INSECT MICROBIOME: FROM DIVERSITY TO APPLICATIONS

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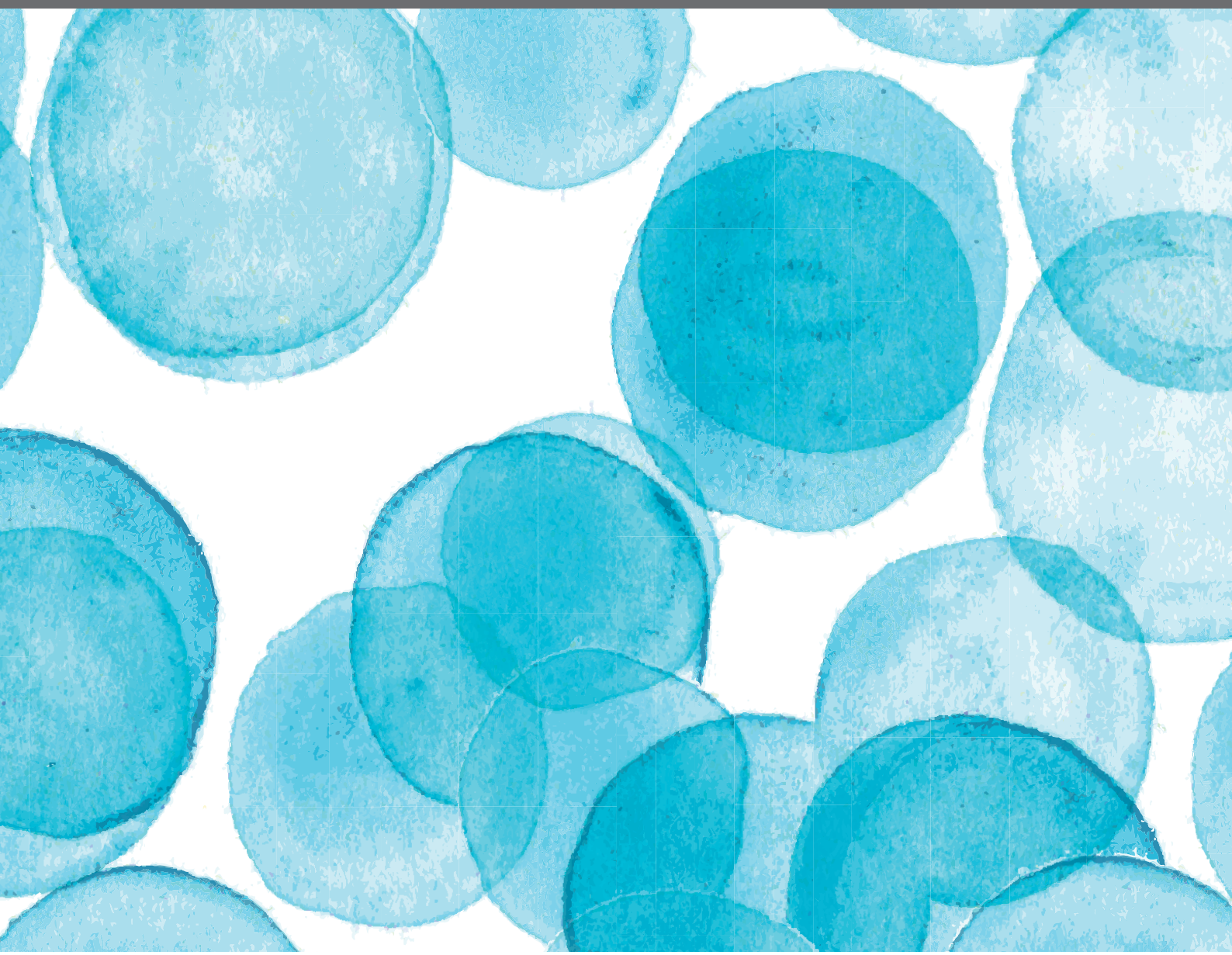

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INSECT MICROBIOME: FROM DIVERSITY TO APPLICATIONS

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Taxonomy, Diet, and Developmental Stage Contribute to the Structuring of Gut-Associated Bacterial Communities in Tephritid Pest Species

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Insect-symbiont interactions are receiving much attention in the last years. Symbiotic communities have been found to influence a variety of parameters regarding their host physiology and fitness. Gut symbiotic communities can be dynamic, changing through time and developmental stage. Whether these changes represent real differential needs and preferential relationships has not been addressed yet. In this study, we characterized the structure of symbiotic communities of five laboratory populations that represent five Tephritidae species that are targets for pest control management through the sterile insect technique (SIT), namely *Bactrocera oleae, Anastrepha grandis, Anastrepha ludens*, and two morphotypes of *Anastrepha fraterculus* (sp.1 and the Andean lineage). These populations are under artificial or semi artificial rearing conditions and their characterization was performed for different developmental stages and age. Our results demonstrate the presence of a symbiotic community comprising mainly from different Enterobacteriaceae genera. These communities are dynamic across developmental stages, although not highly variable, and appear to have a species-specific profile. Additional factors may contribute to the observed structuring, including diet, rearing practices, and the degree of domestication. Comparison of these results with those derived from natural populations could shed light to changes occurring in the symbiotic level during domestication of Tephritidae populations. Further studies will elucidate whether the changes are associated with modification of the behavior in laboratory strains and assess their effects in the quality of the mass rearing insects. This could be beneficial for improving environmentally friendly, species-specific, pest control methods, such as the SIT.

Keywords: *Anastrepha*, *Bactrocera*, sterile insect technique, pest control, laboratory domestication, 16S rRNA, amplicon sequencing

INTRODUCTION

Tephritidae is a family of Diptera harboring more than 500 genera and 4600 species (Norrbom, 2004; Virgilio et al., 2009, 2014). Among tephritid genera, *Anastrepha, Bactrocera*, *Ceratitis*, *Dacus, Rhagoletis*, and *Zeugodacus* include frugivorous species, with around 100 of them being agricultural pests of economic importance (White and Elson-Harris, 1992; Norrbom, 2004; Virgilio et al., 2009, 2014). Economic damage is due to the oviposition of eggs in the mesocarp, the subsequent reduction of quality of the fruit due to the punctures made during oviposition, and the loss of production due to larval feeding (White and Elson-Harris, 1992).

Among the methods used against insect pests, environmentally friendly control methods have received attention in the last decades, enhanced by the documented environmental and health concerns associated with the extensive use of pesticides. In this respect, area-wide integrated pest management (AW-IPM) utilizes different approaches that synergistically can drastically suppress or locally eliminate the pest population. A major component of IPM for a variety of pests is the sterile insect technique (SIT). SIT is based on the release of sterile insects, preferentially only males, that can mate with the female flies of the natural population, leading to infertile crosses and, ultimately, reduction in population size (Dyck et al., 2005).

One of the previously underestimated factors that can affect the behavior of laboratory strains is their symbiotic communities. Recent studies have shown that symbionts, especially gut microbiota, can affect different parameters that are important for the insects' physiology and life history traits (Bourtzis and Miller, 2003, 2006, 2009; Ben-Yosef et al., 2008, 2014; Douglas, 2009, 2015; Zchori-Fein and Bourtzis, 2011; Engel and Moran, 2013; Minard et al., 2013). Within Tephritidae, many studies have been performed in the Mediterranean fruit fly, *Ceratitis capitata,* providing evidence for the structure of the gut symbiotic community of both natural and laboratory populations (Behar et al., 2008; Lauzon et al., 2009; Ben Ami et al., 2010; Aharon et al., 2012; Augustinos et al., 2015; Malacrino et al., 2018) and the impact of specific bacteria used as probiotics or alternative protein sources (Niyazi et al., 2004; Behar et al., 2008; Ben-Yosef et al., 2008; Ben Ami et al., 2010; Gavriel et al., 2011; Aharon et al., 2012; Hamden et al., 2013; Augustinos et al., 2015; Kyritsis et al., 2017). Besides medfly, studies have been performed in the olive fruit fly, *Bactrocera oleae* (Capuzzo, 2005; Sacchetti et al., 2008; Estes et al., 2012b; Savio et al., 2012; Ben-Yosef et al., 2014; Koskinioti et al., 2019) and to a few other tephritid species, mainly *Bactrocera* (Wang et al., 2011; Prabhakar et al., 2013; Thaochan et al., 2013; Pramanik et al., 2014; Wang A. et al., 2014; Wang H. et al., 2014; Andongma et al., 2015; Hadapad et al., 2015; Deutscher et al., 2017, 2018; Gujjar et al., 2017; Yong et al., 2017; Akami et al., 2019). Fewer studies are available in *Anastrepha* species, focusing on *A. ludens* (Kuzina et al., 2001) and more recently, in wild samples of four different species (Ventura et al., 2018).

The primary target of the insect pest control laboratory (IPCL) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (Seibersdorf, Austria) is to develop the SIT as a component of AW-IPM projects against different insect pest species. Many problems arise from the suboptimal conditions regarding the initial steps of laboratory domestication as well as mass rearing, including artificial diet and artificial substrates for oviposition. In some cases, such constraints can modify the behavior of laboratory strains, affecting therefore the efficiency of SIT (Dyck et al., 2005; Estes et al., 2012b; Rempoulakis et al., 2018). The changes that can happen in the symbiotic communities of laboratory populations due to long established domestication and artificial conditions are worth investigating.

In this study, the gut symbiotic communities of five selected tephritid laboratory populations were analyzed using *16S* rRNA gene-based NGS approaches: two populations representing two morphotypes of the *Anastrepha fraterculus* species complex, *Anastrepha ludens*, *Anastrepha grandis,* and *Bactrocera oleae*. *A. fraterculus* and *A. ludens* are well adapted in artificial conditions, while a totally artificial diet has not yet been achieved for *A. grandis*. Regarding *B. oleae,* its artificial rearing can be considered far from optimal, since many laboratories are either suffering from occasional collapses of their colonies or must follow a semi-artificial rearing, using olive fruit as an oviposition substrate. Whether differences in the performance among laboratory-adapted populations of a given species could be attributed, at least partially, to the changes of the structure of their symbiotic communities has not yet been addressed. Aiming to "dissect" factors that may contribute to the structuring of gut symbiotic communities, samples representing different developmental stages, age, and sex, were collected and analyzed.

MATERIALS AND METHODS

Rearing Conditions, Sample Collection, and Preparation

The laboratory populations studied are currently colonized in IPCL and include *A. fraterculus* sp. 1 (Vera et al., 2006), *A. fraterculus* (Andean lineage) (Hernández-Ortiz et al., 2015), *Anastrepha grandis* (Hallman et al., 2017), *A. ludens* (Eskafi, 1988), and *B. oleae* (Ahmad et al., 2016). The full description of these strains, along with their rearing conditions is summarized in **Table 1**.

Gut Collections and Dissections

Guts were collected for each of the strains from 3rd instar larvae (L), 1-day-old males (1D_M) and females (1D_F) that had not been fed, 5–10-days old males (10D_M) and females (10D_F), and 15–20-days old males (15D_M) and females (15D_F). Samples of guts were collected in batches of five individuals in three biological replicates (a total of 15 individuals). Prior to dissections, flies were immobilized/anesthetized at 4°C and disinfected (surface-sterilized) through dipping in 70% ethanol and subsequently were kept in sterile phosphate-buffered saline of 1(×) concentration (1× PBS). Dissections were also performed in sterile 1x PBS under aseptic conditions. Samples were stored in

TABLE 1 | Strains used, their origin, and rearing conditions.

Species	Symbol	Generations in IPCL	Adult diet	Larval diet[2]	Humidity (%)	Temperature (°C)	Photoperiod
Anastrepha fraterculus sp. 1	Af1	84	1:3 (yeast: sugar)	Carrot	65	25	14 light: 10 dark
Anastrepha fraterculus (Andean lineage)	AfA	10	1:3 (yeast: sugar)	Carrot	65	25	14 light: 10 dark
Anastrepha grandis	Agr	7	1:3 (yeast: sugar)	Specialized[3]	65	25	14 light: 1 dark
Anastrepha ludens	Alu	7	1:3 (yeast: sugar)	Carrot	65	25	14 light: 10 dark
Bactrocera oleae	Bol	117	Specialized[1]	Specialized[4]	65	25	14 light: 10 dark

[1]The specialized adult diet for B. oleae consists of 75% sugar, 19% hydrolyzed yeast, and 6% egg yolk powder without antibiotics. [2]The typical carrot diet consists of 7% brewer's yeast, 0.25% sodium benzoate, 0.2% methylparaben, 0.8% (v/w) HCl, 15% carrot powder, and all dissolved in water. [3]Anastrepha grandis larvae diet is semi-artificial, since young larvae are transferred and reared in zucchini. [4]B. oleae larvae diet consists of 52.08 ml water, 2.08 ml virgin olive oil, 0.78 ml Tween 80 (emulsifier), 0.05 gr potassium sorbate, 0.21 gr methylparaben, 2.08 gr sugar, 7.81 gr brewer's yeast, 3.13 gr soy hydrolyzed, 3.13 ml HCl (2N) and 28.65 gr cellulose powder.

−20°C until DNA extraction. The sampling scheme is presented in **Supplementary Table S1**.

DNA Extraction and 16S rRNA Gene Amplicon Library Preparation and Sequencing

Prior to extraction, guts were homogenized in liquid nitrogen using sterile polypropylene pestles. Subsequently, DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) following manufacturer's instructions for total DNA purification from animal tissues. Negative controls were included in DNA extraction. DNA quality and quantity were measured using the NanoDrop 1000 Spectrophotometer (Thermo scientific). A total of 105 samples were analyzed using 454 NGS for the 16S *rRNA* gene with the following primers, targeting the V6-V8 hypervariable region: 926F (AAA CTY AAA KGA ATT GAC GRC GG) and 1392R (ACG GGC GGT GTG TRC) (Rinke et al., 2013). PCRs were performed by Macrogen[1], after linking the primer with decamer multiplex identifier (MID) sequences and adaptors for the GS FLX Titanium Chemistry to facilitate library multiplexing in the 454-sequencing system. In brief PCR was performed under the following conditions: 94°C for 3 min followed by 32 cycles of 94°C for 30 s; 55°C for 40 s and 72°C for 1 min; and a final elongation step at 72°C for 5 min. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, United States). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents and following manufacturer's guidelines.

Bioinformatics and Data Analysis

Sequences were analyzed and processed using the QIIME package (Caporaso et al., 2010). Briefly, the QIIME pipeline takes all sequences from a single pyrosequencing run and assigns sample IDs using a mapping file and the barcode assigned to each sample. Sequences were excluded from the analysis if they were <200 bp in length, had a quality score of <25, contained ambiguous characters or did not contain the primer sequence. Sickle version 1.200 was used to trim reads based on quality: any reads with a window quality score of less than 20, or which were less than 10 bp long after trimming, were discarded (Joshi and Fass, 2011). BayesHammer was used to correct reads based on quality (Nikolenko et al., 2013). Chimeras were detected and omitted using the program UCHIME with the QIIME-compatible version of the SILVA111 release database (Quast et al., 2013). The 16S *rRNA* gene sequences were clustered using the usearch algorithm (Edgar, 2010) and assigned to operational taxonomic units (OTUs) with 97% similarity. Representative sequences from each OTU were aligned with Pynast (Caporaso et al., 2010) against the Greengenes core reference alignment (version gg_12_10). Taxonomy was assigned using the SILVA 16S *rRNA* gene database. The *16S* rRNA gene sequences reported in this study have been deposited in NCBI under Bioproject number PRJNA525967.

α-diversity indices, as well as indices depicting the population structure, were calculated with the QIIME pipeline (Caporaso et al., 2010) based on the rarefied OTU table at a depth of 5,000 sequences/sample (observed species, PD whole tree, chao1 and simpson reciprocal). Variation between replicates was low since all replicates were a pool of 5 tissues. Inter-sample diversity was calculated using Bray-Curtis distances, and principal coordinate analysis (PCoA) was performed on the resulting distance matrix. These calculations and those for alpha diversity were performed in QIIME version 1.9.1. ANOVA and Tukey-Kramer *post hoc* tests were employed to detect statistical differences (Edgar, 2010). Overall similarities in bacterial community structures were shown using the unconstrained ordination technique, principal coordinate analysis, multidimensional scaling (MDS) analysis, and the multidimensional plots as implemented in PRIMER version 6+ (Anderson, 2001). Permutational multivariate analysis of variance (PERMANOVA) analyses were applied to Bray-Curtis similarity matrices to compute similarities between groups. Differences in community structure were viewed using the constrained ordination technique CAP (canonical analysis of principal coordinates), using the CAP classification success rate and CAP $\text{trace}_{Q\_m'HQ\_m}$ statistics, and were performed with 9999 permutations within PRIMER version 6+ (Anderson and Willis, 2003). CAP analysis was performed using the Bray-Curtis similarity matrices.

Checking for Missing Tenants

Curated 16S *rRNA* gene sequences from the Greengenes and SILVA databases were retrieved, corresponding to genera known

[1]https://dna.macrogen.com/eng/

to be part of tephritid symbiotic communities. Sequences were aligned, and the respective fragment amplified with 926F – 1392R primers was selected *in silico*. These sequences were incorporated in our data set and were analyzed through the QIIME pipeline as described before.

RESULTS

16S rRNA Gene Pyrosequencing

A total of 2,254,978 raw reads were obtained from the 454 processing; 1,544,398 reads passed the filters applied through QIIME for non-eukaryotic sequences, with an average of 14,890 reads/sample. After grouping the three replicates, number of reads per sample ranged from 1351 to 93543 (see **Table 2**). Analysis of alpha diversity measures and respective rarefaction curves show that library coverage was adequate to capture the whole diversity of the gut symbiotic communities for almost all samples (**Supplementary Figure S1**).

Structure of the Gut-Associated Bacterial Communities of the Five Laboratory Populations

The *Anastrepha fraterculus* Af1 laboratory population was mainly dominated by *Proteobacteria* in all samples, followed by *Firmicutes*, *Actinobacteria*, *Bacteroidetes,* and *Cyanobacteria*

TABLE 2 | Number of reads and bacterial diversity indices of all samples.

Sample[1]	Number of reads[2]	Number of OTUs	Species richness indices		Species diversity indices	
			Chao1	Menhinick	Shannon	Simpson
Af1_L_G	450.33	23, 33 ± 7.69	28.61 ± 10.76	1.266 ± 0.07	3.154 ± 0.33	0.823 ± 0.02
Af1_1D_M_G	997.33	33.33 ± 6.06	35.86 ± 5.32	1.225 ± 0.21	4.040 ± 0.23	0.910 ± 0.02
Af1_1D_F_G	1608	18.00 ± 1.73	29.53 ± 9.70	1.223 ± 0.54	2.292 ± 0.80	0.633 ± 0.19
Af_5D_M_G	10904	26.00 ± 9.45	34.66 ± 11.89	0.251 ± 0.08	1.877 ± 0.37	0.602 ± 0.05
Af1_5D_F_G	7260.67	16.66 ± 4.37	19.16 ± 5.80	0.204 ± 0.06	1.903 ± 0.64	0.575 ± 0.17
Af1_15D_M_G	13555.33	9.33 ± 0.33	9.66 ± 0.33	0.094 ± 0.02	1.621 ± 0.34	0.556 ± 0.11
Af1_15D_F_G	24513.33	10.00 ± 0.58	10.6 ± 0.66	0.066 ± 0.01	0.896 ± 0.29	0.280 ± 0.11
AfA_L_G	4614.33	18.00 ± 6.60	19.77 ± 7.80	0.333 ± 0.10	1.108 ± 0.17	0.374 ± 0.05
AfA_1D_M_G	2677.67	34.33 ± 6.43	39.68 ± 4.04	0.881 ± 0.30	2.930 ± 0.61	0.742 ± 0.09
AfA_1D_F_G	11633.67	27.33 ± 6.08	29.58 ± 6.69	0.409 ± 0.17	1.604 ± 0.50	0.490 ± 0.12
AfA_5D_M_G	12361.33	15.66 ± 5.86	16.77 ± 6.76	0.135 ± 0.03	0.970 ± 0.24	0.288 ± 0.10
AfA_5D_F_G	7634.33	18.33 ± 3.57	19.33 ± 3.34	0.211 ± 0.02	1.797 ± 0.28	0.583 ± 0.06
AfA_15D_M_G	16545.00	24.33 ± 6.06	26.44 ± 6.58	0.208 ± 0.02	1.303 ± 0.10	0.404 ± 0.01
AfA_15D_F_G	6400.33	12.33 ± 1.09	16.33 ± 3.54	0.631 ± 0.39	2.029 ± 0.93	0.519 ± 0.18
Alu_L_G	18153.67	14.33 ± 0.98	16.16 ± 0.49	0.103 ± 0.01	0.546 ± 0.26	0.206 ± 0.13
Alu_1D_M_G	30594.67	28.33 ± 2.99	33.66 ± 3.14	0.531 ± 0.20	1.716 ± 0.81	0.421 ± 0.18
Alu_1D_F_G	30072.67	31.66 ± 5.19	39.40 ± 7.65	0.760 ± 0.24	2.135 ± 0.75	0.545 ± 0.19
Alu_5D_M_G	18493.67	18.00 ± 2.87	20.08 ± 2.42	0.132 ± 0.04	0.957 ± 0.55	0.282 ± 0.17
Alu_5D_F_G	22066.67	15.66 ± 1.08	16.05 ± 1.05	0.088 ± 0.01	0.437 ± 0.06	0.111 ± 0.01
Alu_15D_M_G	16340	20.00 ± 0.94	22.64 ± 2.65	0.291 ± 0.06	1.273 ± 0.39	0.391 ± 0.13
Alu_15D_F_G	20527.67	29.33 ± 0.27	32.44 ± 2.81	0.276 ± 0.03	2.341 ± 0.27	0.679 ± 0.07
Agr_L_G	19733.33	39.66 ± 5.82	48.50 ± 9.57	0.294 ± 0.03	1.515 ± 0.39	0.430 ± 0.13
Agr_1D_M_G	11965.33	14.33 ± 6.43	17.00 ± 7.07	0.098 ± 0.05	0.247 ± 0.19	0.062 ± 0.05
Agr_1D_F_G	8712.33	9.00 ± 1.70	11.33 ± 2.84	0.053 ± 0.01	0.219 ± 0.16	0.088 ± 0.07
Agr_5D_M_G	23014.33	30.66 ± 1.65	31.77 ± 2.45	0.240 ± 0.03	1.699 ± 0.25	0.459 ± 0.07
Agr_5D_F_G	31314.33	39.33 ± 1.44	47.04 ± 4.48	0.291 ± 0.03	1.512 ± 0.49	0.394 ± 0.13
Agr_15D_M_G	7764.67	29.66 ± 1.66	33.83 ± 3.89	0.242 ± 0.02	2.031 ± 0.12	0.605 ± 0.04
Agr_15D_F_G	12023.33	30.00 ± 1.63	31.44 ± 1.69	0.219 ± 0.03	1.943 ± 0.59	0.537 ± 0.15
Bol_L_G	15181.33	5.00 ± 1.70	7.00 ± 2.94	0.147 ± 0.07	0.146 ± 0.10	0.043 ± 0.03
Bol_1D_M_G	11604	9.33 ± 0.27	12.50 ± 1.08	0.305 ± 0.10	0.739 ± 0.18	0.228 ± 0.05
Bol_1D_F_G	22276.67	10.66 ± 2.13	10.50 ± 1.65	0.047 ± 0.01	0.013 ± 0.01	0.002 ± 0.01
Bol_5D_M_G	26805	12.66 ± 3.57	14.27 ± 4.20	0.081 ± 0.02	0.227 ± 0.05	0.057 ± 0.01
Bol_5D_F_G	22400.67	14.00 ± 2.36	14.33 ± 2.37	0.095 ± 0.02	0.697 ± 0.27	0.219 ± 0.08
Bol_15D_M_G	649.67	3.33 ± 1.09	3.66 ± 1.36	0.143 ± 0.05	0.724 ± 0.12	0.311 ± 0.08
Bol_15D_F_G	23949.67	8.33 ± 3.67	9.50 ± 4.42	0.069 ± 0.01	0.933 ± 0.41	0.378 ± 0.16

Af1, A. fraterculus sp. 1; AfA, A. fraterculus (Andean lineage); Agr, A. grandis; Alu, A. ludens; Bol, B. oleae; L, larvae; M: males; F, females; 1D, 1-day old, unfed; 5D, 5–10-days old; 15D, 15–20-days old; G, gut.

which were present at a low relative abundance (RA) (**Supplementary Excel S1** and **Supplementary Figure S2A**). The most dominant class of *Proteobacteria* was Gammaproteobacteria with a RA up to 100% in some samples (**Supplementary Excel S1** and **Supplementary Figure S2B**) with all abundant OTUs present in all replicates. It's worth noting that the symbiotic community was rather dynamic with fluctuations in the species richness and the RA of different genera (**Table 2**, **Supplementary Excel S1**, and **Supplementary Figure S2C**). PCoA analysis indicated the formation of three clusters with the first two axes accounting for the 62.7% of the total variation (**Supplementary Figure S3A**), although intra-sample variability was also evident (**Supplementary Figure S3B**). Permanova analysis indicated that the clusters observed were statistically significant ($p < 0.001$) with the 3rd instar larvae constituting the first cluster, the 1-day old adults forming the second cluster, and the 5–10 day and 15–20 days old adults forming the third cluster.

Proteobacteria was the prevailing Phylum in all samples of the *A. fraterculus* (AfA) laboratory population followed by *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Actinobacteria,* and *Aquificae* (**Supplementary Excel S1** and **Supplementary Figure S4A**). Among *Proteobacteria*, the majority belonged to Gammaproteobacteria (**Supplementary Excel S1** and **Supplementary Figure S4B**). The symbiotic community of this population was also dynamic with fluctuations in the species richness and the RA of different genera (**Table 2**, **Supplementary Excel S1**, and **Supplementary Figure S4C**). Interestingly, two members of the dominant family of Enterobacteriaceae, *Morganella* and *Enterobacter*, seem to have completely different patterns, with the first one being a major component of the symbiotic communities of larvae and 1-day old adults and almost undetectable in the older adult stages, while the second one being almost undetectable in L and 1-day old females and becoming the most (or nearly the most) abundant genus in older flies (**Supplementary Excel S1** and **Supplementary Figure S4C**). PCoA analysis indicated the presence of two distinct clusters, with the first two axes describing the 65.4% of the total variation (**Supplementary Figure S5**). Permanova analysis verified that the 3rd instar larvae and the 1-day old flies formed a distinct group, separate from the 5 to 10 days and 15 to 20 days old flies forming the second group ($p < 0.001$).

Proteobacteria dominated all samples of the *Anastrepha ludens* (Alu) laboratory population, followed by *Firmicutes* 1-day old males (**Supplementary Excel S1** and **Supplementary Figure S6A**). Gammaproteobacteria was the most dominant class with Bacilli being also an abundant component of the symbiotic community, which was characterized by fluctuations in species richness and RA during development (**Table 2**, **Supplementary Excel S1**, and **Supplementary Figure S6B**). In Gammaproteobacteria, members of the family Enterobacteriaceae, such as *Providencia, Enterobacter,* and *Klebsiella*, were the most abundant (**Supplementary Excel S1** and **Supplementary Figure S6C**). The first two axes of the PCoA accounted for the 57.6% of the observed variance (**Supplementary Figure S7A**). Permanova analysis indicated that the developmental stage and age of the adults had a significant role in the formation of the bacterial profile of the gastrointestinal tract ($p < 0.002$) (**Supplementary Figure S7B**). More specifically, the *A. ludens* larval gut bacterial profile was statistically different only from the 1-day and 15 days old flies ($p < 0.033$, and $p < 0.01$, respectively). All other combinations were not statistically different.

Proteobacteria was the prevailing phylum in the laboratory population of *Anastrepha grandis* (Agr), followed by *Bacteroidetes, Firmicutes*, and *Actinobacteria*, although not present in RA higher than 1% in all samples (**Supplementary Excel S1** and **Supplementary Figure S8A**). Gammaproteobacteria was the dominant class in this symbiotic community, which was characterized by changes in the species richness and RA during development (**Table 2**, **Supplementary Excel S1**, and **Supplementary Figure S8B**). Members of the family Enterobacteriaceae seemed to play a major role with *Providencia* being quite abundant followed, in some stages, by *Klebsiella*, *Morganella*, and *Enterobacter* (**Supplementary Excel S1** and **Supplementary Figure S8C**). These differences are displayed in PCoA analysis, which captured 71.9% of the observed variance (**Supplementary Figure S9A**). Permanova analysis indicated that developmental stage and age of adults were significant factors affecting the bacterial profile in the gastrointestinal tract ($p < 0.002$) (**Supplementary Figure S9B**). The 1-day old flies formed a group distinct from the 10 to 15 days and 15 to 20 days old flies but also from the 3rd instar larvae ($p < 0.003$, $p < 0.002$, and $p < 0.011$, respectively).

The *Bactrocera oleae* (Bol) laboratory population was also dominated by *Proteobacteria* ranging between RA 85% and 100% in the different stages. *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* followed at rather low RA (less than 1% in the majority of the samples) (**Supplementary Excel S1** and **Supplementary Figure S10A**). The great majority of *Proteobacteria* belonged to Gammaproteobacteria (reaching up to 100% in some of the samples) (**Supplementary Excel S1** and **Supplementary Figure S10B**). Interestingly, *Morganella* was a major component in all samples, since it ranged from 62 to 98%, while *Providencia, Acinetobacter, Enterobacter*, and *Klebsiella* were also present at low RA (**Supplementary Excel S1** and **Supplementary Figure S10C**). PCoA failed to provide a clear clustering of the samples based either on developmental stage, sex or age, although it captures 79.5% of the observed variance (**Supplementary Figure S11**). Based on Permanova analysis, adult age and the developmental stage of *B. oleae* did not play a significant role in the formation of the gut bacterial profile ($p < 0.115$), however, the 1-day old females were quite differentiated from all other samples.

Comparison of the Gut-Associated Bacterial Communities of the Different Developmental Stages of the Five Laboratory Populations

Trying to "dissect" consistent similarities and differences among the laboratory populations of the different species, we excluded the differences that could derive from changes happening during

development. To do so, samples of the same developmental stages of the different laboratory populations were compared.

During the larval stage, the five populations exhibited different levels of bacterial OTU diversity with Agr having the highest one (**Table 2**). PCoA analysis demonstrated that there was a significant difference among the symbiotic communities of the larval guts of the five laboratory populations, consistent with their taxonomy ($p < 0.001$) (**Supplementary Figure S12**). The gut symbiotic communities of the three Bol replicates cluster together, apart from other samples ($p < 0.001$). No statistically significant differences are evident among the larval stages of the *Anastrepha* species, although there is a tendency for clustering per species. The larval bacterial profile of the Af1 colony is not statistically different from that of the AfA colony ($p < 0.104$). Alu samples cluster together, and close to the remaining Agr and AfA samples ($p < 0.09$ and $p < 0.099$, respectively).

The symbiotic communities of 1-day old flies of Af1, AfA, and Alu, were more diverse than those of Agr and Bol (**Table 2**). PCoA analysis demonstrates that there are significant differences among the symbiotic communities of the 1-day old guts of the five colonies that can be attributed to their different origin (species, geographic origin) (**Supplementary Figures S13A,B**) ($p < 0.001$). Samples representing the gut symbiotic communities of Bol and Agr ($p < 0.002$) are clearly differentiated from each other and all other samples, while samples from the three remaining populations (Alu, AfA, and Af1) are not clearly differentiated from each other ($p < 0.085$).

The symbiotic communities of 5–10 days old flies of Agr were more diverse than those of all other populations (**Table 2**). *Morganella* was the most abundant symbiont in the Bol population but was either undetected or in RA <2% in all other samples. *Klebsiella* (Enterobacteriaceae) and *Streptococcus* (Streptococcaceae) were the main components of the Alu gut symbiotic community contributing together 99% and 65% of the RA for the females and males of this population, respectively. However, these genera were either undetected or found at very low RAs (less than 0.1%) in all other populations (**Supplementary Excel S1**). PCoA analysis demonstrates that there are significant differences among the symbiotic communities of the 5–10 days old adult guts of the five colonies that can be attributed to their different origin (species and geographic origin) and/or to the different rearing practices followed ($p < 0.001$) (**Supplementary Figures S14A,B**). The gut symbiotic communities of Bol and Alu are clearly differentiated from each other ($p < 0.005$) and from all others ($p < 0.006$ Bol/AfA, $p < 0.005$ Bol/Agr, $p < 0.005$ Bol/Af1, $p < 0.01$ Alu/AfA, $p < 0.005$ Alu/Agr, $p < 0.004$ Alu/Af1) while, Agr, AfA and Af1 samples cluster together ($p < 0.412$ AfA/Af1, $p < 0.056$ AfA/Agr, $p < 0.076$ Af1/Agr).

The overall symbiotic diversity of the 15–20 days old fly samples was low (**Table 2**). Clustering of the 15–20 days old samples is not as clear as in the previous developmental stages and ages. PCoA analysis demonstrated that the gut symbiotic communities of Bol cluster apart from all *Anastrepha* samples (Permanova; $p < 0.001$), while no obvious clustering was evident within *Anastrepha* samples (**Supplementary Figures S15A,B**).

Analysis of Possible Factors Influencing the Structure of the Gut Microbiome

Species Effect Plus Phylogenetic Distance and/or Rearing Conditions

As evident from CAP analysis (**Figure 1A**) there was a clear clustering of olive fruit fly samples against all others [tr(Q_m′HQ_m): 2.41921 P: 0.0001] and, after the removal of olive fruit fly from the analysis, *A. grandis* samples clustered together, although not very well separated from the remaining *Anastrepha* samples [tr(Q_m′HQ_m): 1.89387 P: 0.0001] (**Figure 1B**). After the removal of the Agr samples from the analysis, the remaining three *Anastrepha* colonies highly overlapped, although a tendency of the Alu samples to form a different cluster was observed (**Figure 1C**). When all populations were tested together, the Permanova analysis performed pointed to the statistically significant contribution of host species in the observed clustering ($p = 0.001$, $F = 4.86$, df: 4), which remained statistically significant after the removal of the Bol samples species ($p = 0.014$, $F = 2.46$, df: 3).

Developmental Stage and Age

An obvious clustering in almost all laboratory populations was that of larvae and 1-day old flies (unfed) against older fed flies (Permanova; $p < 0.001$ 1d/Larvae, Permanova; $p < 0.001$ 1-day/10-day, Permanova; $p < 0.001$ 1-day/15-day). A second level of clustering was that of the bacterial profile of the 5–10-days with the 15–20-days old flies (Permanova; $p < 0.483$). This was clear in Af1 (**Supplementary Figure S3**), AfA (**Supplementary Figure S5**), and Agr (**Supplementary Figure S9**) but not so in Alu (**Supplementary Figure S7**) and Bol (**Supplementary Figure S11**). CAP analysis of the different groups based on developmental stage and age indeed verified the separation of larvae and 1-day old adults from the 5 to 10 and 15 to 20 days-old adults [tr(Q_m′HQ_m): 0.7546 P: 0.0057] (**Figure 2**).

Fly Sex

Another factor that can contribute to differences among symbiotic communities is the fly sex. Our data were not in favour of this hypothesis. An MDS analysis was performed, assuming two different groups: males and females. As evident in **Figure 3**, there was no obvious clustering based on the sex and the Permanova analysis gave no statistical support (Permanova; $p < 0.984$). Therefore, at least for the laboratory populations studied, sex cannot be considered as a factor contributing to the differences observed among the gut symbiotic communities.

Key Players Constituting the Gut Symbiotic Communities of the Laboratory Populations

Although five different laboratory populations were tested, representing five different species from two different genera, with samples across life cycle, only a limited number of bacterial OTUs displayed high RAs, as shown in the heat map of the OTUs (**Figures 4A–C**). Despite the presence of more than 400 different OTUs, only 53 had a RA of >1% in the different samples (**Figure 4A**). The 13 most abundant OTUs accounted

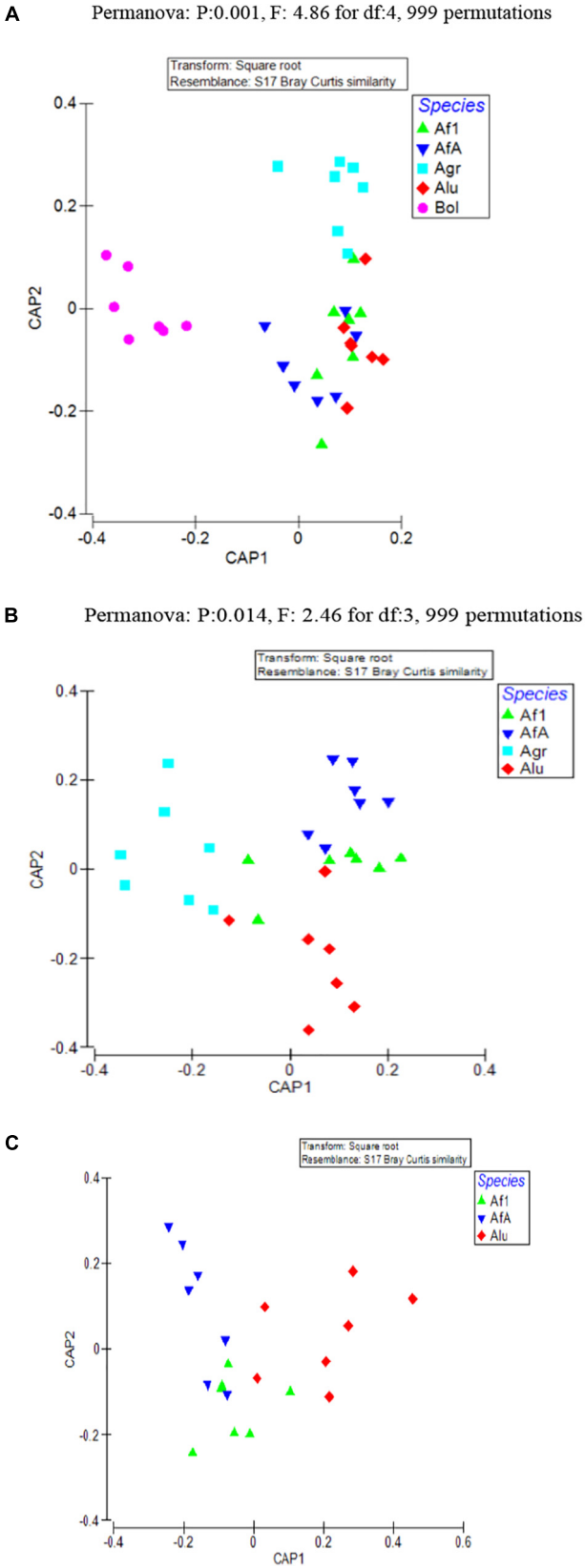

FIGURE 1 | Continued

FIGURE 1 | CAP was used to find axes that best discriminate the groups of interest. In this analysis, clustering at insect species level was examined. **(A)** All samples representing the five laboratory populations are included **(B)** Only the four *Anastrepha* laboratory populations (*Anastrepha grandis, Anastrepha ludens,* and *Anastrepha fraterculus*) are included. **(C)** Only the three laboratory populations from the *A. fraterculus* intergeneric group (*A. ludens* and *A. fraterculus*) are included. Af1, *A. fraterculus* sp. 1; AfA, *A. fraterculus* (Andean lineage); Agr, *A. grandis*; Alu, *A. ludens*; Bol, *B. oleae.*

for the 90% of the overall sequences detected, ranging from 64 to 100% per sample (**Figure 4B**). Unambiguously, the most abundant OTUs are *Providencia, Enterobacter*, and *Morganella* (**Figure 4C**), which account for the 32%, 20%, and 16% of the total sequences, respectively. However, *Morganella's* abundance is mainly restricted to all olive fruit fly samples, where it can reach up to 100%, while *Providencia* and *Enterobacter* have a more "universal" distribution, present in high RAs in all species analyzed.

Did We Miss Somebody? the Mock Experiment of "Expected" Tenants

Available sequences representing 28 different bacterial genera previously reported to be present in the gut symbiotic communities of different Tephritidae were downloaded and incorporated in our data set. QIIME was used to assign them to respective OTUs (**Supplementary Table S2**). In most of the taxa, all sequences were assigned to the correct OTU (**Supplementary Figures S16A,B**).

DISCUSSION

The present analysis indicates that the laboratory populations of the five targeted taxa harbored a varying degree of gut bacterial community diversity. Although the major "players" belonged to Enterobacteriaceae and the overall diversity can be considered rather low, compared to available data from natural populations of some of the species, there were clear differences among our samples. The structure of the communities can be considered as "dynamic," since there were clear intra-population differences, based on the developmental stage and age. Moreover, there were clear inter-population differences, which can be attributed to a variety of factors, such as the original microbiome of the wild populations at the moment of colonization, the degree of adaptation and rearing conditions, but not sex.

