 16SrRNA 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. At the place of collection, insects were externally sterilized by washing three times with 75% ethanol and immediately conserved in 96% ethanol for DNA isolation. Three independent samples of immatures (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 hypervariable region V3V4 of the bacterial 16SrRNA 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 16SrRNA amplicon Illumina 250PE libraries were sequenced using NovaSeq platform (Illumina, San Diego, CA, USA) at Novogene Corporation Inc. (Sacramento, CA, USA).

Processing of 16SrRNA sequence data and taxonomic classification:
Demultiplexed raw 16SrRNA 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. When NCBI-BLAST and EzBiocloud resulted in contradictory Genus best-hits at >=97% identify each, the original GreenGenes identification taxon level was maintained. 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 based on Bray-Curtis distance and 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:
From the *M. velazangeli* individuals collected in this study, abdomen samples were separately dissected from nymphs or adults under sterile conditions in a stereoscope 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 step of denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 40 sec; 55°C for 30 sec and 72°C for 40 sec, 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*) laboratory 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 contigs (isolates) using GeneStudio v.2.2.0 (https://sourceforge.net/projects/genestudio/). The DNA contig sequences were compared with 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 with 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 were assessed using the aLRT test (SH-Like) (Anisimova and Gascuel 2006).

Results:

Microbial 16SrRNA sequence data:
After removing putative contaminant sequences, a total of 491,802 denoised, non-chimeric merged sequences for the V3V4 variable region of the bacterial 16SrRNA gene were produced among all samples (nymph and adult). From these sequences, 123 ASVs were inferred, with a mean 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). Statistically, no 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). Since these two statistical tests are sensitive to differences in dispersion (variance) within groups, we assessed this with PERMDISP (Anderson and Walsh 2013). This analysis showed that there is homogeneity of multivariate dispersions between nymph and adult samples (PERMDISP: F-value: 0.1958; p-value: 0.681).

Since the removal of *Wolbachia*-associated sequences is a regular practice for microbiome analysis in insects when they are detected in bacterial 16SrRNA libraries (Chandler et al. 2014; Rudman et al. 2019), we also analyzed this procedure in our 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 91.9% and 4.8% abundance, 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 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 that were present in all samples of either life stage. The remaining 21 ASVs were only present in one or the other life stage. 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 primarily 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 *M. velezangeli* 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 collection 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 of PCR quality based on the successful amplification of a DNA band for the arthropod 28SrRNA gene target. This indicates that lack of *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.

Sanger sequencing was performed for 10 randomly selected *wsp* DNA amplicons derived from *M. velezangeli* samples. All Sanger 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 a 78% similarity between (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 to two distant subclades, respectively; along with their corresponding *wsp* BLASTn top-hits (see above).

Discussion:

We used 16SrRNA 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, here we focused the bacteria screening on a population feeding on coffee plants in Colombia. In this study, 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 relative to 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 on 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 16SrRNA 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 throughout 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 V3V4 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 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*, representing 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* endosymbiont was also confirmed by PCR screening in *M. velezangeli* samples and DNA sequence analysis of wMvel *wsp* isolates indicates they belong to *Wolbachia* B supergroup. Insect-infecting *Wolbachia* strains with major biological effects have been mostly associated with host reproductive disturbances (e.g. CI, parthenogenesis, male-killing and feminization) (Serbus et al. 2008; Werren et al. 2008; Kaur et al. 2021) and recently with effects on 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). In the hematophagous bed bugs *Cimex lectularius* and *Cimex hemipterus* (Hemiptera: Miridae), *Wolbachia* infection creates a obligate mutualism that is essential for normal insect growth and reproduction via provisioning of B vitamins (Hosokawa et al. 2010; Laidoudi et al. 2020). *Wolbachia* infections in insects have been mainly associated to the 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 (Casper-Lindley et al. 2011; Pietri et al. 2016; Diouf et al. 2018) and also as habitant of bacteriocytes (Hosokawa et al. 2010). The presence of this parasitic endosymbiont in *M. velezangeli* raises new questions about the possible biological implications, if any, 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 would not be the case for *M. velezangeli* - *Wolbachia* relationship.