Key Players Constituting the Gut Symbiotic Communities of Tephritids

The data presented above are in line with previous studies in laboratory and natural populations of different tephritids. Up to now, the tephritid species model for such studies is the medfly, followed by the olive fruit fly and other *Bactrocera* spp. Previous studies have addressed several questions regarding laboratory and natural populations of the Mediterranean fruit fly. Marchini et al. (2002), using culture dependent approaches and classical microbiological techniques, suggested that *K. oxytoca*

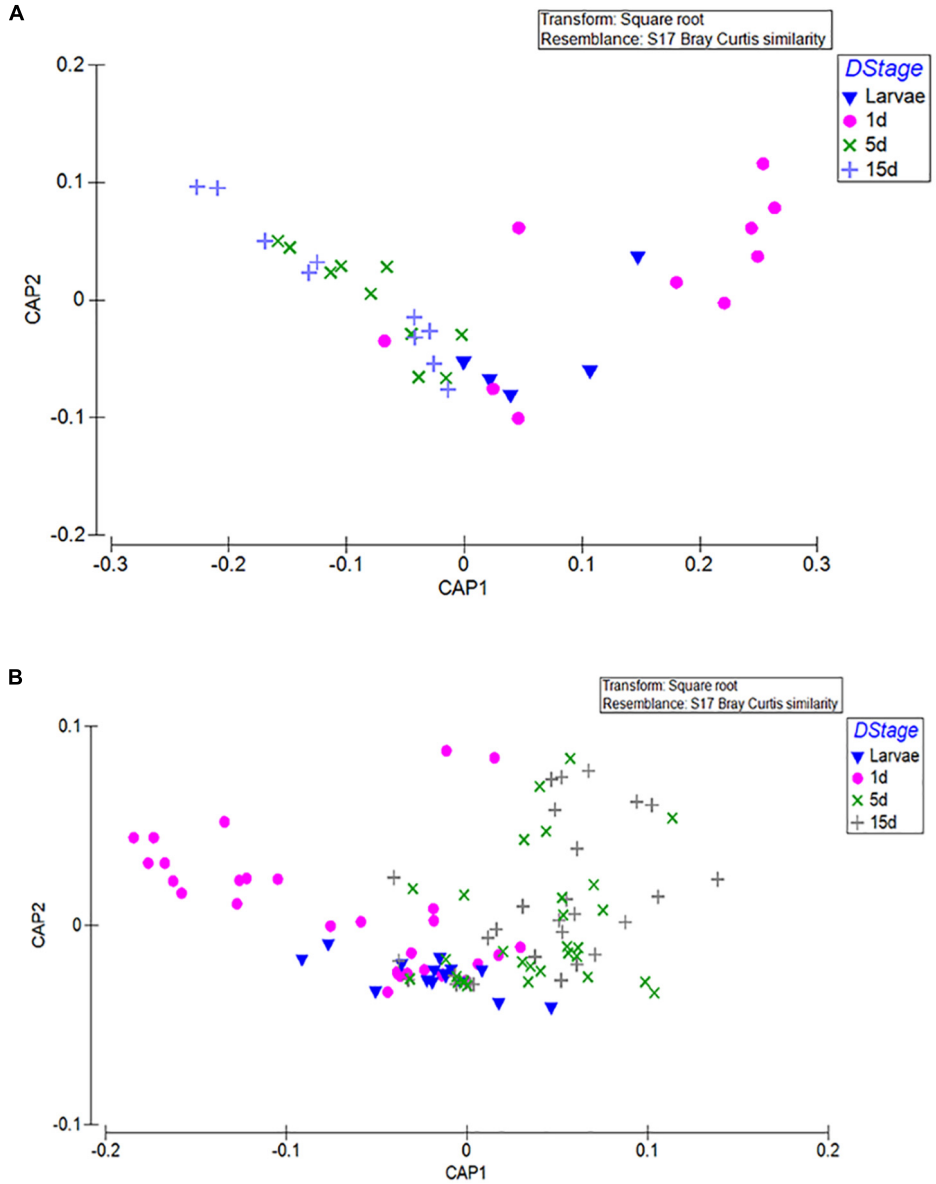

FIGURE 2 | CAP was used to find axes that best discriminate the groups of interest. In this analysis, clustering based on developmental stage and age was tested. **(A)** The mean values of the 3 replicates for the male and female samples are displayed. **(B)** All replicates are displayed. L, larvae; M, males; F, females; 1D, 1-day old, unfed; 5D, 5–10-days old; 15D, 15–20-days old.

is dominating natural populations while *Enterobacter* sp. is dominating laboratory populations of the species. Several studies from another research team (Behar et al., 2005, 2008; Ben-Yosef et al., 2008; Ben Ami et al., 2010; Gavriel et al., 2011; Aharon et al., 2012) provided interesting findings that can be summarized as: (a) Enterobacteriaceae was shown to be the dominant community in the medfly gut, with relatively few genera being present in varying RAs, (b) *Klebsiella* is believed to be a key genus, important for the fitness of medfly and is mainly found in the wild populations of the species, (c) gut symbiotic community is dynamic, depending mainly on the developmental stage and age of adults and, (d) wild populations seem to harbor more polymorphic symbiotic communities than long adapted laboratory strains, although no direct comparison has been performed. More recently, it has been shown that plant host and instar stage are among the factors that shape gut symbiotic communities of natural populations (Malacrino et al., 2018). The reduced diversity of the gut symbiotic communities of laboratory populations of the Mediterranean fruit fly has been addressed by other groups, especially for the VIENNA 7 and VIENNA 8 genetic sexing strains (GSS) (Hamden et al., 2013; Augustinos et al., 2015). As an extreme example of reduced symbiotic diversity, Morrow et al. (2015) managed to retrieve only *Enterobacter*

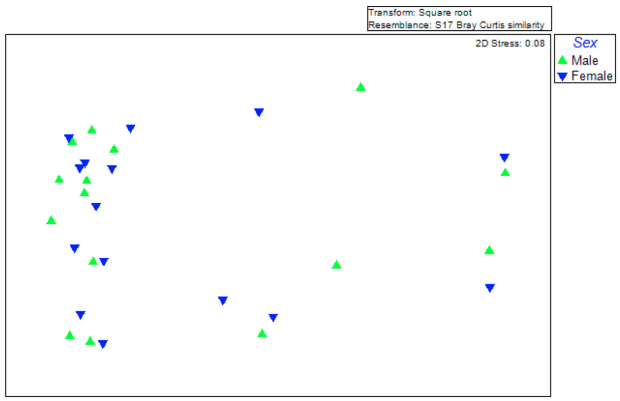

FIGURE 3 | Multidimensional scaling (MDS) plot for a graphical illustration of changes in the bacterial community structure. Green triangles represent males while inverted blue triangles represent females. Similarities in ribotype profiles were calculated by the Brey-Curtis algorithm.

sp. from the VIENNA 7 GSS (a line reared in a mass rearing facility in Australia), even though high throughput 454 NGS sequencing was used.

In the olive fruit fly, all published data suggest that the diversity of the symbiotic community of this species is relatively low, with *Candidatus* Erwinia dacicola dominating wild populations, although other bacterial species, such as *Providencia* sp., *Enterobacter* sp. and *Acetobacter tropicalis* can be detected (Kounatidis et al., 2008; Sacchetti et al., 2008, 2014; Crotti et al., 2010; Estes et al., 2012a; Ben-Yosef et al., 2014; Koskinioti et al., 2019). At the same time, some of these studies presented important findings for the laboratory adaptation of the species, including the loss of *Candidatus* E. dacicola when olive fruit fly is reared on a totally artificial diet (Kounatidis et al., 2009; Estes et al., 2012a) and the increase of *Morganella* sp. in some of the olive fruit fly laboratory populations, which may be pathogenic and is considered as a negative symptom for artificially rearing (Estes et al., 2012b). Our data agree with what is expected for domesticated populations reared on totally artificial diet, since we did not retrieve *Candidatus* Erwinia dacicola sequences and *Morganella* sp. was dominating all samples of our olive fruit fly laboratory population (**Supplementary Figure S10**).

Studies regarding gut symbiotic communities in *Anastrepha* species are limited. The analysis of new and old laboratory populations of *A. ludens,* using culture dependent approaches, gave a total of 18 bacterial species belonging mainly to Enterobacteriaceae, with *Enterobacter*, *Providencia*, *Serratia*, and *Staphylococcus* being the most abundant genera (Kuzina et al., 2001). More recently a study in four *Anastrepha* species, namely *A. ludens, A. obliqua, A. serpetina,* and *A. striata,* using 454 pyrosequencing and samples collected from the nature, provided further insight in the symbiotic communities of this genus (Ventura et al., 2018). Four phyla were identified, with *Proteobacteria* being the dominant phylum. A total of 27 bacterial genera were identified, with *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, and *Raoultella*, being the most abundant. Our data are in line with previous studies, at least regarding the "key" players, which seem to be mainly few genera belonging mainly to Gammaproteobacteria.

Besides these, the structure of the gut symbiotic communities has been addressed in few other *Bactrocera* species (Wang et al., 2011; Prabhakar et al., 2013; Khan et al., 2014; Pramanik et al., 2014; Reddy et al., 2014; Wang A. et al., 2014; Wang H. et al., 2014; Andongma et al., 2015; Deutscher et al., 2017, 2018), suggesting that few genera are present in their gut symbiotic communities.

Factors Shaping the Symbiotic Profile of Domesticated Populations

If phylogenetic distances of the species are responsible (to a certain extent) for the observed differences, one major clustering should be the one of *Anastrephas* against samples from the olive fruit fly, since they belong to different tephritid genera. Moreover, they have a completely different geographic distribution (*Anastrepha* species derive from Latin America, while the olive fruit fly derives from Europe). On a second level, after the removal of olive fruit fly from the analysis, *A. grandis* should cluster apart from the remaining *Anastrephas,* since this species belongs to a different intrageneric group, while both *A. fraterculus* and *A. ludens* belong to the *A. fraterculus* intrageneric group. Although our data are in line with this scenario, interpretation of these results is not easy and straightforward, since different factors highly overlap with each other (for example, phylogenetic differences overlap with different rearing practices). Olive fruit fly that had the most divergent symbiotic community is indeed the most distant phylogenetically and its rearing protocol is again different from all others. Finally, we must keep in mind that olive fruit fly is the only one of these species that is considered as strictly monophagous at the larval stage, while all others are polyphagous (with *A. grandis* having preference for cucurbitaceous fruits), which may influence the "build-up" of preferential symbiotic relationships in nature and through this, the original symbiotic "load" that was transferred in the laboratory and the potentially any novel symbiotic relationships established thereafter. The same applies for the remaining samples after the removal of the olive fruit fly from the analysis. *A. grandis,* which is phylogenetically distant from the other three *Anastrepha* colonies, also had a differentiated symbiotic community profile. However, larval stage diet of this species is still semi-artificial, differing from the other *Anastrepha* populations. Finally, the other three laboratory populations share phylogenetic proximity, long established adaptation, and common rearing practices.

Our analysis showed that developmental stage and age are important factors shaping symbiotic communities. Larvae and 1-day old adults have a different profile from older flies (5–10 and 15–20 days old). This has been previously shown in other studies dealing with the changes of symbiotic communities during development and/or age in the medfly (Ben Ami et al., 2010; Hamden et al., 2013; Augustinos et al., 2015; Malacrino et al., 2018). On the other hand, our data do not support a possible

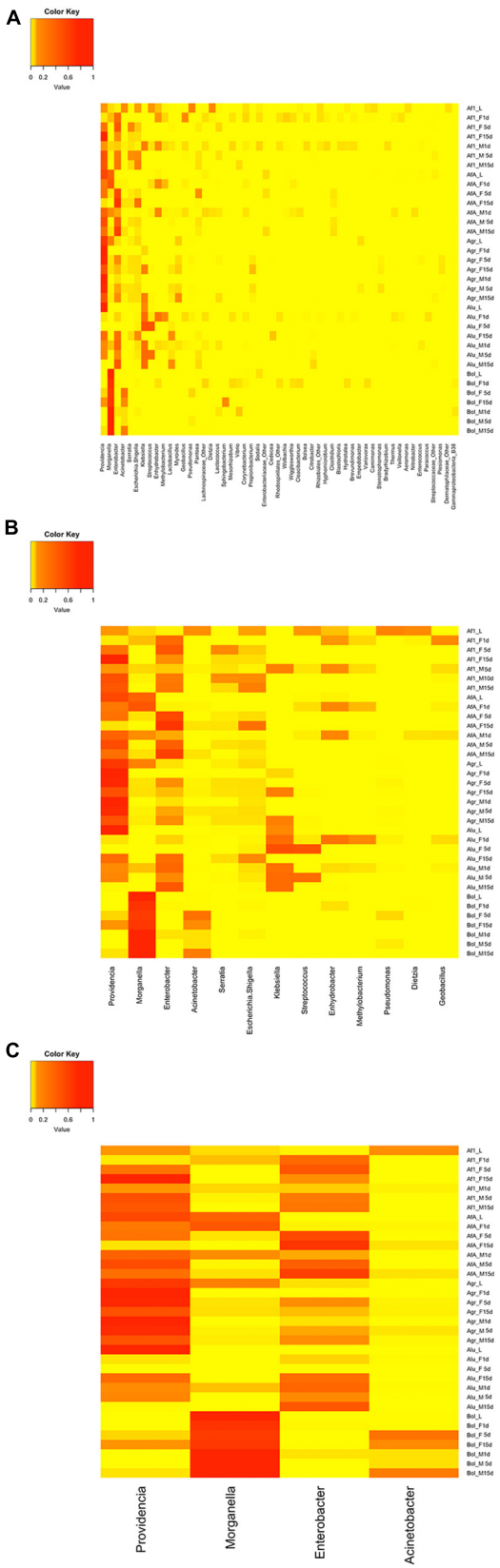

FIGURE 4 | Continued

FIGURE 4 | Heat maps showing the RA of the different OTUs, at genus level. **(A)** OTUs with a RA >1% **(B)** the thirteen most relative abundant OTUs **(C)** the four most relative abundant OTUs. Af1, *A. fraterculus* sp. 1; AfA, *A. fraterculus* (Andean lineage); Agr, *A. grandis*; Alu, *A. ludens*; Bol, *B. oleae*; L, larvae; M, males; F, females; 1D, 1-day old, unfed; 5D, 5–10-days old; 15D, 15–20-days old; G, gut.

effect of the sex on gut symbionts, and there is still lack of studies addressing this factor in tephritids.

Laboratory vs. Natural Populations – Who to Trust?

Laboratory populations have certain limitations regarding the deduction of generalized conclusions. High selective pressure, bottleneck effects, genetic drift and inbreeding are known to affect the genetic structure of population in the laboratory, mainly through reducing diversity. Although not tested directly yet, this could be the case for the symbiotic communities as well. Moreover, the limited "resources" for acquiring bacteria and the specific rearing practices (both in larvae and adult diet but also in oviposition) can lead to a laboratory symbiotic community much different than the one of the wild populations. Preferential adaptation of specific members of the symbiotic community, abundance of specific taxa in the restricted diet and redundancy of previously important symbionts in these new, stringent conditions could also lead to significant changes in the symbiotic status. An extreme example, also pointed out in this study (and previous ones), is the relationship of the olive fruit fly with *Candidatus* E. dacicola. All studies up to now suggest that this bacterium is necessary in natural populations or laboratory populations reared on olive fruits but becomes "unnecessary" in a totally artificial diet and gradually disappears (Capuzzo, 2005; Kounatidis et al., 2009; Estes et al., 2012a; Ben-Yosef et al., 2014; Koskinioti et al., 2019).

On the other hand, data collected from flies in nature could be considered as a "snapshot" of the symbiotic community, and we cannot be sure whether they correctly represent the "core" symbiotic needs of the different species. The random acquisition of bacteria from the different hosts, even if they are not essential, can also compromise the final conclusions. Colonized material enables organizing exact collections schemes, generating the replicates necessary for robust conclusions and allows "revisiting" in the future. Unfortunately, such experiments cannot be easily performed on natural populations. A combination of different approaches, starting from well characterized "wild" material and follow up for many generations in the laboratory will provide a more complete picture of the changes occurring in the symbiotic communities during laboratory domestication.

Comparing Apples and Oranges: How to Correlate With Previous Findings?

As discussed above, there are several studies addressing the structure of gut symbiotic communities in different tephritids. However, a direct comparison is very difficult to be done, since there are many methodological differences among them, as explained in the Introduction. Apart from those, there are other

factors that make comparison even more difficult. The first is the material being used: there are studies using material derived from the wild, others are using populations colonized in semi-artificial conditions and others, like ours, are using laboratory populations adapted in totally and semi-artificial rearing. A second factor that should be considered is the samples used for these studies. Samples can be larvae, pupae, emerging flies or flies of specific age and specific sex. In cases where adult flies were collected directly from the field, age could not be accurately specified. Our data are in line and, to some extent, directly verify some of the previous reported findings for laboratory populations. More specifically, our analysis shows that: (a) members of the *Enterobacteriaceae* are dominating the gut symbiotic communities of the studied colonies, (b) only few key players are present, (c) although of relatively low diversity, gut symbiotic communities are dynamic, since we observed clear changes in the RA of the different bacterial genera, evident mainly after young flies start to feed and, (d) the profile of these communities and the profile of their change through time can be influenced by a variety of parameters, such as insect host (species, its geographic origin, host plant, etc.) and rearing practices (both diet and oviposition substrates). However, other suspected parameters, such as sex were not found important in our study.

CONCLUSION

The present study clearly indicates that insect species, including those which are under artificial or semi-artificial laboratory rearing conditions, can establish in their gut sophisticated symbiotic associations with diverse bacterial species. Most of the gut-associated bacterial species in all five-insect species studied were members of Enterobacteriaceae. The overall bacterial diversity observed in our samples was low when compared to the diversity observed in natural populations. Taxonomy, diet, and developmental stage were found to be key factors influencing the structure of the symbiotic communities. The role of rearing conditions, the degree of laboratory adaptation, and the original microbiome of the wild populations at the moment of colonization may also be critical. These potential factors deserve additional investigation to assess the potential improvement in a cost-effective manner the rearing efficiency and the biological quality of mass reared insect species, which may be the target of AW-IPM strategies with a SIT component.

DATA AVAILABILITY

The datasets generated for this study can be found in NCBI, PRJNA525967.

AUTHOR CONTRIBUTIONS

AA designed and performed the experiments, analyzed the data, and drafted part of the manuscript. GT analyzed the data and drafted part of the manuscript. CC interpreted the data and critically revised the manuscript. AMA designed the experiments, interpreted the data, and critically revised the manuscript. KB conceived and designed the experiments, interpreted the data, and drafted part of the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02004/full#supplementary-material

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Impact of Sample Preservation and Manipulation on Insect Gut Microbiome Profiling. A Test Case With Fruit Flies (Diptera, Tephritidae)

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High-throughput sequencing (HTS) techniques are of great value for the investigation of microbial communities, and have been extensively used to study the gut microbiome. While most studies focus on the human gut, many others have investigated insects. However, because of the rapid spread of HTS techniques, a lot of variation exists in the protocols for sample preparation. In the present study, we investigated the impact of two widely adopted sample-processing procedures preceding library preparation, i.e., preservation of insect tissue in 70% ethanol (EtOH) and sample dissection. We used the fruit fly *Ceratitis capitata* (Diptera: Tephritidae) as a model organism and set up two experiments, one comparing the effects of sample manipulation and preservation across life stages and the other across fruit samples from different sources. The results of this study showed no major effects of dissection on the outcome of HTS. However, EtOH preservation did have effects on the recovered gut microbiome, the main effect being a significant reduction of the dominant genus, *Providencia*, in EtOH-preserved samples. Less abundant bacterial groups were also affected resulting in altered microbial profiles obtained from samples preserved in 70% EtOH. These results have important implications for the planning of future studies and when comparing studies that used different sample preparation protocols.

Keywords: amplicon sequencing, gut microbiome, methodology, Tephritidae, *Ceratitis capitata*

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INTRODUCTION

Microbial communities are an integral part of the functioning and survival of all ecosystems and all living organisms (Holguin et al., 2001; Rosenberg et al., 2007; Zilber-Rosenberg and Rosenberg, 2008). In recent decades, the emergence of high-throughput sequencing (HTS) techniques has revolutionized the study of these communities (Metzker, 2010; Morey et al., 2013). These methods allow large amounts of information about microbiological communities to be collected in a relatively short time, without the need of specialized microbiological techniques. However, the fast emergence of this technique led to a large diversity of protocols applied.

Using HTS techniques, increasing amounts of information have been collected describing the gut microbiome and its potential benefits to the host fitness. While a lot of this research has been focused on the human gut microbiome (NIH HMP Working Group et al., 2009; Kinross et al., 2011), several studies have addressed other organisms, including insects (Dillon and Dillon, 2004; Alma, 2008; Engel and Moran, 2013; Raymann and Moran, 2018). These studies have linked microbiological activity to improved digestion of indigestible components, including toxins, increased reproductive output, and many other factors benefiting the host. However, recent studies have indicated potential large effects of different sample preparation protocols in the study of microbiological communities (Hale et al., 2015; Hammer et al., 2015; Song et al., 2016). In fecal samples, one of the most crucial aspect emerging from these studies is preservation, as multiple studies have reported large effects of different preservation protocols on the microbiological community (Vlčková et al., 2012; Hale et al., 2015; Song et al., 2016). Therefore, the use of a strictly standardized protocol for sample processing is of utmost importance.

Tephritid fruit flies (Diptera: Tephritidae) are well-known pests in agricultural- and horticultural crops. Worldwide there are more than 4600 species of fruit flies (White and Elson-Harris, 1992; Uchôa, 2014), of which a large part utilizes fruits for larval development, as suggested by their name. This causes enormous losses, both directly, by damaging the fruit tissue, and indirectly, by accelerating the rotting process and infestation by other insects, fungi, and bacteria. Because of this, infestations by fruit flies can have huge economic impacts on the agricultural sector. The Mediterranean fruit fly (Medfly), *Ceratitis capitata*, is one of the most wide spread and notorious fruit fly species (De Meyer et al., 2008; Dominiak and Daniels, 2012). While native to sub-Saharan Africa, it has spread throughout the Mediterranean region, Latin-America, and Western Australia, with occasional records from North-America (De Meyer et al., 2008). One of the main reasons behind this widespread occurrence is its highly polyphagous nature, enabling it to exploit a wide variety of plant species. The host species can thus vary depending on the available plant species in a particular region or time. Currently, *C. capitata* infestations have been found in more than 260 plant species. Many of these host species are agricultural crops, and therefore the control of these species is of uttermost importance. The gut microbiome of tephritid fruit flies has increasingly been studied in the recent years, revealing the presence and role of many microorganisms. Examples include bacteria helping to overcome pesticides (Cheng et al., 2017) and host defenses (Ben-yosef et al., 2015) or generally increasing longevity of fruit flies (Behar et al., 2008b). Nevertheless, the overall knowledge of the fruit fly microbiome is still very fragmented and lacking in many areas.

In the study of the insect gut microbiome variation in the preparation protocol has only been studied to a limited extent. Although, as for fecal samples, we can suspect effects of the choice of preparation protocol in this kind of samples, empirical evidence for this is still lacking. In the present study, we set out to analyze two aspects of sample processing for HTS study of the gut microbiome of insects, using *C. capitata* (Diptera: Tephritidae) as a model organism. The first aspect of the sample processing protocol that was analyzed is the effect of dissection of the gut from the insect body. The removal of the gut (or other insect organs) is a well-established part of the protocol of many gut microbiome studies (Husseneder and Grace, 2005; Ami et al., 2010; Gavriel et al., 2011; Colman et al., 2012; Augustinos et al., 2015; Clarke, 2016; Zhang et al., 2016). However, since dissecting the gut from bodies of tiny insects, such as fruit flies, is challenging and time consuming, this step is often omitted from the sample preparation protocol (Wong et al., 2011; Ceja-Navarro et al., 2015; Garofalo et al., 2017). Although dissection might have a large impact on the gut microbiome assemblage recovered, to our knowledge, no studies have previously assessed this effect. Our hypothesis is that dissection in fruit flies, and in particular larvae, has no significant effect on the gut microbiome profiles recovered through HTS and can therefore be omitted from the protocol. The second aspect that was studied is the effect of storing individual insects in 70% ethanol (EtOH). As EtOH is a product that is easily acquired and transported, it is ideal for the collection and storage of fruit flies, even in countries where fast cooling is less evident. Additionally, preserving insects in 70% EtOH has the benefit of keeping insects flexible enough to make manipulations, such as removal of the gut, feasible, while this is not actually possible with specimens preserved in 100% EtOH, which become more fragile and tend to break during dissection. In fecal samples, 70% EtOH has been reported to have some effect on the microbiome recovered and its use is advised against (Vlčková et al., 2012; Hale et al., 2015; Song et al., 2016). Therefore, identifying and quantifying how EtOH preservation affects the gut microbiome in insect bodies should provide valuable information. Our hypothesis is that preserving insect samples in 70% EtOH for long periods will have no major effects on the gut microbiota and major patterns in microbiome composition will still emerge even after preservation.

Like many insects, tephritid fruit flies go through a significant metamorphosis during their development to adults. It has been shown in previous studies that this metamorphosis in insects, including tephritid fruit flies, can have major effects on the gut microbiome (Morales-Jiménez et al., 2012; Aharon et al., 2013; Andongma et al., 2015). Additionally, recent studies have shown that there are major differences in the gut microbiome between different populations within the same fruit fly species (Wang et al., 2011, 2014). In the present study, we aim at verifying if widely adopted insect preservation and manipulation procedures might significantly bias the HTS profiling of their gut microbial communities. This will provide important baseline information to interpret and compare data from different studies.

MATERIALS AND METHODS

Experimental Setup

In a first experiment, we investigated the effects of gut dissection and sample preservation across different life stages of *C. capitata*. Reared specimens were provided by the Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA

Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency (IAEA). We sampled third-instar larvae, tenerals (i.e., newly emerged adults) and mature adults from a long-established laboratory colony population (>30 years; >400 generations) of Greek origin. More than 60 individuals were collected for each life stage, 30 of which were processed immediately (see below) and the remaining individuals were stored in 70% EtOH at −20°C for a period varying from 12 to 18 weeks.

In a second experiment we evaluated the effects of sample dissection across EtOH-preserved third-instar larvae from different sources. We sampled three different laboratory colonies of *C. capitata*, provided by the IPCL. This included the long-established colony population (>30 years; >400 generations) of Greek origin, an intermediate established colony population (eight generations) of Australian origin and a newly established colony population (one generation) of Argentinian origin. Larvae collected from a wild population from Italy were also included in the experimental setup. Before processing the wild population, the identity of each larva was confirmed via DNA barcoding (see **Supplementary Table S1**) as described in Virgilio et al. (2012). Each sample was composed of more than 30 larvae that were immediately stored in 70% EtOH (see **Supplementary Tables S2**, **S3** for details on the experimental design and sample collection).

Laboratory Procedures

Before sample processing, the body surface of all insect specimens was sterilized in 70% EtOH for 30 s and then rinsed once in phosphate-buffered saline (PBS) water. Guts were dissected by removing, with sterilized tools, the whole gut from crop to anus. Undissected, full bodies were directly crushed with a sterilized pestle. In order to minimize biases due to inter-individual variability, five dissected guts or crushed bodies were pooled per sample and DNA was extracted from each pool using the Qiagen DNAEasy extraction kit as per the manufacturer's protocol. Before genomic library preparation, DNA concentrations of samples were determined using a Qubit Fluorometer (Thermofisher). Samples with DNA concentration <1 ng/μl were discarded and DNA extraction repeated on a novel set of specimens. The genomic library preparation targeted the V3–V4 region of the 16S ribosomal RNA gene [rRNA, insert size 465 bp, 341 F, and 806R primers (Takahashi et al., 2014)] and relied on the Nextera XT kit (including Illumina sequencing adapters, and dual-index barcodes) as per the manufacturer's protocol. DNA was amplified in two steps and, for most samples, the second amplification was repeated to increase DNA yield. A final quality check of fragment size distributions was performed using an Agilent 2100 Bioanalyzer system. A mock community including DNA of 18 bacterial strains from the BCCM/LMG Bacteria Collection[1] and a blank were also included in the Illumina run as positive and negative controls, respectively. Libraries were sequenced on an Illumina MiqSeq platform [300 bp paired end (PE) sequencing, performed by Macrogen].

[1]http://bccm.belspo.be/

Analysis of Data

Read quality was preliminarily assessed in FastQC (Andrews, 2014) and data filtering implemented via the DADA2 pipeline (Callahan et al., 2016) in R. This pipeline is based on a self-learning algorithm that compiles a parametric error model fitting the raw data, which is then used to infer sequencing errors. After trimming, demultiplexing, and filtering, paired reads were assigned to operational Taxonomic Units (OTUs) according to the Bayesian classifier method implemented by DADA2 (Wang et al., 2007). Results for the mock community and blanks were used to determine quality of the analysis. The Silva reference database v132 (Pruesse et al., 2012) was used for taxonomic assignment of OTUs (percentage of identity = 97% similarity, p-min-consensus = 0.51) and the robustness of taxonomic assignment was double-checked using the RDP (Cole et al., 2014) and Greengenes databases (DeSantis et al., 2006; data not shown). The complete analytical pipeline is detailed in **Supplementary Table S4**. Before further analysis singletons and double tons were removed from the data and OTUs with a significant presence in the blanks were deleted. For comparison between samples data scaling, based on the median sample number of reads, was implemented (de Cárcer et al., 2011).

Downstream analyses were done in R, using the Phyloseq (McMurdie and Holmes, 2013) and Vegan (Oksanen et al., 2017) packages. Differences in univariate patterns of alpha diversity [as estimated by the Reverse Simpson index (Lande, 2016)], calculated from OTU data, were tested via analysis of variance (ANOVA), with life stage (larva, teneral, and adult), preservation (fresh vs. EtOH preserved), and dissection (dissected gut vs. full body) as fixed, orthogonal factors for the first experiment and dissection (dissected gut vs. full body) and sample origin (colony strain Greece, colony strain Australia, colony strain Argentina, wild population Italy) as fixed, and random orthogonal factors, respectively, for the second experiment. *A posteriori* pairwise comparisons of significant factors were implemented via Tukey's honestly significant difference (HSD) test (Abdi and Williams, 2010).

Multivariate differences in OTU abundance and composition were tested using permutational multivariate analysis of variance (PERMANOVA; Anderson, 2017) and permutational multivariate analysis of dispersion (PERMDISP; Anderson, 2006) on scaled data as implemented by the programs PERMANOVA and PERMDISP. For PERMANOVA we relied on the same three-way factorial setup described above for univariate analyses, while for PERMDISP, which only applies to two-ways experimental designs, we tested the effects of life stage and preservation. *A posteriori* pairwise comparisons of multivariate significant factors were then implemented using the permutational *t*-statistics of PERMANOVA and PERMDISP. Probability values of repeated *a posteriori* tests were corrected for Type I errors using the false discovery rate procedure (Benjamini and Hochberg, 1995) with experiment-wise probability $p = 0.05$. Multivariate patterns were visually interpreted via scaled and centered principal coordinates analyses (PCoAs) based on Bray–Curtis distance (Bray and Curtis, 1957) as implemented by the R package ggplot2 (Wickham, 2009).

RESULTS

Overall Bacterial Diversity Associated With *C. capitata*

The Illumina Miseq run yielded more than 13×10^6 PE reads across the 54 samples considered. After assessing the quality of the reads in FastQC (Andrews, 2014), forward and reverse reads were trimmed to 230 and 200 bp, respectively. Based on read quality, a strict error rate (max *N*s = 0, max error rate = 1, see **Supplementary Table S4**) was applied. After quality control, demultiplexing, pairing, and filtering, we obtained a total of 3.6×10^6 reads, corresponding to a total of 848 unique OTUs. Taxonomic assignment of OTUs yielded 155 genera from 13 phyla (**Supplementary Table S6**). The phylum Proteobacteria was by far the most dominant (91.64% of reads) and comprised a few dominant genera: *Acinetobacter* (19.03%), *Pluralibacter* (11.31%), *Morganella* (7.40%), *Klebsiella* (3.36%), *Serratia* (2.06%), and *Enterobacter* (1.44%). The second most important phylum, Firmicutes (8.21%), included different genera from the orders Bacillales [mainly represented by *Bacillus* (2.15%), *Staphylococcus* (1.77%), and *Salinicoccus* (1.19%)], and Lactobacillales [mainly including *Lactococcus* (1.08%) and *Lactobacillus* (0.78%)]. Genera from the phyla Bacteroidetes (0.44%) and Actinobacteria (0.22%) could also be identified. The remaining phyla represented <0.01% of the total reads. A full overview of the OTU composition can be found in **Supplementary Table S5**. Comparison between samples used not the total read output as described above but a scaled dataset.

Impact of Sample Preservation and Manipulation Across Life Stages

On average, samples from the first experiment had 47.26 OTUs (SD = 28.30) and a reverse Simpson index of 0.43 (SD = 0.26). ANOVA on the reverse Simpson index obtained from the OTU data showed no effects of dissection or life stage on diversity (**Table 1A** and **Supplementary Table S7a**). However, a highly significant effect of preservation, with lower diversity in fresh compared to EtOH-preserved samples was found (**Figure 1**).

Permutational multivariate analysis of variance revealed a highly significant interaction of preservation and life stage (at $P < 0.001$) as well as a significant interaction of preservation and dissection (at $P = 0.047$) (**Table 1A** and **Supplementary Table S8a**). The *a posteriori* comparisons showed that EtOH preservation always had a significant effect on the multivariate patterns of microbial assemblages. Interestingly, significant differences across life stages were detected across all EtOH-preserved specimens, while in the microbial assemblages of fresh specimens, significant variations were only observed when comparing tenerals to adults (**Supplementary Table S8a**). Effects of dissection were only detected in fresh samples ($P = 0.040$) (**Supplementary Table S8a**).

Permutational multivariate analysis of dispersion showed a significant interaction of life stage and preservation (**Table 1A** and **Supplementary Table S9a**). Pairwise *a posteriori* tests (**Supplementary Table S9a**) revealed that the multivariate patterns of dispersion of fresh samples were comparable (with average within group dissimilarities ranging from 14.717 to 26.374) and not significantly different, while in EtOH-preserved samples the multivariate dispersion of tenerals, adults, and larvae significantly differed with average within group dissimilarities of, respectively, 58.335, 31.031, and 2.091.

TABLE 1 | Summary table for ANOVA, PERMANOVA, and PERMDISP testing for differences in patterns of alpha diversity (as estimated by the Reverse Simpson index calculated from OTU data) across **(A)** dissection procedures, sample preservation methods, and life stages of *C. capitata* and **(B)** dissection procedures on different populations of *C. capitata*.

	ANOVA	PERMANOVA	PERMDISP
(A)			
Life stage (li)	n.s.	***	n.s.
Preservation (pr)	***	***	n.s.
Dissection (di)	n.s.	n.s.	
Li × pr	n.s.	***	***
Li × di	n.s.	n.s.	
Pr × di	n.s.	*	
Li × pr × di	n.s.	n.s.	
(B)			
Origin (or)	n.s.	***	*
Dissection (di)	n.s.	n.s.	n.s.
Or × di	n.s.	n.s.	n.s.

*Full tables available in **Supplementary Table S7**. n.s., not significant; *, significant at P < 0.05, ***, at P < 0.001.*

Overall, the first two axes of the PCOA (**Figure 2**) explained 71.9% of variation (47.7 and 23.8% for PC1 and PC2, respectively). The visual inspection of graphs again suggested a lack of major differences between full body and dissected gut samples. EtOH preservation showed distinct differences where fresh individuals of all life stages combined, while EtOH-preserved samples form distinct groups corresponding to their life stage. Fresh samples of tenerals and adults were markedly less dispersed around their group centroid compared to their EtOH-preserved counterparts. Interestingly, fresh larvae have a remarkably higher dispersion compared to EtOH-preserved larvae.

The relative abundance of the most common taxa across treatments did not suggest any obvious effect of dissection in either fresh or EtOH-preserved samples (**Supplementary Table S10**). However, as indicated by PERMANOVA there were some effects of dissection within fresh samples. In taxon composition this was apparent as a lower dominance of *Providencia* in full body samples (gut: 93.84%, SD: 7.00%; full: 78.46%, SD: 18.66%). Conversely, there was a slightly higher relative abundance of many other genera in full body samples. However, there were only two genera, *Serratia* and *Klebsiella*, where this difference exceeded 1% relative abundance. Conversely, EtOH preservation heavily affected the composition of the gut microbiome with not consistent effects from life stage to life stage (**Figure 3**). Overall, we could observe a general trend from dominance of *Providencia* in fresh samples (larvae: 92.57%, SD: 12.81%; tenerals: 80.66%, SD: 19.27%; adults: 85.22%, SD: 15.04%) to a strong decline of *Providencia* (larvae: 0.14%, SD: 0.19%; tenerals: 35.83%, SD: 35.82%; adults: 0.90%,

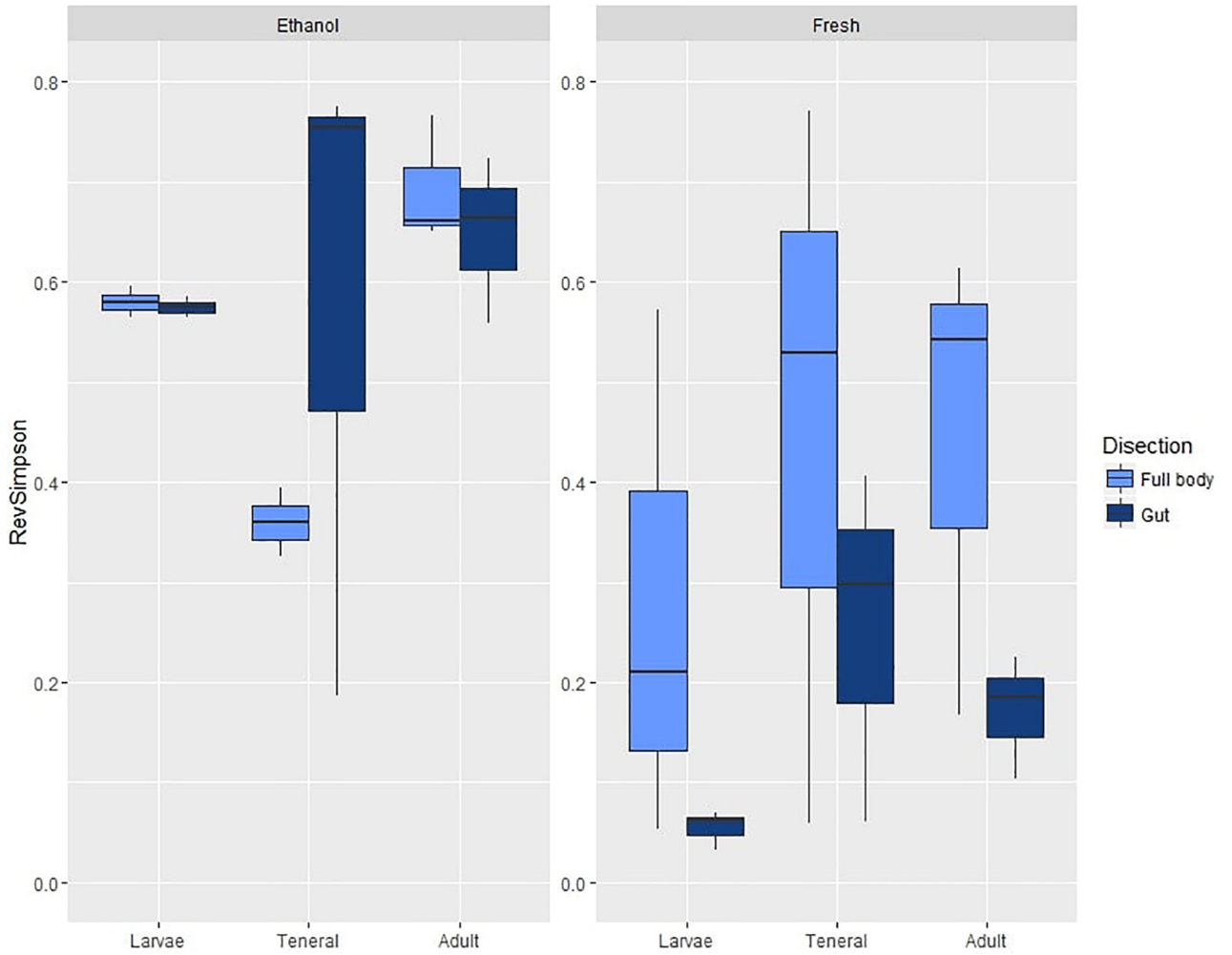

FIGURE 1 | Box plots of OTU diversity (as estimated by the Reverse Simpson index) of microbial assemblages in ethanol preserved/fresh and gut/full body specimens of *C. capitata*. Standard deviations are shown as error bars.

SD: 0.96%) and dominance of one or more other genera in EtOH-preserved samples. In EtOH-preserved larvae, we observed *Acinetobacter* as the dominant genus (fresh: 0.05%, SD: 0.05%; EtOH: 98.63%, SD: 1.11%). In EtOH-preserved tenerals, *Providencia* (fresh: 80.66%, 19.27%; EtOH: 35.83%, SD: 35.83%), *Salinicoccus* (fresh: 1.35%, SD: 1.1%; EtOH: 32.60%, SD: 27.23%), and *Staphylococcus* (fresh: 1.92%, SD: 1.74%; EtOH: 23.29%, SD: 35.59%) are the most abundant taxa. In EtOH-preserved adults, *Pluralibacter* (fresh: 0.15%, SD: 0.2%; EtOH: 61.41%, SD: 11.45%) was dominant, followed by *Acinetobacter* (fresh: 0.60%, SD: 1.03%; EtOH: 14.63%, SD: 21.2%), *Serratia* (fresh: 3.43%, SD: 5.9%; EtOH: 6.52%, SD: 7.02%), *Klebsiella* (fresh: 3.01%, SD: 6.36%; EtOH: 3.73%, SD: 1.87%), *Cronobacter* (fresh: 0.02%, SD: 0.03%; EtOH: 3.28%, SD: 2.14%), and *Enterobacter* (fresh: 0.01%, SD: 0.01%, EtOH: 2.96%, SD: 2.18%). Additionally, even if the qualitative composition of within group replicates was remarkably similar (particularly for the dominant groups, see **Supplementary Table S10**), quantitative differences and differences of low abundant genera could also be observed (see error bars of **Figure 3**). Details about the taxa relative abundance and variability are provided in **Supplementary Table S10**.

Impact of Sample Manipulation Across Fruit Fly Samples From Different Sources

For the second experiment, we observed an average diversity per sample of 44.42 OTUs (SD = 19.62) and an average reverse Simpson index of 0.57 (SD = 0.20). ANOVA (**Table 1B** and **Supplementary Table S7b**) did not show significant differences in species diversity between dissection methods or across populations (**Figure 4**).

However, PERMANOVA (**Table 1B** and **Supplementary Table S8b**) did reveal significant variability across populations from different origin, with significant differences in all pairwise comparisons, while it did not detect differences between the multivariate patterns of dissected and non-dissected samples.