The extremely high abundance of ASV sequences identified as *Wolbachia* in our samples (~92% overall abundance) could be introducing 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 NGS 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 research work (Wilches et al. 2021; Henry and Ayroles 2021) have 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 specially relevant when comparing infected samples versus non-infected. In our study 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 as seen when these sequences are included. Although this comparison involves two sample groups (nymph and adult) with *Wolbachia*-infection, it is likely that this may not be the case when comparing infected versus non-infected samples.

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 registered in the microbiota of guts from several vertebrate animals (Gerritsen et al. 2014, 2017; Ricaboni et al. 2016; Johnson et al. 2019b) and also insects (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, with the exclusion of *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, being dominated within *P*. *seriatus* by bacteria *Diaphorobacter*, *Lactococcus*, *Pseudomonas*, *Pantoea* and *Izhakiella*; and within *A*. *suturalis* 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*, which contain mostly aerobic species; 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 (Ratcliffe et al. 2022) and especially amenable under aerobic culturable conditions for greater ease in the engineering and experimentation processes. Future attempts for selection of culturable bacterial isolates from the insect gut tissues will reveal what microbes have these characteristics in *M. velezangeli'*s microbiota.

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:

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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).

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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

19

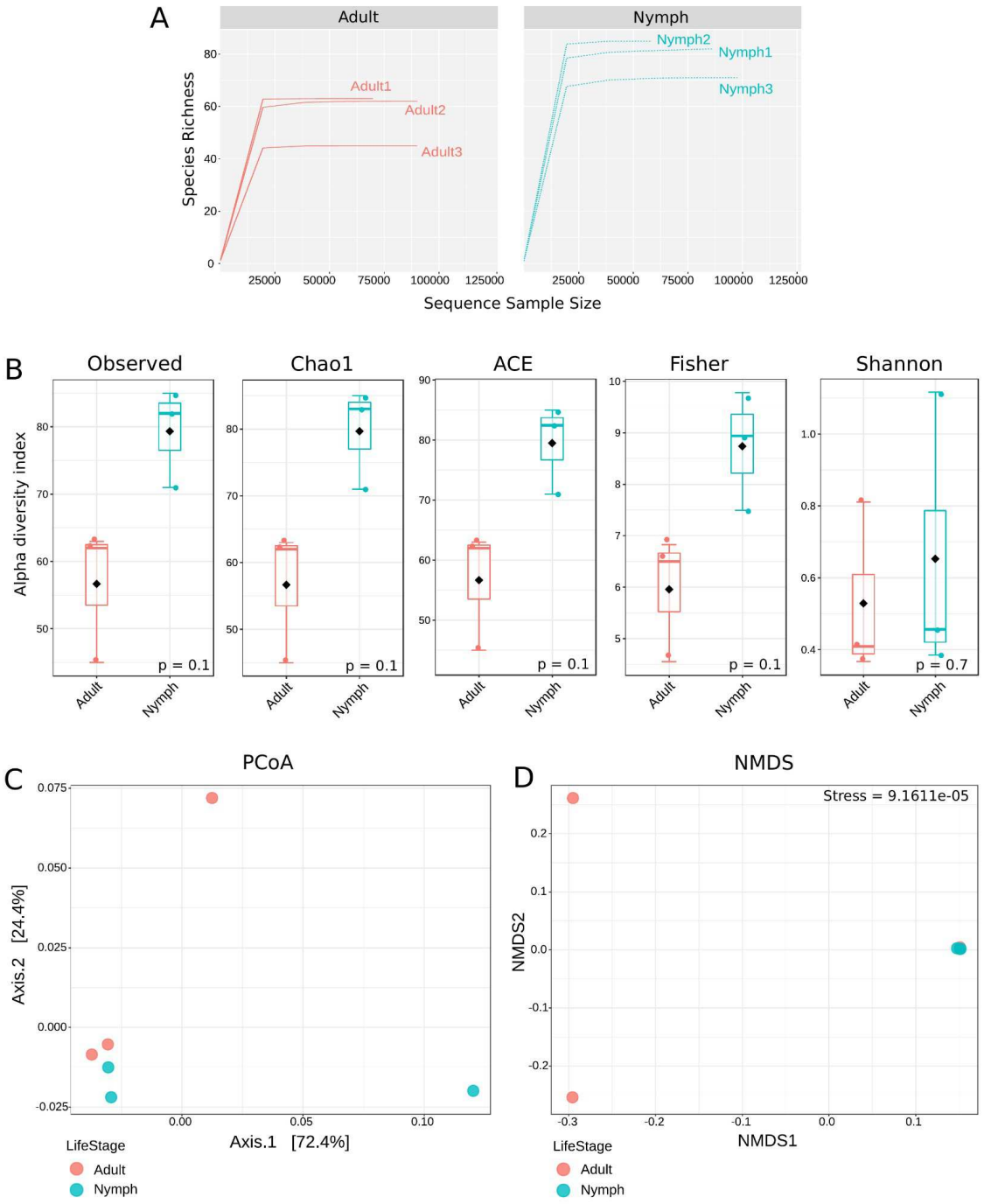
Table 3. Bacterial genus annotations for ASVs considered as members of the core microbiota in *Monalonion velezangeli*.