Similarly, PERMDISP (**Table 1B** and **Supplementary Table S9b**) showed highly significant differences across

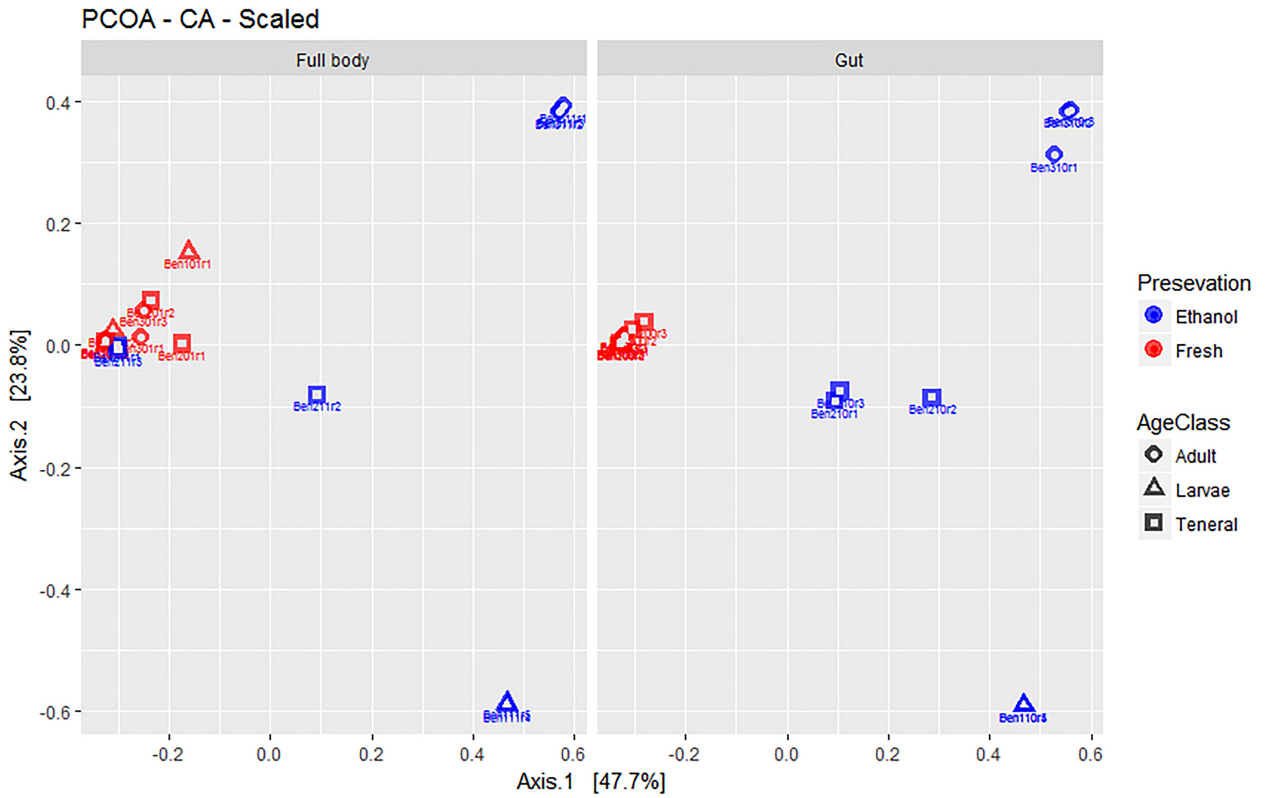

FIGURE 2 | Multivariate ordination (PCOA) of gut microbial assemblages in fresh and ethanol preserved specimens of larvae, teneral, and adults of *C. capitata*. For clarity, the same PCOA is represented twice, either with full body **(left)** or gut-dissected samples **(right)** indicated. Colors refer to preservation (blue: ethanol preserved and red: fresh) and shapes to life stage (circle: adult, triangle: larvae, square: teneral).

populations, with average within group dissimilarities ranging from 16.824 (Greece) to 76.975 (Argentina). Significant differences occurred in comparisons between all populations, except between the Italian and Argentinian population. PERMDISP also did not detect significant differences between dissected guts or full bodies.

Overall, the first two axes of the PCOA (**Figure 5**) explained 54.3% of variation (30.1 and 24.1% for PC1 and PC2, respectively). The visual analysis of **Figure 5** further suggested the lack of relevant differences between the microbial assemblages obtained from full body and gut-dissected samples. Populations from Greece (with remarkably low dispersion around the corresponding group centroid) and Australia formed separate groups while the Argentinian and Italian populations grouped closer together.

Analyzing the taxon compositions across populations (**Figure 6**) further confirmed minor differences related to the dissection protocol. The microbiome compositions of abundant genera were remarkably similar in full body and gut samples with only some genera having major quantitative differences. The most notable of these are the genera *Lactococcus* (gut: 54.71%, SD: 47.32%; full body: 1.56%, SD: 2.58%) and *Providencia* (gut: 39.35%, SD: 48.34%; full body: 77.51%, SD: 41.41%) in the Argentinian population and *Lysinibacillus* (gut: 1.77%, SD: 1.08%; full body: 12.46%, SD: 7.7%) in the Australian population. Beside this, there are only differences in low abundance genera (details are provided in **Supplementary Table S11**). Conversely, we observed considerable variation across populations. Argentina samples were dominated by *Providencia* (58.43%) and *Lactobacillus* (28.13%) complemented with a number of genera in low abundance. Australian samples are dominated by *Bacillus* (71.91%) followed by *Staphylococcus* (11.94%) and *Lysinibacillus* (7.11%). Samples from Greece were dominated by *Acinetobacter* (98.63%). Italian samples had the most even spread, dominated by *Morganella* (46.59%), and *Klebsiella* (18.58%) but having multiple genera with a significant presence [*Providencia* (5.27%), *Enterobacter* (4.36%), *Lactobacillus* (3.30%), etc.]. Only a few genera, *Providencia*, *Acinetobacter*, *Morganella*, and *Klebsiella*, were presented across all populations. Details about the taxa relative abundance and variability are provided in **Supplementary Table S11**.

DISCUSSION

This study explored the effects of commonly used sample processing protocols on the gut microbiome of *C. capitata* recovered by amplicon sequencing of 16S *rRNA* genes across life stages and samples from different sources. Our results showed that the gut microbiome of the target *C. capitata* colonies

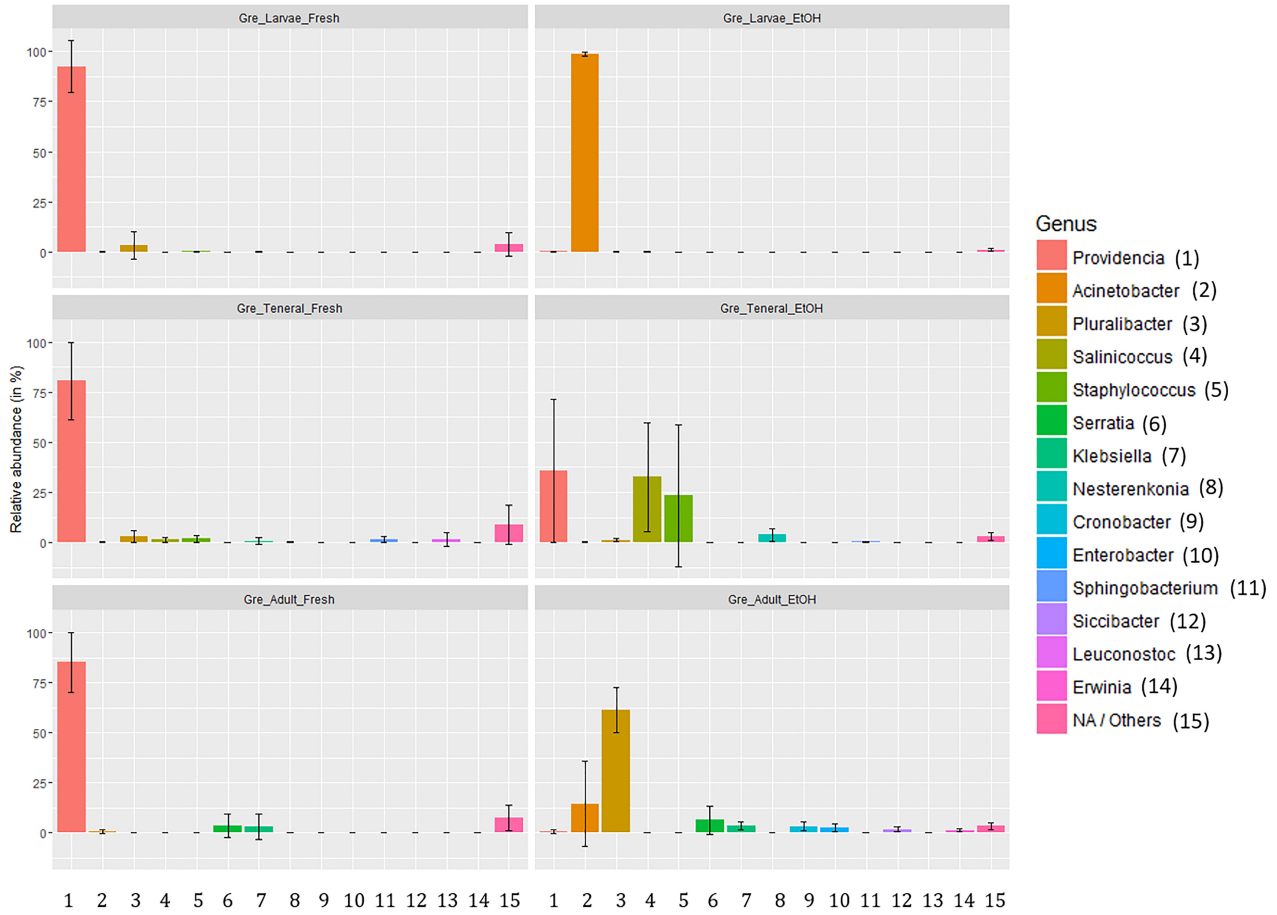

FIGURE 3 | Relative abundance of gut symbionts (genus level classification) in fresh and ethanol preserved specimens of larvae, teneral, and adults of *C. capitata* from the colony strain of Greek origin.

mainly consisted of members of the Proteobacteria (>91% of reads) and Firmicutes, and to a lesser extent, Bacteroidetes and Actinobacteria. The phylum of Proteobacteria was mainly composed of members of the Enterobacteriaceae family. This composition is compatible with other studies done in *C. capitata* and other tephritid fruit flies (Behar et al., 2008b; Prabhakar et al., 2013; Andongma et al., 2015; Yong et al., 2016). Comparing microbiome composition at genus level, we find that most samples studied in this experiment are dominated by only one or two genera. Although there is considerable variation across samples, one of the most dominant bacterial genera was *Providencia* (**Supplementary Table S2**). This genus has been commonly detected as a part of the gut microbiome of *C. capitata* and other fruit fly species (Allwood and Drew, 1996; Behar et al., 2008a; Ami et al., 2010; Wang et al., 2011; Augustinos et al., 2015; IAEA, 2016). In contrast to many other members from the Enterobacteriaceae family, this genus consists mainly of opportunistic pathogenic species (Boemare et al., 1996; Juneja and Lazzaro, 2009; Galac and Lazzaro, 2011), so it would be interesting to investigate the potential role of *Providencia* in medfly and find out if it acts as a beneficial or parasitic partner. Other dominant genera included *Bacillus*, *Acinetobacter*, *Staphylococcus*, and *Morganella*. All of these genera have been recorded in multiple earlier studies of the fruit fly gut microbiome (Kuzina et al., 2001; Alma, 2008; Thaochan et al., 2010; Wang et al., 2011; Yuval et al., 2013; Hadapad et al., 2015; IAEA, 2016; Liu et al., 2016; Yong et al., 2016).

From our comparison of different sample treatment and preservation protocols, we can draw multiple conclusions regarding the microbiome composition. First of all, we found that dissection of the gut seems to have little impact on the microbiome profiling. Across all methods used to compare gut dissection against the use of full bodies (e.g., diversity indexes, PERMANOVA, PERMDISP, PCOA, and taxonomic composition), we only found a significant differences between gut and full body microbiome profiles in fresh samples from Experiment 1 (see **Supplementary Table S8a**). However, analysis of the PCOA (**Figure 2**) and visual inspection of taxon composition (see **Supplementary Table S10**) suggested that this difference was related to a lower relative abundance of the most dominant genus, *Providencia*, in full body samples. While there were only minor differences in relative abundance in many

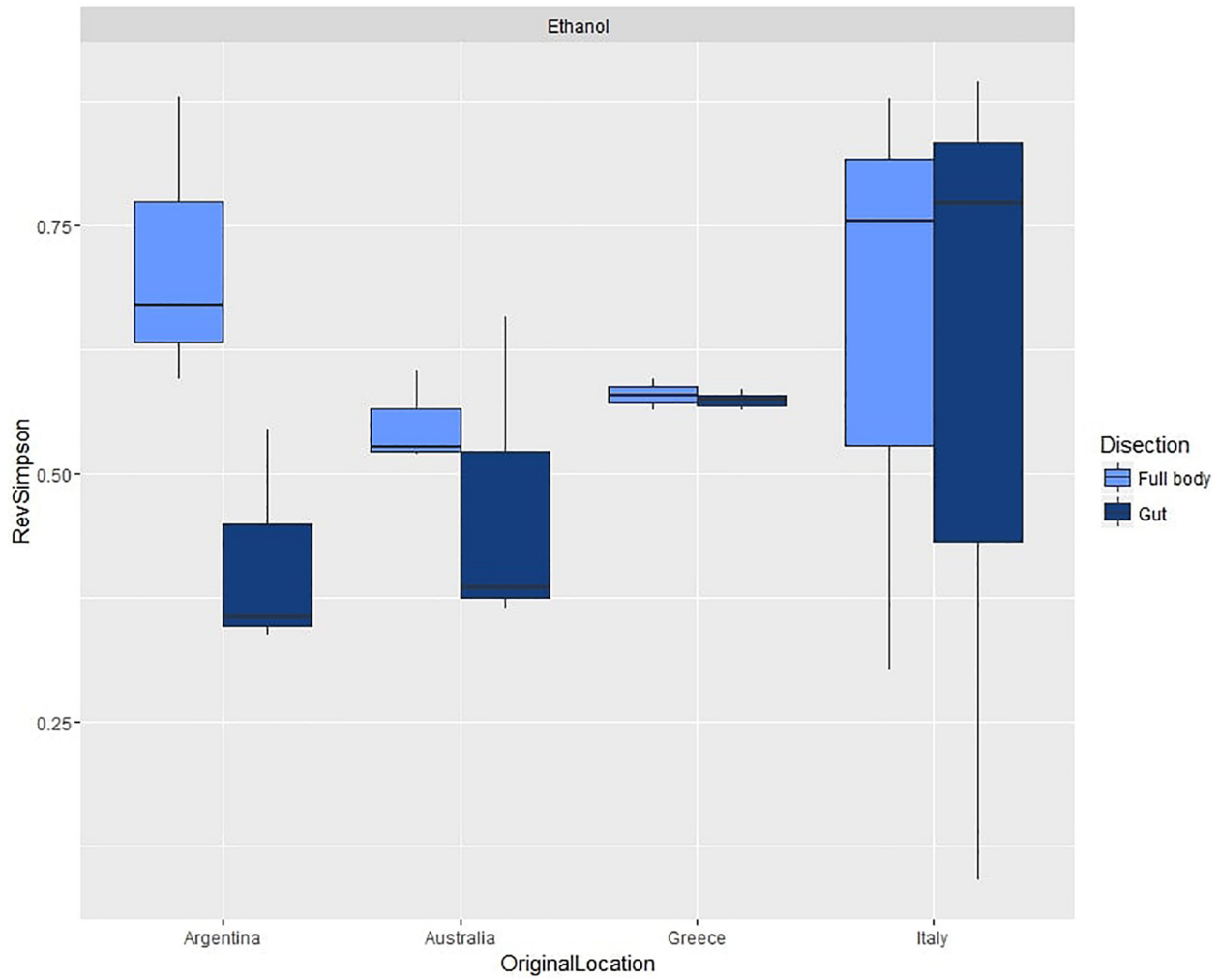

FIGURE 4 | Box plots of average reverse Simpson indexes (+SD) calculated from gut and full body samples across different populations of *C. capitata*.

other genera. This results in similar patterns of composition to be found between gut dissection and use of full bodies. This relatively little difference observed between gut and full body microbiome profiles suggests that the gut bacterial community is predominant or outnumber bacteria occurring in the rest of the body and/or that bacterial communities end up mixing during dissection.

In contrast to the limited effect of dissection, preserving fruit flies in 70% EtOH strongly affected the microbiota composition as revealed through 16S *rRNA* gene amplicon sequencing. This is consistent with recent studies of the effects of EtOH preservation of different fecal samples (Hale et al., 2015; Sinha et al., 2016; Song et al., 2016). To our knowledge this is the first time the effects of 70% EtOH preservation have been shown in insect tissue. The most prominent effect found in this experiment was an increase in the evenness of EtOH-preserved samples, as indicated by the Simpson index, through the decrease in abundance of *Providencia*, i.e., the most dominant genus observed in fresh specimens. This was consistently observed in all different life stages and is reflected in the comparison of the reverse Simpson index (see **Table 1A** and **Figure 1**). Seeing we encountered *Providencia* in much lower relative abundance in all EtOH-preserved samples, we detected other genera with higher relative abundances. *Staphylococcus*, *Salinicoccus*, *Acinetobacter*, *Serratia*, and *Klebsiella*. All had a low abundance in fresh samples but dominate EtOH-preserved samples. Beside these major changes, we also found minor changes for many genera. For some, this was a small decrease in relative abundance for EtOH-preserved samples, while for others there was a small increase. For a number of genera, with a very low abundance, these small changes made the difference between being detected or not. It is likely that genera that have a decreased relative abundance in EtOH-preserved samples, such as *Providencia*, are negatively affected by EtOH preservation more than the other genera, while this decrease makes it easier for other genera to be detected. Hale et al. (2015) reported that in fecal samples preservation methods could exhibit this kind of bias toward or against certain microbiological groups. EtOH preservation does not only affect

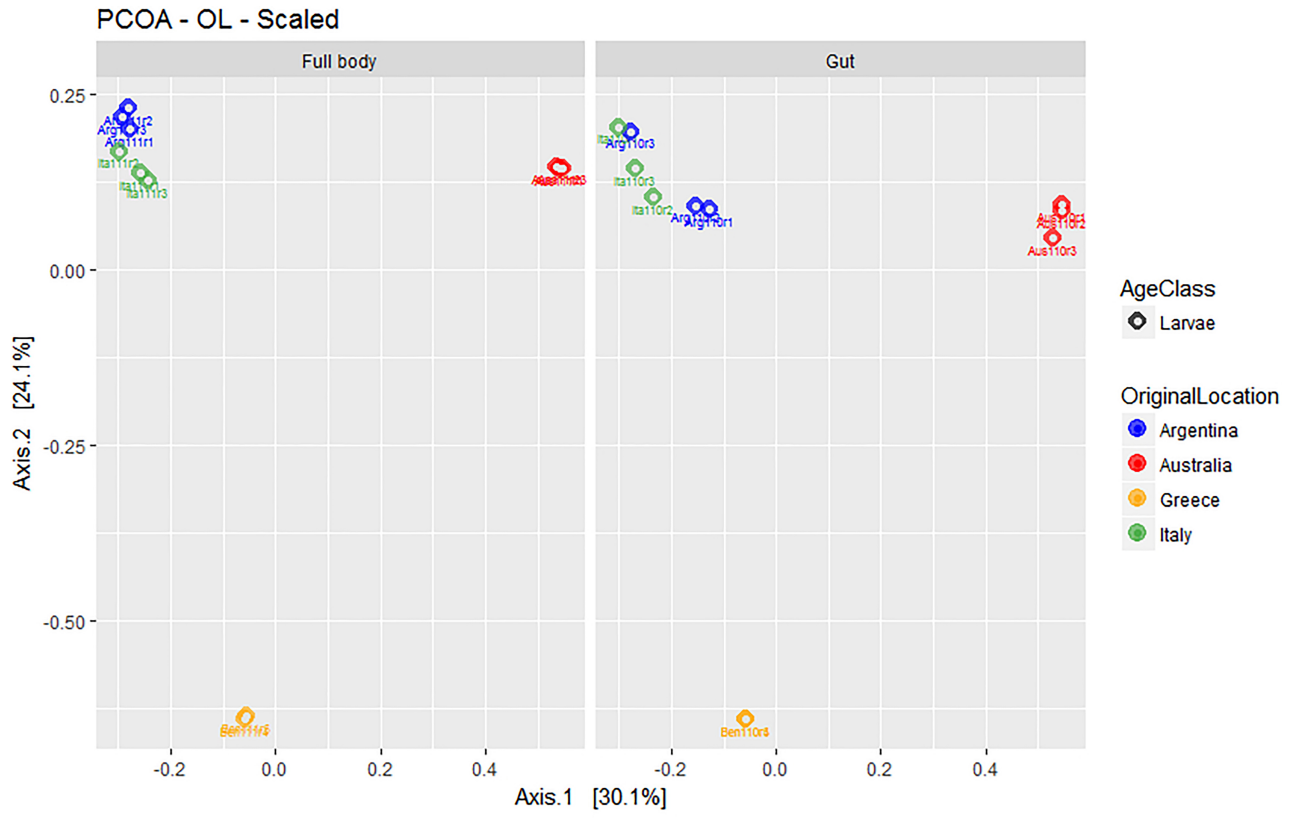

FIGURE 5 | Multivariate ordination (PCOA) of gut microbial assemblages in ethanol preserved third instar larvae of *C. capitata* with different origin (colony – Greece, colony – Argentina, colony – Australia, wild – Italy). For clarity, the same PCOA is represented twice, either with full body **(left)** or gut-dissected samples **(right)** indicated. Colors represent original origin of samples (blue: Argentina, red: Australia, yellow: Greece, green: Italy).

microbiome composition but also affects the variation between replicate samples.

In general, comparison of the different life stages showed that the gut microbiota of larvae is not only different in composition, but also have a lower diversity and variation when compared to adults and tenerals. This is not unexpected when taking in account the different feeding, lower mobility, and interaction with the environment of larvae in comparison with adults. However in larvae, we found that the multivariate dispersion in EtOH-preserved samples was much lower than in fresh samples. This is in contrast to what was found in tenerals and adults where fresh samples showed a lower dispersion. It is likely that this is linked to the complexity of the gut microbiome. In larvae, we found that after EtOH preservation samples are again completely dominated by one genus, *Acinetobacter*, while in tenerals and adults there is a more even spread with multiple genera being dominant. Additionally, in all EtOH-preserved samples differences in the relative abundances of a number of taxa could also be observed. The non-consistent patterns observed suggest that the use of 70% EtOH as preservative might produce unpredictable effects on the microbiome profiles of samples, including not consistent shifts in the relative proportion of the less abundant bacterial taxa. However, further experimental validation is necessary to verify this hypothesis.

In all fresh samples we found comparable microbiome compositions even across life stages, which were all consistently dominated by members of the genus *Providencia*. As expected, many differences can be found when looking at the less abundant bacterial taxa and this seems the reason of the difference in microbial profiles between fresh tenerals and adults (see **Supplementary Table S8a**). Conversely, in EtOH-preserved samples we detected major differences across the different life stages. We hypothesize that the reduced abundance *Providencia* after EtOH preservation allows less abundant taxa to dominate or become detectable in the gut microbiome profiles from EtOH-preserved samples. This, and the added unpredictability, might magnify the differences between life stages, and showing no consistent differences in gut microbiome composition. Therefore, our tests on EtOH-preserved specimens seem to confirm earlier studies that shifts in the gut microbiome profiles occur across fruit fly development stages (Aharon et al., 2013; Andongma et al., 2015). These results should be taken cautiously as compositional changes observed in fresh specimens seem to be far less impressive.

The second experiment allowed us to verify the consistency of patterns observed for the first experiments on an heterogeneous group of fruit fly samples from different sources. In this experiment we observed high variability in both diversity and

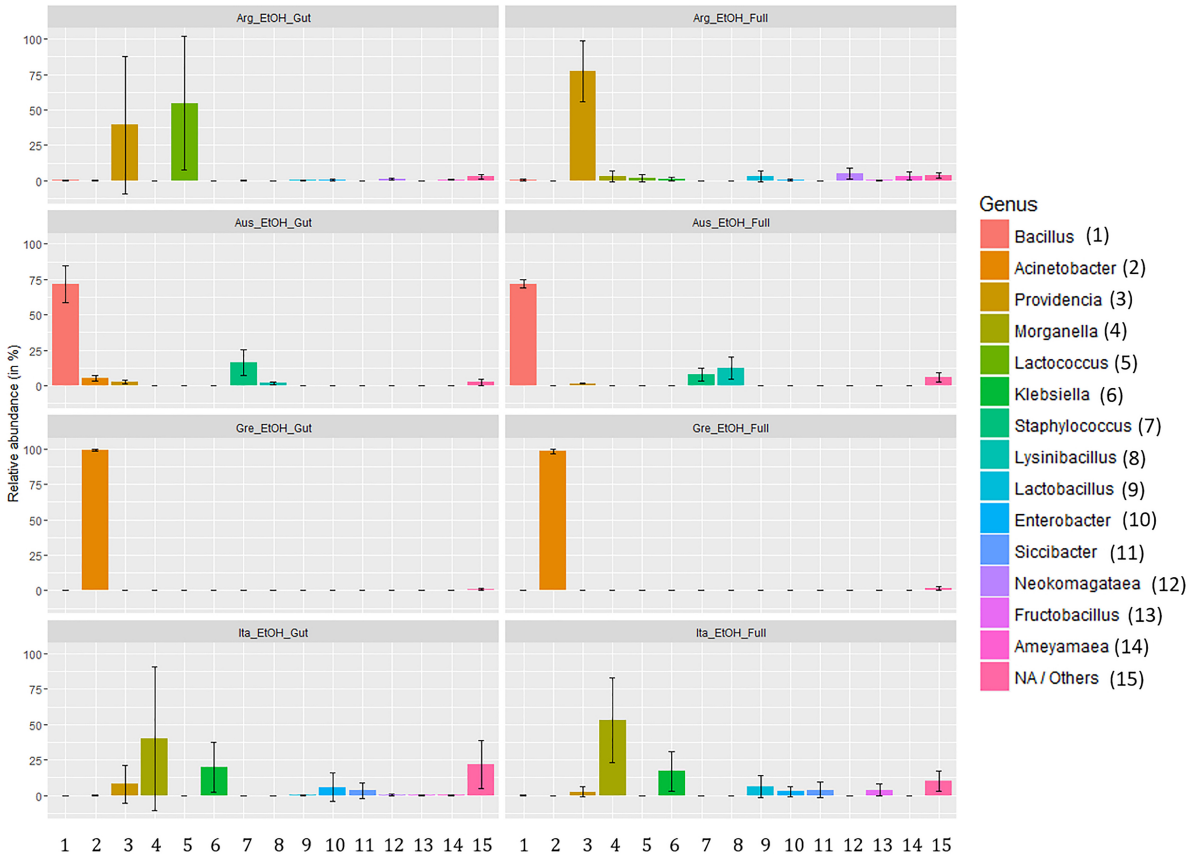

FIGURE 6 | Relative abundance of gut symbionts (genus level classification) in ethanol preserved third instar larvae of *C. capitata* from different samples (colony – Greece, colony – Argentina, colony – Australia, wild – Italy).

composition of microbiome profiles (**Table 1B**). Even the Greek, Australian, and Argentinian laboratory populations, which were reared with an identical diet and similar environmental conditions, still had very distinct gut microbiome compositions. It is however difficult to speculate about the combination of processes responsible for the observed patterns. The geographical origin of samples seems to have a very strong effect on the gut microbiome composition; however, there also seemed that there is a correspondence between the colony age and the diversity within/variation between samples. The larvae from the (long established) Greek colony populations showed the lowest variation, followed by the (intermediately established) Australian colony population. The variation in the Italian (wild) population and (recently established) Argentinian colony population was much higher (see **Supplementary Table S9b**). This result seemed to be in line with results of previous studies comparing diversity and composition of the gut microbiome from colony and wild fruit fly populations (Tsiropoulos, 1983; Konstantopoulou et al., 1999; Ben-yosef et al., 2015; Morrow et al., 2015; Deutscher et al., 2018; Malacrinò et al., 2018). To adequately disentangle the effects of colony age and colony origin, targeted experiments with adequately replicated samples of similar ages and/or origins are needed.

CONCLUSION

In conclusion, we found that differences in gut microbial profiles obtained from gut dissected and non-dissected samples were only minor with patterns that were stable across all life stage and samples from different sources. In contrast, preservation of samples in 70% EtOH had a major effect on the resulting gut microbiome profiles and was associated to higher inter-replicate variability and not consistent changes across life stages. These results shed new light on how samples preparation protocols can affect the results of HTS experiments and will help us interpreting and cross-compare the results of future and past studies. This study suggests that standardizing wet-lab procedures will increase the consistency, reliability, and repeatability of microbiomic research. A recommendation could be made against the use of 70% EtOH, a widely used preservatives in entomology, as the proportion of water is still probably too high to guarantee efficient gut microbiome fixation and preservation. When possible, fresh material or of more efficient preservative approaches (tentatively including deep freezing and absolute EtOH) should be preferred. Further experiments comparing different sample preparation protocols, different preservation techniques, on different model organisms, might give more insight in the use alternative sample

preparation and preservation protocols. These studies will help us to further identify the effects of variations in the sample preparation and help pave the way a more comprehensive understanding of the insect gut microbiome.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) and can be accessed with the accession numbers: SRR8732251–SRR8732302.

AUTHOR CONTRIBUTIONS

MD, AW, MV, and PV designed the research and secured the funding. MD, AA, KB, and MV designed and performed the experiments. MD and MV analyzed the data with input from all other authors. MD wrote the manuscript. All authors proofread, edited, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02833/full#supplementary-material

TABLE S1 | Results of DNA barcoding identifications implemented on wild samples; identified species: Top matched species, Top%: highest similarity with the identified species, Low%: lowest similarity with the identified species.

TABLE S2 | Overview of the experimental setup: Experiment 1: three life stages (larvae, tenerals, and adults), two forms of preservation (fresh and preserved in 70% ethanol), and two forms of samples preparation (dissected and full body) across one population of *C. capitata*. Experiment 2: one life stage (larvae), two forms of preservation (fresh and preserved in 70% ethanol), and two forms of samples preparation (dissected and full body) across three different colony strains and one wild population.

TABLE S3 | List of the considered *C. capitata* samples for this study.

TABLE S4 | Detailed analytical pipeline implemented for data filtering and taxon assignment.

TABLE S5 | Overview of sequencing data and taxon assignment after data filtering.

TABLE S6 | Relative composition of groups in all samples of *C. capitata* included in this study. Inner circle: phylum composition; outer circle: most abundant genera.

TABLE S7 | ANOVA and *a posteriori* comparisons (Tukey's HSD test) testing for differences in patterns of alpha diversity (as estimated by the Reverse Simpson index calculated from OTU data) across **(A)** dissection procedures, sample preservation methods, and life stages of *C. capitata* and **(B)** dissection procedures on different populations of *C. capitata*. df, degrees of freedom; MS, mean square estimates; F, pseudo-*F*; P, *p*-value; n.s., not significant; and ***, at $P < 0.001$. Tukey's HSD test: diff, difference in test scores; lwr–upr, lower and upper limits of 95% confidence interval; *p*-adj, false discovery rate-adjusted *p*-value.

TABLE S8 | PERMANOVA and *a posteriori* comparisons (*t*-tests) verifying differences in OTUs abundance and composition across **(A)** dissection procedures, sample preservation methods, and life stages of *C. capitata* and **(B)** dissection procedures on different populations of *C. capitata*. df, degrees of freedom; MS, mean square estimates; F, pseudo-*F*; P, *p*-value; n.s., not significant; and *, significant at $P < 0.05$, **, at $P < 0.01$, ***, at $P < 0.001$.

TABLE S9 | PERMDISP and *a posteriori* comparisons (*t*-tests) verifying differences in variation within samples across **(A)** sample preservation methods and life stages of *C. capitata* and **(B)** dissection procedures on different populations of *C. capitata*. df, degrees of freedom; MS, mean square estimates; F, pseudo-*F*; P, *p*-value; n.s., not significant; and *, significant at $P < 0.05$, **, at $P < 0.01$, ***, at $P < 0.001$.

TABLE S10 | Overview of average relative abundance (+SD) of the 15 most abundant genera in fresh and ethanol preserved specimens of reared larvae, teneral, and adults from the Greek laboratory colony of *C. capitata*.

TABLE S11 | Overview of average relative abundance (+SD) of the 15 most abundant genera in fresh and ethanol preserved third instar larvae of *C. capitata* with different origin.

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The Microbial Diversity of Cabbage Pest *Delia radicum* Across Multiple Life Stages

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The cabbage root fly *Delia radicum* is a worldwide pest that causes yield losses of many common cabbage crops. The bacteria associated with *D. radicum* are suggested to influence the pest status of their host. In this study, we characterized insect-associated bacteria of *D. radicum* across multiple life stages and of their diet plant (turnip, *Brassica rapa* subsp. *rapa*) by sequencing the V3–V4 region of 16S rRNA genes using the Illumina MiSeq platform. In total, over 1.2M paired-end reads were obtained, identifying 1006 bacterial amplicon sequence variants (ASVs) in samples obtained from the eggs, larvae, pupae and adults of *D. radicum*, as well as turnips that were either fresh or infested with *D. radicum* larvae. The microbial community in *D. radicum* was dominated by *Wolbachia*, a common endosymbiont of arthropods which we found in all of the investigated insect samples, with the pupal stage having the highest relative abundance. Moderate amounts of Firmicutes were found only in adult *D. radicum* flies, but not in previous life stages. Actinobacteria were mostly found on the eggs and on the skin of fresh plants on which the eggs were deposited. These plants also harbored a large amount of *Pseudomonas*. The bacterial diversity of the healthy turnip was low, whereas the microbial community of decaying turnips that were heavily infested by *D. radicum* larvae and showing symptoms of advanced soft rot was characterized by a high bacterial diversity. Taken together, this work provides insights into the bacterial communities associated with the cabbage pest *D. radicum* and its associated disease symptoms.

Keywords: 16S rRNA sequencing, microbial diversity, cabbage root fly, *Delia radicum*, community profiling, DADA2, phyloseq

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INTRODUCTION

Studies that characterize the microbial community profiles of different insect species are gaining increasing attention, yet not much is known about the microbiome of the cabbage root fly *Delia radicum*. This crop pest can cause devastating yield losses on many popular vegetable crops, e.g., cabbage, kohlrabi or rapeseed (Soroka et al., 2004; Dosdall et al., 2012). The females of this species deposit their eggs onto or near the stem of cruciferous plants and emerging larvae bore into root tissue. The larvae generally stay inside the plant tissue, only to move into the soil just before pupation and to subsequently emerge as adults (Smith, 1927). Metamorphosis causes drastic changes in the anatomy and physiology of the insects, and studies on other insects are reporting

that it can also have drastic effects on the associated microbiome (Geib et al., 2009; Hammer et al., 2014; Hammer and Moran, 2019).

The importance of symbiotic bacteria has long been well established in a multitude of Eukaryotic models. Also in insects, symbionts have been shown to have important functions in host physiology, e.g., by supplying essential amino acids, conferring resistance against insecticides, or even preventing predation by other insects (Buchner, 1965; Oliver et al., 2005; Kikuchi et al., 2012). The majority of the associations between insect guts and bacteria are of a facultative nature and appear to perform context-dependent functions (Mason et al., 2018). Gut microbes are thus suggested to mediate insect interactions with plant defensive compounds, effectively improving herbivore performance in real time, and between generations (Mason et al., 2018). Diet is an important factor in determining the fitness of any insect, and is a factor that can easily be manipulated in laboratory cultures. A near-aseptic laboratory culture of the diamondback moth (*Plutella xylostella*) showed increased fitness after being treated with antibiotics and then inoculated with symbiotic bacteria that were resistant to that antibiotic (Somerville et al., 2019). Although this study showed that certain gut symbionts of *P. xylostella* can have significant impact on fitness, the intestinal tracts of caterpillars are typically characterized by short transit times and a high pH which make microbial colonization difficult, and wild Lepidoptera (caterpillars) have been suggested to typically lack resident gut symbionts (Hammer et al., 2017).

Regarding the microbiome of *D. radicum*, a comprehensive analysis of the gut-associated microbial community associated to different developmental life stages compared to the healthy and invaded feed plant is lacking. A 2006 study by Lukwinski and co-workers (Lukwinski et al., 2006) was the first to analyze the gut microbial community of eggs, larval midgut and feces using a culturing approach. They found that the culturable midgut microbial community was primarily composed of Gammaproteobacteria. The microbial communities of eggs and fecal material were only analyzed regarding their colony forming units, and no further identification was performed. Subsequently, Welte et al. (2016a) performed a metagenome analysis of the larval gut microbial community which provided a more detailed view, largely congruent with the findings by Lukwinski et al. (2006), showing Gammaproteobacteria as the most abundant group of gut-associated microorganisms and several others (*Wolbachia, Bacteroidetes, Firmicutes, Actinobacteria,* Betaproteobacteria) in lower abundance. Bili et al. (2016) analyzed the microbial communities associated to adult flies of two different populations of *D. radicum*. They found that both *D. radicum* populations showed *Wolbachia* as most dominant associated microbial group. Both populations also harbored a low amount of Firmicutes ($\leq$1% abundance), and one of the two populations' microbiomes showed an abundant fraction of *Gluconacetobacter* (18% abundance).

In this study, our objective was to compare the microbial communities associated to the different life stages of *D. radicum* as well as healthy and invaded food sources. We profiled the microbial community of the eggs, larvae, pupae and adults. We also identified the major differences of bacterial communities that were associated with healthy the feed plants *Brassica rapa* subsp. *rapa* pre-predation, compared to plants that were macerated after being invaded by *D. radicum* larvae. This rot only developed in turnips that were used as egg laying substrate by *D. radicum*, suggesting that it is an effect caused either directly by the larvae or by larva-associated bacteria, which is a mechanism that is well described in *Drosophila* (Blum et al., 2013). Bacterial isolates from *D. radicum* have already shown the potential for metabolizing secondary metabolites that are associated with the diet plant of *D. radicum* (Welte et al., 2016a). Our results lead to the description of the microbial communities of multiple life stages of *D. radicum* as well as that of the environment that is created in the diet plant of the larvae. This work provides novel data for the research of symbioses in largely unstudied holometabolous insect groups, and may ultimately even aid in identifying transmission routes of insect and plant-associated bacteria as well as potential novel targets in microbial pest control.

MATERIALS AND METHODS

Delia radicum Rearing

The eggs that formed the starting colony of *D. radicum* were obtained from Wageningen University (Laboratory of Entomology, Prof. Joop van Loon). Flies were kept in an entomology cage (60 × 60 × 120 cm) with *ad libitum* access to water and a 1:1:1 mixture of dry food consisting of yeast extract, skim milk powder and sucrose. Cages were kept in a laboratory without humidity control at room temperature and under natural lighting. For breeding purposes, a small plastic container (12 × 12 × 6 cm, now referred to as egg box) was filled with 1 cm of wetted river sand upon which a turnip (*B. rapa* subsp. *rapa*) was placed to facilitate egg deposition. Egg boxes were moved out of the entomology cage after a week and the larvae were left to feed on the supplied turnip for approximately 4 weeks until pupation. Pupae were then separated from the river sand by flooding the egg box and sieving the water, after which the pupae were placed back into an entomology cage until eclosion, marking the beginning of a new generation.

Sample Acquisition

Turnip (TURN1-5)

Skin of turnips (*B. rapa* subsp. *rapa*) was cut superficially with a surface-sterilized razor blade, approximately 2 mm thick. Approximately 5 g of this material was homogenized in liquid nitrogen by the use of mortar and pestle. Approximately 0.25 g of homogenous material was used downstream with PowerSoil DNA extraction.

Decaying Turnip Tissue (PULP1-5)

Approximately 200 mg samples of macerated tissue from a *D. radicum* maggot-infested turnip were transferred directly to a PowerSoil tube with a surface-sterile spatula.

Eggs (EGGS1-5)

Approximately 200 *D. radicum* eggs were collected by flooding the egg boxes with (non-sterilized) demiwater, and collecting the

runoff in a thin-necked volumetric flask in order to concentrate the floating eggs at the top of the flask. They were then collected with a Whatman filter, and placed in a filter holder. Another Whatman filter was placed on top and the eggs were washed with 5 mL of the following solutions using a syringe: milliQ water, 6.5% bleach, 70% ethanol, and milliQ water. Subsequently, the Whatman filters containing the eggs were homogenized by freezing in liquid nitrogen and then crushing by mortar and pestle. The homogenate was used downstream in DNA extraction using the PowerSoil kit.

Larvae (MAGG1-10)

Twenty larvae were collected with sterile forceps and stored at −20°C until DNA extraction. Prior to DNA extraction, all were washed in 5 mL sterile MQ water, then in 5 mL 70% ethanol, then in 5.5–7.5% active chlorine bleach, and lastly rinsed in 5 mL MQ again. Subsequently, all whole larvae were homogenized by mortar and pestle in liquid nitrogen.

Pupae (PUPA1-5)

40-100 pupae were collected by flooding an egg box with tap water, and running the top liquid containing the pupae through a generic sieve. Prior to DNA extraction, all pupae were surface-sterilized in an identical fashion to the larval samples. Subsequently, all whole pupae were homogenized by mortar and pestle in liquid nitrogen.

Flies

30-40 flies were collected from entomology cages with an aspirator and subsequently killed with chloroform. Flies were sexed based on the morphology of the abdomen; flies with swollen abdomens that are characteristic for gravid females (FLYF1-5) were designated as females and flies with shriveled abdomens (FLYM1-5) were designated as males. Individuals with inconclusive exteriors were discarded. Flies were surface-sterilized in an identical fashion to the larval samples and subsequently homogenized by mortar and pestle in liquid nitrogen. For detailed descriptions on corresponding origins and amounts of biological material that was used per sample, as well as the DNA concentrations after extraction, we refer to the metadata table (**Supplementary Table 1**).

Sequencing and Analysis of the 16S rRNA Gene V3–V4 Region

After sample preparation, all 40 samples were weighed and DNA was extracted using the DNeasy PowerSoil kit (Qiagen) according to the manufacturer's protocol. Due to low DNA concentrations, the samples were concentrated using a SpeedVac Vacuum concentrator. Samples were submitted to BaseClear (Leiden, the Netherlands) for paired-end sequencing of the V3–V4 region on the Illumina MiSeq system, where the primers CCTACGGGNGGCWGCAG and GACTACHVGGGTATCTAATCC were used for the generation of the V3–V4 region amplicon (Klindworth et al., 2013). 80 paired-end FASTQ read sequence files (two per sample) were generated using bcl2fastq2 version 2.18 and initial quality assessment was based on data passing the Illumina Chastity filtering. Reads containing PhiX control signal were removed using an in-house filtering protocol by BaseClear. Second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.5. Raw reads were delivered demultiplexed and without non-biological nucleotides (i.e., sequencing primers, adapters, linkers), and the primers used for amplification of the V3–V4 region were manually removed by deleting the first 17 NTs from forward reads and the first 21 NTs from reverse reads using simple unix commands prior to preprocessing. Preprocessing of the sequencing data was done using the DADA2 pipeline (Callahan et al., 2016). Taxonomic assignment of the reads was done up to the species level with DADA2 using the Silva non-redundant database version 128 (Yilmaz et al., 2014). A phylogenetic tree of the 1006 ASVs that were resolved by the DADA2 pipeline was created by RAxML (Stamatakis, 2006). Data visualization and analysis were performed using the phyloseq package (McMurdie and Holmes, 2013). Chao1 was used for a measure of estimated richness, whereas the Shannon-Weaver index provides more information about community composition and evenness by considering relative abundances (Kim et al., 2017). In order to compare sample groups and test the null hypothesis that the dispersion of the groups as defined by measure space are equivalent for all groups, we performed permutational multivariate analysis of variance (PERMANOVA) using adonis from the 'vegan' package in R (Oksanen et al., 2019). Distance matrices were built with the Bray-Curtis method of vegdist and multilevel pairwise comparisons were performed in 999 permuations with a wrapper for adonis (Martinez Arbizu, 2019).