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

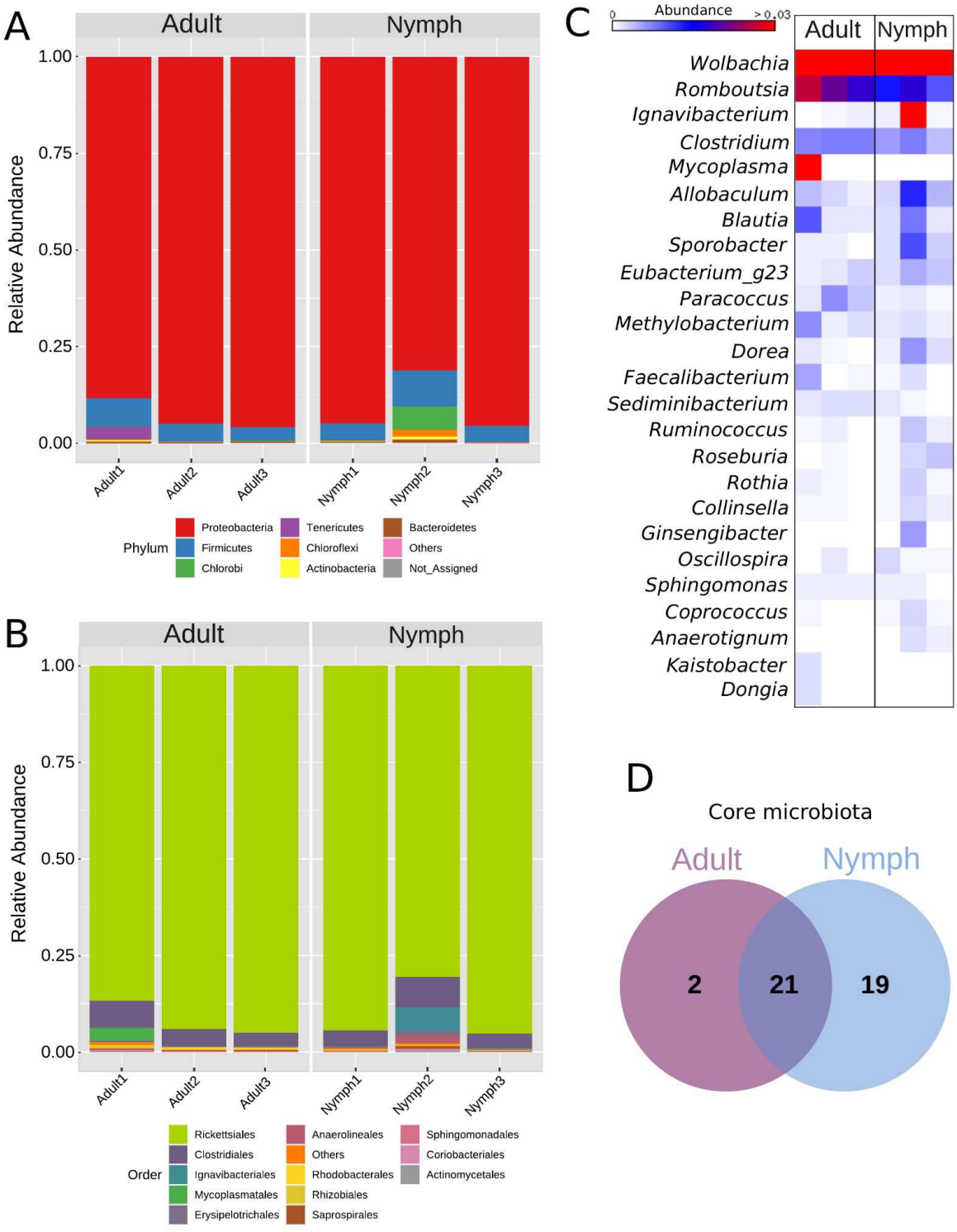
identity for their top hits (taxa denoted with asterisk [*]). Further details can be found in the Methods section.