RESULTS

Preprocessing

Paired-end sequencing of the V3–V4 region of the 16S rRNA gene resulted in a total of 1,249,018 reads from 40 samples. Quality score profiles were typical for MiSeq sequencing, with high quality scores for >250 cycles in the forward direction and ∼200 cycles in the reverse direction (**Supplementary Figure 1**). Rarefaction curves indicated that the coverage of the bacterial diversity was sufficient and we therefore chose not to subsample to an equal depth per sample. Recently developed methods allowed us to resolve amplicon sequence variants (ASVs) instead of resorting to the more classical construction of molecular operational taxonomic units (OTUs). The benefits of this method include higher resolution and reproducibility, and allows for simple merging between independently processed datasets (Callahan et al., 2017). Unless stated otherwise, the data and analyzes of this manuscript excludes reads that were taxonomically assigned to mitochondria, chloroplast, or where the taxonomic assignment was not resolved at the phylum level.

Bacterial Community Composition

A total of 1006 amplicon sequence variants (ASVs) were identified in the entire dataset. Filtering ASVs with a relative abundance mean smaller than 5e−5 left a total of 357 abundant ASVs across ten phyla (**Figure 1A**). All *D. radicum* samples,

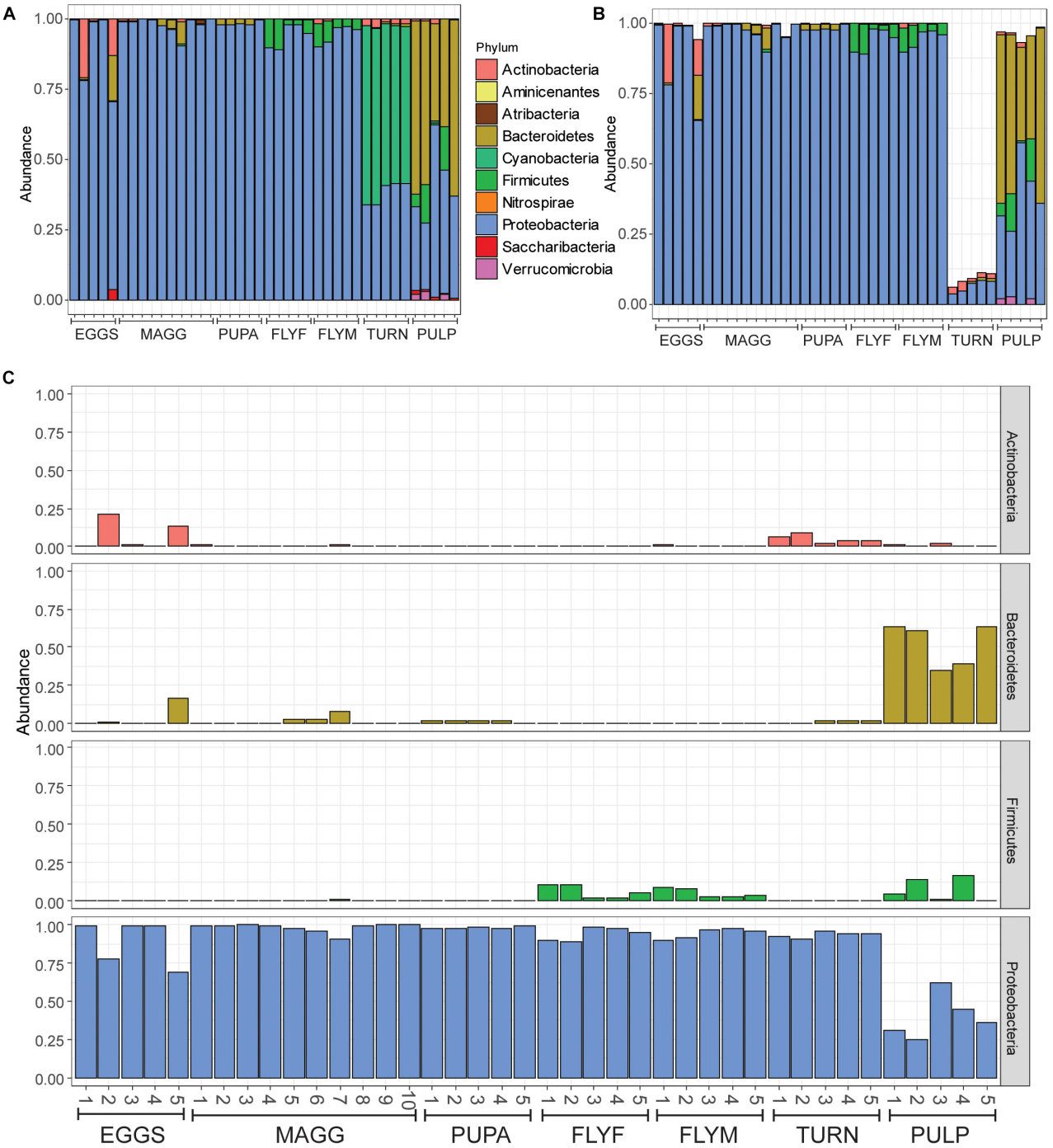

FIGURE 1 | (A) All reads represented as relative abundance, after filtering of low-abundance reads (mean(x) > 5e–5). **(B)** Bar plot after removal of reads where Class was designated as "Chloroplast" or "Unidentified," or where Family was designated as "Mitochondria." Note that over 80% of reads were removed from samples in the "turnip" sample group, which can be explained by the high number of chloroplasts within the tissue. **(C)** Relative reads of Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria in all samples, faceted by phylum. EGGS, *Delia radicum* eggs; MAGG, *Delia radicum* larvae; PUPA, *D. radicum* pupae; FLYF, Gravid female adults of *D. radicum*; FLYM, Male adults of *D. radicum*; TURN, Skin of fresh, non-infested *Brassica rapa* subsp. *rapa*; PULP, Macerated tissue of a *D. radicum*-infested individual of *B. rapa* subsp. *rapa*.

regardless of life stage, were largely dominated by Proteobacteria. Most of the sequence data gathered from the samples of intact turnips (∼90%) consisted of ASVs that came from chloroplasts and were thus not suitable for the determination of its microbiome (**Figure 1B**). Moderate amounts of Firmicutes appeared in adult *D. radicum* flies, but not in previous life

stages (**Figure 1C**). The remaining turnip ASVs were mostly classified as Proteobacteria and Actinobacteria. Samples of decaying turnip tissue contained high numbers of Bacteroidetes as well as Proteobacteria (**Figure 1C**), and uniquely harbored Verrucomicrobia ASVs (not shown). Contamination from chloroplast was not apparent in these samples. An overview of the 20 most abundant genera across all samples is presented in **Figure 2A**, and a heatmap depicting the relative abundances of different bacterial families across sample groups can be seen in **Figure 2B**.

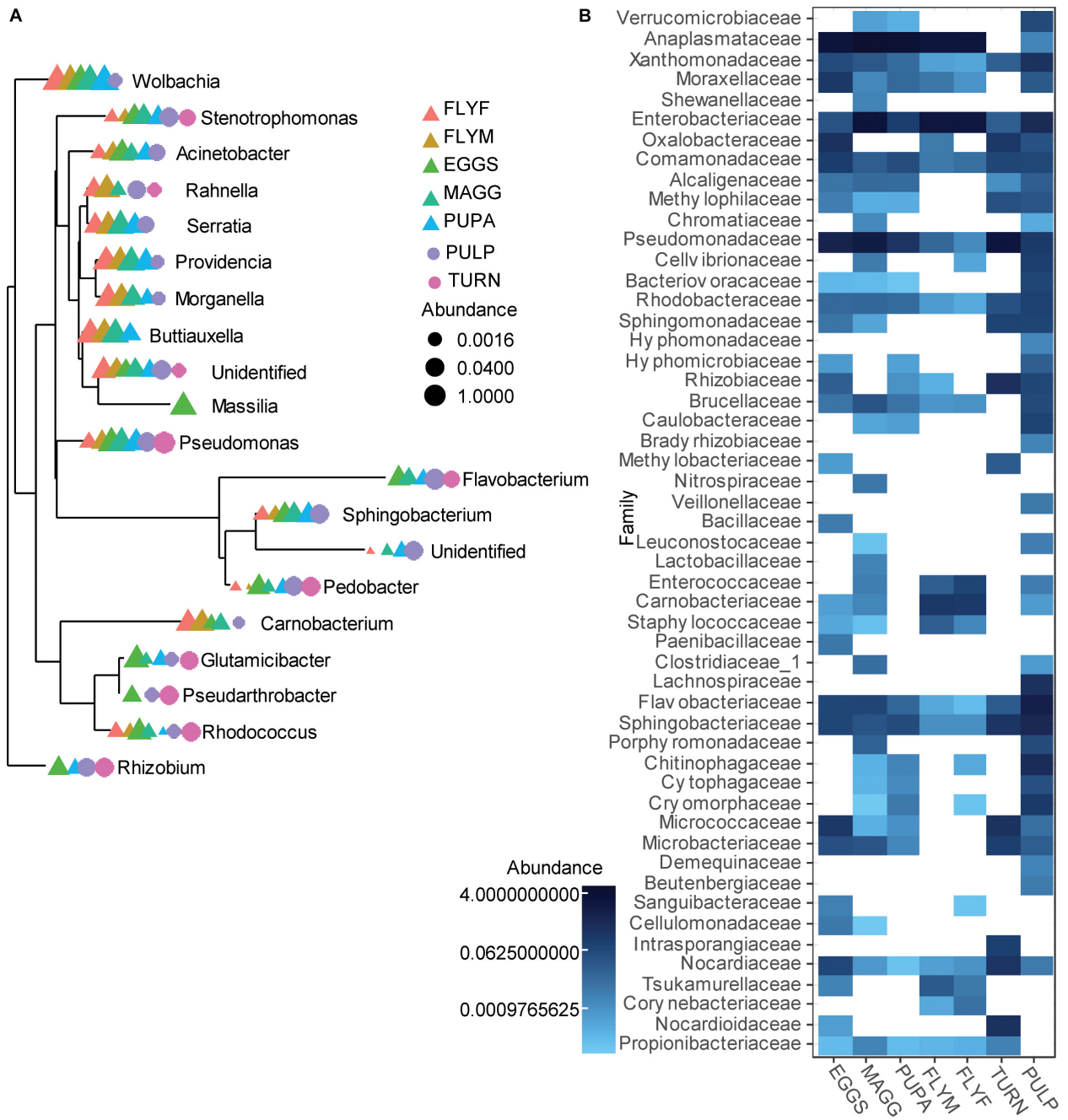

FIGURE 2 | (A) Overview of the 20 most abundant genera associated with a laboratory culture of *Delia radicum* including samples from multiple life stages, and from fresh and decayed turnips. Data was first agglomerated at the genus level, then merged per sample. **(B)** Heatmap that shows the relative abundance of reads per sample groups, where abundances were merged at the Family level. EGGS, *D. radicum* eggs; MAGG, *D. radicum* larvae; PUPA, *D. radicum* pupae; FLYF, Gravid female adults of *D. radicum*; FLYM, Male adults of *D. radicum*; TURN, Skin of fresh, non-infested *Brassica rapa* subsp. *rapa*; PULP, Macerated tissue of a *D. radicum*-infested individual of *B. rapa* subsp. *rapa*.

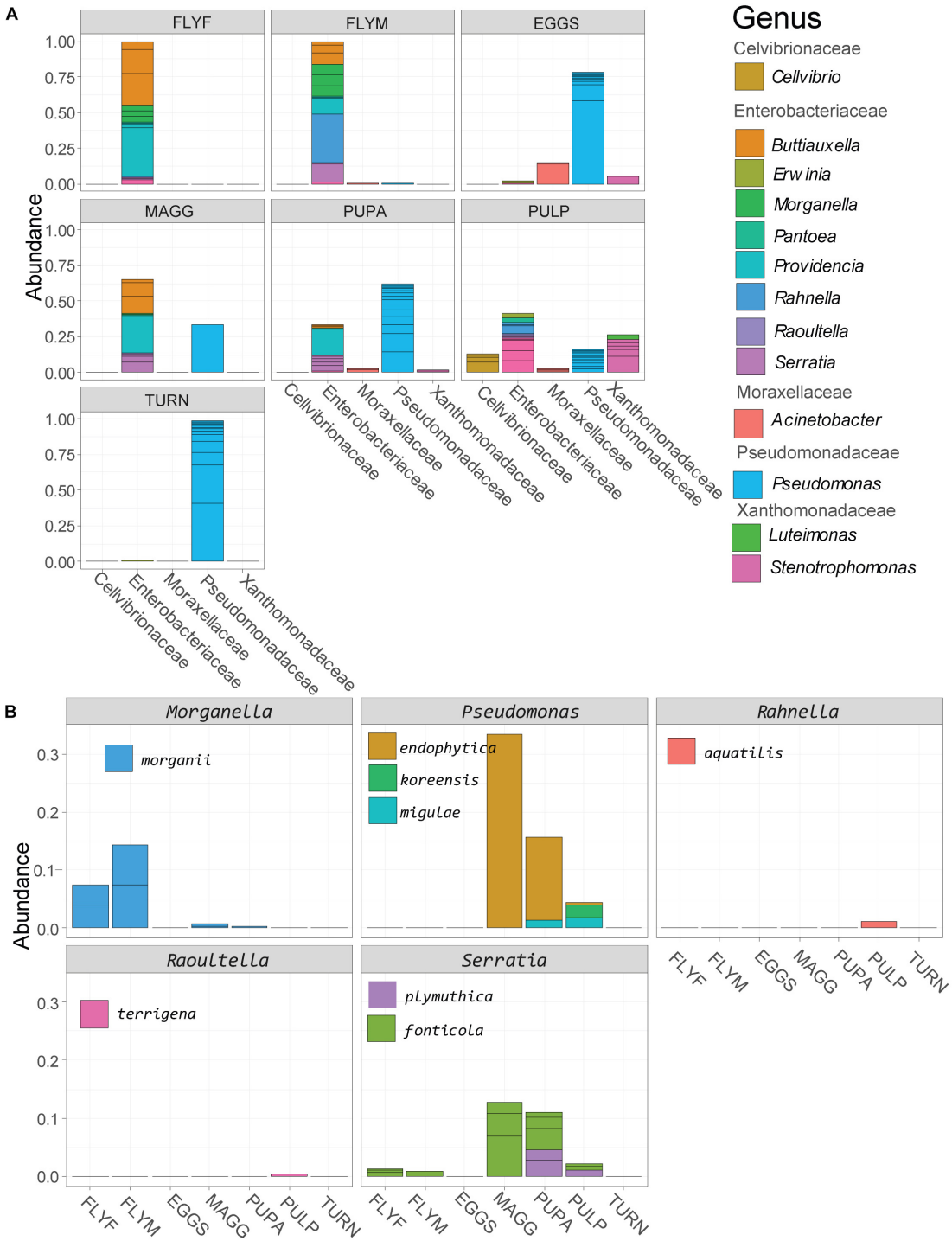

FIGURE 3 | (A) Relative abundance of reads in the class of Gammaproteobacteria, agglomerated by sample group. **(B)** Gammaproteobacterial strains that could be identified to the species level based on sequencing of the V3–V4 region. MAGG, *Delia radicum* larvae; PUPA, *D. radicum* pupae; FLYF, Gravid female adults of *D. radicum*; FLYM, Male adults of *D. radicum*; TURN, Skin of fresh, non-infested *Brassica rapa* subsp. *rapa*; PULP, Macerated tissue of a *D. radicum*-infested individual of *B. rapa* subsp. *rapa*.

Gammaproteobacteria

Gammaproteobacterial reads made up 31% of the reads in the dataset and we decided to use this subset for a deeper analysis (**Figure 3A**). We found that almost all adult fly ASVs in the Gammaproteobacteria class were affiliated with the family Enterobacteriaceae, whereas larvae and pupae also contained

considerable amounts (30-62%) of *Pseudomonas* reads. High relative abundances (>75%) of ASVs in the *Pseudomonas* genus were found in *D. radicum* egg samples and in fresh turnip skin samples. The Gammaproteobacterial ASVs found in the decayed turnip tissue represented all five families. One of the advantages of generating amplicon sequence variants with the DADA2 pipeline rather than 97%-identity-based OTU clustering is that this higher-resolution method allows for the taxonomic assignment of species-level and sometimes even strain-level variants. Strain-level variants can be a source of functional diversity and can represent specialists adapted to particular hosts or environments, the identification of which would otherwise be obscured by OTU clustering (Kwong et al., 2017). The taxonomy of 34 ASVs could be determined to the species level, of which 22 were Gammaproteobacteria, and 8 of these were categorized as abundant (relative abundance > 5e-4) (**Figure 3B**). *Morganella morganii* was found primarily in the adult flies and in small amounts in larvae and pupae. *Morganella* sp. were also found among the 20 most abundant genera (**Figure 2A**), so it can be concluded that different species of this genus were present in all samples, among which *Morganella morganii*. The genus *Rahnella* was also detected among the 20 most abundant genera (**Figure 2A**) found in all life stages of *D. radicum* apart from eggs. One ASV identified down to the species level was *Rahnella aquatilis* that was primarily found in the decayed turnip tissue, but was previously also reported inside the gut of certain species of longicorn beetles in Korea (Park et al., 2007). Three *Pseudomonas* species (*P. endophytica, P. migulae, P. koreensis*) and two species of *Serratia (S. fonticola* and *S. plymuthica)* could be identified in larvae, pupae and decayed turnip tissue. Representatives of both genera were also found to be very abundant in all life stages of *D. radicum* (**Figure 2A**) but the methods employed in this study did not allow for deeper taxonomic identification. Although sequencing of the V3–V4 region did not allow strain-level resolution, it is interesting to note that the strain *Serratia plymuthica* 3Rp8 was previously isolated from the rhizosphere of *Brassica napus* L. (Adam et al., 2016).

Wolbachia

The genus *Wolbachia* comprises endosymbiotic bacteria and is known to infect a wide variety of arthropods and nematodes (Serbus et al., 2008). In our dataset, ASVs that were taxonomically assigned to the genus *Wolbachia* made up 52% of the total reads, which includes ten non-insect samples, nine of which were devoid of *Wolbachia* reads. Since we extracted the DNA of multiple individuals for every *D. radicum* sample, the infection rates of the culture cannot be determined with this data and it is possible that not 100% of individuals carry *Wolbachia*. One out of five samples of decaying turnip ("PULP4") yielded 34 reads of *Wolbachia*, which is most likely due to the accidental inclusion of larval tissue during sampling. A total of nine different ASVs were designated as *Wolbachia*, one being represented by 478723 reads, whereas the other eight contained only between 2 and 25 reads. In all likelihood, the detection of the latter variants was the result of sequencing errors and/or artifacts in the ASV-calling algorithm of DADA2. Between-sample variation of relative abundance of *Wolbachia* reads in *D. radicum* life stages is presented in **Figure 4**.

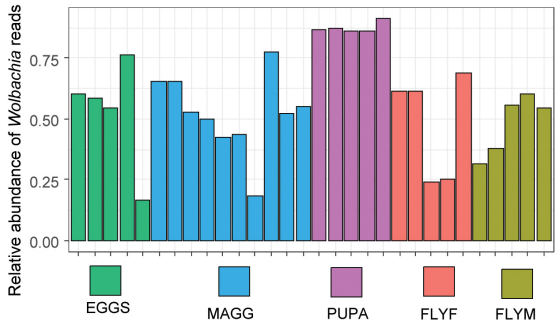

FIGURE 4 | Relative abundance of reads assigned to the genus of *Wolbachia* in *Delia radicum* samples. MAGG, *D. radicum* larvae; PUPA, *D. radicum* pupae; FLYF, Gravid female adults of *D. radicum*; FLYM, Male adults of *D. radicum*; TURN, Skin of fresh, non-infested *Brassica rapa* subsp. *rapa*; PULP, Macerated tissue of a *D. radicum*-infested individual of *B. rapa* subsp. *rapa*.

Sample Diversity

The complexity within samples (alpha diversity) was analyzed by two diversity metrics (Chao1 and Shannon-Weaver) (**Figure 5A**). Low-abundance reads were included in these analyzes in order to obtain a better indication of sample complexity. The macerated tissue of *D. radicum*–infested turnip showed particularly high complexity by both Chao1 and Shannon-Weaver indices. Bacterial species richness in the microbiome of *D. radicum* is highest in the larval stage, most likely due to the presence of a gut that is filled with decaying plant matter that is, as we show here, characterized by a highly complex bacterial community. The Shannon diversity of pupae was particularly low and can be explained by the high abundance of *Wolbachia* that decreases the evenness of the bacterial community in these samples. The between-sample diversity was visualized by performing principal coordinate analysis (multidimensional scaling), using both weighted and unweighted UniFrac distances (**Figure 5B**). Assuming a cut-off *p*-value of 0.05, we found significant differences that explained between 25% and 71% of the variation between all sample groups except between the female and male adult fly groups (**Table 1**). From these results we can conclude that each of the life stages of *D. radicum* has an identifiably unique microbiome. We did not observe significant differences between the microbiome of male and female flies.

DISCUSSION

In this study we explored the diversity of the microbial community that is associated with the eggs, larvae, pupae and adult life stages of the cabbage root fly *D. radicum*. All developmental life stages contained considerable amounts of *Wolbachia* reads, but also contained a diverse microbial community of other bacteria. Since diet and environment are also factors that influence the microbiome, we included the

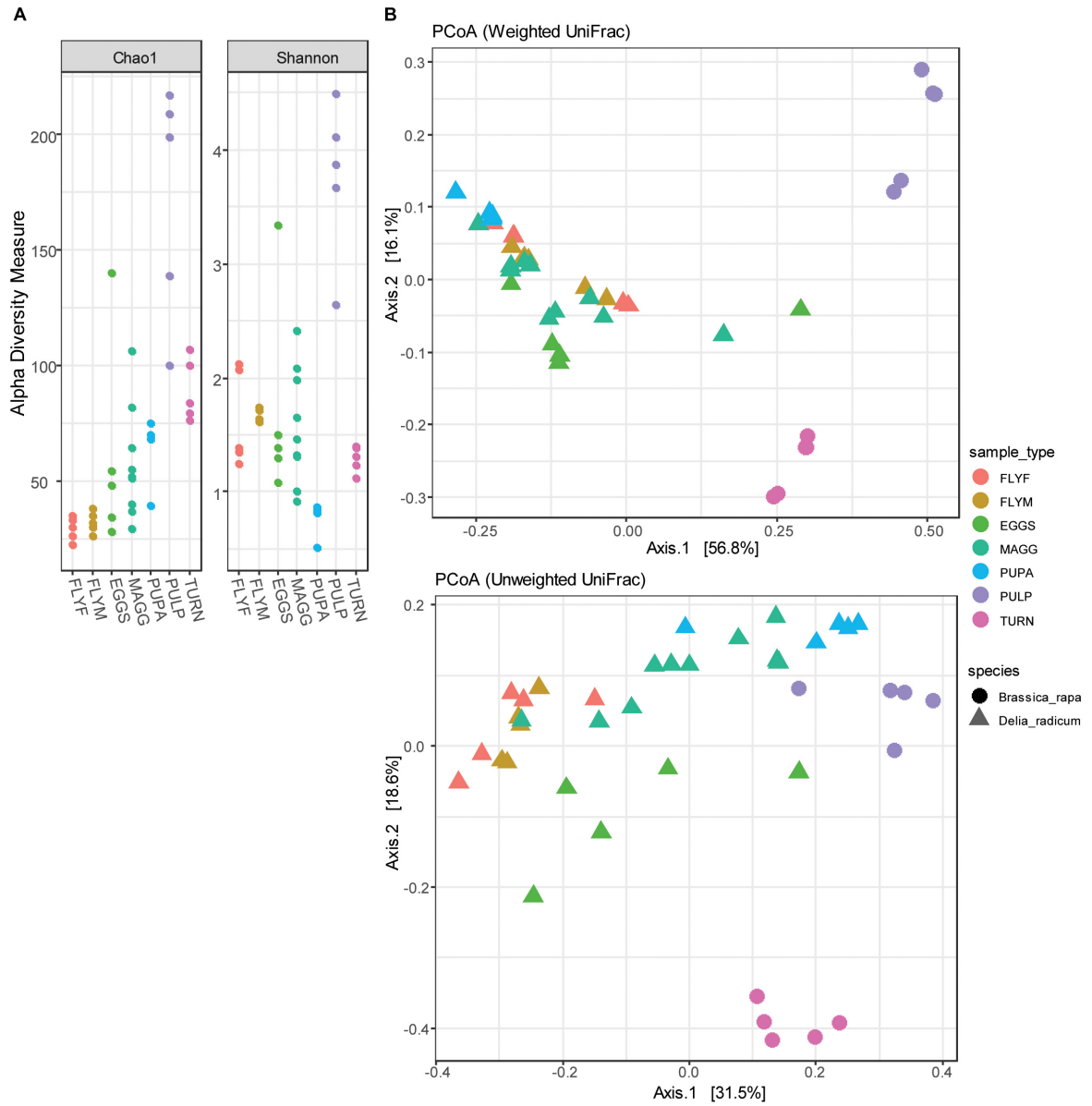

FIGURE 5 | (A) Dot plots of the alpha diversity (Chao1 and Shannon-Weaver indices) of *Delia radicum*-associated microbiota and their diet substrates pre feeding (turnip_skin) and post feeding by *D. radicum* larvae (turnip_pulp). **(B)** Principle coordinate analysis of 30 *D. radicum* and 10 *Brassica rapa* samples, based on unweighted and weighted UniFrac distances. MAGG, *D. radicum* larvae; PUPA, *D. radicum* pupae; FLYF, Gravid female adults of *D. radicum*; FLYM, Male adults of *D. radicum*; TURN, Skin of fresh, non-infested *B. rapa* subsp. *rapa*; PULP, Macerated tissue of a *D. radicum*-infested individual of *B. rapa* subsp. *rapa*.

microbial diversity of the host plant (turnip), and of decayed host plant material that had been infested with *D. radicum* larvae. The decayed plant material was characterized by a very high alpha diversity.

Fresh and macerated turnip samples were furthermore considerably different from the microbial communities of *D. radicum* in structure and composition, but also shared a number of taxa that are possibly transferred between the environment and the host, or vice versa.

Brassica rapa subsp. *rapa* is a member of the Brassicaceae family of plants which employ a method of defense against herbivores and pathogenic bacteria by producing the secondary metabolite isothiocyanate (Tierens et al., 2001). Our initial expectation was that the high concentration of isothiocyanates within damaged turnip tissue would only allow isothiocyanate-resistant bacteria to thrive in this environment. Furthermore, many isothiocyanate-resistant strains are within genera that are also shown to have cell wall degrading properties (Basset et al., 2000; Welte et al., 2016b). Instead of a low diversity of strains that are typical cell wall degraders such as *Erwinia*, *Pectobacterium*, or *Pantoea*, these samples contained a flourishing community of Bacteroidetes and Gammaproteobacteria from all different

TABLE 1 | Pairwise multilevel comparisons using adonis with 999 permutations.

Pr(>F)	*D. radicum* Eggs	*D. radicum* Larvae	*D. radicum* Pupae	*D. radicum* Adult females	*D. radicum* Adult males	Macerated turnip
D. radicum Larvae	0.002**					
D. radicum Pupae	0.009**	0.001***				
D. radicum Adult females	0.010**	0.007**	0.010**			
D. radicum Adult males	0.016*	0.001***	0.012*	0.206		
Macerated turnip	0.009**	0.001***	0.012*	0.007**	0.011*	
Fresh turnip	0.008**	0.003**	0.010**	0.010**	0.011*	0.009**

The levels compared are the sample groups that distinguish the Delia radicum life stages. p-values denoted as (0.010–0.05), (0.001–0.010)**, or (0–0.001)***.*

families. Isothiocyanates are volatile and the bioavailability can be expected to differ significantly between the initial moment of *D. radicum* attack and the late-stage infestation marked by complete maceration of the turnip tissue. It is possible that early colonizers are capable of overcoming isothiocyanate-based defenses of the plant, and that a multitude of opportunistic taxa colonize the tissue after the plant defenses have sufficiently diminished over time. The *Serratia* and *Pseudomonas* species that were identified in larval samples could potentially be of great nutritional aid for their hosts. *Serratia plymuthica* 3Rp8, isolated from the rhizosphere of *Brassica napus* L. (Adam et al., 2016), for example, contains two copies of the *saxA* gene (Van den Bosch et al., 2018) that has been implied in overcoming isothiocyanate-based plant defenses in plant pathogenic bacteria (van den Bosch et al., 2019).

All life stages of *D. radicum* were characterized by high abundances of Proteobacterial symbionts, but each was shown to have unique characteristics that could be resolved by comparing various diversity metrics.

Comparisons with other studies that sequenced the 16S rRNA gene of insect-associated gut bacteria show some interesting parallels, as well as dissimilarities.

Bili et al. (2016) previously analyzed the bacterial community of *D. radicum* adults of three different geographic locations in France using 454 pyrosequencing of the V4-V5 region of the bacterial 16S rRNA gene (2016). They reported a relatively low-complexity microbiome dominated by *Wolbachia* or *Wolbachia* and *Gluconacetobacter*, depending on the geographical origin of the flies. The abundance of Firmicutes was low in the fly samples of their study, aligning well with the data presented here. In contrast to the study performed by Bili et al. (2016), we found high abundances of Enterobacteriaceae and we were able to identify multiple Gammaproteobacterial reads which had not been reported previously. *Morganella morganii* is found as a resident of the gut microbiome of the common house fly *Musca domestica* (Gupta et al., 2012) and in the microbiome of healthy bees (Erban et al., 2017) but is also designated as a lethal pathogen in Mexican fruit flies [*Anastrepha ludens* (Loew)] and sand flies [*Lutzomyia longipalpis* (Lutz and Neiva)] (Salas et al., 2017). In our dataset, *Morganella* sp. were abundant in all life stages of *D. radicum*, most of which could not be assigned to a specific species due to the limitation of the sequencing method. In the oriental fruit fly *Bactrocera dorsalis,* it was shown that Proteobacteria dominated immature stages, whereas adult stages were dominated by Firmicutes (Andongma et al., 2015). Although Firmicutes did not quite dominate in *D. radicum* adults, this life stage was the only one where moderate amounts of Firmicute reads were present. In the house fly *M. domestica*, Gammaproteobacteria are dominating larval and adult microbiomes (Gupta et al., 2012; Zhao et al., 2017). Also the microbiomes of other flies harbor a high amount of Gammaproteobacteria, e.g., as found in *Drosophila melanogaster* (Corby-Harris et al., 2007; Cox and Gilmore, 2007). Furthermore, the genus *Comamonas* was reported to be the most abundant in pupae and completely absent in adults of *B. dorsalis* (Andongma et al., 2015). This trend was also observed in the *D. radicum* dataset. 4-6% of non-*Wolbachia* reads in all pupae samples were *Comamonas,* but fly samples contained at most 1%.

For future studies it might be interesting to combine sequencing results from a different laboratory-reared insect species to see whether the variance of microbial diversity is larger between different life stages of one insect, or between two insect species at the same life stage. Studies on house flies have shown that geographical origin and laboratory rearing can have a considerable impact on the microbiome (Park et al., 2019).

The presence of *Wolbachia* has been shown to play a role in determining the microbiome composition in *Drosophila* (Simhadri et al., 2017).

We observed *Wolbachia* reads in all *D. radicum* samples regardless of life stage. In the adult stages, the number of reads assigned to this genus ranged from 25 to 65%, which is markedly lower than the 97% and 80% reported previously (Bili et al., 2016). The vertical transmission rate of *Wolbachia* is 100%, and there is no evidence of reproductive manipulation phenotypes such as feminization, parthenogenesis, male-killing or cytoplasmic incompatibility in *D. radicum* (Lopez et al., 2018). We found particularly high relative abundances of *Wolbachia* in the pupal samples. One possible explanation for this is that larvae shed their gut lining before pupation, evacuating a large part of the gut-associated bacteria (Hammer and Moran, 2019). The distribution of reads over the nine *Wolbachia* ASVs suggests that the presence of multiple genotypes of *Wolbachia* seems unlikely. The artificial OTU richness may stem from technical artifacts such as PCR and/or sequencing errors, or to the limitation of the 16S rRNA gene for taxonomically resolving *Wolbachia* specifically (Ellegaard et al., 2013). The age-dependency of the relative abundance of *Wolbachia* in the gut of the termite *Nasutitermes arborum* was recently described for the first time (Diouf et al., 2018). The relative abundance of *Wolbachia*

was shown to be negatively correlated with alpha diversity, suggesting a mutual exclusion from the same environment. Although our sampling methods included homogenization of whole insects, previous studies have indicated that communities from such samples can closely resemble communities that were sampled from the gut alone (Hammer et al., 2014). We therefore suggest that the removal of *Wolbachia* reads *in silico* resulted in a dataset that is a closer representation of the gut lumen of *D. radicum*. For future microbiome analyzes of *Wolbachia*-positive *D. radicum* samples one should consider a more cost-effective approach for reducing the number of *Wolbachia* reads in a sample by specific restriction digestion of the *Wolbachia* 16S rRNA gene, as was recently done in *Drosophila* (Simhadri et al., 2017).

Whether the taxa reported in this study are transient or resident, or even dead or alive, cannot be resolved by the methods presented in this work. Future studies could elucidate the potential of nutritional mutualisms between *D. radicum* and resident microbes.

This work has enabled a deeper understanding of the bacterial players associated with *D. radicum* at different life stages, and of the bacterial nature of the plant rot that is associated with *D. radicum* infestation. As such, it could potentially provide new clues on symbiotic bacteria that could be exploited in biocontrol programs. Elucidation of the transmission patterns and the specific functions of these bacterial players species are interesting platforms for further research.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are available on NCBI under the BioProject PRJNA573643. Additional data such as the code for generating figures will be made available, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

TB and CW designed the research. TB conducted the research, analyzed the data, and wrote the manuscript with input from CW.

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ORIGINAL RESEARCH
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Multiple Factors Determine the Structure of Bacterial Communities Associated With *Aedes albopictus* Under Artificial Rearing Conditions

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Insect symbionts are major manipulators of host's behavior. Their effect on parameters such as fecundity, male mating competitiveness, and biological quality in general, can have a major influence on the effectiveness of the sterile insect technique (SIT). SIT is currently being developed and applied against human disease vectors, including *Ae. albopictus*, as an environment-friendly method of population suppression, therefore there is a renewed interest on both the characterization of gut microbiota and their exploitation in artificial rearing. In the present study, bacterial communities of eggs, larvae, and adults (both males and females) of artificially reared *Ae. albopictus,* were characterized using both culture-dependent and culture-independent approaches. Mosquito-associated bacteria corresponding to thirteen and five bacteria genera were isolated from the larval food and the sugar solution (adult food), respectively. The symbiont community of the females was affected by the provision of a blood meal. *Pseudomonas* and *Enterobacter* were either introduced or enhanced with the blood meal, whereas *Serratia* were relatively stable during the adult stage of females. Maintenance of these taxa in female guts is probably related with blood digestion. Gut-associated microbiota of males and females were different, starting early after emergence and continuing in older stages. Our results indicate that eggs contained bacteria from more than fifteen genera including *Bacillus*, *Chryseobacterium*, and *Escherichia–Shigella*, which were also main components of gut microbiota of female adults before and after blood feeding, indicating potential transmission among generations. Our results provided a thorough study of the egg- and gut-associated bacteria of artificially reared *Ae. albopictus*, which can be important for further studies using probiotic bacteria to improve the effectiveness of mosquito artificial rearing and SIT applications.

Keywords: *Aedes albopictus*, gut, microbiota, artificial rearing, SIT, culture-dependent, culture-independent

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INTRODUCTION

The tiger mosquito, *Aedes albopictus*, is known to carry dengue, chikungunya, and Zika viruses. Together with its closely related species *Aedes aegypti* they represent, in addition to significant biting nuisance, a serious health threat to more than three billion people in over 120 countries (Brady et al., 2012; WHO, 2017). The population control of these mosquito species is challenging due to the inefficiency of current conventional control methods, which are largely based on the removal of breeding sites and use of insecticides (Bourtzis et al., 2016; Pang et al., 2017; Flores and O'Neill, 2018). Several novel population suppression methods have been developed against *Aedes* species and some of them, including the sterile insect technique (SIT), the incompatible insect technique (IIT), the combined SIT/IIT and the Release of Insects carrying Dominant Lethals (RIDL), are currently being tested in small scale pilot trials (Bourtzis et al., 2016; Flores and O'Neill, 2018; Zheng et al., 2019).

All these methods should ideally be used as a component in area-wide integrated pest management programs (AW-IPM) (Bourtzis et al., 2016; Bouyer and Marois, 2018). They all depend on the mass rearing, sex separation, sterilization (or lethality), handling, packing, transportation and continuous release, at overflooding ratios, of males in the field to suppress a target population (Lees et al., 2015; Bourtzis et al., 2016). So, large production of high-quality males is required since they should be released at large numbers and should also be able to compete with wild males for mating with wild females. However, the mass rearing process, handling, packing, transportation and release as well as the irradiation (SIT), the infection with a bacterial symbiont such as *Wolbachia* (IIT or combined SIT/IIT) or the insertion of a transgene (RIDL) may have a negative effect in the life history traits of the mass reared insect strain and the overall biological quality of the released males (Lees et al., 2015; Bourtzis et al., 2016).

Many bacterial species have established long evolutionary and intimate symbiotic associations with insects affecting many aspects of their biology, ecology and evolution including nutrition, metabolism, immune function, physiology as well as reproduction and behavior (Dillon and Dillon, 2004; Bourtzis and Miller, 2009; Zchori-Fein and Bourtzis, 2011; Engel and Moran, 2013). There have been several studies which have focused on the characterization of the bacterial communities associated with insects, their role in the host biology as well as their potential exploitation to develop and/or enhance novel strategies to control populations of pests and disease vectors including mosquitoes (Douglas, 2015; Berasategui et al., 2016; Arora and Douglas, 2017; Flores and O'Neill, 2018).

As previous studies in fruit flies have shown, the biological quality and the overall ecological fitness of the insects highly depends on their associated bacterial and other microbial partners (Shelly and McInnis, 2003; Niyazi et al., 2004; Ben-Yosef et al., 2008; Ben Ami et al., 2010; Gavriel et al., 2011; Storelli et al., 2011; Blum et al., 2013; Hamden et al., 2013; Sacchetti et al., 2014; Augustinos et al., 2015; Kyritsis et al., 2017; Cai et al., 2018; Khaeso et al., 2018; Akami et al., 2019). Therefore, insect symbiotic bacterial species can be used as probiotics to improve key parameters of population suppression strategies, including the productivity of mass reared strains as well as the mating performance and longevity of sterile males (Niyazi et al., 2004; Ben Ami et al., 2010; Gavriel et al., 2010; Yuval et al., 2013; Augustinos et al., 2015; Kyritsis et al., 2017; Cai et al., 2018).

There is an increasing interest in the structure of mosquito-associated microbiota as well as the interactions between the host and the microbes (for recent reviews see Guegan et al., 2018; Strand, 2018; Scolari et al., 2019), due to the role of the associated microbes in the biology of their hosts and the immunity and pathogen interference. The latter is critical for the development and application of novel population suppression and population modification strategies against major mosquito vector species (Flores and O'Neill, 2018; Guegan et al., 2018). Many studies have investigated the role of the environment and the breeding sites on microbial acquisition in *Aedes*, *Anopheles,* and *Culex* mosquitoes and have shown that there is a clear overlap of bacterial composition between mosquito species, developmental stages, and habitats (for a recent review see Guegan et al., 2018). In addition, the concept of mosquito core- and pan-microbiota has been investigated and recent data clearly indicate that environmental factors and food resources play a major role in the environmental bacterial acquisition and this may determine key biological properties of the mosquito hosts (Pastoris et al., 1989; Lindh et al., 2005; Rani et al., 2009; Wang et al., 2011, 2018; Minard et al., 2013a,b; Guegan et al., 2018). Sex, female size, and genetic diversity have been shown to affect the associated microbiota in *Ae. albopictus* with the overall bacterial diversity of both *Ae. albopictus* and *Ae. koreicus* being significantly lower in recently invaded regions (Minard et al., 2018; Rosso et al., 2018).

Interestingly, some bacterial species (such as *Wolbachia, Asaia,* and *Elizabethkingia*) may have interstadial transmission, for example from pupae to adults or via maternal transmission (Kittayapong et al., 2002; Favia et al., 2007; Akhouayri et al., 2013; Ngwa et al., 2013). Differences in the bacterial diversity between males and females have been partly attributed to differences in the flight dispersal and blood feeding (Foster, 1995; Demaio et al., 1996; Pumpuni et al., 1996; Zayed and Bream, 2003; Gusmao et al., 2010; Kumar et al., 2010; Benoit et al., 2011; Gaio et al., 2011; Wang et al., 2011; Zouache et al., 2011). A number of studies has also shown that mosquito-associated bacterial species may affect both metabolism and life history traits including blood and sugar digestion, supply of vitamins and amino acids, body size, oviposition site choice and egg production, longevity, sex ratio, larval development as well as virus dissemination (Chouaia et al., 2012; Mitraka et al., 2013; Sharma et al., 2013; Coon et al., 2014, 2016a,b, 2017; Muturi et al., 2016; Dickson et al., 2017; Guegan et al., 2018).

The Insect Pest Control Laboratory of the Joint FAO/IAEA Division of Nuclear Applications in Food and Agriculture has been developing the SIT-based approaches for the population control of *Ae. albopictus* and guidelines for mass-rearing laboratory populations of insects that are targets for SIT. Given the importance of the gut-associated microbiota for the rearing process and the overall biological quality of artificially produced insects for SIT-based applications, the present study focused on the characterization of the gut-associated bacterial

species of a laboratory strain of *Ae. albopictus* under artificial rearing conditions for potential SIT-based applications. The characterization was performed using culture-dependent and culture-independent approaches, throughout the mosquito developmental stages (egg, larva and adult), at different adult ages (both young and old males and females) and female feeding regimes (sugar or blood). The density levels of key bacterial partners were assessed, and the overall findings are discussed from an applied perspective towards the use of SIT-based approaches for the population suppression of this major mosquito vector species.

MATERIALS AND METHODS

Mosquito Strains and Maintenance

The experiments were conducted at the Joint FAO/IAEA Insect Pest Control Laboratory (hereafter IPCL), Seibersdorf, Austria. *Aedes albopictus* wild type strain (Guangzhou, China), known as GUA strain (Zhang et al., 2015) at F13 generation, was used in these experiments. Egg hatching, larval rearing, and adult maintenance were carried out as previously reported (Zhang et al., 2015). Laboratory-reared cyclic colony was maintained under at 26 ± 1°C, 60 ± 10% RH with a photoperiod of 12: 12 h (L: D).

Sample Collection and Dissection

Blood meals were provided to the GUA females aged 7- to 9-days post emergence. Two days after the blood meal, a plastic 250-ml beaker containing 100 mL sterilized deionized water and a strip of sterilized white filter paper (white creped papers IF C140, Industrial Filtro S.r.l., Cologno Monzese, Italy) were placed in the cage (30 × 30 × 30 cm, BugDorm 1, MegaView, Taichung, Taiwan). Fresh eggs (less than 4 h) were collected and counted (approximately 100 for each replicate) as materials for bacterial culture. The rest of the eggs were maintained in the adult rearing room for 7 days for maturation and then hatched as previously described (Zhang et al., 2015). Larvae were fed on modified IAEA liquid larvae diet (Balestrino et al., 2014). The whole gut of the 3rd instar larvae (L3) was dissected and was used as source for the isolation of cultivable bacterial species. Pupae were collected and separated using an improved Fay-Morlan separator (Dame et al., 1974; Focks, 1980).

Male and female pupae were reared separately in sterilized deionized water and placed in cage for emergence. Non-fed less than 24 h old adults (both males and females) were collected and were used for the dissection of whole guts. The rest of the adults were supplied with 10% sucrose solution. Part of the females were offered with defibrinated pig blood (Rupert Seethaler, Vienna, Austria) in sausages (EDICAS, Girona, Spain) from 7th day to 9th day after emergence. Whole guts of blood-fed females were dissected at the 14th day after emergence to allow full digestion of blood. In parallel, whole guts of males and non-blood fed females were also collected. Ventral diverticulum and Malpighian tubules were removed from all gut samples.

Samples from seven groups, including eggs (EGG), dissected guts of larvae (LAR), up to 1-day old non-fed males (1DM), up to 1-day old non-fed females (1DF), 14 days old sucrose-fed males (14DM), 14 days old non-blood fed (sucrose-fed) females (NBF), and 14 days old blood-fed females (BFF) were included in both culture-dependent and culture-independent approaches. Fresh eggs were collected with sterilized dissecting needles. Alive adults which had been anesthetized at 4°C and alive larvae were surface disinfected by dipping in 70% ethanol for 1 min, placed into 1 × PBS (phosphate buffer saline) for rinsing, and then dissected in PBS under a binocular microscope with sterilized needles to get whole guts under aseptic conditions. One hundred fresh eggs or 5 whole guts were pooled to create one sample (replicate).

Culture-Dependent Approaches

For the isolation of cultivable gut bacteria, samples were collected in 1.5 mL Eppendorf tubes with 200 μL sterile LB medium (Invitrogen). Samples were mechanically crushed using sterile pestles and 800 μL LB were added to make a total volume of 1,000 μL. The homogenate was serially diluted (from 10^0 to 10^{-4}) and plated on three types of agar media, one non-selective (LB Agar, Life technologies) and two types of selective media, Chromocult Agar ES and XLD Agar (Merck Millipore). Three replicates with 100 μL solution were used for each medium. Plates were incubated in incubator (IPP110, Memmert, United States) at 26°C for 16 h (or until bacterial colonies were visible but incubation did not exceed 48 h). From the dilution series, those plates with 10 to 300 bacterial colonies were used and the number of colony-forming units (CFU) in the original solution was calculated.

Appropriate controls were also prepared, including (a) EGG-liquid: one hundred eggs were washed in 200 μL LB medium by vortex and gently centrifuged to get 100 μL supernatant as potential source of bacteria; (b) Larval food: this control was prepared with larvae diet (30 mL/L) which was maintained in a rearing room for 48 h and (c) Sugar solution: this was collected from both females' and males' cages at the 14th day post emergence.

Twenty bacterial isolates for each sample treatment and 12 for each control sample (eggs, larval food or sugar solution) were selected based on bacterial colony characteristics such as color, size, shape, opacity, margin, elevation and viscosity. Three rounds of streaking and isolation were performed on the corresponding medium to ensure that the bacterial isolates were pure cultures. Purified isolates were cultivated in LB medium at 26°C for 16–20 h, and then stored in 25% glycerol at −80°C.

Colony Characterization Using 16S *rRNA* Gene-Based RFLP Assay

PCR was performed on 1 μL fresh culture liquid using 2 × Taq mix (Qiagen) and the universal 16S *rRNA* gene primers 27F/1492R (Edwards et al., 1989; Weisburg et al., 1991) with MJ Research Tetrad PTC-225 Thermal Cycler (GMI, United States). The PCR cycling conditions were template denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 45 s, primer annealing at 55°C for 1 min, and primer extension at 72°C for 2 min; a final extension step of 10 min at 72°C was also included. In case that amplification failed, PCR was carried

out again with DNA extracted using Dneasy Blood and Tissue Kit (Qiagen) after lysozyme (SIGMA) digestion (resuspended in the lysis buffer with 10 mg/mL lysozyme and digested at 37°C for 2 h). Part of each PCR reaction (5/50 μL) was electrophoresed on 1.5% agarose gels, and all amplicons of the expected size were individually digested with restriction endonucleases *Taq*I, *Eco*RI, *Hae*III and *Rsa*I (Thermo) according to manufacturer's suggestions. Specifically, 5–15 μL per amplicon were digested, using 2 μL 10 × buffer and 3–5 μL enzyme, in a final volume of 20 μL. Reactions were kept for 3–4 h at 37°C, then inactivated at 80°C and electrophoresed on 2% agarose gels. In case the selected bacterial isolates with different colony morphology characteristics were found to present quite similar RFLP patterns for all 4 enzymes, digestion with an additional restriction enzyme (*Asu*II, *Mfe*I or *Hga*I, Thermo) was included for its genotyping or their 16S *rRNA* gene was sequenced double stranded (see below).

16S *rRNA* Gene Sequencing and Data Analyzing

From each bacterial group and taking into consideration the RFLP pattern and colony morphology, 2–7 bacterial isolates were selected for sequencing of almost the entire 16S *rRNA* gene. PCR products were purified using the High Pure PCR product purification kit (Roche, Germany) and sequenced double stranded (VBC, Austria by using the 27F-1492R initial primer set and 4 internal primers: 519R, 596F, 960R and 1114F (Reed et al., 2002).

All 16S *rRNA* gene sequences were assembled in Sequencher 4.14, aligned with Clustal X 1.83 and BioEdit 7.01. Isolates with identical sequences were recognized with a different number. Sequences were examined for chimeras using the DECIPHER's web tool[1] and USEARCH 6.0[2]. Taxonomy assignment was performed using BLASTN[3] and the closest relative was assigned using RDP classifier[4]. Alignment of sequences was carried out using MUSCLE (Edgar, 2004). A phylogenetic tree, based on the distance matrix method, was constructed using the software package Geneious 8. Evolutionary distances were calculated using the Jukes-Cantor model, and topology was inferred using the "neighbor-joining" method. A phylogenetic tree calculated by maximum parsimony, using the PAUP phylogenetic package, was also generated. Sequences with 1180 bp length were used for tree constructions.

Culture-Independent Approaches-Next Generation Sequencing (NGS) and Statistical Analysis

Eggs and guts were mechanically crushed using sterile pestles in liquid nitrogen. DNA was extracted following the protocol of Dneasy Blood and Tissue Kit. If required, DNA was concentrated into >15 ng/μL, and three replicates per sample were prepared and sent for 16S *rRNA* gene sequencing using the MiSeq Illumina platform to the IMGM Laboratories GmbH (Martinsried, Germany) targeting two different regions with primers U341F (5′-CCTACGGGRSGCAGCAG-3′) and 805R (5′-GTGCCAGCMGCCGCGGTAA-3′) and 909F (5′-ACTCAAAKGAATWGACGG-3′) and 1391R (5′-GACGGGCGGTGWGTRCA-3′). The 16S *rRNA* gene sequences reported in this study have been deposited in NCBI under Bioproject number PRJNA575054, while the Sanger generated sequences have been deposited under accession numbers MN540103 to MN540125.

De-multiplexing and conversion to FASTQ format was performed using Qiime 1.9.1 (Caporaso et al., 2010b). Pair-end reads were assembled, trimmed and corrected for error using PandaSeq (Masella et al., 2012). Unassembled reads and reads outside the range of 440 to 450 bp once assembled were discarded. All subsequent analyses were conducted in QIIME version 1.9.1 (Caporaso et al., 2010b). Sequences were clustered into Operational Taxonomic Units (OTUs) using USEARCH (Edgar, 2010) by open-reference OTU picking. Chimeras were detected and omitted using the program UCHIME (Edgar et al., 2011) with the QIIME-compatible version of the SILVA 111 release database (Quast et al., 2013). The most abundant sequence was chosen as the representative for each OTU. Taxonomy was assigned to representative sequences by BLAST (Altschul et al., 1990) against the SILVA 111 release database (Quast et al., 2013). Representative sequences were aligned against the SILVA core reference alignment using PyNAST (Caporaso et al., 2010a). Alpha-diversity indices, as well as indices depicting the population structure, were calculated with the QIIME pipeline (Caporaso et al., 2010b) based on the rarefied OTU table at a depth of 15,000 sequences/sample (observed species, PD whole tree, chao1 and simpson reciprocal). Inter-sample diversity was calculated using Bray-Curtis distances while Principal Coordinate Analysis (PCoA) and multidimensional scaling (MDS) plot (Anderson, 2001) was performed on the resulting distance matrix. These calculations and those for alpha diversity were performed in QIIME version 1.9.1. ANOVA and Tukey–Kramer *post hoc* tests were employed to detect statistical differences (Edgar, 2010). Permutational Multivariate Analysis of Variance (PERMANOVA) analyses were applied to Bray–Curtis similarity matrices to compute similarities between groups (Anderson, 2001). Community structure differences were viewed using the constrained ordination technique CAP (Canonical Analysis of Principal Coordinates), using the CAP classification success rate and CAP trace$_{Q_m'HQ_m}$ statistics, and were performed with 9999 permutations within PRIMER version 6+ (Anderson and Willis, 2003).

Semi-Quantitative Analysis of Main Gut Bacteria Groups

qPCR-based semi-quantitative analysis was performed for some of the most relatively abundant genera (*Aeromonas*, *Asaia*, *Elizabethkingia* and *Chryseobacterium, Enterococcus,* and *Wolbachia*) detected by the 16S *rRNA* gene next generation sequencing (NGS). The qPCR analysis was carried out in three replicates on the same samples used for the NGS. It was difficult

[1]http://www2.decipher.codes/index.html

[2]http://fungene.cme.msu.edu/FunGenePipeline/chimera_check/form.spr

[3]http://www.ncbi.nlm.nih.gov/BLAST/

[4]http://rdp.cme.msu.edu/classifier/classifier.jsp

to design genus specific primers for *Elizabethkingia*, so this genus was studied as a group together with *Chryseobacterium*. The sequence of the primers and other relevant information for the qPCRs are presented in **Supplementary Table 1**. The *Ae. albopictus* ribosomal protein S6 (*rps*6) gene was used as control (for calibration).

A gradient PCR was initially performed to standardize the PCR conditions. PCR amplification was performed with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57–64°C for 30 s, and extension at 72°C for 45 s, and kept under 72°C for 10 min. PCR products were analyzed on agarose gel electrophoresis to confirm the presence of the specific amplicon. These conditions were then used to perform qPCR analysis (**Supplementary Table 1**). The amplification was performed using iQ™ SYBR® Green Supermix (Bio-Rad, United States). The reaction mixture (15 μL) consisted of 5 ng DNA template, 2–5 pmol of each primer and 7.5 μL of 2 × Supermix. qPCR was performed with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, United States). Due to the different annealing temperatures (**Supplementary Table 1**), the reactions for the target genes and the housekeeping gene were put on different plates. An initial denaturation at 95°C for 2 min was followed by 40 cycles consisting of denaturation at 95°C for 10 s, at annealing temperature for 60 s. To check and confirm the quality of amplification, a melting profile was generated for the amplicon over a temperature range of 65°C to 95°C. Melting curves for each sample were analyzed after each run to check the specificity of amplification. Cq of products was calculated using CFX Manager™ Software (Bio-Rad Laboratories, Inc.). Relative density of selected bacterial taxa was determined by using the $2^{-\Delta\Delta CT}$ calculation method. Three biological replicates were employed for each sample (with the exception of 14DM for which only 2 replicates were used for the *Asaia* and *Chryseobacterium-Elizabethkingia* group reactions), and 2 technical replicates of qPCR were carried out for each reaction.

Statistical Analysis of Data

Statistical analysis were carried out with JMP 14.3.0 (2018, SAS, Cary, NC, United States). The average of relative density of 2 technical replicates was used for each biological replicate. The mean and standard error (SE) of relative density was calculated, and comparisons of relative density of selected bacterial taxa between sample groups were assessed using ANOVA followed by Tukey–Kramer HSD.

RESULTS

Isolation and Characterization of Cultivable Bacterial Species During the Development of *Aedes albopictus*

Using three different microbiological media (LB Agar, Chromocult Agar ES and XLD Agar), 462 bacterial isolates were isolated in total including 73 isolates from the three control samples. The selection of the colonies was mainly based on colony morphology since our goal was to isolate as many different bacterial species as possible for further investigation. A 16S *rRNA* gene-based PCR-RFLP assay was employed for the initial characterization and grouping of the bacterial isolates. Restriction endonucleases *Eco*RI, *Asu*II, *Mfe*I and *Hga*I exhibited up to maximum one recognition site, whereas *Taq*I, *Hae*III and *Rsa*I displayed multiple ones (**Supplementary Table 2**). According to the PCR-RFLP data, the bacterial isolates were placed in 27 groups. Up to two representatives from each group were selected for 16S *rRNA* gene sequencing and the sequencing data revealed the presence of 23 unique bacterial isolates, belonging to *Proteobacteria* (mainly members of Gammaproteobacteria), *Firmicutes* and *Bacteroidetes* (mainly members of Flavobacteriaceae) (**Table 1** and **Figure 1**). Members of the *Acinetobacter*, *Bacillus*, *Cedecea*, *Chryseobacterium*, *Comamonas*, *Elizabethkingia*, *Enterobacter*, *Escherichia*, *Pseudomonas*, *Serratia*, *Staphylococcus,* and *Stenotrophomonas* genera, were retrieved from eggs and the digestive track of adult mosquitoes. Members of the *Raoultella* genus, as well as members of the *Acinetobacter* and *Pseudomonas* (but different from the ones detected in the eggs and the digestive track of adult mosquitoes), were isolated only in larval food, but not in adult mosquitoes (**Table 1**).

Although the initial selection of colonies from all three microbiological media was based on morphological criteria and only up to 20 colonies per sample were selected, it's still worth noting the following: (a) representatives of nine genera were isolated from LAR samples while only one bacterial strain, *Elizabethkingia* sp., was isolated from 14DM; (b) some genera were represented by multiple representatives (in some cases, they may represent different species); (c) some bacterial isolates were recovered from just a single developmental stage while others from multiple ones and (d) representatives of several bacterial genera were isolated from both mosquito and control samples including *Acinetobacter* sp., *Bacillus* sp., *Cedecea* sp., *Elizabethkingia* sp., *Enterobacter* sp., and *Pseudomonas* sp. isolates (**Table 1**).

16S *rRNA* Gene-Based Taxonomic Composition of Artificially Reared *Aedes albopictus*

The sequence data obtained for the two targeted regions of the 16S *rRNA* gene (amplified by the primer pairs U341F/805R and 909F/1391R) were compared in selected samples and no statistically significant differences were observed (**Supplementary Figures 1–5**). For this reason, all results presented below and in the respective figures and tables have been prepared by combining the raw data of the two 16S *rRNA* gene regions.

In total, seven samples (EGG, LAR, 1DM, 1DF, 14DM, NBF, and BFF with three replicates each) were sequenced producing 546,136 reads for bioinformatic analysis with an average of 78,014 reads per sample (**Table 2**). Based on alpha-diversity indices, the larval samples were the most species rich in *Ae. albopictus* (**Table 2**). Males and females fed with sucrose and/or blood together with the teneral females

TABLE 1 | Bacterial isolates obtained from *Ae. albopictus*; and cultivable close phylogenetic relatives (identity > 97%) from Aedes, other mosquito or other insects.

Sample code	Accession Number	Stage/Medium (Times isolated)	Phylogenetic linkage	Phylogenetic relative and Isolation source	References	Accession No.	Identity(%)
1_150	MN540103	Sugar solution from females' cage-CC, LB, XLD (24)	*Enterobacter* sp.	*Enterobacter* sp., *Ae. albopictus* male adult, wild-caught	Zouache et al., 2011	GU726184.1	98.84
		BFF-CC (1)					
		LAR-CC, LB, XLD (24)					
		Larval food-CC, LB, XLD (19)					
2_71	MN540104	LAR-LB (1)	*Escherichia* sp.	*Klebsiella pneumoniae*, *Ae. aegypti* (Rockefeller) female adult, lab-rearing	Terenius et al., 2012	JN201948.1	97.54
				Escherichia hermannii (non-mosquito source)		JN644551.1	100
3_272	MN540105	BBF-CC, XLD (3)	*Enterobacter* sp.	*Enterobacter* sp., *Ae. albopictus* male adult, wild-caught	Zouache et al., 2011	GU726182.1	97.54
		1DF-CC, LB (26)					
		1DM-CC, LB (5)					
4_48	MN540106	EGG-CC (5)	*Cedecea* sp.	*Enterobacter* sp., *Ae. albopictus* male adult, wild-caught	Zouache et al., 2011	GU726183.1	97.75
				Cedecea neteri (non-mosquito source)		MH362698.1	100
		EGG-liquid-CC (3)					
		LAR-LB (1)					
5_149	MN540107	Larval food-XLD (2)	*Raoultella* sp.	*Serratia marcescens*, *Ae. aegypti* (Rockefeller) female adult, lab-rearing	Terenius et al., 2012	JN201947.1	97.75
				*Raoultella ornithinolytica_*		HQ259705.1	100
6_380	MN540108	NBF-CC, LB, XLD (47)	*Serratia* sp.	*Serratia marcescens*, *Ae. aegypti* (Rockefeller) female adult, lab-rearing	Terenius et al., 2012	JN201947.1	99. 52
		EGG-CC, LB, XLD (41)					
7_336	MN540109	BFF-CC, LB, XLD (35)	*Serratia* sp.	*Serratia* sp., *Delia radicum* larvae, lab-rearing	Welte et al., 2016	KP836246.1	97.95
		NBF-CC (1)					
		1DF-CC (2)					
8_200	MN540110	1DF-CC, LB, XLD (5)	*Aeromonas* sp.	*Aeromonas hydrophila*, *An. maculipennis*, stage unknown, wild-caught		GU204971.1	98.64
		1DM-LB, XLD (32)					
		LAR-XLD (2)					
9_139	MN540111	Larval food-CC, LB (9)	*Pseudomonas* sp.	*Pseudomonas* sp., *Ae. albopictus*, female adult, lab-rearing	Zouache et al., 2009	FJ688378.1	97.40
10_230	MN540112	1DM-CC (6)	*Acinetobacter* sp.	*Acinetobacter beijerinckii*, *Cx. Quinquefasciatus*, female adult, wild-caught	Chandel et al., 2013	JN644620.1	97.34
11_158	MN540113	Larval food-CC (1)	*Acinetobacter* sp.	*Acinetobacter* sp., *Glossina pallidipes*, stage unknown, wild-caught		MG162615.1	98.15

(Continued)

TABLE 1 | Continued

Sample code	Accession Number	Stage/Medium (Times isolated)	Phylogenetic linkage	Phylogenetic relative and Isolation source	References	Accession No.	Identity(%)
12_85	MN540114	1DM-CC (3)	*Acinetobacter* sp.	*Acinetobacter* sp. *Ae. albopictus*, female adult, lab-rearing	Zouache et al., 2009	FJ688379.1	99.25
		LAR-CC, LB (11)					
13_119	MN540115	LAR-CC (1)	*Stenotrophomonas* sp.	*Stenotrophomonas maltophilia*, *An. gambiae*, female adult, lab-rearing	Lindh et al., 2008	EF426435.1	99.45
14_229	MN540116	1DF-LB (1)	*Comamonas* sp.	*Comamonas* sp., *Ae. albopictus*, female adult, lab-rearing	Zouache et al., 2009	FJ688377.1	100
		1DM-CC, LB (7)					
15_124	MN540117	LAR-CC, LB (4)	*Comamonas* sp.	*Comamonas odontotermitis*, *Odontotermes formosanus*, wild-caught	Chou et al., 2007	NR_043859.1	99.93
16_39	MN540118	EGG-LB (2)	*Staphylococcus* sp.	*Staphylococcus saprophyticus*, *Cx. Quinquefasciatus*, female adult, wild-caught	Chandel et al., 2013	JN644617.1	99.46
17_468	MN540119	Sugar solution from females' cage-LB (6)	*Bacillus* sp.	*Bacillus cereus*, *Ae. albopictus* female adult, wild-caught	Zouache et al., 2011	GU726173.1	99.66
		Sugar solution from females' cage-LB (2)					
		LAR-LB (1)					
		Larval food-LB (2)					
18_74	MN540120	LAR-CC, LB (14)	*Chryseobacterium* sp.	*Chryseobacterium* sp., *Cx. Quinquefasciatus*, female adult, wild-caught	Chandel et al., 2013	HQ154575.1	97.98
19_244	MN540121	BFF-LB (14)	*Elizabethkingia* sp.	*Elizabethkingia meningoseptica*, *Ae. aegypti* (Rockefeller) female adult, lab-rearing	Terenius et al., 2012	JN201943.1	97.92
		NBF-LB (12)					
		14DM-CC, LB (40)					
		EGG-CC, LB (12)					
		EGG-liquid-CC (4)					
20_287	MN540122	1DF-CC, LB (21)	*Elizabethkingia* sp.	*Elizabethkingia meningoseptica*, *Ae. aegypti* (Rockefeller) female adult, lab-rearing	Terenius et al., 2012	JN201943.1	98.34%
21_129	MN540123	Larval food-LB (3)	*Pseudomonas* sp.	*Pseudomonas* sp., *Ae. albopictus*, female adult, lab-rearing	Zouache et al., 2009	FJ688378.1	97.26
22_97	MN540124	Larval food-XLD (1)	*Aeromonas* sp.	*Aeromonas hydrophila*, *An. maculipennis*, stage unknown, wild-caught		GU204971.1	98.57
24_363	MN540125	BFF-CC, LB (7)	*Pseudomonas* sp.	*Pseudomonas ficuserectae*, *Cx. Quinquefasciatus*, female adult, wild-caught	Chandel et al., 2013	JN644593.1	97.61

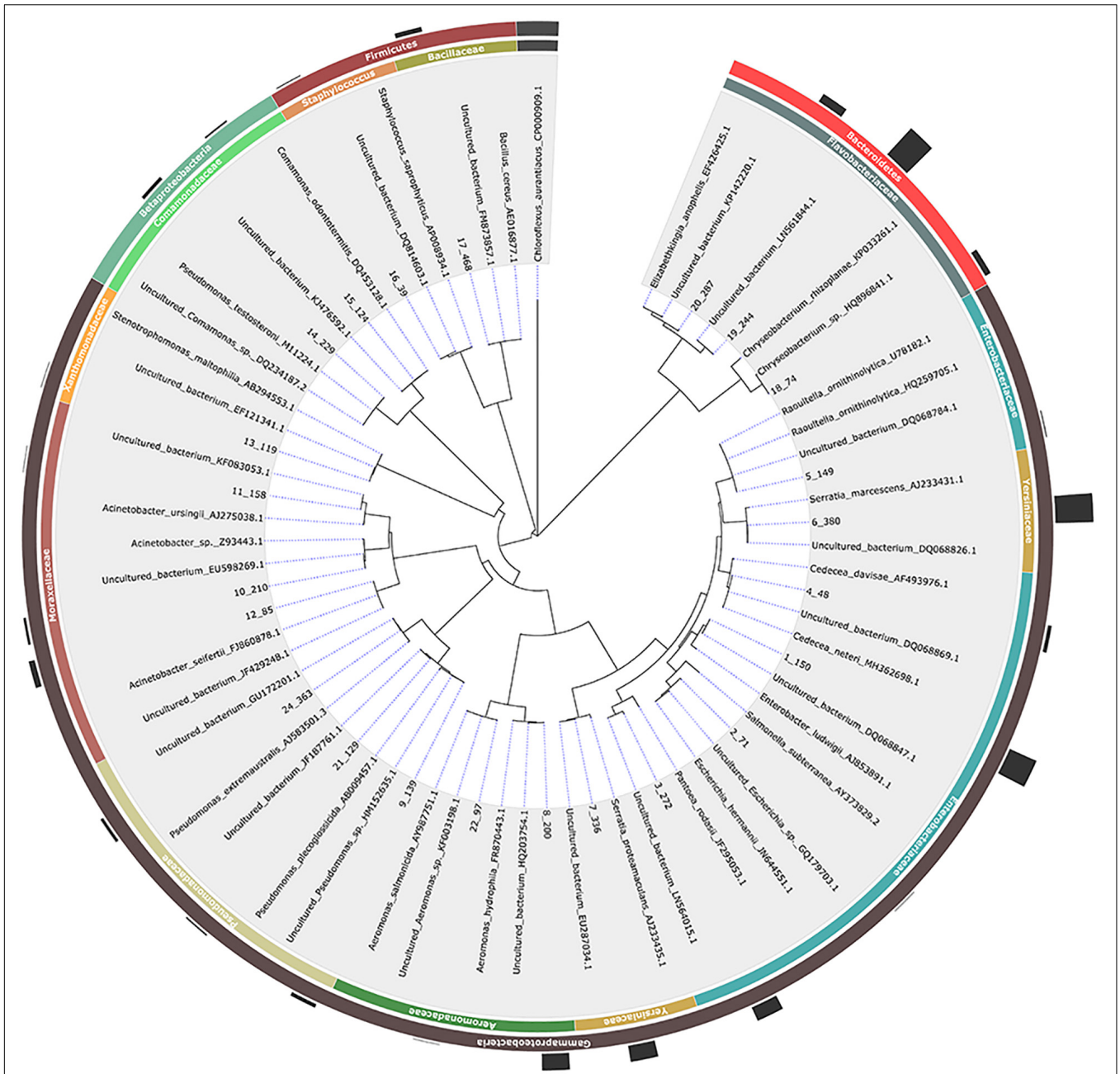

FIGURE 1 | Phylogenetic relationships based on 16S *rRNA* gene sequence analysis of members of the bacterial communities associated with *Aedes albopictus*. In the cases where no published mosquito-derived sequences belonging to the same bacterial species were available, the closest hit deriving from mosquitos and the closest hit regardless of origin were included (to avoid misinterpretation of results). Evolutionary distances were calculated using the method of Jukes and Cantor and the topology was inferred using the neighbor-joining method. A representative isolate per each phylotype with their taxonomy assigned is indicated in the outer circle while the number of isolates is graphically represented. The 16S *rRNA* gene sequence of *Chloroflexus aurantiacus* was arbitrarily chosen as outgroup.

exhibited the lowest species richness and diversity indices (**Table 2**). Interestingly, teneral mosquitoes exhibited a sex specific differentiation (**Table 2**). Richness indices like Chao1 were within the range of the number of OTUs indicating, like the rarefaction analysis (**Supplementary Figure 5**), that sampling of each treatment has reached saturation, which was supported by the Good's coverage index (**Table 2**). The dominant OTUs that were identified in the present study were classified into 5 phyla, 8 classes, 17 orders, 23 families, and 29 genera (**Table 3**).

Our amplicon sequence analysis indicated that the EGG samples were dominated by Alphaproteobacteria (89.5%), mainly by *Wolbachia*, followed by Firmicutes and Bacteroidetes at 5.8% and 1% respectively (**Figures 2A,B**).

TABLE 2 | Richness and diversity estimation of the 16S *rRNA* gene libraries of *Aedes albopictus* through the amplicon sequence analysis.

Samples			Number of OTUs	Good's coverage	Number of reads	Species richness indices		Species diversity indices	
Stage /Sex	Age	Diet				Chao1	ACE	Shannon	Simpson
Eggs	Eggs	BFA	114.13 ± 7.30^{a}	0.998	111,107	138.37 ± 9.61^{a}	137.7 ± 9.38^{a}	2.57 ± 0.03^{a}	0.72 ± 0.007^{a}
Larva	Larva	LLD	318.13 ± 9.86^{b}	0.995	62,223	362.61 ± 9.45^{b}	363.9 ± 9.41^{b}	5.21 ± 0.15^{b}	0.91 ± 0.009^{b}
Males	1 days	Teneral	142.47 ± 3.43^{c}	0.998	54,555	157.50 ± 3.68^{c}	157.45 ± 4.09^{c}	2.64 ± 0.05^{c}	0.64 ± 0.021^{c}
Females	1 days	Teneral	61.20 ± 2.69^{d}	0.999	82,200	72.63 ± 3.65^{d}	69.97 ± 2.93^{d}	0.84 ± 0.06^{d}	0.17 ± 0.014^{d}
Males	14 days	Sucrose	67.17 ± 7.61^{d}	0.999	101,620	76.74 ± 8.10^{d}	76.61 ± 7.47^{d}	2.22 ± 0.16^{e}	0.57 ± 0.044^{e}
Females	14 days	Sucrose	61.57 ± 3.01^{d}	0.998	57,246	86.67 ± 4.72^{e}	81.43 ± 3.15^{e}	1.59 ± 0.13^{f}	0.50 ± 0.043^{e}
Females	14 days	Blood	61.70 ± 2.29^{d}	0.998	77,145	81.27 ± 3.54^{e}	80.48 ± 3.32^{e}	1.02 ± 0.02^{g}	0.30 ± 0.002^{f}

For each diversity index, ANOVAs followed by the Tukey HSD test, ($p < 0.05$) were performed. Significant differences are indicated by different letters.

In LAR samples, bacterial diversity was high and was evenly distributed between Bacteroidetes (31%), Firmicutes (28.8%), and Actinobacteria (20.8%), followed by Betaproteobacteria (10.6%), Gammaproteobacteria (6.8%), and Alphaproteobacteria (3.3%) (**Figure 2A**). The most dominant genera were those of *Chryseobacterium*, and *Clostridium* and to a lesser degree *Microbacterium*, *Comamonas*, *Rhodococcus*, and *Acinetobacter* (**Figure 2B**). The 1DM samples were dominated by Gammaproteobacteria (72%) followed by Alphaproteobacteria (16.6%), Firmicutes (4.3%), Actinobacteria (3%), Betaproteobacteria (2.2%), and Bacteroidetes (1%), while the 1DF samples were displaying a more even distribution between Gammaproteobacteria (31.1%), Firmicutes (32.2%), and Bacteroidetes (33.2%) (**Figure 2A**). This clear differentiation was also reflected at the genus level. The 1DM samples were dominated by *Aeromonas* sp. followed by *Wolbachia* and *Serratia* sp., while the 1DF samples were dominated by *Elizabethkingia* sp., *Enterococcus* sp. and *Aeromonas* sp. (**Figure 2B**). The 14DM samples, the bacterial diversity was evenly distributed between Gammaproteobacteria (30.4%), Alphaproteobacteria (28.8%), and Bacteroidetes (32.5%), followed by Firmicutes (5.1%) and Actinobacteria (2.6%) while the NBF exhibited a similar diversity but with the Gammaproteobacteria being abundant (62.4%) followed by Alphaproteobactreria (22.6%), and Bacteroidetes (13.1%) (**Figure 2A**). Interestingly, the BFF were almost exclusively dominated by Bacteroidetes (96.4%) (**Figure 2A**). At the genus level, the 14DM samples were characterized by the presence of *Elizabethkingia* sp. followed by *Asaia* sp., *Serratia* sp., *Enterobacter* sp., *Providencia* sp., and *Wolbachia* sp., the NBF samples were dominated by *Serratia* sp. and *Asaia* sp. followed by *Elizabethkingia* sp., while the BFF samples were dominated by *Elizabethkingia* sp., (95.4%) and *Chryseobacterium* sp. (0.94%) (**Figure 2B**).

Non-metric multi-dimensional scaling (MDS) and principal coordinates analysis (PCoA) indicated that the samples examined were clustered mainly based on the developmental stage and the diet used (**Figures 3**, **4**). In more detail, the clusters between eggs and larvae were statistically significant (PERMANOVA, $P < 0.001$), as were those between the guts of 1-day and 14-day old adults (PERMANOVA, $P < 0.001$). Interestingly, there was a statistically significant difference between the gut samples of 14-day old *Ae. albopictus* females fed with sucrose (NBF) or blood (BFF) (PERMANOVA, $P < 0.001$).

Relative Density of Selected Bacterial Taxa During *Aedes albopictus* Development

Based on the NGS data, we selected some of the most abundant bacterial taxa, *Aeromonas, Asaia*, *Chryseobacterium-Elizabethkingia* groups, *Enterococcus* and *Wolbachia*, to investigate their relative density by qPCR (**Figure 5** and **Supplementary Table 3**). The data clearly indicated that: (a) *Wolbachia* was dominant in the EGG samples and it could also be detected at lower densities in 1DM, 14DM, NBF and BFF samples (Tukey HSD, $P < 0.0001$); (b) *Asaia* was detected at higher densities in the NBF samples and at much lower densities in 14DM samples (Tukey HSD, $P < 0.0001$); (c) *Aeromonas* was detected in the 1DM samples and in one of the biological replicates of the 1DF samples (Tukey HSD, $P = 0.0950$); (d) the *Elizabethkingia–Chryseobacterium* group was present in high densities in BFF samples, and it was also detected in some biological replicates of the LAR, 1DF, 14DM as well as in the NBF samples; however, there was no statistically significant difference among the different groups (Tukey HSD, $P = 0.0496$). It is worth noting that, based on the NGS data, *Elizabethkingia-Chryseobacterium* was only detected in BFF, NBF, 14DM, 1DF but not in LAR, and (e) *Enterococcus* was detected at higher densities in one replicate of the 1DF samples and at lower densities in one replicate of the NBF samples (Tukey HSD, $P = 0.0950$).

DISCUSSION

By employing a cultivation-dependent and a cultivation-independent approach, significant information on the composition of gut-associated microbiota of lab-reared *Ae. albopictus* along developmental stages, sex and feeding regimes was obtained. Although our study provides useful information for a colonized population of an SIT targeted species, the results can not be generalized. However, this information can be used to improve the effectiveness of mosquito population control strategies such as SIT, IIT and others through, for

TABLE 3 | Taxonomic composition of the 16S *rRNA* gene sequencing data in the three analyzed populations.

Phylum	Class	Order	Family	Genus	EGG	LAR	1DM	1DF	14DM	NBF	BFF
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*							
			Pseudomonadaceae	*Pseudomonas*							
		Aeromonadales	Aeromonadaceae	*Aeromonas*							
		Legionellales	Legionellaceae	*Legionella*							
		Enterobacterales	Enterobacteriaceae	*Enterobacter*							
			Morganellaceae	*Providencia*							
			Yersiniaceae	*Serratia*							
		Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*							
	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Asaia*							
		Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*							
		Rickettsiales	Anaplasmataceae	*Wolbachia*							
	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Schlegelella*							
				Simplicispira							
				Acidovorax							
				Comamonas							
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*							
				Brevibacillus							
			Bacillaceae	*Bacillus*							
			Staphylococcaceae	*Staphylococcus*							
		Lactobacillales	Enterococcaceae	*Enterococcus*							
	Clostridia	Clostridiales;	Clostridiaceae	*Clostridium*							
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Chryseobacterium*							
				Cloacibacterium							
				Elizabethkingia							
Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Corynebacterium*							
			Nocardiaceae	*Rhodococcus*							
		Micrococcales	Microbacteriaceae	*Microbacterium*							
		Propionibacteriales;	Propionibacteriaceae	*Propionibacterium*							
Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	*Deinococcus*							

Cells with a RA more than 1% they appear gray.

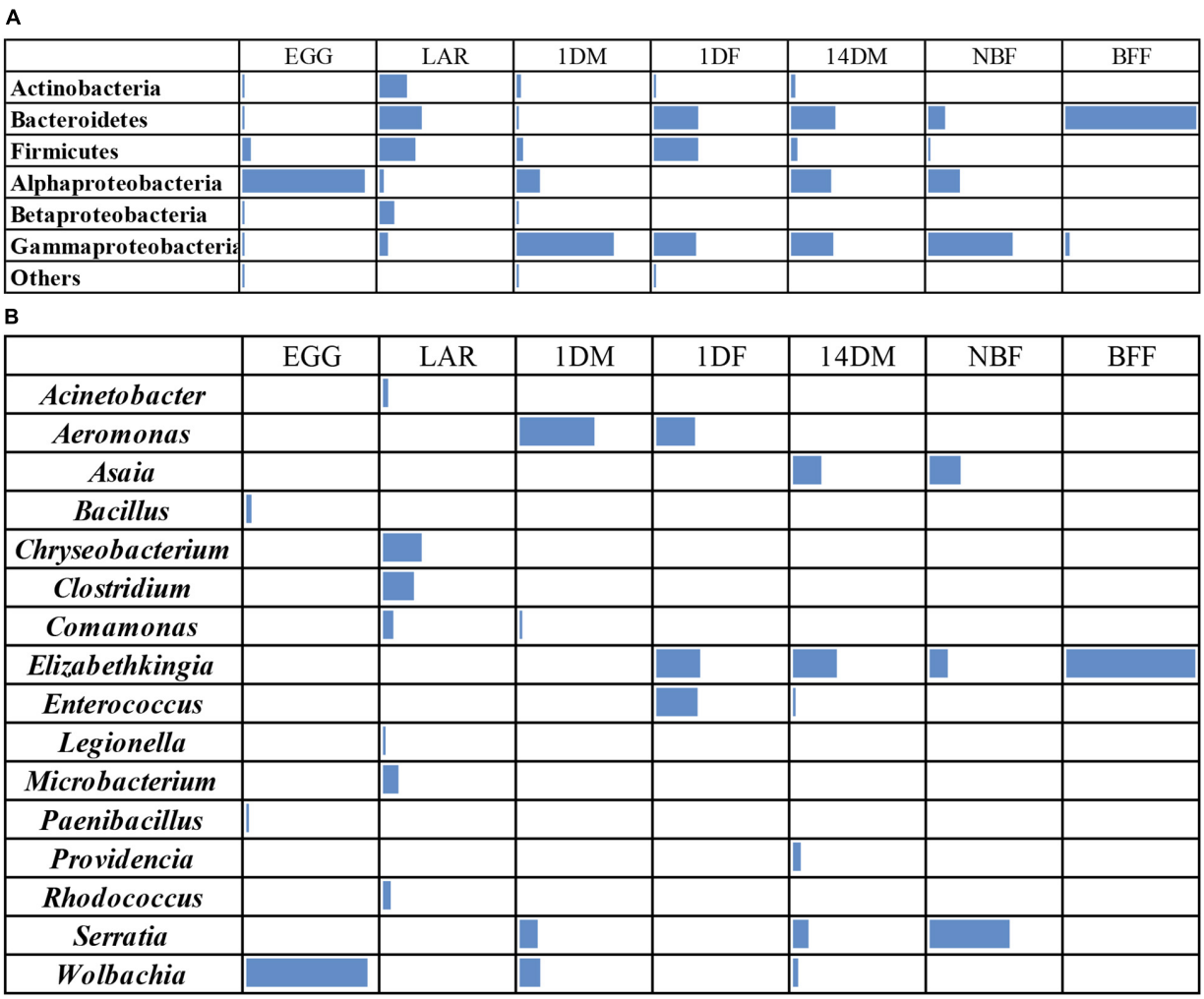

FIGURE 2 | Heatmaps of bacterial taxa identified by 16S *rRNA* gene sequence analysis in *Aedes albopictus* eggs (EGG), guts of larvae (LAR), and guts of 1-day old teneral males (1DM), 1-day old teneral females (1DF), 14-day old sugar-fed males (14DM), 14-day old sugar-fed females (NBF) and 14-day old blood-fed females (BFF). Taxa were grouped at **(A)** phylum level, except Proteobacteria that were grouped at the class level and **(B)** genus level. Cells with a RA less than 1% they appear empty.

example, the assessment of cultivable bacteria as potential probiotics to enhance the rearing efficiency and quality of mass-produced insects.

The Composition of Gut-Associated Microbiota of Lab-Reared *Aedes albopictus*

The cultivation-dependent approach resulted in the isolation of mosquito-associated bacteria which were assigned to 13 different genera (**Table 1**). The majority of them were Gram-negative, and mainly Gammaproteobacteria of the Enterobacteriaceae family, which have been previously described in other mosquito species such as *Ae. triseriatus*, *An. albimanus*, *An. gambiae*, *An. stephensi*, *C. pipiens*, *C. quinquefasciatus,* and *C. tarsalis* (Chao and Wistreich, 1959; Demaio et al., 1996; Pumpuni et al., 1996; Straif et al., 1998; Fouda et al., 2001; Gonzalez-Ceron et al., 2003; Pidiyar et al., 2004; Lindh et al., 2005), as well as in *Ae. albopictus* (Zouache et al., 2011; Otta et al., 2012; Minard et al., 2013b, 2014; Valiente Moro et al., 2013; Yadav et al., 2015), suggesting that they are widespread and constantly associated with mosquitoes.

Some gut-associated bacteria genera detected in the present study have been related with host's biological process. *Acinetobacter* play a role in both blood digestion and nectar assimilation of *Ae. albopictus* (Minard et al., 2013b). *Asaia* may provide *An. stephensi* with vitamins (Crotti et al., 2010). *Asaia* and *Elizabethkingia* have been considered as candidates in paratransgenic approaches for mosquito control (Favia et al., 2007; Chen et al., 2015). *Serratia* and *Enterobacter* contain hemolytic enzymes and play a role in blood digestion of *Ae. aegypti* (Gaio et al., 2011). *Serratia* also play a role in chitin degradation of puparium (Iverson et al., 1984). However, *S. marcescens* was reported as pathogen of artificial-reared insects

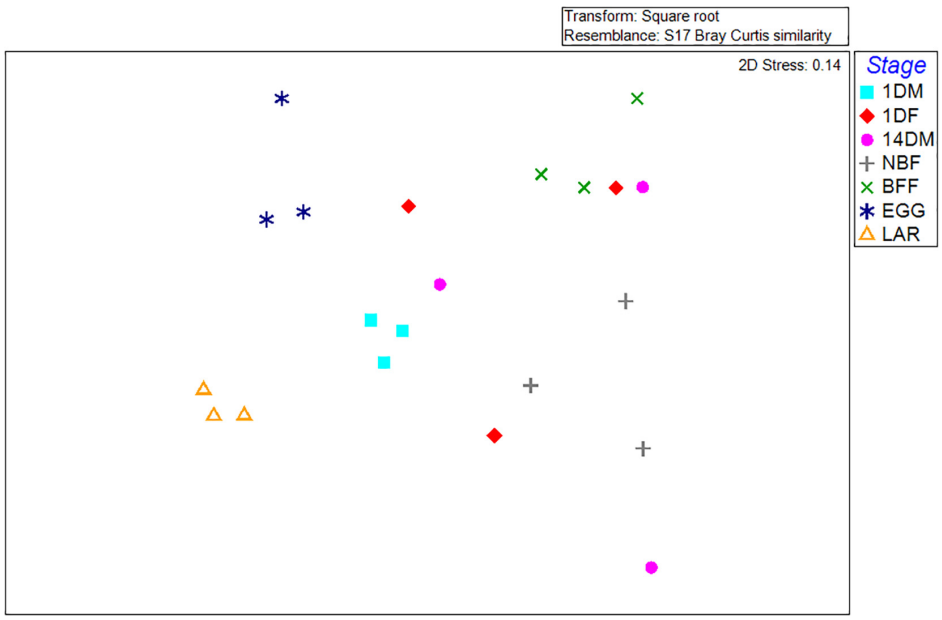

FIGURE 3 | Non-metric multidimensional scaling (MDS) plot based on Bray–Curtis dissimilarities visualizes differences in bacterial community structures according to age, developmental stage, sex and diet. Orange triangles inverted blue and cyan squares, red diamonds, pink circles, gray crosses, and green multiplication sign represent *Aedes albopictus* eggs (EGG), guts of larvae (LAR), and guts of 1-day old teneral males (1DM), 1-day old teneral females (1DF), 14-day old sugar-fed males (14DM), 14-day old sugar-fed females (NBF) and 14-day old blood-fed females (BFF), respectively.