21



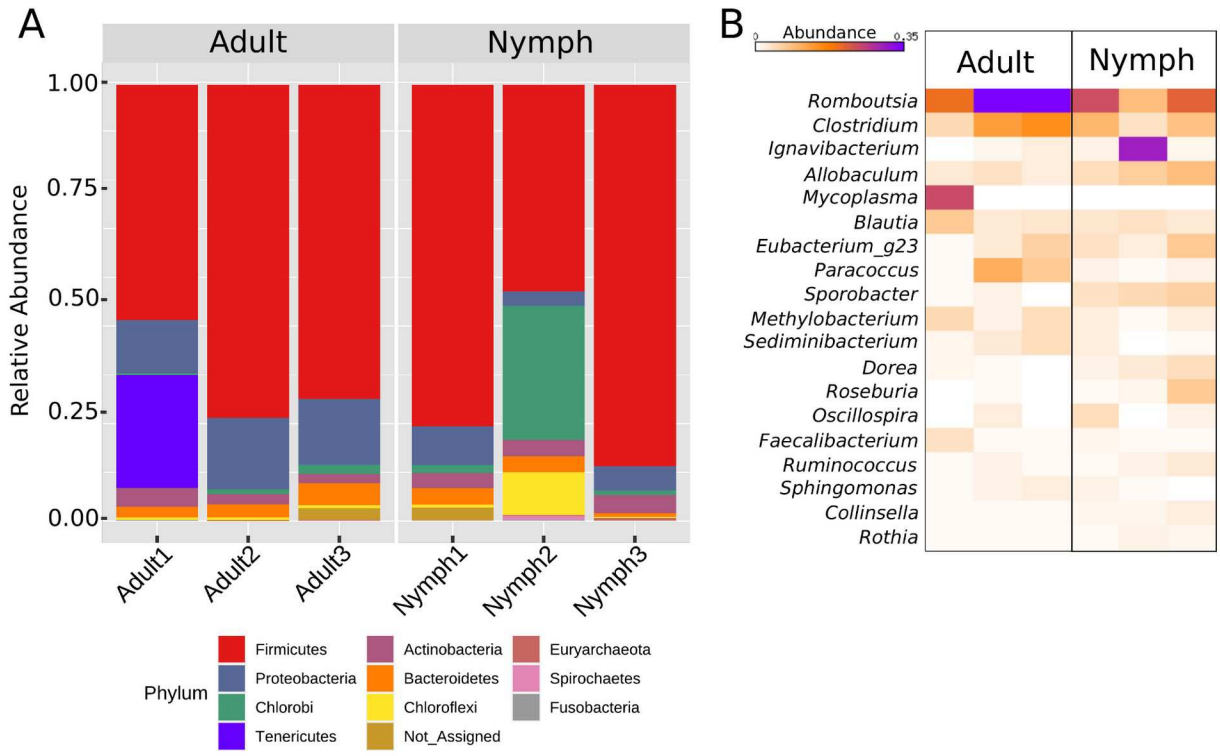
688 **Figure 1**. Diversity and community structure of the bacterial microbiota in adult and nymph life
689 stages of *Monalonion velezangeli*. (A) Rarefaction curves. (B) Alpha diversity indices and their
690 corresponding p-value of the Mann-Whitney U test. (C) Principal coordinate analysis (PCoA)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