(Grimont and Grimont, 2015). As strains of *Enterobacter* have been proved candidates of probiotic for the Mediterranean fruit fly (Augustinos et al., 2015), isolation of these bacteria from *Ae. albopictus* provide potential material for further probiotic studying. *Asaia* played a specific role in the larval development of *An. stephensi* and reduced the developmental time before pupation (Crotti et al., 2010). It's also worth noting that *Cedecea* spp. have been reported in the midgut of *Psorophora columbiae* (Demaio et al., 1996) and both field-collected and lab-reared *An. gambiae* (Pumpuni et al., 1996; Straif et al., 1998) while *Cedecea* spp. was also detected in the midgut of *Ae. aegypti* females (Gusmao et al., 2010). Interestingly, we isolated *Cedecea* spp. from larval guts as well from the egg surface of lab-reared *Ae. albopictus* suggesting a potential route for their inter-stadial transmission and these isolates may also represent potential probiotic candidates.

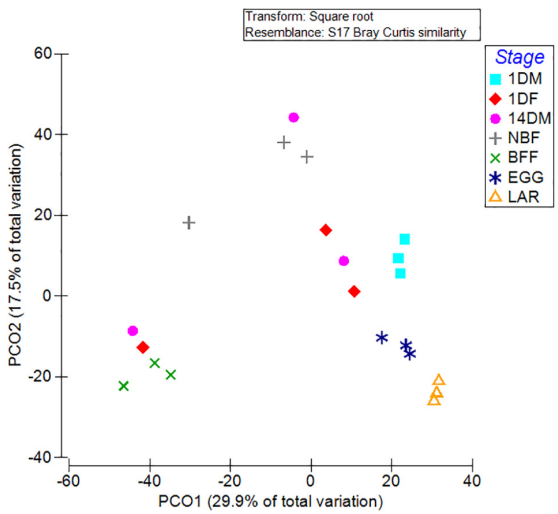

FIGURE 4 | Principal coordinate analysis (PCoA) of bacterial communities based on the relative abundance of OTUs with ordinations from *Aedes albopictus* eggs (EGG), guts of larvae (LAR), and guts of 1-day old teneral males (1DM), 1-day old teneral females (1DF), 14-day old sugar-fed males (14DM), 14-day old sugar-fed females (NBF) and 14-day old blood-fed females (BFF). Variance explained by each PCoA axis is given in parentheses, while the main taxa that affect ordination clustering is presented.

Previous studies in *Anopheles* showed lower diversity of gut-associated bacteria in lab-reared mosquitoes than wild populations (Gonzalez-Ceron et al., 2003; Rani et al., 2009). *Klebsiella*, which has been proven to be an effective probiotic of the Mediterranean fruit fly (Ben Ami et al., 2010; Gavriel et al., 2011; Kyritsis et al., 2017), has also been reported as a component of gut-associated bacterial communities in mosquitoes (Chao and Wistreich, 1960; Demaio et al., 1996; Straif et al., 1998; Terenius et al., 2012) including wild populations of *Ae. albopictus* (Crotti et al., 2009; Gusmao et al., 2010; Zouache et al., 2011; Valiente Moro et al., 2013; Yadav et al., 2015). However, this group was not detected in our study through both the cultivation-dependent and the cultivation-independent assays. Since there are documented differences among populations (Demaio et al., 1996; Zouache et al., 2011), this could be attributed to its absence from the original wild population. However, it could also be a result of a domestication

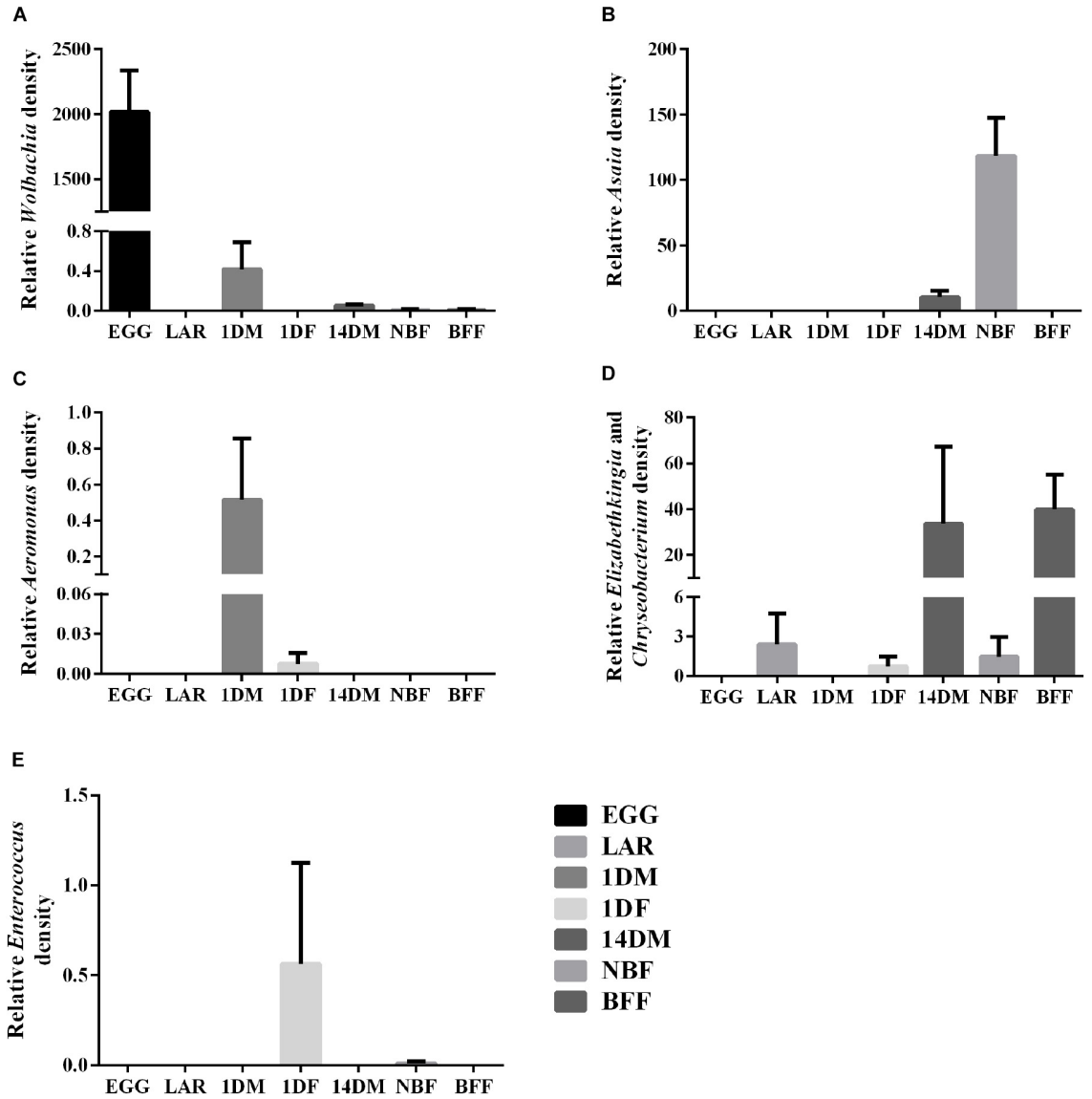

FIGURE 5 | Relative abundance of five bacterial groups based on qPCR. **(A)** *Wolbachia*; **(B)** *Asaia*; **(C)** *Aeromonas*; **(D)** *Elizabethkingia-Chryseobacterium* group; **(E)** *Enterococcus*.

process and the continuous artificial rearing. Similar to genetic changes, symbiotic changes may happen due to phenomena such as bottlenecks and small effective population size. Moreover, symbionts that are not crucial in the new environment may disappear and new may emerge, depending on the new rearing conditions.

Some of the bacterial isolates, such as *Enterobacter*, *Cedecea* (Enterobacteriaceae), *Stenotrophomonas* (Xanthomonadaceae), *Pseudomonas* (Pseudomonadaceae), and *Staphylococcus* (Staphylococcaceae) were not very abundant groups in NGS. It is well known that many factors including selectivity of culture-medium for specific bacterial taxa, growth rate and small colony number bias could influence the isolation process (Jannasch and Jones, 1959; Amann et al., 1995). NGS based culture-independent methods give relatively complete, albeit semi-quantitative, profile of the bacteria community. However, bias of Illumina could be induced too, for example, by PCR amplification protocols, primer choice and short read lengths (Claesson et al., 2010; Schloss et al., 2011; Pinto and Raskin, 2012; Tremblay et al., 2015; Gohl et al., 2016). It is interesting to note, however, that our NGS study provided similar Shannon diversity indices with those of previous studies which investigated either laboratory populations or populations of recent invasions and clearly lower when compared with that observed in established wild populations (Coon et al., 2016b; Hegde et al., 2018; Rosso et al., 2018). The bacterial profiles including the *Aeromonas* and *Serratia* co-occurrence pattern detected in the present study have also been previously observed (Hegde et al., 2018).

Impact of Sex, Age and Diet on Gut-Associated Bacteria

Our study clearly shows that there are differences between males and females, and age and diet may also contribute to these differences. Clustering analysis suggested that the effect of diet was more significant than that of sex. After the full digestion of blood meal, bacteria diversity decreased significantly in accordance with previous study in *An. gambiae* (Wang et al., 2011). Over 95% of the gut-associated bacteria were *Elizabethkingia* and *Chryseobacterium*, mostly the former one. This closely related group may possess a competitive advantage over the other bacteria. *Elizabethkingia* was dominant in the guts of both sugar-fed and blood-fed females (Wang et al., 2011). It has been reported that *Elizabethkingia anophelis* could contribute nutrients by participating in erythrocyte lysis in the mosquito midgut (Chen et al., 2015).

Among cultivable strains, *Serratia* could not be isolated after blood feeding, in contrast to *Enterobacter* and *Pseudomonas*. These genera have also been reported in field-caught female *Anopheles* and *Culex* mosquitoes (Gonzalez-Ceron et al., 2003; Pidiyar et al., 2004; Lindh et al., 2005; Rani et al., 2009; Chavshin et al., 2012). In wild-caught *An. stephensi*, *Cryseobacterium*, *Pseudomonas* and *Serratia* were identified only in females (Rani et al., 2009). Our results indicated that *Pseudomonas* and *Enterobacter* may be enhanced with blood meal in *Ae. albopictus*, and a similar observation has been made in *An. gambiae* (Wang et al., 2011). On the other hand, *Serratia* were relatively stable during the adult stage of *Ae. albopictus* females while in *An. gambiae*, it increased after blood meal and decreased later (Wang et al., 2011). Interestingly, *Enterobacter* sp. and *Serratia* sp. showed strong hemolytic activity among bacteria isolated from *Ae. aegypti* midgut while *Serratia* was found to be dominant in all isolations during blood digestion in the same species (Gaio et al., 2011) (Gusmao et al., 2010). It's also worth noting that Azambuja and colleagues (Azambuja et al., 2004) isolated a *S. marcescens* strain which was able to lyse erythrocytes from guts of blood feeding *Rhodnius prolixus*. The nutrient composition of food sources was considered an explanation of the differential bacterial population structure between sexes (Minard et al., 2013b). Our results suggested that there was a strong effect of adult diet, like blood meal, on the structure of gut-associated bacterial communities.

Our study indicated that age also plays an important role in shaping the bacterial communities. For example, *Wolbachia* and *Aeromonas* were found at high densities in 1-day old males but their densities were drastically decreased in older males. On the other hand, the *Elizabethkingia-Chryseobacterium* group was essentially absent in young males and drastically increased in 14-days old males.

Dynamics and *Trans*-Stadial Transmission of Gut-Associated Bacteria Under Lab-Rearing Conditions

The present study identified several genera shared among control groups and mosquito samples. The isolates from larval food and sugar solution belonged to Acinetobacter, Bacillus, Enterobacter, Pseudomonas and Raoultella bacterial genera. In previous studies, *Enterobacter* sp. was shown to immigrate successfully in both larval and adult stages, *Bacillus* sp. to settle through food into larval guts whereas *Raoultella* sp. and *Pseudomonas* sp. to fail to settle in larval guts or to be maintained in a detectable level. In addition, the *Acinetobacter* sp. was found not only in mosquito guts but also in breeding sites and food sources (reviewed in Minard et al., 2013b).

Our study also provided a view of the bacterial dynamic among life stages of *Ae. albopictus* clearly indicating that gut-associated bacteria diversity changed significantly between eggs and larvae, teneral and 14-day old adults. Food (larval food, sugar solution, blood) is certainly a contributing factor for these changes and the transmission of some of these bacteria. *Wolbachia, Asaia,* and *Elizabethkingia* have been shown to pass on to eggs from parents (Kittayapong et al., 2002; Favia et al., 2007; Akhouayri et al., 2013). Gusmao and colleagues (Gusmao et al., 2010) identified *Asaia* sp. and *Enterobacter* sp. from *Ae. aegypti* eggs and considered them to be probably transovarially transmitted. Based on our NGS data, it was shown that *Ae. albopictus* eggs contained bacteria from more than 15 genera including *Bacillus*, *Chryseobacterium* and *Escherichia-Shigella* which were among the main groups in female adults both before and after blood feeding. *Cedecea* sp. and *Elizabethkingia* sp. were isolated from *Ae. albopictus* eggs and detected outside the eggs, suggesting that they are transmitted vertically via "egg smearing." Akhouayri et al. (2013) externally treated embryos of *An. gambiae* with antiseptic solution and dramatically reduced the melanotic pathology which was caused by *Elizabethkingia meningoseptica* suggesting that it could be transmitted to embryos via "egg smearing." In *An. gambiae*, one of the transmission routes of *Asaia* was also reported to be "egg smearing" (Damiani et al., 2010). Further studies are necessary to get knowledge on how these taxa are transmitted.

In conclusion, using culture-dependent and culture-independent approaches, we characterized mainly the microbiota associated with a laboratory strain of *Ae. albopictus*, which is reared under artificial rearing conditions for potential SIT-based applications, throughout the mosquito developmental stages (egg, larva and adult), at different adult ages (both young and old males and females) and female feeding regimes (sugar or blood). It is worth noting that a relatively high diversity of bacteria was detected in eggs (a stage understudied in mosquitoes), some of which were also found in adult females. Overall, our study clearly shows that developmental stage and diet are the key factors shaping the microbiota. The density levels of some of the most abundant taxa (*Aeromonas, Asaia*, *Chryseobacterium-Elizabethkingia* groups, *Enterococcus,* and *Wolbachia*) in different developmental stages and diets were determined by PCR. However, this should in the future be extended to other abundant bacterial taxa such as *Serratia*, *Clostridium,* and *Providencia*. Our study identified several species which may worth be investigated about their potential probiotic properties including members of the Enterobacteriaceae (*Aeromonas*, *Elisabethkingia*, *Enterococcus*, *Enterobacter*, *Providencia*) and Acetobacteriaceae (*Asaia*) families to enhance production and

improve quality of mass-reared mosquito species which are to be used for SIT and other related population suppression programs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the 16S rRNA gene sequences reported in this study have been deposited in NCBI under Bioproject number PRJNA575054, while the Sanger generated sequences have been deposited under accession numbers MN540103 to MN540125.

AUTHOR CONTRIBUTIONS

SC performed the experiments and drafted the manuscript. DZ performed the experiments, analyzed the data, and drafted the manuscript. AA performed the experiments and critically revised the manuscript. VD and NB performed the bioinformatic analysis and critically revised the manuscript. GT performed the bioinformatic analysis, interpreted the data, contributed to the drafting and critical revision of the manuscript. HM performed gut samples dissection and collection and critically revised the manuscript. KB conceived the study, designed the experiments, interpreted the data, contributed to the drafting, and critically revised the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00605/full#supplementary-material

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ORIGINAL RESEARCH
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Influence of Diet, Sex, and Viral Infections on the Gut Microbiota Composition of *Spodoptera exigua* Caterpillars

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The gut microbiota plays essential roles in processes related with metabolism, physiology, and immunity in all organisms, including insects. In the present work, we performed a broad analysis of the *Spodoptera exigua* gut microbiota, a major agricultural pest. We analyzed the influence of multiple parameters such as diet, geographic location, sex, or viral infections on *S. exigua* caterpillar gut microbiota composition. Our study revealed a high variability in bacterial composition among individuals, and a major influence of environmental bacteria (including those acquired through diet) on the gut microbiota composition, supporting previous studies that claim resident microbiota are lacking in caterpillars. Previous studies with laboratory-reared insects showed that changes in caterpillar gut bacterial composition affect the insecticidal properties of entomopathogenic viruses and bacteria. Our study revealed different microbiota composition in field insects carrying a natural viral infection with *Spodoptera exigua nucleopolyhedrovirus* (SeMNPV) and/or *Spodoptera exigua iflavirus 1* (SeIV1). Few taxa can be specifically associated with the infection, suggesting microbiota influence the infective process of these natural pathogens, and providing new strategies for insect pest management.

Keywords: lepidoptera, microbiota, *Spodoptera exigua*, 16S rRNA, viral infection

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INTRODUCTION

The Lepidoptera *Spodoptera exigua* (Hübner) (Noctuidae) is an important polyphagous agricultural pest, polyphagous and worldwide distributed. Traditionally, chemical insecticides have been used to control this pest but, extensive use over a long time period has led to the emergence of resistant populations (Brewer and Trumble, 1989; Moulton et al., 2000; Osorio et al., 2008; Ahmad and Arif, 2010; Che et al., 2013). Therefore, biological insecticides such as baculovirus and *Bacillus thuringiensis* (Bt) represent an effective and safer alternative for pest control.

Gut microbiota is described as the complex community of microorganisms living in the digestive tracts of human and other animals in a symbiotic relationship. Many animals, including insects, are colonized by microbial symbionts, which can regulate host processes related with development, immunity, and metabolism (Dillon and Dillon, 2004; Engel and Moran, 2013). Most caterpillars are herbivorous, and their gut bacteria can play important roles in nutrition and host adaptation.

For instance, gut microbiota in caterpillars can contribute to plant cell wall digestion (Mason et al., 2015; Xia et al., 2017), detoxification of toxic compounds synthesized by the plants they feed on (Shao et al., 2011; Hammer and Bowers, 2015), or even suppress plant defense mechanisms (Acevedo et al., 2017). In recent years, researchers investigated if caterpillars depend on their gut microbes for feeding and/or development, but this question remains unclear. While some studies have shown that diet affects the insect microbiome composition (Berman et al., 2018; Jones et al., 2019), others have reported that diet does not have a clear effect on gut microbiota composition (Whitaker et al., 2016; Hammer et al., 2017). Moreover, the great variability usually observed in lepidopteran bacterial composition also indicates that many factors influence the final gut bacterial composition of these insects (reviewed in Mereghetti et al., 2017).

Furthermore, the Lepidoptera gut microbiota seems to influence the host interaction with pathogenic microorganisms. Several studies on different lepidopteran species revealed changes in gut microbiota composition after intoxication with Bt toxins (Broderick et al., 2004; Caccia et al., 2016), and insect susceptibility alteration to Bt and its toxins (Broderick et al., 2004) due to gut microbiota changes. In addition, Xia et al. (2013) suggested that certain bacterial taxa can confer Bt-resistance in *Plutella xylostella* larvae. In the case of *S. exigua*, our previous studies showed an increase in Bt tolerance associated with an increase in the gut bacterial load (Hernández-Martínez et al., 2010). Regarding viral pathogens such as baculovirus, a previous study from our laboratory revealed an increase in gut bacterial load after infection of a laboratory-reared *S. exigua* colony with its baculovirus, *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV) (Jakubowska et al., 2013). Such change in bacterial load was associated with an increase in baculovirus virulence, pathogenicity, and dispersion.

Baculoviruses are DNA viruses highly specific against different invertebrate species which cause larval death after viral ingestion. In addition to the oral infection caused by baculovirus, the virus can asymptomatically persist on the insects and be vertically transmitted to the offspring (Virto et al., 2014). In the case of *S. exigua*, 54% of field adult insects are covertly infected with its baculovirus, SeMNPV (Virto et al., 2014). In addition, some field insects can also carry a second covert infection with RNA viruses from the *Iflaviridae* family, which apparently do not cause lethal infection. *Spodoptera exigua Iflavirus 1* (SeIV1) (Millán-Leiva et al., 2012) was present in about 13.1% of field insects and 6.1% of insects were simultaneously infected with SeMNPV and SeIV1 (Virto et al., 2014). These covertly infectious viruses can be activated by different stress factors, leading to a lethal infection and finally killing the insect.

Based on the previous observations mentioned above, we hypothesized the possible role of gut microbiota composition in modulating *S. exigua* interaction with its naturally occurring viruses in the field. In the present work, as a first step in this investigation, we characterized the gut microbiota composition of laboratory and field collected *S. exigua* caterpillar, and studied the influence of dietary regimen, geographic location, and caterpillar sex on the gut microbiota composition. In a second part of the study, we analyzed viral infection interaction and gut microbiota composition, identifying certain bacterial groups which could shape viral-host interaction.

MATERIALS AND METHODS

Insects

In this study, samples came from *S. exigua* larvae with different backgrounds and dietary regimens. Field larvae were obtained from pepper greenhouses located in the Almería province (Spain). Third to fifth instar larvae were collected during September and October 2015 and sent to our laboratory in Valencia (Spain). The insects were sent in individual plastic bottles containing the pepper leaf from where they were collected, and the larvae were dissected and processed immediately upon arrival. The insects reared on standard artificial diet (AD) (Elvira et al., 2010) came from our laboratory colony, which has been maintained for more than 200 generations. The colony is maintained at 25 ± 3°C with 70 ± 5% relative humidity and a 16/8 h (light/dark) photoperiod. We also used *S. exigua* larvae from our laboratory colony fed a plant-based diet (PBD), which was prepared with lyophilized plant leaves in 2% agar and supplemented with 5% AD. For the PBD studies, fourth instar larvae previously reared on AD were transferred to the PBD and reared for 48 h before dissection. In parallel, a group of larvae were kept only on AD and dissected simultaneously. The plants employed for the PBD studies were pepper (dulce de España variety) and two different tomato varieties [Ailsa (A) and Money Maker (MM)]. Plants were grown in greenhouse facilities and harvested before floriation.

The *S. exigua* larvae were dissected and processed as follows. The whole gut (including the gut content) of each individual larva from the field, and pools of three guts per sample from an AD and PBD were dissected and homogenized in Luria-Bertani (LB) medium supplemented with 10% glycerol and frozen at −80°C for DNA extraction. For each sample, half of the remaining body was directly frozen at −80°C for subsequent DNA extraction, and the other half was frozen in RNAzol® RT reagent (Sigma Aldrich, St Louis, MO, United States) at −80°C for RNA extraction.

DNA Extraction and Sequencing

A fraction of each homogenized gut was used for total DNA extraction using the MasterPure™ Complete DNA & RNA Purification Kit (Epicenter, Madison, WI, United States) according to the manufacturer's instructions. Purified DNA was quantified in QUBIT and 10 ng/ul were used for amplification and sequencing of the 16S rRNA gene from 77 samples. The amplicon sequencing protocol targets the 16S gene V3 and V4 regions (459 bp), with the primers designed surrounding conserved regions (Klindworth et al., 2013). Following the Illumina amplicon libraries protocol, DNA amplicon libraries were generated using a limited cycle PCR: initial denaturation at 95°C for 3 min, followed by 25 cycles of annealing (95°C 30 s, 55°C 30 s, 72°C 30 s), and extension at 72°C for 5 min, using a KAPA HiFi HotStart ReadyMix (KK2602). Then Illumina sequencing adaptors and dual-index barcodes (Nextera XT index kit v2, FC-131-2001) were added to the amplicon. Libraries

were then normalized and pooled prior to sequencing. The pool containing indexed amplicons was then loaded onto the MiSeq reagent cartridge v3 (MS-102-3003) spiked with 10% PhiX control to improve base calling during sequencing, as recommended by Illumina for amplicon sequencing. Sequencing was conducted using a 2 × 300-pb paired-end run on an Illumina MiSeq sequencing system.

Sex Determination

Field larvae sex was molecularly determined by *kettin* gene copy number relative quantification. *Kettin* is a sex-linked gene without dosage compensation as previously described for *Bombyx mori* (Suzuki et al., 1999), and was recently used to determine sex in different Lepidopteran larvae species (Belousova et al., 2019). In this study, specific primers for *S. exigua kettin* gene amplification were designed (**Supplementary Table 1**) using the same region published previously for *B. mori* (Koike et al., 2003). Primers were previously validated on thorax DNA obtained from *S. exigua* male and female adults.

Total DNA was extracted from field larvae carcasses using the MasterPure™ Complete DNA & RNA Purification Kit (Epicenter, Madison, WI, United States), and the isolated DNAs (50 ng) were used for *kettin* quantification by quantitative PCR (qPCR) using the specific primers and *ATP synthase* as a reference. The qPCR was performed with 5x HOT FIREPOL EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) following standard protocols, and the DNA amplification was measured in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). The relative quantification was represented as $2^{-\Delta\Delta Ct}$ calculated according to the method described in Livak and Schmittgen (2001) and Belousova et al. (2019), were $\Delta\Delta Ct = (Ct_{kettin} - Ct_{ATP\ synthase}) - Avg(Ct_{kettin} - Ct_{ATP\ synthase})_{female}$.

RNA Extraction and Virus Quantification

The presence of the SeIV1 RNA virus and the active replication of the SeMNPV DNA virus in field insects were determined by qPCR using specific primers (**Supplementary Table 1**). Total RNA was isolated from the carcass of each larvae using RNAzol® RT reagent (Sigma Aldrich, St Louis, MO, United States) following the manufacturer's protocol, and used for cDNA synthesis using the PrimeScript RT Reagent kit (TaKaRa Bio Inc., Otsu Shiga, Japan). The qPCR was performed as described in the previous section. The amplification curve of each sample was examined, and $\Delta Rn \geq 1$ values were considered as positive viral infections.

Total Bacterial Load Quantification

The total bacterial load was quantified in the field samples by qPCR using 16S rRNA universal primers (Nadkarni et al., 2002). The qPCR was performed with total DNA (50 ng) isolated from the gut of each larvae in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, United States). The bacterial concentration was calculated by comparison with a standard curve of known bacterial DNA quantities, and then statistically analyzed for viral presence with a Student's *t*-test (GraphPad Prism version 7.00) as described in Gasmi et al. (2019).

Data Analysis

Data-mining and statistical analysis were performed with the open-source software QIIME (v. 1.9) (Caporaso et al., 2010) and the online Calypso pipeline (v. 8.84) (Zakrzewski et al., 2017). Quality assessment of obtained reads was carried out with the prinseq-lite program (Schmieder et al., 2011) with defined parameters (i.e., min_length, 50; trim_qual_right, 20; trim_qual_type, mean; trim_qual_window, 20). Paired reads from Illumina sequencing were joined using fastq-join from the ea-tools suite (Aronesty, 2011). Potential chimeric sequences were removed using USEARCH 6.0 available at RDPipeline (Edgar et al., 2011). Filtered and demultiplexed sequences were then processed with QIIME software using default parameters. First, the sequences were clustered into operational taxonomic units (OTUs) using *de novo* OTU picking (pick_de_novo_otus.py script) based on 97% identity and filtering the unassigned, mitochondria, chloroflexi, and cyanobacteria taxa using the QIIME's filter_taxa_from_otu_table.py script, to visualize the most abundant phyla in all samples. Next, to analyze the samples more deeply to look for microbiotia differences, the generated OTU table was filtered to include only the four most abundant phyla. Bacterial composition was analyzed through the summarize_taxa_through_plots.py script and it was represented as the relative abundance of the 20 most abundant genera in a bar graphic using Excel software. We also identified the core microbiome (compute_core_microbiome.py) as those OTUs present in at least 50% of all samples.

The OTU table including only the most abundant phyla was filtered in Calypso software to remove the samples with less than 1000 reads from the analysis, and the resulting data was transformed by CSS (cumulative-sum scaling)+log with total sum normalization (TSS). Then, canonical correspondence analysis (CCA) was used to estimate differences in microbiota (at the genus level) according to different factors (diet, sex, or viral infections). Alpha diversity and richness were determined at the genus level using the Shannon index and Chao1 index, respectively. The Wilcoxon rank test was employed to identify differentially abundant bacteria associated with viral presence. Spearman correlation analysis using GraphPad Prism (version 7.00) were used to examine the relationship between the total bacterial loads and the diversity or richness in field samples.

RESULTS

S. exigua Caterpillar Microbiota Compositions

A total of 3,450,084 reads were obtained from 16S rRNA gene Illumina Miseq sequencing of 77 *S. exigua* samples (45 samples from field captures, 15 from AD, and 17 from the PBD) (detailed on **Supplementary Tables 2,3**). After cleaning and chimera filtration, the remaining 2,795,707 reads led to the identification of 42,601 OTUs. After removing some host *S. exigua* contaminant sequences (designated as

unassigned), mitochondria, chloroflexi, and cyanobacteria taxa, 21,197 OTUs remained. These OTUs were assigned mostly to Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria phyla (98% total phyla coverage) showing different distribution across the analyzed groups (**Figure 1**). Proteobacteria was the most abundant phyla in all groups, representing around 50% or more OTUs in the different groups. The phylum Firmicutes was almost as abundant as Proteobacteria in the AD group but decreased to around 20% in the larvae fed PBD group. Bacteroidetes was the least abundant in all groups, although its presence was slightly higher in field samples than from laboratory larvae. Actinobacteria was more abundant in samples from two tomato varieties. Additionally, 8 samples (5 field samples, 1 AD, 1 from tomato (MM), and 1 from pepper) were removed from the analysis, since they contained less than 1000 reads. Although some variability in the richness and Shannon indices between the individual samples was observed (**Supplementary Figures 1A,B**), rarefaction curves tended to reach a plateau indicating that the sequencing depth was enough to capture the majority of microbial diversity (**Supplementary Figure 1C**).

Changes in Gut Microbiota Associated With Different Diets

Influence of diet on the bacterial community composition was further characterized according to their genus distribution (**Figure 2**) by analyzing bacterial composition changes in caterpillar reared on AD, and then transferred to a PBD for 48 h. A multivariate canonical correspondence analysis (CCA) at the genus level revealed significant differences ($P = 0.002$) among the different diet types. Accordingly, changes in diet composition produced a quick shift in gut microbial composition, suggesting that *S. exigua* larvae microbiota composition was strongly influenced by ingested food. Composition differences were observed even among larvae fed (for 48 h) with two different tomato varieties (**Figure 2A**).

The bacterial diversity measured by the Shannon index at the genus level was almost identical for larvae fed a PBD, showing very little variability among samples (**Figure 2B**). In contrast, those larvae fed an AD showed an apparent greater variability among each sample. Nevertheless, no significant differences in bacterial diversity were observed between the different diets tested. The Chao1 index for richness estimation also did not show differences between diets (**Figure 2C**). Further bacterial composition characterization was carried out by relative abundance comparison of the 20 most abundant genera for each sample (**Figure 2D**). Great genus composition heterogeneity was observed and, consequently, no clear association of specific genera to the different diets could be established.

Gut Microbiota Composition in Field *S. exigua* Larvae

S. exigua bacterial composition was also evaluated in individual larvae collected from pepper plants from different greenhouses in the Almería province (Spain). Relative abundance analysis of the 20 most abundant genera revealed great similarity among individuals collected from the same greenhouse (**Figure 3**). However, samples from different greenhouses were highly diverse in composition. These results suggest a major influence of environment (including the ingested plant) on *S. exigua* larvae gut bacterial composition.

TABLE 1 | The most common OTUs from the gut microbiota of *S. exigua* larvae.

OTU	Prevalence (%)
p__Bacteroidetes__f__Enterobacteriaceae_1157	92.75
p__Bacteroidetes__f__Enterobacteriaceae_1648	92.75
p__Proteobacteria__g__Rhizobium__s__leguminosarum_2017	91.30
p__Proteobacteria__g__Delftia_3407	79.71
p__Proteobacteria__g__Acinetobacter_4337	76.81
p__Proteobacteria__g__Erwinia_4576	73.91
p__Proteobacteria__g__Pseudomonas_8097	72.46
p__Proteobacteria__g__Staphylococcus_8177	72.46
p__Proteobacteria__f__Enterobacteriaceae_12431	71.01
p__Proteobacteria__g__Sphingomonas_12862	71.01
p__Proteobacteria__g__Delftia_13306	69.57
p__Proteobacteria__g__Bacillus__s__flexus_14993	68.12
p__Proteobacteria__g__Sphingomonas__s__yabuuchiae_17824	68.12
p__Proteobacteria__g__Klebsiella_20803	68.12
p__Proteobacteria__g__Enterococcus_22758	63.77
p__Proteobacteria__g__Propionibacterium__s__acnes_23146	63.77
p__Proteobacteria__g__Ralstonia_23680	57.97
p__Proteobacteria__g__Lysinibacillus__s__boronitolerans_27244	56.52
p__Proteobacteria__g__Agrobacterium_36803	55.07
p__Proteobacteria__f__Oxalobacteraceae_37375	52.17

Core Microbiome

A core microbiome was defined as those OTUs present in at least 50% of all samples. Taking all samples individually into account, a core that comprises 20 OTUs belonging to phyla Proteobacteria was identified (**Table 1**). However, no bacteria (OTU) were found in common for all samples analyzed. In addition, a core microbiome was also analyzed in a more restricted sample group, the field-collected insects. In that case, only one OTU, belonging to the *Pseudomonas* genus, was found in common in all the field individuals. Results obtained with the two sample sets analyzed revealed the lack of a true core microbiome in the *S. exigua* caterpillar, since no OTUs were found in common between all the analyzed samples.

Sex Influence on Gut Microbiota Composition

Although caterpillars do not show obvious sex dimorphism, the immature reproductive organs are already present at the larval stage. We wondered if caterpillar sexual destination could influence microbiota composition. To test such hypothesis, the gut microbiota composition from field collected *S. exigua* larvae were also analyzed according to their sex. Sex determination was performed by *kettin* gene quantification in field samples, and 12 males and 23 females were identified (5 samples could not be sexed due lack of proper DNA) (**Figure 4A**). The bacterial community was examined using a CCA at the genus level to assess

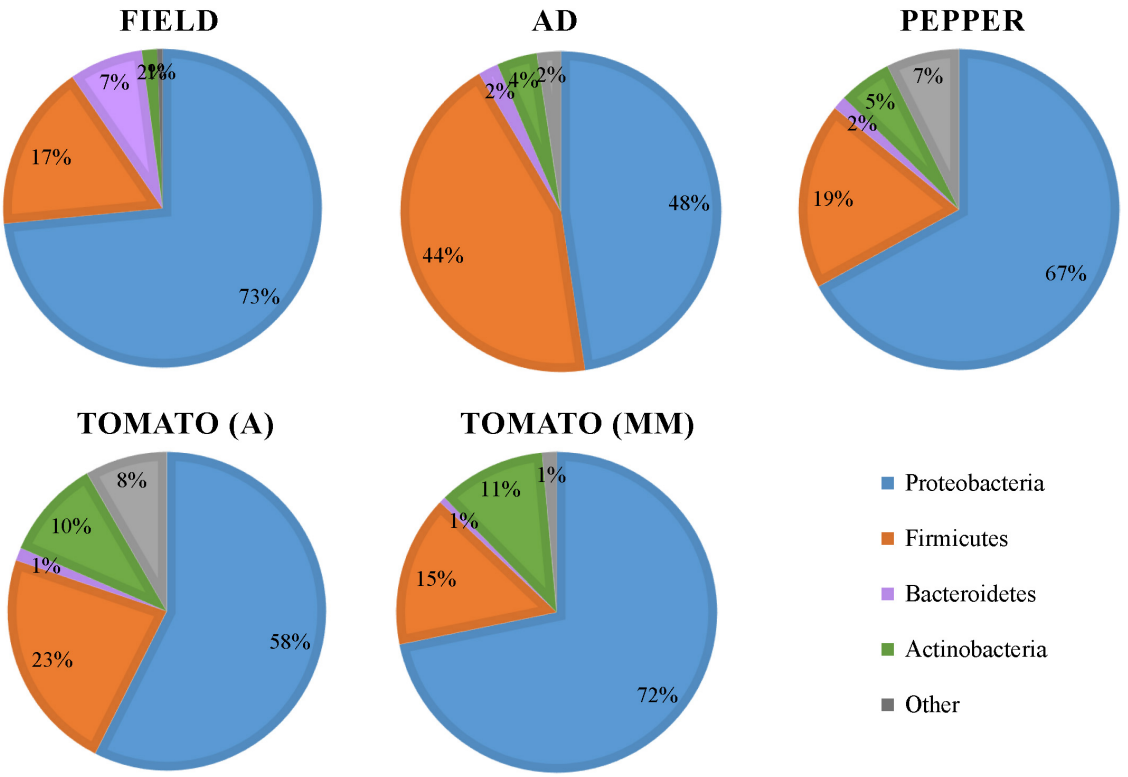

FIGURE 1 | Bacterial phyla distribution in the gut of *S. exigua*. Pie charts represent the relative abundance in percentages of the main phyla found in the gut of *S. exigua* larvae collected on the field from pepper greenhouses and the laboratory colony fed with different diets (AD: artificial diet; A: Ailsa variety; MM: Money Maker variety).

if larvae microbiota differs depending on sex, but no significant differences were found (**Figure 4B**). Additionally, the diversity and richness indices also did not show differences between sexes (**Figures 4C,D**).

Gut Bacterial Composition and Viral Infections

Naturally present viruses in the *S. exigua* field samples were determined by qPCR amplification of specific viral genes. We found an infectious rate around 50%, which was distributed as follows: 6 larvae infected with BV, 9 larvae infected with SeIV1, and 6 larvae simultaneously infected by both viruses, whereas 19 larvae were classified as virus-free. The gut microbiota was then examined at the genus level according to the presence or absence of viral infection. Although the Shannon and the Chao1 indices estimated that diversity and richness, respectively, were roughly the same for virus-infected and non-virus-infected samples (**Figures 5B,C**), the CCA analysis showed a significant difference ($P = 0.003$) in microbiota composition (**Figure 5A**).

An additional analysis revealed the existence of 9 OTUs significantly associated ($P < 0.05$) and more abundant (at least twice) on the viral infected samples (**Table 2** and **Supplementary Figure 2**), the sequences of which are found in the supplementary data. Six of the identified OTUs belong to phylum Proteobacteria (three from the genus *Acinetobacter*), two OTUs were identified as bacteria from genus *Pedobacter* (phylum Bacteroidetes), and another from phylum Firmicutes. An interesting observation is that all OTUs which showed significant abundance changes were overrepresented in the virus-infected group. A moderate increase in abundance of less than 10-fold was found for all the identified OTUs, except for the p__*Proteobacteria*__g__*Acinetobacter*_12048 which is 36,120-fold more abundant in the virus-infected group. Nevertheless, no differences in bacterial load were observed between the viral-infected and non-infected caterpillars (**Figure 5D**). Interestingly, a deeper analysis revealed a significant negative correlation between the bacterial load and the diversity index for both groups (**Figure 5E**). However, the relationship between bacterial load and richness only showed a significant positive correlation for the viral infected samples (**Figure 5F**), which suggests that an increase in microbiota in viral-infected samples leads to the presence of a greater number of bacterial taxa.

DISCUSSION

In this work, we performed a comprehensive characterization of *S. exigua* larvae gut bacterial microbiota composition, and determined how aspects such as diet, location, sex, and viral presence influence it. Taxonomic analysis revealed that

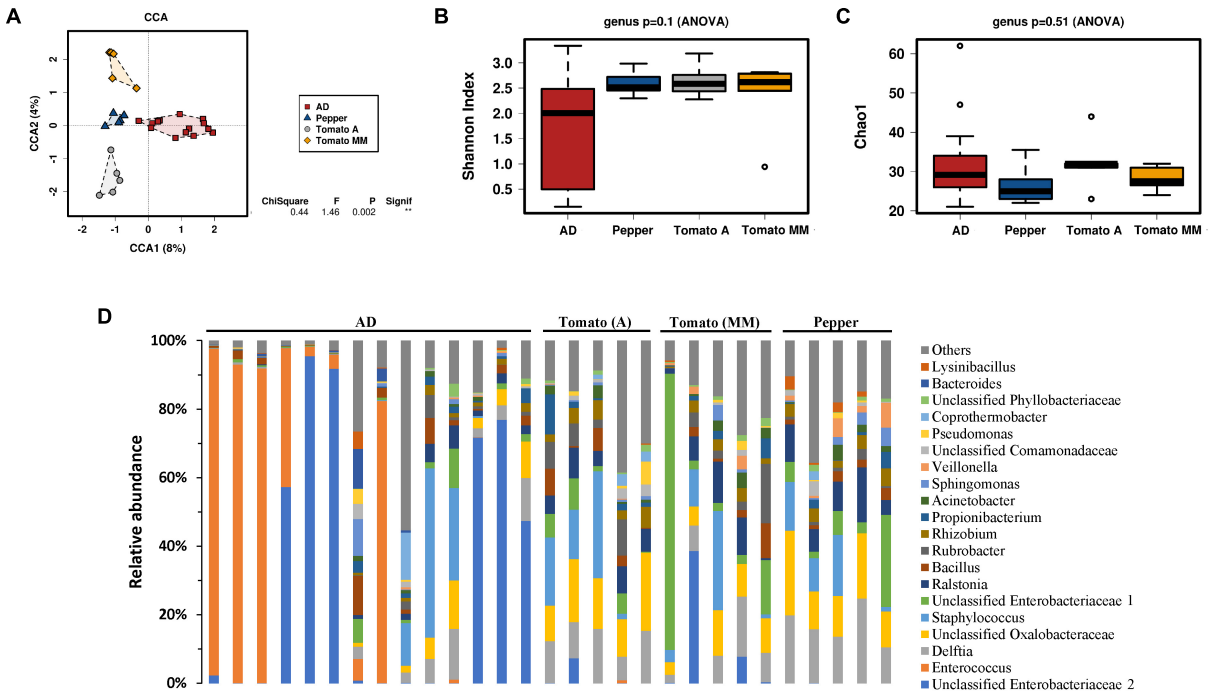

FIGURE 2 | Microbiota of *S. exigua* larvae according the diet. Canonical correspondence analysis (CCA) **(A)**, estimation of the Shannon index diversity **(B)**, the Chao1 index of richness **(C)** and the relative abundance of the 20 most abundant genera in *S. exigua* larvae from the laboratory colony fed with different diets **(D)** at a genus level.

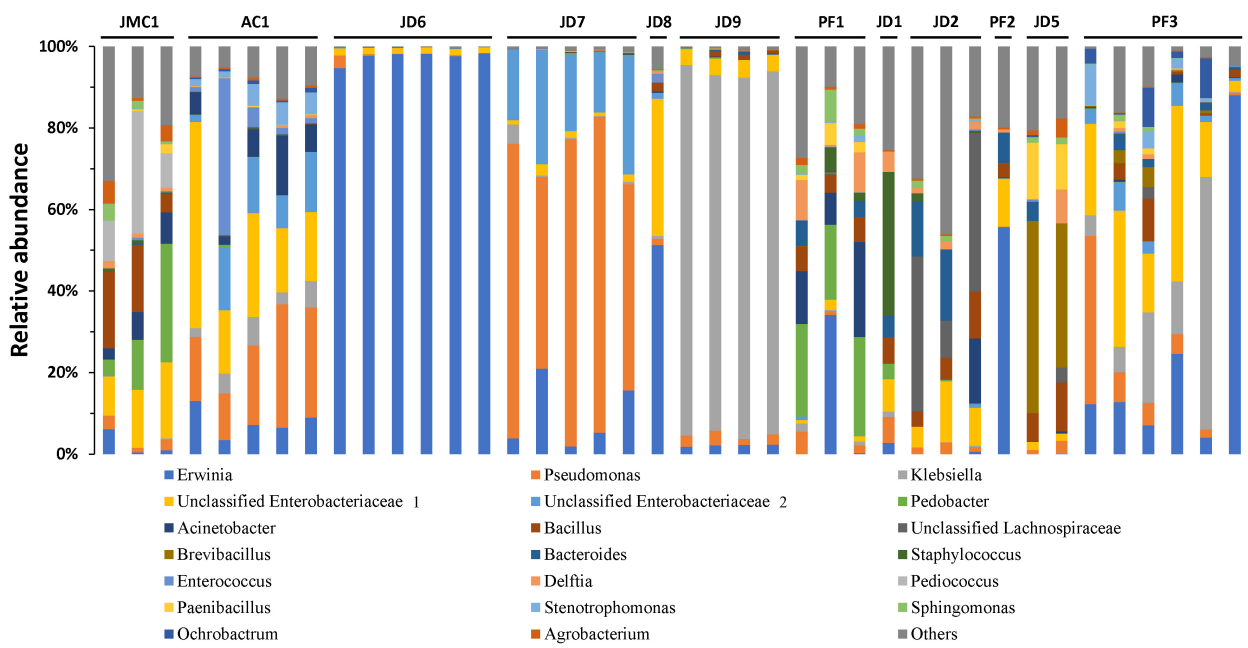

FIGURE 3 | Microbiota composition of *S. exigua* according the location. Relative abundance of the 20 most abundant genera in *S. exigua* larvae from field. The codes of numbers and letters refer to the different greenhouses in which larvae were collected.

the *S. exigua* gut bacterial community independent of the feeding conditions (diet and origin), was mainly composed of Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, as previously described by Gao et al. (2019). These four phyla were also the most common found in other lepidopteran species (Whitaker et al., 2016; Xia et al., 2017; Chen et al., 2018;

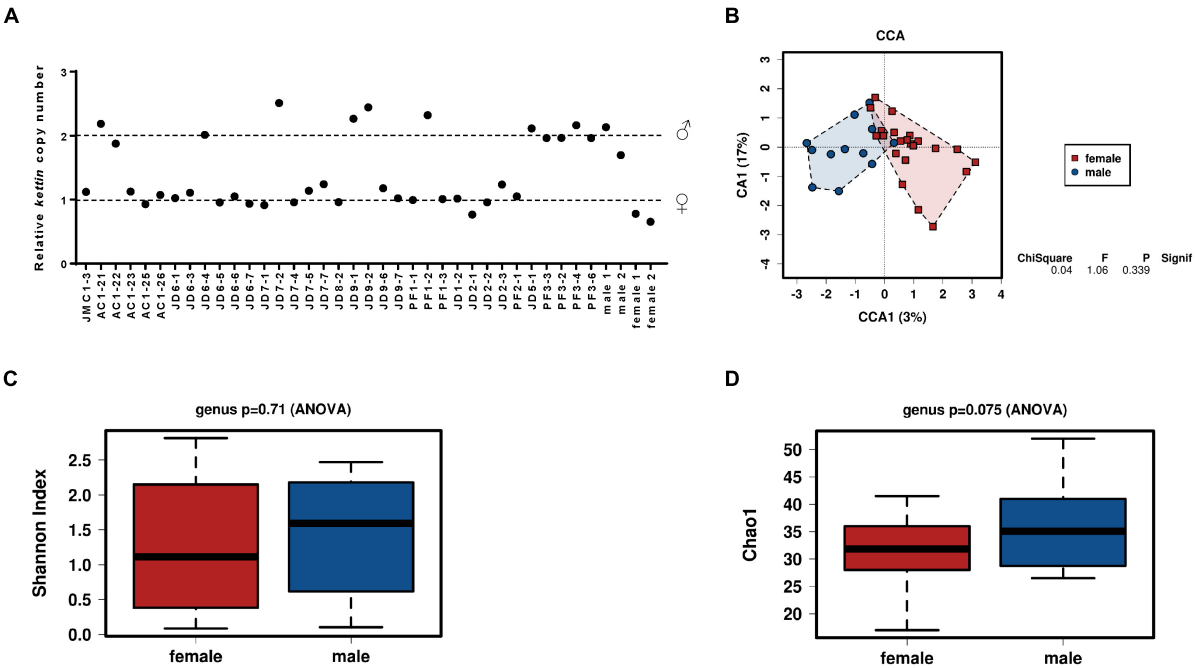

FIGURE 4 | Influence of sex on the microbiota of *S. exigua*. Sex determination of the *S. exigua* larvae from field by quantification of the *kettin* gene copy number **(A)**. Canonical correspondence analysis **(B)**, the estimation of the Shannon index diversity **(C)** and the Chao1 index for richness **(D)** at a genus level according the sex of the collected larvae.

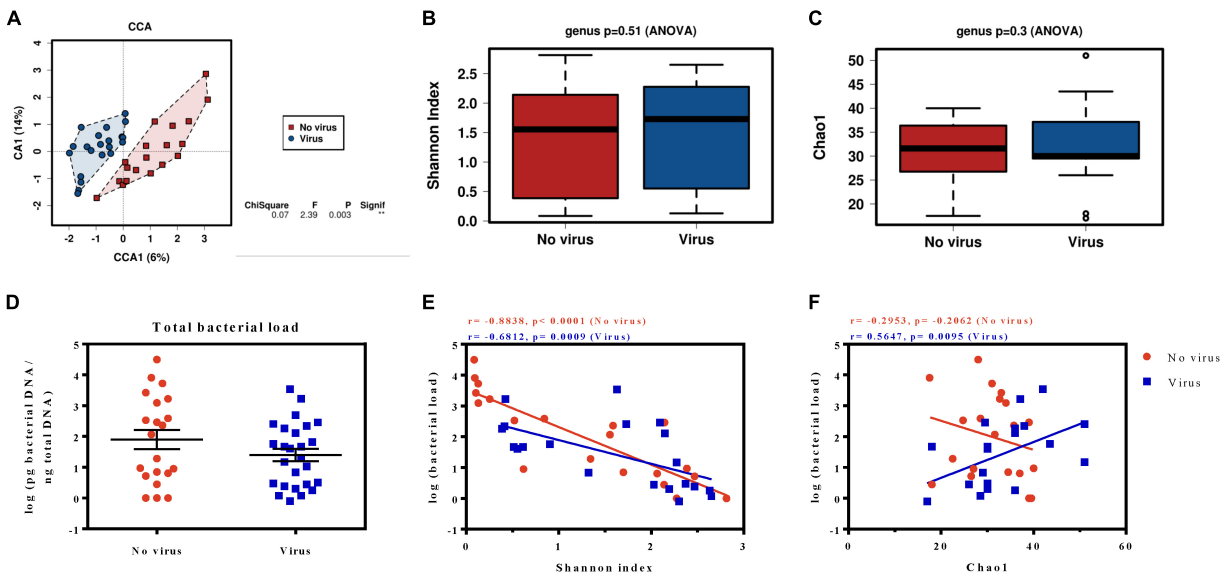

FIGURE 5 | Microbiota analysis of *S. exigua* larvae according their viral infection status. Canonical correspondence analysis **(A)**, the estimation of the Shannon index diversity **(B)** and the Chao1 richness index **(C)** at a genus level in field *S. exigua* larvae naturally infected and non-infected with viruses. Calculation of the total bacterial load of *S. exigua* larvae from viral-infected and viral-free field samples **(D)** and their relationship with diversity **(E)** and richness **(F)**. The best-fit lines, the Spearman r correlations and the p-values of the regression are shown for each analysis.

Phalnikar et al., 2018; van Schooten et al., 2018), as well as in other insects (Yun et al., 2014). Slight abundance differences were observed between the different groups tested, but likely the main difference was the increased presence of the phylum Firmicutes in larvae reared on AD in comparison with those reared on a PBD (**Figure 1**). This observation suggests that diet could influence *S. exigua* larvae microbial gut composition. Thus, the bacterial composition was further analyzed.

TABLE 2 | Differentially abundant OTUs associated to viral infection.

Otu	Fold change	*P*-valor
p__Bacteroidetes__g__Pedobacter_3455	2.1	0.0020
p__Bacteroidetes__g__Pedobacter_1816	2.8	0.0038
p__Proteobacteria__g__Acinetobacter__s__johnsonii_15272	9.5	0.0065
p__Proteobacteria__g__Acinetobacter_12048	36,120	0.0270
p__Proteobacteria__g__Acinetobacter_15052	3.1	0.0330
p__Firmicutes__g__Staphylococcus_1362	9.9	0.0250
p__Proteobacteria__g__Sphingobium_40602	2.8	0.0250
p__Proteobacteria__f__Xanthomonadaceae_11563	2.8	0.0092
p__Proteobacteria__f__Enterobacteriaceae_14126	3.2	0.0370

In a more detailed analysis with respect to gut bacterial composition we focused on laboratory colony larvae fed different diet types (AD, tomato (A and MM varieties), and pepper). A rapid shift in gut bacterial composition was associated with diet changes. Although the relative abundance analyses of the most abundant genera showed great heterogeneity and variability, even among samples from the same diet group, a significant difference was observed in the multivariate analysis (CCA) indicating that larval gut microbiota composition is strongly influenced by diet. No significant differences were observed neither in diversity nor in richness at the genus level, however, the samples reared on AD showed great variability, while samples reared on a PBD were more homogeneous (**Figure 2**). This observation could be because those insects came from different generations collected at different periods. In addition, although the AD recipe was not changed, the ingredients with it is made of can be slightly differ from one batch to another and affect someway the larvae gut microbiota. Other differences between larvae reared on AD or PBD can be extracted from this study. For example, the genus *Enterococcus* and others from the *Enterobacteriaceae* family were more abundant in samples from AD, as previously shown for *S. exigua* and other lepidopteran species (Broderick et al., 2004; Xiang et al., 2006; Raymond et al., 2009; Hernández-Martínez et al., 2010; Jakubowska et al., 2013). However, in these previous studies *Enterococcus* was reported as a bacterium present in all samples as the most abundant, while in our analysis *Enterococcus* genus abundance was highly variable among samples. Although a fast shift in microbiota composition had been observed after only 48 h of shifting diet, we cannot discard that longer time (e.g., at least one complete generation) could show stronger effects. In addition, previous Lepidoptera studies revealed apparent contradictions with respect to diet influence on gut microbiota. Some studies claimed diet significantly influenced lepidopteran gut microbiota composition (Yun et al., 2014; Berman et al., 2018; Phalnikar et al., 2018; Jones et al., 2019), whereas other authors reported diet had little or no effect on microbiota (Whitaker et al., 2016; Chaturvedi et al., 2017; Minard et al., 2019). This apparent contradiction seems to reveal the lack of caterpillar specific gut microbiota as reported by Hammer et al. (2017). Consequently, any diet change (and the microbiota present in the diet) or in the environment would produce a shift in gut microbiota composition without being diet specific.

In agreement with that, field larvae samples also showed great variability in gut bacterial composition, even though the caterpillars were all collected from pepper plants. Nevertheless, some general microbial composition patterns were observed when samples were grouped according to their location (**Figure 3**), suggesting a stronger contribution from environmental bacteria in the final larval gut composition than bacteria associated with PBD. This is consistent with a recent study by Hannula and colleagues (Hannula et al., 2019), in which they demonstrated that *Mamestra brassicae* larvae microbiota depends on the soil microbiome. So, the variability observed among the different greenhouses could be attributed to differences in foliar microbiota of each pepper plant and the greenhouse soil microbiota in which the caterpillars were collected. This explanation is also supported by previous studies in which the larval gut bacteria did not totally correspond with dietary associated leaf bacteria (Whitaker et al., 2016; Hammer et al., 2017; Minard et al., 2019). Thus, the authors suggested larval gut microbiota is composed of transient bacteria instead of resident microbiota, due to the intrinsic insect gut physiology, such as their high pH, simple tube structure, and rapid digestion that prevent gut colonization by bacterial uptake with food (Hammer et al., 2017). In agreement with this hypothesis, we observed the absence of resident microbiota. Although we found about 20 OTUs present in at least 50% of the samples, we were unable to find a core microbiome, and no OTU was shared by all the analyzed samples. The lack of a resident microbiota was also described in caterpillar from other species such as *Choristoneura fumiferana* (Landry et al., 2015), and also by Hammer et al. (2017) in a study analyzing the gut microbiota of several Lepidoptera species.

Several Lepidoptera studies revealed differences in microbiota community between male and female adults. *Spodoptera litorallis* adults differ in their microbiota community composition, since female microbiota is composed mainly of *Enterococcus*, *Klebsiella*, and *Pantoea* genera, while the male microbiota is dominated by *Klebsiella* (Chen et al., 2016). van Schooten et al. (2018) also reported significant abundance differences between sexes in several *Heliconius* species, but only for 13 rare OTUs, as similarly occurs for a few *Melitaea cinxia* larvae taxa (Minard et al., 2019). Recent *S. exigua* studies by Gao et al. (2019) described the absence of differences in microbiota composition between male and female adults. In our study we compare for the first time the microbiota composition between males and females at the larval stage, and the results showed no sex influence on caterpillar gut bacterial composition, diversity, or richness (**Figure 4**). Whether this lack of differences can be attributed to the larval stage or to the studied species would need further investigation.

Previous studies in our laboratory revealed that baculovirus infection increases the gut microbiota load in *S. exigua* larvae, and that increase also benefits the virus, enhancing their virulence, pathogenicity, and dispersion in laboratory conditions (Jakubowska et al., 2013). However, the relationship between viral infections in the field and microbiota composition had never been addressed. As occurs for many lepidopteran field

populations (Williams et al., 2017), *S. exigua* field insects are naturally infected by different viruses (Virto et al., 2014). This is the first time that SeMNPV and/or SeIV1 presence was related with *S. exigua* larvae microbiota composition. Interestingly, although no differences were observed in diversity or richness, the results obtained with the multivariant analysis carried out with field samples showed a different gut bacterial composition associated with the presence or absence of viral infections (**Figure 5**). The bacterial load also did not show differences for infected and non-infected field larvae. However, a negative correlation between bacterial load and diversity levels was observed for both groups. So, the greater total bacterial loads in the *S. exigua* gut can be explained by the increased abundance of only a few bacterial groups, as occurs with *Enterococcus* in laboratory *S. exigua* populations (Jakubowska et al., 2013). In the case of richness, a positive correlation was observed only for the viral-infected samples, which means that the larval microbiota is composed of a greater number of bacterial species. In addition, significant abundance differences were observed for 9 OTUs, which were always more prevalent in infected *S. exigua* larvae than in the virus-free samples. Half of these OTUs belong to genera *Acinetobacter* and *Pedobacter*, suggesting these bacterial genera could have active functions in viral-host interaction. *Staphylococcus*, *Sphingobium*, and unclassified bacteria from families *Xanthomonadaceae* and *Enterobacteriaceae* are also differentially abundant OTUs when larvae are virally infected.

Wolbachia is an intracellular bacterium commonly found in insects, and present in about 80% of lepidopteran species (Ahmed et al., 2015). Graham et al. (2012) reported that *Wolbachia* increased *S. exempta* susceptibility to baculovirus, becoming a potential biological control agent. However, it was absent from our field larvae samples. *Wolbachia* is usually found in reproductive tissues, and was described as a parasite that manipulates reproduction in Lepidoptera (Hiroki et al., 2002; Werren et al., 2008), but it can also be found in other tissues, even in the gut (Narita et al., 2007; Whitaker et al., 2016). Since our work focused on gut microbiota, we cannot discard *Wolbachia* presence in other larval tissues. Nevertheless, our study identified certain bacterial groups that could influence the infection cycle, and perhaps increase susceptibility to viral infections, or even trigger covert viral activation, which could be key to developing new pest control strategies through insect microbiota manipulation.

CONCLUSION

S. exigua larvae microbiota is mainly composed of Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria phyla. Although some differences in gut bacterial composition were observed according the different diets analyzed, the results showed that gut microbiota is highly variable and strongly influenced by environmental bacteria (including those acquired through diet), supporting previous studies reflecting the lack of resident microbiota in caterpillars. In addition, microbiota study related to field larvae natural infection status revealed that the microbiota composition is significantly different when larvae present covert infections, and individual taxa could be associated specifically with the infection. Our observations suggested the possibility that microbiota composition influences the infective process of these *S. exigua* larvae pathogens. Thus, these results offer valuable information that will be useful for insect pest management using entomopathogenic viruses.

DATA AVAILABILITY STATEMENT

The raw Illumina sequences generated and analyzed for this study can be found in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA603888.

AUTHOR CONTRIBUTIONS

MM-S and SH designed the project. MM-S conducted the research. MM-S and MC analyzed the data. MM-S and SH wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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CORRECTION
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Corrigendum: Influence of Diet, Sex, and Viral Infections on the Gut Microbiota Composition of *Spodoptera exigua* Caterpillars

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ORIGINAL RESEARCH
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The Core Gut Microbiome of Black Soldier Fly (*Hermetia illucens*) Larvae Raised on Low-Bioburden Diets

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An organism's gut microbiome handles most of the metabolic processes associated with food intake and digestion but can also strongly affect health and behavior. A stable microbial core community in the gut provides general metabolic competences for substrate degradation and is robust against extrinsic disturbances like changing diets or pathogens. Black Soldier Fly larvae (BSFL; *Hermetia illucens*) are well known for their ability to efficiently degrade a wide spectrum of organic materials. The ingested substrates build up the high fat and protein content in their bodies that make the larvae interesting for the animal feedstuff industry. In this study, we subjected BSFL to three distinct types of diets carrying a low bioburden and assessed the diets' impact on larval development and on the composition of the bacterial and archaeal gut community. No significant impact on the gut microbiome across treatments pointed us to the presence of a predominant core community backed by a diverse spectrum of low-abundance taxa. *Actinomyces* spp., *Dysgonomonas* spp., and *Enterococcus* spp. as main members of this community provide various functional and metabolic skills that could be crucial for the thriving of BSFL in various environments. This indicates that the type of diet could play a lesser role in guts of BSFL than previously assumed and that instead a stable autochthonous collection of bacteria provides the tools for degrading of a broad range of substrates. Characterizing the interplay between the core gut microbiome and BSFL helps to understand the involved degradation processes and could contribute to further improving large-scale BSFL rearing.

Keywords: *Actinomyces*, animal feedstuff, waste valorization, circular economy, microbial communities, larval metabolism, 16S amplicon sequencing

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INTRODUCTION

Diet is known to shape structure and function of an organism's gut microbiota (David et al., 2014). In many cases, a major part of metazoan digestive capabilities root in its gut-residing aggregation of a mostly prokaryotic microbiota (Rajagopal, 2009; Ursell et al., 2012; Engel and Moran, 2013). Within a taxonomic entity, very often a microbial core community may be identified, ensuring essential catabolic aptitudes for health and survival (Raymann and Moran, 2018).

Maintaining the stability of such a community may provide protection against extrinsic microbial advances, facilitate degradation, and support the thriving of an organism (Dillon and Dillon, 2004; Engel and Moran, 2013).

The Black Soldier Fly (*Hermetia illucens*; BSF) is known as a workhorse when it comes to valorizing biodegradable organic wastes (Clariza Samayoa et al., 2016). Rapid growth, broad degradation capabilities, and non-competence as a vector for human diseases make the fly ideal for industrial applications (Tomberlin et al., 2002; Wang and Shelomi, 2017). Larvae transitioning to the prepupal stage move out of the humid organic waste and seek a dry spot for pupation. This "self-harvesting" property facilitates the automatization of larva collection (Sheppard et al., 2002). The larvae shine with a high fat (>35%) and protein (>40%) content featuring a favorable amino acid spectrum for application as animal feed (Cullere et al., 2016; Surendra et al., 2016; Ushakova et al., 2016). Thus, larvae and products thereof increasingly find use in pisciculture and poultry farming (Wang and Shelomi, 2017), and more concrete EU-wide regulations regarding the use of insects and derivatives as animal feed are in the works (European Parliament and of the Council, 2015).

The larvae's ability to produce antimicrobial peptides repressing the number of pathogenic and supposedly other bacteria in their environment is still an object of study but could act as a barrier for extrinsic microbial colonization of the larvae and supports the maintenance of an inborn microbial gut community (Erickson et al., 2004; Choi et al., 2012; Park et al., 2015). The growing insect industry and increasing use of insect products demand an in-depth understanding not only of the fly's biology but also of the involved microbial networks to standardize rearing methods, improve waste degradation, maximize biomass output, and meet the hygienic standards (Pastor et al., 2015; Boccazzi et al., 2017; De Smet et al., 2018). Recently, Wynants et al. (2018) observed strong variations in the composition of microbial gut communities in larvae across different rearing locations. Although many biotic and abiotic factors influence the specific microbiome structure, these authors were able to detect bacteria like *Morganella* sp., *Enterococcus* sp., *Pseudomonas* spp., *Providencia* sp., and Bacillaceae in multiple independent samples.

In this study, we investigated the impact of three diets [chickenfeed (CF), grass-cuttings (GC), fruit/vegetables (FV)] carrying a low bioburden, compared with waste-related substrates, on the microbial community composition during larval development. Besides CF as a control diet, we selected these diets because produce and green waste account for a large fraction of common municipal household wastes (European Environment Agency, 2013) and should be considered for BSF treatment. We hypothesized that larvae would perform differently with respect to biomass gain and developmental progress and presumed that a diet-independent association of microbial gut colonizers could support versatile larval growth. Our analysis focused on the characterization of a core microbial community uninfluenced by time or diet, which acts as a foundation for degradative activities in the larval gut.

MATERIALS AND METHODS

Rearing of Larvae and Experimental Set-Up

Larvae of *H. illucens* were obtained from Illucens (Ahaus, Germany) in 2014 and were kept as laboratory population under stable environmental conditions in a Fitotron® SGC 120 (Weiss Technik, United Kingdom) climate chamber [27°C and 60% relative humidity (RH), 16 h photoperiod with light settings as described in Heussler et al., 2018] in Innsbruck, Austria. The population was maintained on a diet based on a 2:3 mixture of ground CF (Grünes Legekorn Premium, Unser Lagerhaus WHG, Austria) processed with a Fidibus flour mill, Komo Mills, Austria) and water. Larvae were kept in 30 × 15 × 5 cm non-transparent plastic boxes, covered with nets for aeration and containing sterilized pine humus (PH; *ad libitum*) as litter for humidity regulation. Until eclosion, pupae were stored in non-transparent plastic cups covered with nets containing straw litter. Flies were kept for oviposition in 50 × 30 × 25 cm transparent plastic boxes with corrugated plastic strips. Two-hundred 6-day old larvae were used for each replicate (three replicates per treatment). After hatching, the larvae were fed with CF for 6 days before being introduced to the two new diets (GC and FV mix). Feeding with CF continued in a control group consisting of three replicates.

Substrate Preparation and Feed Amounts

Chickenfeed was used as control diet, prepared as a 2:3 mixture of ground CF and water as for the standard population maintenance. For GC, freshly cut grass from a lawn mower collector box was further shredded using a Vitamix TNC® electric blender (Vitamix, Germany). The FV diet consisted of cucumber, tomato, orange, and apple, prepared in a ratio of 0.5:1:1:1 (fw/fw), and the ingredients were manually minced. Prior to use, the water content of the substrates was equalized based on their dry weight. Feed was added every 3 days and the feed amounts were set to 100, 300, and 400 mg larva^{-1} for CF, FV, and GC, respectively, based on the organic content of the substrate. The efficiency of substrate degradation and the conversion to larval biomass were calculated based on (1) and (2).

$$\mathrm{WRI} = \frac{\mathrm{D}}{\mathrm{t}} \times 100 \quad \mathrm{D} = \frac{\mathrm{W} - \mathrm{R}}{\mathrm{W}} \tag{1}$$

Waste reduction index (WRI): W = total amount of organic material, R = residue after time t, t = days larvae were fed with material (Diener et al., 2009).

$$\mathrm{ECD} = \frac{\mathrm{B}}{(\mathrm{I} - \mathrm{F})} \quad \mathrm{B} = (\mathrm{I} - \mathrm{F}) - \mathrm{M} \tag{2}$$

Efficiency of conversion of digested food (ECD): B = assimilated food used for growth (measured as prepupal biomass), I = total food offered, F = residues in boxes, M = metabolized food (based on mass balance) (Diener et al., 2009).

Sampling

Twenty larvae were removed every 3 days from each box and constituted a sample. Larval fresh, dry, and organic dry matter were determined, and the gut was extracted (for details, see section "Gut Removal and DNA-Extraction"). Simultaneously, 1 g aliquots of homogenously mixed residual matter (containing substrate residues, pine humus, and excrements) were taken from each box for the determination of physicochemical parameters.

Physicochemical Parameters

During the experiment, a temperature of 27°C and RH of 60% were maintained and constantly monitored. The residual-matter samples were suspended in 9 ml 0.0125 M $CaCl_2$ for the determination of ammonium (NH_4^+) and in 9 ml distilled water for pH measurement. The suspensions were agitated at 150 r/min for 60 min (Controlled Environment Incubator Shaker, New Brunswick Scientific, United States) and subsequently filtered through a folded filter (MN 615 1/4 150 mm, Macherey-Nagel™). The extracts were stored at 4°C overnight. Ammonium concentration was measured every 3 days until the termination of the experiment using the NANOCOLOR® Ammonium 50 Kit (Macherey-Nagel, Germany). The pH was measured using a Metrohm 744 pH Meter (Metrohm Inula, Switzerland).

Initial Substrate Characterization

Dry matter and water content were determined by calculating the difference between the fresh weight of samples and the sample weight after oven-drying at 105°C for 24 h. Organic dry matter was determined by combusting finely ground dried samples in a muffle furnace (Carbolite, CWF 1000) at 550°C for 5 h and determining the residual weight.

Aliquots of the oven-dried samples were used to determine total carbon and nitrogen with a Leco TruSpec CHN Elemental Determinator (Leco, United States) following the manufacturer's protocol.

As preparation for the measurement of unbound fat, substrate samples were weighed into 15-ml plastic tubes (Sarstedt, Germany), mixed with 5 ml water, and centrifuged at 11,000 × *g* for 20 min. The liquid phase was recovered, oven-dried at 105°C for 24 h, and the residues were weighed. The fat content was calculated as follows (3):

$$\text{Fat } (\%) = \frac{\text{liquid phase} - \text{sample dry weight}}{\text{sample fresh weight}} * 100 \quad (3)$$

To determine chemical oxygen demand (COD) and ammonium and protein content, 10-g aliquots of substrate samples were mixed with 25 ml of deionized water and vortexed for a few seconds. After a 30-min incubation at room temperature, the samples were shaken at 25°C and 120 r/min (Controlled Environment Incubator Shaker, New Brunswick Scientific, United States) for another 30 min before subsequent filtration (MN 615 1/4 150 mm folded filters, Macherey-Nagel™). The COD and ammonium concentration in the filtrates were measured using the NANOCOLOR® COD 1500 kit and Ammonium 50 kit (Macherey-Nagel, Germany), respectively. Protein content was determined by the Lowry (alkaline copper reduction) assay according to Noble and Bailey (2009).

The DNA extraction of the filtered substrate samples was carried out using the NucleoSpin® Soil-Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. The extracts contained 18.2 ± 10.3 ng μl^{-1} DNA and were stored at 4°C until sequencing.

Gut Removal and DNA-Extraction

A total of 36 gut samples were collected during the feeding experiments. They were extracted in triplicates at five time points ($Day_{0,3,9,15,21}$), whereas samples of Day_0 derived from the collective initial population before the separation of larvae and the introduction of new diets. At Day_{21}, only guts from GC and FV treatments were extracted as CF fed larvae already transitioned to prepupal stage at Day_{15}. Prior to the removal of the gut, the larvae were kept at −20°C for 30 min for devitalization. After disinfecting the thawed larval surfaces with 70% ethanol, a few millimeters of the anterior part were cut off using a sterile scalpel. The guts were pulled out using sterile forceps, transferred into a sterile microcentrifuge tube (minimum 0.05 g gut tissue/replicate), and snap frozen until further use.

After thawing the gut samples, the DNA was extracted using the NucleoSpin® Soil-Kit (Macherey-Nagel Germany) following the manufacturer's protocol with some modifications: one additional glass (Ø = 1.0 mm) and three additional silicon beads (Ø = 0.5 mm) were added to each bead tube and subsequently autoclaved. To each tube, 0.05 g of gut sample, 700 µl Buffer SL1 and 150 µl Enhancer SX were added before incubating them at 97°C and 400 r/min for 20 min. After this homogenization-step, the tubes were vortexed at high speed for 5 min and centrifuged at 11,000 × *g* for 2 min. Next, 150 µl Buffer SL3 were added and the tubes were vortexed for 5 s. After another incubation-step at 4°C for 5 min, the samples were centrifuged for 1 min at 11,000 × *g*. The steps for binding and washing were executed as described in the manual. The gut samples contained 22.8 ± 9.6 ng μl^{-1} DNA and were stored at 4°C until sequencing.

Next Generation Sequencing

Illumina MiSeq amplicon sequencing using the 2 × 250 base pairs paired-end approach was performed by Microsynth AG (Balgach, Switzerland) with universal bacterial/archaeal primers 515f (GTGCCAGCMGCCGCGGTAA) and 806r (GGACTACHVGGGTWTCTAAT) targeting the V4 region on the 16S rRNA of 36 gut samples (Caporaso et al., 2011). Samples of each time-point and treatment were analyzed in triplicates. Microsynth AG provided library preparation based on Nextera two-step PCR including purification and pooling, demultiplexing, removal of adaptors and primers, and stitching of trimmed reads.

Data Processing

Trimmed raw reads obtained from Illumina MiSeq amplicon sequencing were analyzed using mothur 1.40.0. (Schloss et al., 2009), and sequences were aligned to a V4-trimmed version of the SILVA reference database v.132 (Quast et al., 2013). Following the MiSeq SOP (Kozich et al., 2013), ambiguous bases were

removed and a maximum number of eight homopolymers was allowed to reduce sequencing errors. Chimeric sequences were removed using the chimera uchime algorithm implemented in mothur (Edgar et al., 2011). For the construction of the bacterial OTU dataset, all other lineages (eukaryotes, chloroplasts, mitochondria, archaea, unknown) were removed, and bacterial sequences were subsequently *de novo* clustered based on 97% similarity. For the taxonomic focus, closed-reference phylotype clustering on genus level was applied. For the archaeal dataset, all sequences assigned to archaea were filtered from the dataset and further *de novo* clustered on 97% similarity and genus level phylotypes. OTUs not present in at least five samples were removed from the datasets prior to statistical analysis.

All statistical tests were carried out at a significance level of $\alpha = 0.05$. Analyses of molecular variance (AMOVA) and homogeneity of molecular variance (HOMOVA) were performed in mothur on grouped triplicates of each sampling time-point, separated by treatment. One- and two-way non-parametric multivariate analysis of variance (NPMANOVA) were calculated in PAST (v.2.17c; Hammer et al., 2001) to detect significant differences between sampling time-points and substrates and to investigate the general influence of substrate and time variables on the spread of data. Alpha diversity measurements based on Chao1 species richness estimator and Shannon diversity index were conducted in R v.3.6.0 (R Core Team, 2018) using the phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2018) package. Mantel test (vegan package, Oksanen et al., 2018) was applied to confirm whether differences between distant matrices of physicochemical properties and microbial communities of substrates are statistically significant (number of permutations = 999). To investigate the spread of data and detect clusters of OTUs, non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity and fitted into two dimensions ($k = 2$) was done in PAST as well as R using the vegan package. To assure an appropriate representation of the data, only plots with a stress level < 0.2 were considered for further interpretation. The respective number of each replicate (1–3), the type of substrate, and the numeration of sample were included as explanatory variables. The core microbiome calculated by the get.coremicrobiome command in mothur was construed based on OTUs that are present in at least 80% of the samples. The sequences corresponding to the core OTUs were extracted from the dataset and manually assigned to the taxonomy using the standard nucleotide basic local alignment search tool (BLAST®, Altschul et al., 1990) and the search and classify function in SINA (Pruesse et al., 2012) using the latest SILVA database (v.132) and RDP II classifications (July 2017) (Cole et al., 2005). Figures were produced in R using the ggplot2 package (Wickham, 2016).

RESULTS

Substrate Characteristics, Maturation, and Microbial Colonization

The pH as well as protein and fat contents were higher in CF compared with the other two substrates (**Table 1**). In addition, dry matter content was markedly greater in CF than in GC and FV. The concentration of nitrogen compounds including ammonium and total nitrogen was approximately 50% lower in FV. After the substrates were administered to the feeding experiment, ammonium concentrations and pH therein were strongly affected by larval and microbial activity but showed no significant diet-dependent variation (NH_4^+: $p = 0.876$, pH: $p = 0.334$; **Figure 1**). The initial pH values (Day_0) of the substrates were different and ranged from 4.3 in FV to 6.51 in CF. Both ammonia concentration and pH increased during the experiment with the highest mean NH_4^+ at 128 mg l^{-1} in CF and highest pH of approximately 8 in GC. Ammonium concentrations peaked in FV treatments on Day_{15}, while they temporarily decreased in the other two treatments. The equations from linear trend-lines fitted to the curves delineated a stronger ammonium increase in CF treatments ($k = 6.763$) compared with the other two treatments (GC: $k = 4.079$, FV: $k = 3.121$), while the pH developed in a similar way (**Supplementary Figure S1**).

Despite different matrices, the microbial profiling of the three diets used in the feeding experiment resulted in comparable sequence numbers (CF: $8.6 \pm 0.5\ 10^4$ reads, FV: $8.02 \pm 0.90\ 10^4$ reads, and GC: $6.7 \pm 1.1\ 10^4$ reads). Data from initial diet samples before amendment into boxes were only subsampled and not further filtered due to the already low sequence numbers and were only used to deduce information about the prevalent microbiota in absence of larvae. In the CF and FV diets, a similar group of bacterial classes including Gammaproteobacteria (Enterobacteriaceae, *Morganella* sp.), Bacteroidia (*Dysgonomonas* sp.), and Bacilli (*Enterococcus* sp., *Lactococcus* sp.) account for most of the found sequences. In GC however, Bacilli (*Lactococcus* sp., *Lactobacillus* sp., *Weissella* sp.) and Gammaproteobacteria (Enterobacteriaceae, *Pantoea* sp.) were most abundant (**Figure 2**). The physicochemical characteristics and the microbial profile of substrate samples were compared using hierarchical clustering and significant differences between the matrices were confirmed by Mantel test ($p = 0.001$) (**Supplementary Figure S2**). CF and GC were more similar in their physicochemical properties in contrast to FV, but on the microbial community level, CF and FV showed fewer dissimilarities compared with GC.

Larval Development Based on Distinct Diets

The transitional phase before pupation was reached in the CF-treated replicates at Day_{15} and in the FV- and GC-treated replicates at Day_{21} (**Figure 3**). Larvae raised on CF outperformed the other two treatments, resulting in a higher biomass, more efficient degradation, and a higher pupation rate (**Table 1**). Larvae fed on CF showed a nearly three times higher gain in biomass compared with FV and GC, while the larvae raised on the two fresh substrates reduced and metabolized a higher proportion of substrate [$input_{total}$ - ($biomass_{output}$ + $residues_{total}$) = $metabolism_{total}$] but formed less biomass. Moreover, over the course of the feeding experiment, the efficiency of substrate degradation (WRI) was at least 15% higher, and the efficiency to convert

TABLE 1 | Substrate characteristics, mass balance, degradation parameters, and mean compositional indicators of larvae and pupae (n = 3).

		Diet					
		Chickenfeed		Grass-cuttings		Fruit/vegetables	
		Mean	SD	Mean	SD	Mean	SD
Substrate characterization	pH	6.1	0.02	4.8	0.02	4.0	0.02
	Dry matter [%]	90.1	0.2	21.1	0.8	9.4	0.1
	Chemical oxygen demand [g l^{-1}]	18.0	0.5	4.2	0.4	23.1	0.2
	Ammonium [mg l^{-1}]	9.2	1.4	11.2	1.7	5.1	0.3
	Total carbon [%]	41.0	0.7	39.8	0.6	46.5	0.1
	Total nitrogen [%]	3.0	0.1	3.0	0.1	1.2	0.0
	Protein [mg ml^{-1}]	3.6	0.2	0.6	0.1	2.0	0.1
	Fat [%]	3.6	0.2	0.7	0.1	2.9	0.2
Mass balance	Residues (Feed and feces)	38.6%	2.7%	50.2%	3.9%	40.0%	5.0%
	Larval and pupal biomass	28.0%	1.6%	9.6%	0.2%	12.2%	0.9%
	Larval metabolism	33.4%	2.5%	40.2%	3.9%	47.8%	5.0%
Degradation parameters	Waste reduction index (WRI)	1.7	0.1	1.2	0.1	1.5	0.1
	Efficiency of conversion of digested feed (ECD)	49.8%	2.0%	20.5%	1.8%	21.5%	1.8%
Larvae	Mean organic dry matter content	84.8%	1.4%	75.9%	0.5%	91.8%	1.8%
	Mean water content	67.9%	0.7%	71.4%	1.8%	66.0%	0.3%
Pupae	Pupation rate	94.1%	2.2%	70.7%	5.6%	83.8%	5.5%
	Mean organic dry matter content	83.8%	0.2%	76.2%	2.3%	88.7%	1.2%
	Mean water content	65.6%	0.6%	69.2%	2.3%	65.2%	1.1%

The pupation rate was calculated based on total initial larvae pro box less the larvae removed for sampling. SD = standard deviation.

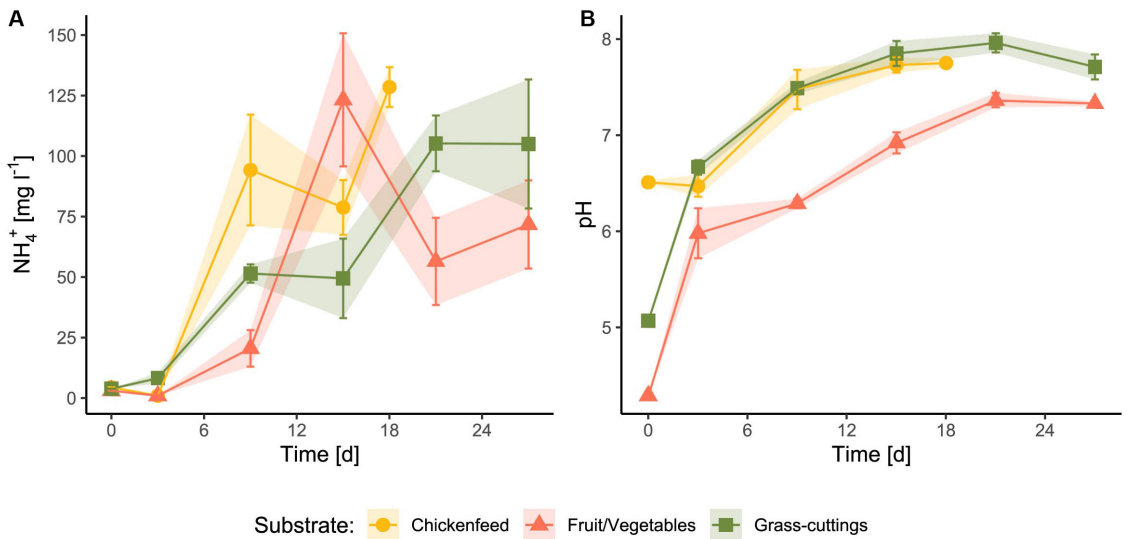

FIGURE 1 | Changes in ammonium concentration **(A)** and pH levels **(B)** of the substrate after administration as feed for larvae (n = 3). Due to the larval activity, the substrate was mixed with indigestible residues, larval feces, and shavings.

substrate to larval biomass (ECD) was at least twice as high in CF fed larvae than in those fed with FV or GC. The amounts of residual matter at the end of the experiment were similar in CF and FV and were around 10% higher in GC. Water content in larvae and pupae was stable at approximately 67% across all treatments, while the organic matter was similar only between larval and pupal stage within each respective treatment. Larvae from the GC treatment exhibited a 8–10% lower content of organic dry matter in both developmental phases.