22



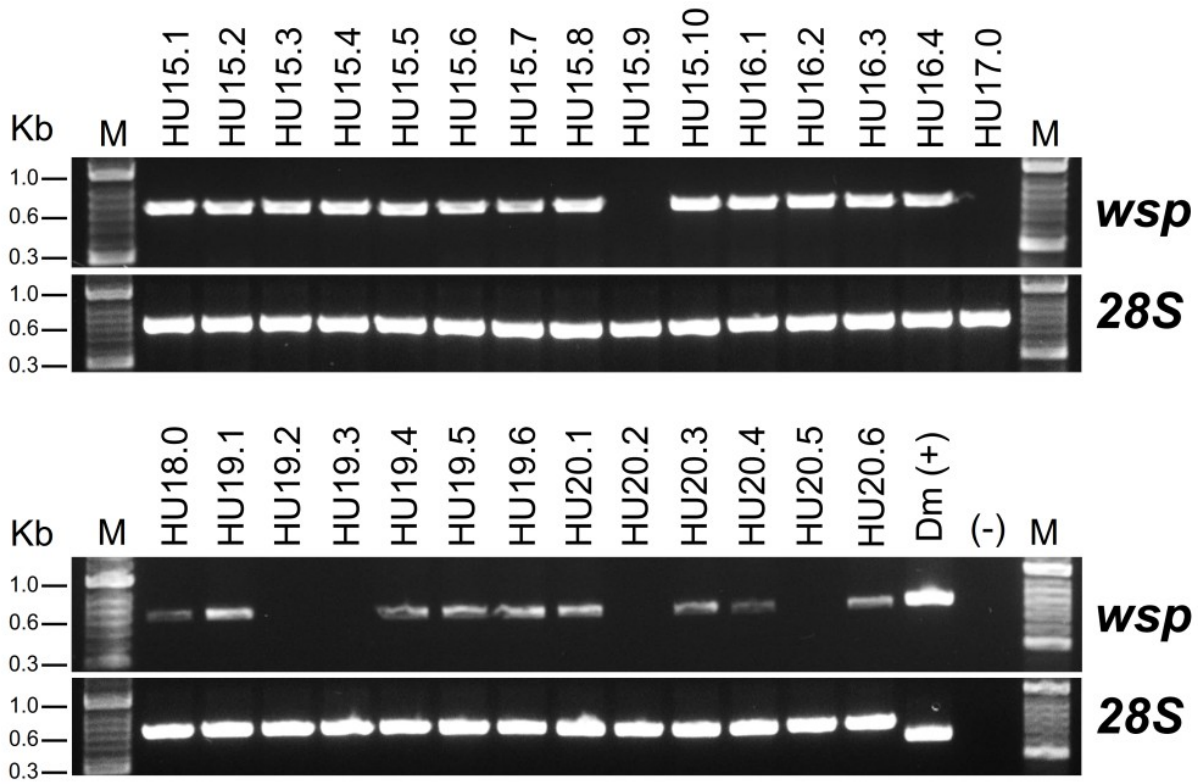
695 **Figure 2**. Taxonomic composition of the bacterial microbiota in nymphs and adults of
696 *Monalonion velezangeli*. (A) Relative abundance at Phylum level. (B) Relative abundance at
697 Order level. (C) Heatmap for relative abundances at genus level. (D) Number of ASV
698 sequences consistently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

23



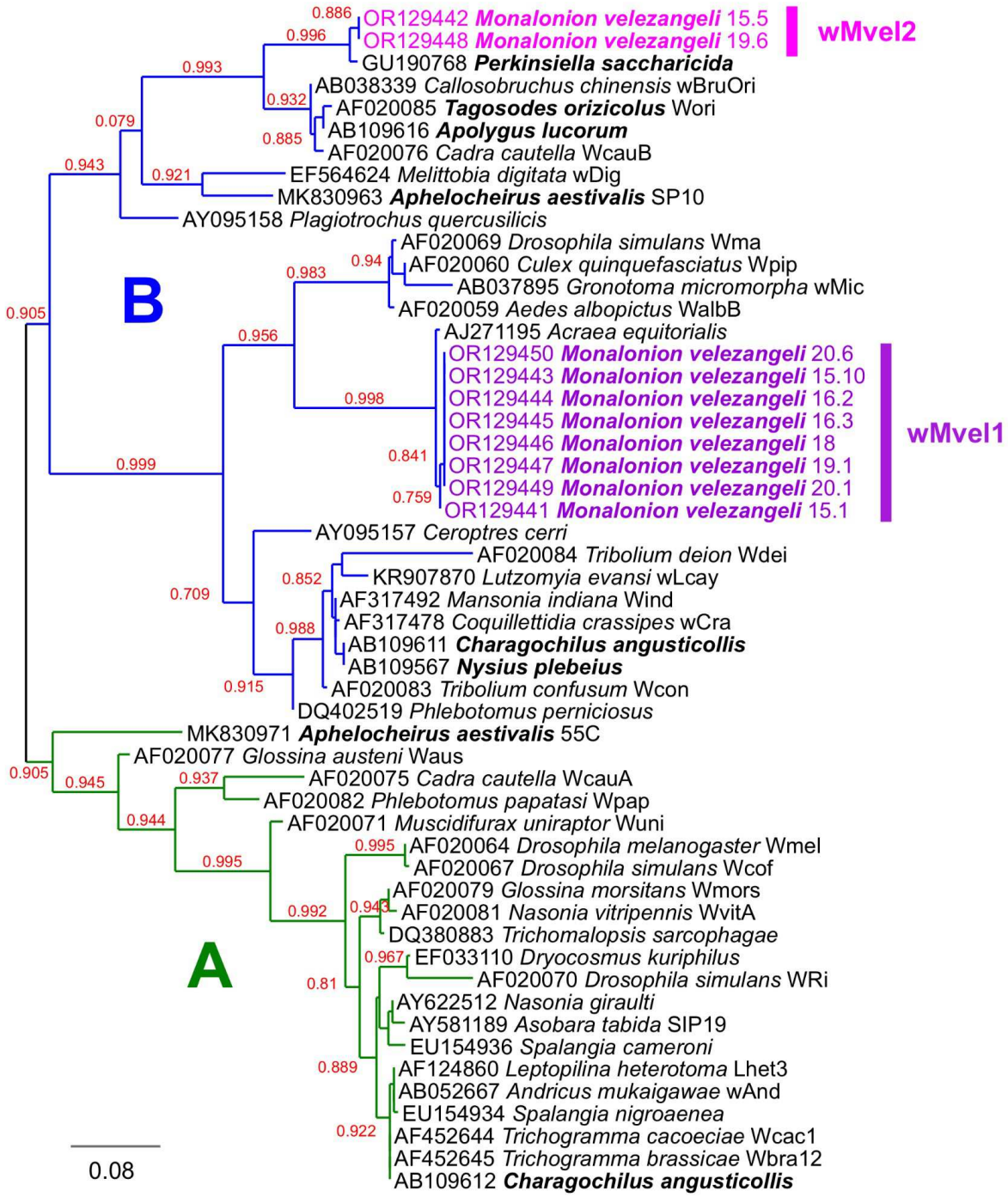
700 **Figure 3**. Relative abundance of the bacterial microbiota, with the exclusion of *Wolbachia*-
701 associated sequences, for nymphs and adults of *Monalonion velezangeli*. (A) Relative
702 abundances at Phylum level. (B) Heatmap of relative abundances at genus level.

24



703 **Figure 4.** Molecular screening for presence of *Wolbachia* endosymbiont in *Monalonion*
704 *velezangeli* samples. DNA samples from single insects (HU15.1 to HU20.6) were tested for
705 PCR amplification of the *Wolbachia wsp* gene using wsp81F and wsp691R primers. Quality of
706 DNA was tested by amplification of the 28SrRNA (28S) gene fragment (~700 bp). DNA from a
707 *Drosophila melanogaster* (Dm) population was used as positive control for *Wolbachia* infection,
708 and water (-) as negative control.

25



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

Supplementary Files

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- Supplementarydatav5.pdf