Larval Gut Microbiome Dynamics and Identification of Core Community

The Illumina MiSeq read numbers of the homogenized larval gut samples ranged from 5.03 10^4 to 1.63 10^5 reads and

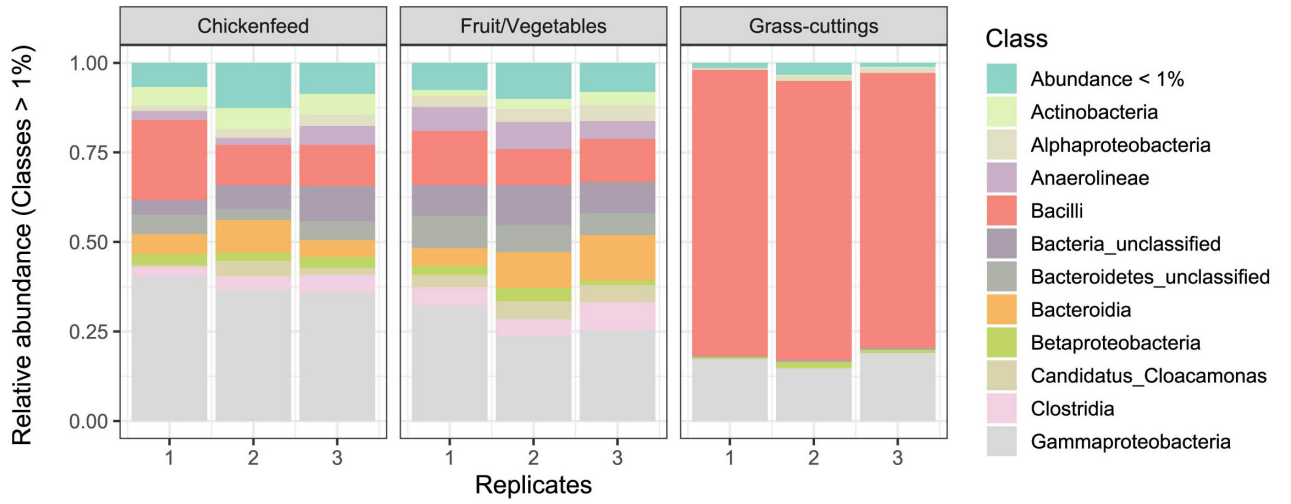

FIGURE 2 | Relative abundance of bacterial classes in diets used as treatments (n = 3). OTUs with a relative abundance lower than 1% were summarized. Note that the significance of differences between sample groups was tested at fine scale delinetation, i.e., the level of genus and 97% OTUs. The community composition on class level is shown for simplicity instead of a more detailed scale.

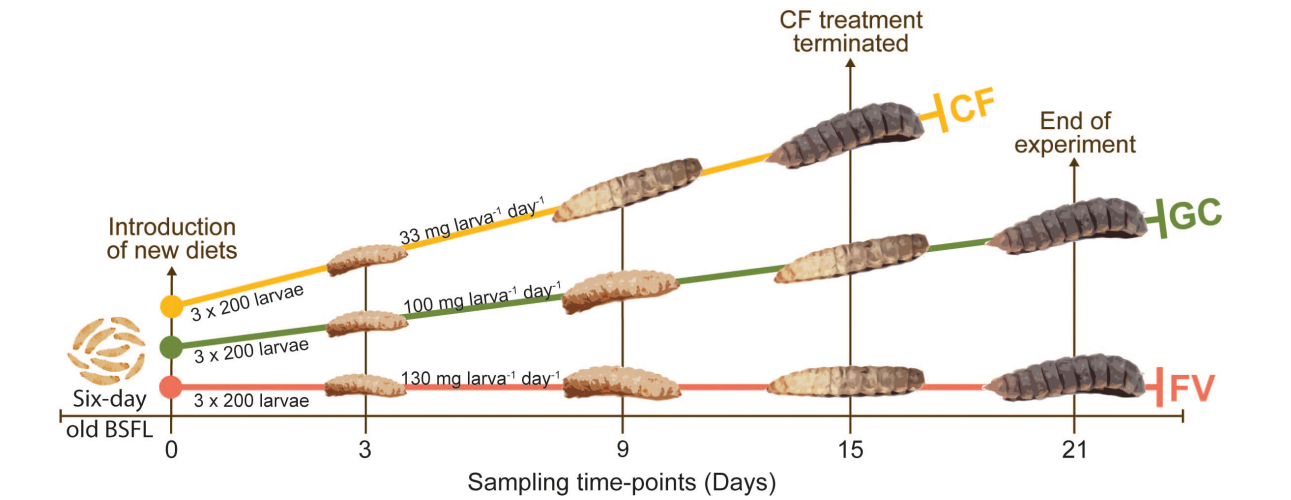

FIGURE 3 | Illustration of the experimental design. Larvae were collectively raised on chickenfeed (CF) for 6 days. At Day_0, 3 × 200 6-day old larvae were manually counted for each of the three diets and put into separate boxes. The two new diets (GC = grass-cuttings and FV = fruit/vegetable mix) were introduced to a triplicate of boxes each, while one triplicate was further fed with CF as control. The amount of feed per larva and day was adapted based on the respective organic and water content. CF treatment was terminated at Day_{15} as all larvae transitioned to prepupal stage. GC and FV treatment further continued until Day_{21}.

were subsampled to the smallest sample size (4.5 10^4 reads). Since removal of rare sequences from the bacterial data (OTUs with less than two sequences in all samples) did not suffice to denoise the subsampled dataset, a second step utilizing stronger filter removing all OTUs not present in at least five samples was applied.

Initial exploration of α-diversity (**Figure 4**) underlined a general impact of diet on the bacterial gut communities based on Chao1 species richness estimation (p = 0.002; **Figure 4A**) and Shannon diversity index (p = 0.012; **Figure 4B**). Both species richness and diversity decreased in CF-fed larvae between intermediate (Day_9) and advanced (Day_{15}) developmental stages. The same trend was found in community diversity of GC fed larvae, while species richness and diversity in FV treatments remained stable. Compositional deviations from the initial population (Day_0) were not uniform throughout the treatments, and neither of the two indices pointed out significant temporal changes in the larval gut microbiomes.

A more detailed comparison of gut communities was carried out by analyzing the β-diversity. Triplicate gut samples taken at the respective time-points from the three treatments were considered as separate groups and statistically compared with each other using AMOVA, HOMOVA, and one-way NPMANOVA. To allow a confident description of

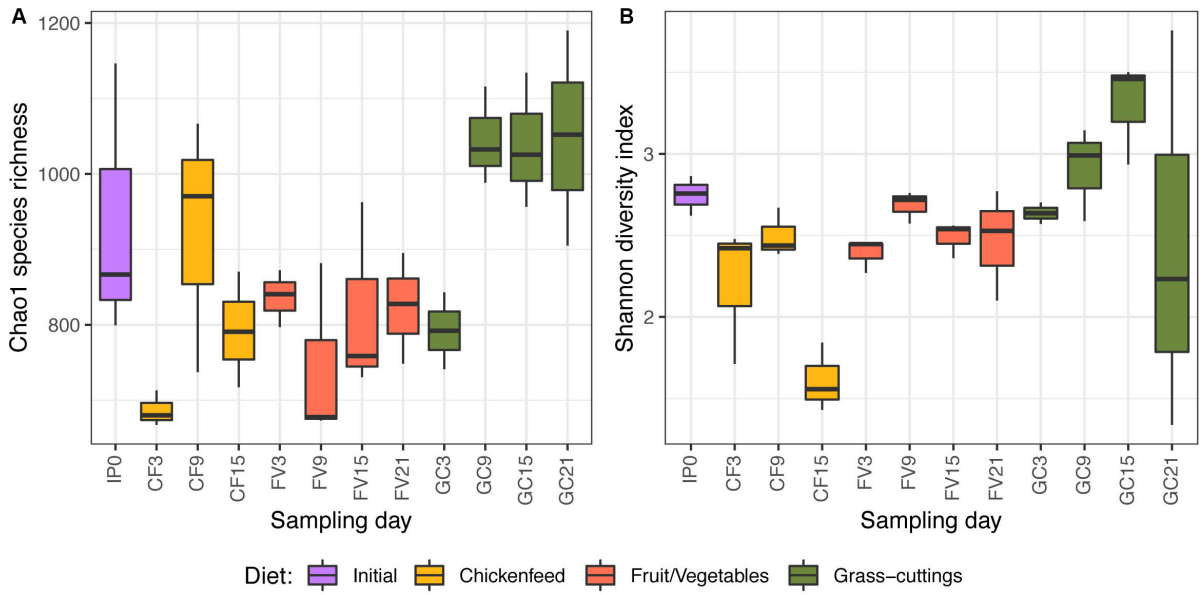

FIGURE 4 | Chao1 species richness **(A)** and Shannon diversity index **(B)** of bacterial gut communities over time (n = 3). The lower and upper limitations of each box define the 25th and 75th percentile and the median is represented by the black lines. Labels on the x-axis describe the treatment (IP = initial population, CF = chickenfeed, FV = fruit/vegetable mix, GC = grass-cuttings) and the day of sample collection (0–21) (e.g., IP0 = gut samples from the initial population collected at t_0).

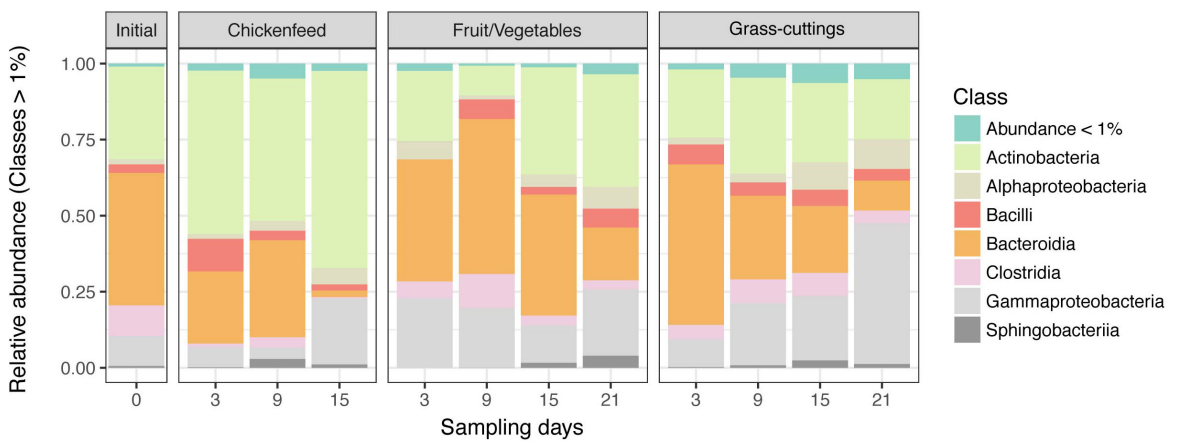

FIGURE 5 | Relative abundance of bacterial classes in gut samples collected during the development of Black Soldier Fly larvae. Operational taxonomic units (OTUs) with a relative abundance lower than 1% were summarized. Note that the significance of differences between sample groups was tested at fine scale delineation, i.e., the level of Genus and 97% OTUs. The community composition on class level is shown for simplicity instead of a more detailed scale. The microbiome composition of the initial population is derived from 6-day old larvae. All guts were analyzed in triplicates (n = 3).

relationships among variables, only statistical results confirmed by all three tests were considered valid. These pair-wise comparisons of groups resulted in no meaningful patterns in terms of statistically significant differences. Therefore, no substantial time- or diet-associated changes in the gut microbiota of the larvae can be deduced from the OTU data on this scale. Although the tests were able to detect single significant differences between some groups, they did not support biological deductions related to larval development and/or health. Predominant classes like Actinobacteria, Bacteroidia, and Gammaproteobacteria demonstrated time-dependent changes in their abundance. Especially in CF and GC, Gammaproteobacteria accumulated during larval development and reached a maximum at the last sampling time-point Day_{21}, whereas Bacteroidia sequence numbers declined (**Figure 5**; for clarity, the community composition is presented on class level. A finer resolution on genus level can be found in **Supplementary Figure S3**). In the CF and FV treatment, changes in abundances of Bacteroidia and Gammaproteobacteria could be partly connected to the ingested substrates, since both diets

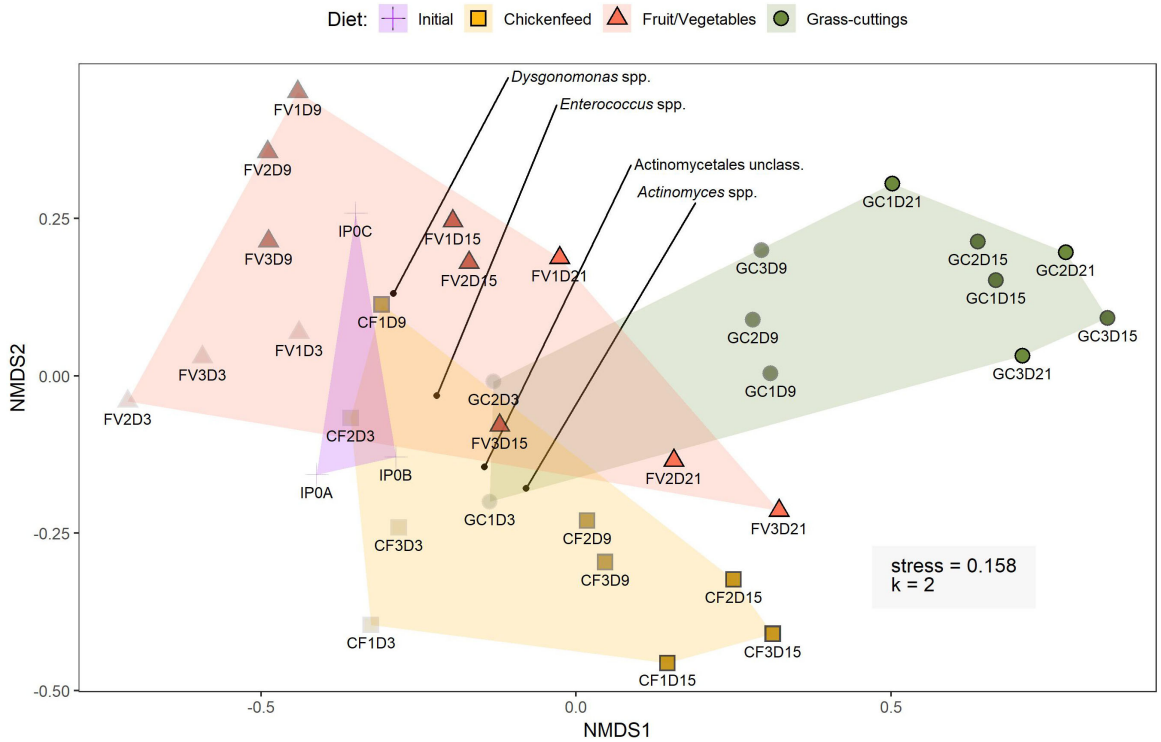

FIGURE 6 | Bray–Curtis based non-metric multidimensional scaling of operational taxonomic units (OTUs) present in at least five samples. OTUs were clustered based on 97% similarity. The gradual increase in shading of data points indicates the progression from early to late sampling time-points (light = early, dark = late). Initial samples represent the initial larval gut microbiome composition at t_0 before the introduction of new diets. The four black points indicate the distribution of core OTUs present in at least 80% of the samples. Sample labels consist of diet (IP = initial population, CF = chickenfeed, FV = fruit/vegetable mix, GC = grass-cuttings), the day of sample collection (0–21), and the replicate (1–3) (e.g., CF1D3 = chickenfeed fed larvae, first replicate collected at t_3).

contained up to 40% reads of Gammaproteobacteria and 12% reads of Bacteroidia.

In a broader perspective, NMDS analysis ($k = 2$, stress = 0.158) on bacterial sequence data with sampling time-point and diet as grouping factors led to strong overlaps between the clustered treatment groups (**Figure 6**). The findings from these analyses are consistent in both *de novo* clustered OTU and reference-based phylotype datasets. Gut samples from 6-day old larvae (IP0A-C), representing the initial gut community before being subjected to new diets, are located near the center of the overlap and form a starting point for the divergent development of gut communities. Following an interpretation of the Pareto principle adapted to the context of microbial community dynamics (Dejonghe et al., 2001), bacterial OTUs present in at least 80% of the gut samples across the three treatments were considered members of the core community (**Figure 7**). Their location in the NMDS is indicated by black dots (**Figure 6**). These OTUs accounted for 44% of all sequences and were identified as *Actinomyces* sp., *Dysgonomonas* sp., and *Enterococcus* sp. and an unclassified representative of the order Actinomycetales. When lowering the threshold to OTUs present in at least 60% of the samples, the aforementioned list was extended by *Morganella* spp. and unclassified Enterobacteriaceae where this congregation accounted for 78% of all sequences. The results from the analysis were further confirmed by extracting the sequences corresponding to the OTUs and manually identifying them by BLAST®, SILVA, and RDP database search, which resulted in congruent results.

Archaeal sequences were present in very low numbers and made up approximately 0.02% of all sequences; therefore, neither subsampling nor filtering steps were applied prior to identifying the present archaeal representatives. They mainly were affiliated to the classes of Methanomicrobia (35%; *Methanosarcina* sp., *Methanoculleus* sp., *Methanotrix* sp., *Methanocorpusculum* sp., *Methanoregula* sp., *Methanospirillum* sp., *Methanosphaerula* sp., *Methanolinea* sp.), Methanobacteria (18%; *Methanobrevibacter* sp., *Methanobacterium* sp., *Methanosphaera* sp.), Thermoplasmata (16%; *Methanomassiliicoccus* sp., *Thermoplasmata* uncl.), and Nitrosopumilales (11%; *Nitrosopumilus* uncl.).

DISCUSSION

The aim of this study was to investigate the influence of three distinct diets (CF, GC, and FV mix) on the development of BSF larvae (BSFL) and to assess temporal changes in the composition of their microbial gut communities. The observations during this 21-day timeframe clearly showed that the composition of the diet strongly affected the thriving of BSFL in terms of developmental progress and biomass gain but did scarcely affect the composition

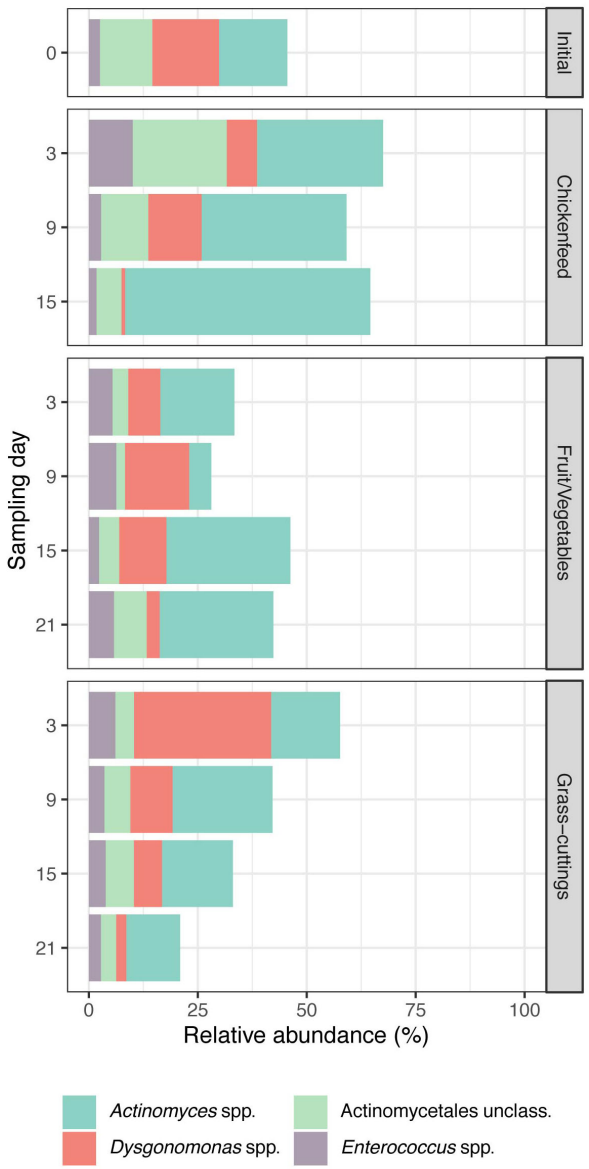

FIGURE 7 | Relative abundances of OTUs identified as bacterial core community in the guts of Black Soldier Fly larvae and present in at least 80% of all samples (n = 3). Together, they account for approximately half of the reads generated during sequencing.

of the gut microbiota. The use of 6-day old instead of freshly hatched larvae was a compromise between easier handling in counting and separation with the larvae still being in a susceptible stage of early development.

Balanced Substrates Yield Higher Larval Biomass Gain

Due to its consistent commercial quality, CF represents a very balanced substrate ideal for providing a stable supply of a well-defined spectrum of nutrients and, therefore, for the maintenance of a population as well as for the use as control substrate. In previous studies, the benefits of using such diets to raise BSFL under laboratory conditions and to provide a reference substrate for feeding experiments have been described (Diener et al., 2009; Gold et al., 2018). Although all three diets used in our study were administered in the same intervals and contained an equalized amount of organic matter and water, larvae fed with FV and GC lagged behind in biomass gain, pupation rate, and degradation efficiency (**Table 1**). This indicates that a similar moisture and organic content were not sufficient to provide a foundation for developmental success. A well-adjusted content of macro- and micronutrients in the organic fraction of the diet is necessary for high degradation efficiency and biomass gain (Beniers and Graham, 2019). Although organic wastes and different types of manure are known for their heterogeneous composition, a guideline of optimal feeding rates, as defined by Diener et al. (2009) for standard fodder, should be advocated also for waste-derived substrates.

Diminished degradation, as also highlighted by a lower WRI, led to greater amounts of residues (up to 12% in GC) due to a higher content of recalcitrant components like cellulose especially found in grass (Rehman et al., 2017). This led to a reduced pupation rate (>90% in CF compared to 70% in GC) and a lower proportion of organics in both larvae and pupae when kept on a plant-based diet. On the contrary, larvae fed with FV exhibited the highest turnover of substrate. Compared with CF, FV diet ended up in similar amounts of residues suggesting an easy digestibility, although a lesser proportion was converted to biomass and was rather used for metabolism (**Table 1**). Depending on the amount of accumulating insect frass and residues (mixture of undigested substrates, excrements, and larval shavings), downstream treatment thereof in anaerobic digestion, pyrolysis for biochar production, or traditional composting could add value to insect rearing on a larger scale (Lalander et al., 2018; Yang et al., 2019). A similar sequential operation yielding an increased economic value from agricultural waste treatment by BSFL with subsequent composting of residues has been investigated by Zhu et al. (2012), while the direct use of the frass fraction as substitute to mineral fertilizer or vermicompost (Sarpong et al., 2019) has also been proposed as a viable option (Choi et al., 2009).

Both waste-related diets (GC and FV) could not keep up with the larval development and the biomass yield in CF. The high degradability and high protein content of CF rapidly affected the larval environment. Ammonia concentrations (**Figure 1A**) in CF-treated boxes increased fastest and were accompanied by a higher initial pH due to the innate properties of the raw feed (**Figure 1B**). Especially ammonium plays an important role during BSFL rearing, since it largely derives from organic nitrogen excreted by larvae and can further be transformed by associated gut microbes (Green and Popa, 2012). Partly due to this efficient microbial assimilation of nitrogen, emissions of nitrogen in form of greenhouse gases have been shown to be much lower in insect rearing compared with livestock breeding and traditional composting (Oonincx et al., 2010; Mertenat et al., 2019).

Based on hierarchical clustering, the characteristics of the diets varied, and they group differently at the physicochemical and

microbial level. CF and GC were more similar to each other when compared by their physicochemical properties (**Supplementary Figure S1A**), while GC formed the outgroup on microbial community level (**Supplementary Figure S1B**). This is further illustrated in **Figure 2**, where especially Gammaproteobacteria made up for the major share of reads found in CF and FV but were outcompeted by Bacilli, mostly *Lactococcus* sp., *Lactobacillus* sp., and *Weissella* sp., in GC substrates.

Jiang et al. (2019) found that microbial communities in the added substrates were reshaped by gut bacteria excreted by larvae, leading to a similar microbiome in larval guts and their environment over time, while compositional changes within the gut were mainly induced by the ingestion of fresh substrates. In our trial, the composition of microbial communities was only determined in the fresh substrates to infer its direct relationship to the larval gut microbiome, and no monitoring of microbial substrate colonization in boxes during the experiment was carried out.

Diet Drives Development of Larval Gut Microbiomes

Compared with direct effects on larval growth, diet effects were comparably small when looking at the larval gut microbiota. However, alpha diversity indicated a dietary impact on the gut microbiota both when based on Chao1 species richness estimation (**Figure 4A**) and Shannon diversity (**Figure 4B**). Not only was the microbial community in the GC diet considerably different from the other diets, but soon after its introduction as a diet it also favored the perpetuation of a comparatively higher species richness. Both GC and CF fed larvae exhibited reduced species diversity before pupation, possibly connected to a physical restructuring in the gut (Bruno et al., 2019).

The relative abundance of bacterial phyla described in this study were in line with previous similar studies (Jeon et al., 2011; Zheng et al., 2013) determining Actinobacteria, Bactoroidetes, Firmicutes, and especially Alpha- and Gammaproteobacteria within the phylum of Proteobacteria to be the major constituents in the BSFL gut (**Figure 5**). Comprehensive time- or diet-dependent changes were not detected at the β-diversity level. Sparse statistically significant dissimilarities in community compositions in larval guts from different time-points and treatments that were supported by all applied statistical tests (AMOVA, HOMOVA, one-way PERMANOVA) did not yield any meaningfully directed biological explanatory power. NMDS was used to spatially map samples in two dimensions and although previous statistical confirmation of dietary and temporal dynamics were weak, gut samples from more advanced developmental stages strayed further away from the state of initial gut microbiomes (**Figure 6**). However, bacteria already present in the gut seemed to compensate for the low microbial abundance in the fresh substrates used in this study. Thereby, the formative impact on the larval gut microbiomes was limited compared with more extensively colonized substrates often used in similar feeding experiments, such as organic wastes, animal manure, or human feces (Bruno et al., 2018; Cai et al., 2018).

The resilience of the resident gut microbiome in larvae from the three treatments could be connected to the age of the larvae when they were first exposed to the new diets. Using 16S rRNA pyrosequencing, Jeon et al. (2011) were able to measure distinguishing effects of various diets on the respective microbial communities in the gut of larvae that were exposed to different feeds directly after hatching. To increase the number of hatched eggs and to standardize the condition of hatched animals, the two new diets used as treatments were introduced into the experiments on the sixth day of the larval development, while a control group was further fed with CF. In this study, the 6 days of CF prior to exposure to new diets seem enough to allow for the establishment of resilient microbial communities through a "priming effect," that is, the establishment of an indigenous population of gut bacteria that is not readily susceptible to colonization by allochthonous microorganisms taken up with the two newly introduced diets (Rillig et al., 2015), despite their measurable and different microbial fingerprint.

Due to the limitations of 16S rRNA amplicon sequencing (Poretsky et al., 2014), only analysis down to the genus level was performed, which could have left differences between treatments on lower taxonomic levels undetected. Other, higher resolution methods, such as whole genome shot-gun sequencing or metabolomic investigations could uncover dietary effects at strain and even functional gene level (Xia et al., 2017; Chen et al., 2018). Moreover, the gut microbiome of wild BSFL has not been studied yet, thereby limiting the possibility of comparing laboratory populations with fly populations accustomed to a changing diet over numerous generations. Larvae deriving from a lab population, and therefore, adapted to a consistent diet, could be less perceptive for the enrichment of transient microorganisms that found their way into their gut, where an observation of dietary effects over a timespan of multiple generations of flies could be necessary to illustrate a more comprehensive insight in gut microbiome dynamics. Microbial gut colonizers are crucial for the thriving of many insects suggesting that mechanisms have to be in place that avoid colonization by pathogens and favor the preservation of vital functional properties (Bahrndorff et al., 2016).

A Microbial Core Community Provides Metabolic Foundation in the Gut

De Smet et al. (2018) and Wynants et al. (2018) recently proposed that, based on several bacterial OTUs found across larval guts exposed to various waste treatments, BSFL establish a core bacterial microbiome. However, they were still susceptible to microbial colonization coming from their surroundings, which was further described as "house flora." The extensive repertoire of pathogen defense mechanisms inherent to BSFL allows the thriving of larvae also in hazardous habitats and is most likely in close relationship with gut-residing microbes (Vogel et al., 2018). To guarantee the dietary flexibility and availability of antimicrobial peptides in different environments, a stable group of bacteria able to provide such services needs to be present within the larvae. From our largely diet-independent community, we identified a core community that was present in at least

80% of the gut samples. Not only very few OTUs fulfilled this requirement but they also accounted for approximately half of all analyzed sequences (**Figure 7**). *Actinomyces* sp., *Dysgonomonas* sp., *Enterococcus* sp., and another unclassified representative of the order of Actinomycetales made up the core community and are well known for the role they play in (insect) guts (Dillon and Dillon, 2004; Engel and Moran, 2013; Dietrich et al., 2014).

The filamentous, high GC, Gram-positive, and mostly facultative anaerobic bacterium *Actinomyces* sp. can degrade a broad spectrum of organic material including lignin and chitin (Wang et al., 2014). It is often found as commensal in the gut of various animals, and the production of a variety of antibiotics inhibiting the growth of other microorganisms additionally represents a benefit for the larvae (Franke-Whittle et al., 2009; Hanning and Diaz-Sanchez, 2015).

Dysgonomonas sp. has mostly been known for its key role in the gut of termites during the degradation of recalcitrant lignocellulose but was also found in high abundances in the gut of BSFL (Yang et al., 2014; Sun et al., 2015). Bruno et al. (2019) highlighted the significance of *Dysgonomonas* in the digestion of complex polysaccharides, which was further emphasized by Jiang et al. (2019) on a functional level, indicating that *Dysgonomonas* sp. in the gut of BSFL is positively correlated with genes for sulfate, carbohydrate, and nitrogen metabolism. Shelomi et al. (2020) recently assessed the effect of post-production and post-consumer wastes on larval gut microbiota and pointed out multiple OTUs assigned to *Dysgonomonas* sp. as representatives of a core community, since they were found in the larval guts irrespective of the waste type. From a metagenomic analysis of the BSFL gut, the origin of a new α-galactosidase gene that makes it possible to break up α-galactoses abundant in non-digestible plant carbohydrates was traced back to a specific *Dysgonomonas* strain (Lee et al., 2018). This anaerobic bacterium is also able to contribute to the biodegradation of pharmaceutical products like ciprofloxacin when appearing in a consortium with other bacteria like, for instance, *Actinomyces* sp., underlining the potential for yet to discover biotechnological applications (Martins et al., 2018).

As a typical commensal gut colonizer, Gram-positive and facultative anaerobe *Enterococcus* sp. is involved in making nutrients accessible for the host and contributing to gut health (Dubin and Pamer, 2017). In other insects like the greater wax moth (*Galleria mellonella*), *Enterococus* dominates the microbial communities in the larval gut and supports their host by providing immunity-related antimicrobial peptides (Krams et al., 2017).

Considering the early stabilization of this core consortium of highly abundant microorganisms and the broad functional competences coming from its representatives, this specific gut microbiome in BSFL can be of great advantage when exposed to various nutrient-poor or even contaminated environments and for stable industrial production. Wynants et al. (2018) compared the BSFL gut microbiota from large-scale cycles and found that bacterial community composition and abundances generally differ among facilities but may in addition also vary between batches produced in those locations. Despite these variations linked to site-specific features including diverse diets, they identified a small group of bacterial genera shared across multiple rearing facilities. Besides *Pseudomonas* sp. and *Providencia* sp., this group also included *Enterococcus* sp. and *Morganella* sp., which our study pointed out as core members (found in 80 and 60% of the samples, respectively). The presence of these two genera further supports our approach to identify core members of the larvae's gut microbiota.

Apart from that, the exposure to substrates that knowingly carry a high microbial bioburden such as organic animal wastes might represent a stronger driving force in shaping the larval gut microbiota than the low bioburden diets used in this study. In a comprehensive investigation, Zhan et al. (2019) identified 16 bacterial phyla of OTUs present in BSFL guts irrespective of having been fed food waste, poultry, dairy, or swine manure. The high abundances of Bacteroidetes and Proteobacteria in larval guts across diets and time coincide with our findings, since *Dysgonomonas* sp., *Actinomyces* sp., and the unclassified Actinomycetales identified in our analysis as members of the gut core community also belong to these two phyla. The absence of Firmicutes in our core community, however, may relate to the predominant function they take over in the degradation of animal manure. It is plausible that this phylum is not *a priori* inherent to BSFL but instead gets acquired when the larvae are exposed to manure.

If these observations are seen from a practical perspective, BSFL still demonstrated a great ability in degrading a broad variety of organic compounds, including various waste substrates, with the only two outputs being larval biomass and organic fertilizer in form of substrate residues and frass (Cickova et al., 2015). So far, no indication of the presence of a fungal core community has been brought forward since this kingdom has been largely neglected in microbiome studies. Observations of Boccazzi et al. (2017), however, suggest that diet is the sole driver of the BSFL gut mycobiome. Experiments sounding out the optimum amount of organic waste combined with commercially available well-balanced feeds such as CF provide room for improving the biological parameters relevant for large scale rearing (e.g., biomass gain, WRI, ECD, developmental time) and waste treatment. Even if the composition of wastes is inconsistent, a steadily available fraction of uniform quality CF in the diet could stabilize the degradation processes and boost the functional performance of the microbiome and the larva itself through the priming of the gut environment.

CONCLUSION

This investigation sets an additional cornerstone in characterizing the gut's microbiome and core community of *H. illucens* larvae, which are known for their ability to degrade a vast array of organic substances. Especially the industrial use of BSFL for animal feed production or waste management could profit from better controllable growth characteristics and a fast adaptation to changing diet compositions to guarantee consistent biomass gain and substrate degradation. Constant degradation rates under stable environmental conditions could allow adapting the number of larvae to the amount of substrate

to be degraded, inhibiting the molding and/or dehydration of substrates. Fresh substrates like GC and FV with a low bioburden (compared with, e.g., matured biodegradable wastes) had no significant shaping influence on the microbial gut communities of 6-day old larvae, whereas BSFL rather maintain a stable gut microbiome already early in their development. This community is dominated by a simple group of highly abundant bacterial species (*Actinomyces* sp., *Dysgonomonas* sp., *Enterococcus* sp., unclassified Actinomycetales) known to play key roles in the degradation of organic substances.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the European Nucleotide Archive (PRJEB33904).

AUTHOR CONTRIBUTIONS

AW and TB conceived the study design. AW performed the experiments together with CH, who also maintained the BSF population. FS, BS-S, and WA assisted in BSF maintenance. TK conducted the statistical and bioinformatical analyses and wrote the manuscript. BS, FS, BS-S, WA, and HI contributed scientific comments to the experimental design and the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00993/full#supplementary-material

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REVIEW
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Wolbachia pipientis Associated With Tephritid Fruit Fly Pests: From Basic Research to Applications

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Members of the true fruit flies (family Tephritidae) are among the most serious agricultural pests worldwide, whose control and management demands large and costly international efforts. The need for cost-effective and environmentally friendly integrated pest management (IPM) has led to the development and implementation of autocidal control strategies. These approaches include the widely used sterile insect technique and the incompatible insect technique (IIT). IIT relies on maternally transmitted bacteria (namely *Wolbachia*) to cause a conditional sterility in crosses between released mass-reared *Wolbachia*-infected males and wild females, which are either uninfected or infected with a different *Wolbachia* strain (i.e., cytoplasmic incompatibility; CI). Herein, we review the current state of knowledge on *Wolbachia*-tephritid interactions including infection prevalence in wild populations, phenotypic consequences, and their impact on life history traits. Numerous pest tephritid species are reported to harbor *Wolbachia* infections, with a subset exhibiting high prevalence. The phenotypic effects of *Wolbachia* have been assessed in very few tephritid species, due in part to the difficulty of manipulating *Wolbachia* infection (removal or transinfection). Based on recent methodological advances (high-throughput DNA sequencing) and breakthroughs concerning the mechanistic basis of CI, we suggest research avenues that could accelerate generation of necessary knowledge for the potential use of *Wolbachia*-based IIT in area-wide integrated pest management (AW-IPM) strategies for the population control of tephritid pests.

Keywords: insect control, symbiosis, endosymbiont, incompatible insect technique, cytoplasmic incompatibility

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INTRODUCTION

The Economic Importance and Management of Tephritid Pest Species

Flies in the family Tephritidae (Diptera) include some of the world's most important agricultural pests. The family is comprised of ∼4,900 described species within 481 genera, of which six (*Anastrepha*, *Bactrocera*, *Ceratitis*, *Dacus*, *Rhagoletis*, and *Zeugodacus*) contain ∼70 major pest species (White and Elson-Harris, 1992; Norrbom, 2004a,b, 2010; Mengual et al., 2017). Pest tephritids represent an enormous economic cost because they cause direct losses to a diversity of crops (fruits, vegetables, and flowers) (White and Elson-Harris, 1992). Furthermore, they hamper the development of agriculture in numerous countries, due to the strict quarantines imposed by countries importing affected crops, and to the huge costs associated with efforts aimed at prevention, containment, suppression, and eradication.

To prevent or minimize the harmful effects of tephritid pests, growers must comply with health and safety standards required by the market, applying an area-wide management approach involving chemical, biological, cultural, and autocidal control practices (Reyes et al., 2000; Enkerlin, 2005). Autocidal refers to methods that use the insect to control itself, by releasing insects that are sterile or induce sterility upon mating with wild insects in the next or subsequent generations (Black et al., 2011; Leftwich et al., 2014; Handler, 2016). Autocidal strategies include the sterile insect technique (SIT) (Knipling, 1955; Hendrichs and Robinson, 2009); one of the most widespread control methods used against fruit flies (reviewed in Dias et al., 2018). SIT relies on the mass-rearing production, sterilization and recurrent release of insects (preferentially males) of the targeted species. Sterilization is typically attained by radiation (Bakri et al., 2005), in a way that does not impair male mating and insemination capabilities. Wild females that mate with sterilized males lay unfertilized eggs. At the appropriate sterile:wild (S:W) ratio, the reproductive potential of the target population can be reduced (Knipling, 1955; Klassen and Curtis, 2005; Cáceres et al., 2007). Historically, at least 28 countries have used the SIT at a large-scale for the suppression or eradication of pests (Hendrichs et al., 1995, 2005; Suckling et al., 2016). SIT has been applied successfully for several non-tephritid insect pests including the New World screw worm *Cochliomyia hominivorax* (Coquerel), several species of tsetse fly (*Glossina* spp.), the codling moth *Cydia pomonella* (L.) (reviewed in Robinson, 2002b; Dyck et al., 2005; Bourtzis and Robinson, 2006), and mosquitoes (Benedict and Robinson, 2003; Lees et al., 2015). Successful SIT programs as part of Area-wide Integrated Pest Management (AW-IPM) strategies have also been implemented for several tephritids: *Ceratitis capitata* (Wiedemann); *Anastrepha ludens* (Loew); *Anastrepha obliqua* (Macquart); *Zeugodacus cucurbitae* (Coquillett); *Bactrocera dorsalis* Hendel; and *Bactrocera tryoni* (Froggatt) (Enkerlin, 2005; Hendrichs et al., 2005; Klassen and Curtis, 2005; Cáceres et al., 2007). SIT is currently being developed for three additional tephritid species: *Anastrepha fraterculus* (Wiedemann) (Cladera et al., 2014); *Dacus ciliatus* Loew (Rempoulakis et al., 2015) and *Bactrocera tau* (Walker) (Du et al., 2016). The advantages of the SIT over other pest control approaches (e.g., use of pesticides) are that it is species-specific and environmentally friendly (Lees et al., 2015; Bourtzis et al., 2016), and resistance is less likely to evolve (but see Hibino and Iwahashi, 1991; McInnis et al., 1996).

Another autocidal strategy where mating between mass-reared and wild insects can be used to suppress pest populations is the incompatible insect technique (IIT); coined by Boller et al. (1976). The earliest successful pilot application of IIT was in *Culex* mosquitoes (Laven, 1967), and interest in applying it to mosquitoes has resurged in recent years (reviewed in Ross et al., 2019b). IIT also relies on the principle of reducing female fertility, but utilizes endosymbiotic bacteria instead of radiation, to induce a context-dependent sterility in wild females. It is based on the ability of certain maternally inherited bacteria (mainly from the genus *Wolbachia*) to induce a form of reproductive incompatibility known as cytoplasmic incompatibility (CI; explained in the section below). Herein we review the current knowledge on taxonomic diversity of *Wolbachia*-tephritid associations and their phenotypic consequences, and identify gaps in knowledge and approaches in the context of potential application of IIT, alone or in combination with SIT, in AW-IPM programs to control tephritid pests.

The Influence of *Wolbachia* on Host Ecology

Insects and other arthropods are common hosts of maternally inherited bacteria (reviewed in Duron and Hurst, 2013). These heritable endosymbionts can have a strong influence on host ecology. Typically, such vertically transmitted bacteria are vastly (or fully) dependent on the host for survival and transmission. Certain associations are obligate for both partners, and generally involve a nutritional benefit to the host. Other heritable bacteria are facultative, with such associations ranging from mutualistic to parasitic from the host's perspective. Among these, *Wolbachia* is the most common and widespread facultative symbiont of insects and arthropods (Hilgenboecker et al., 2008; Zug and Hammerstein, 2012; de Oliveira et al., 2015; Weinert et al., 2015).

Wolbachia is a diverse and old genus (possibly older than 200 million years; Gerth and Bleidorn, 2016) of intracellular Gram-negative Alphaproteobacteria (within the order Rickettsiales) associated with arthropods and filarial nematodes. *Wolbachia* cells resemble small spheres 0.2–1.5 μm, occur in all tissue types, but tend to be more prevalent in ovaries and testes of infected hosts, and are closely associated with the female germline (reviewed by Harris et al., 2010; see also Sacchi et al., 2010). *Wolbachia* is estimated to infect 40–66% of insect species (Hilgenboecker et al., 2008; Zug and Hammerstein, 2012; de Oliveira et al., 2015; Weinert et al., 2015). Within a species or population, the infection prevalence of *Wolbachia* can be quite variable over space (e.g., Kriesner et al., 2016) and time (e.g., Turelli and Hoffmann, 1991, 1995).

The most commonly documented effects of *Wolbachia* on arthropod hosts fall under the category of reproductive parasitism, which involves manipulation of host reproduction

to enhance symbiont transmission and persistence, in general by increasing the relative frequency of *Wolbachia*-infected vs. uninfected females. Females are typically the sex that can transmit *Wolbachia* and other heritable bacteria, although rare exceptions exist (Hoffmann and Turelli, 1988; Moran and Dunbar, 2006; Chafee et al., 2010). *Wolbachia* employs all four types of reproductive manipulation (reviewed by Werren et al., 2008; Saridaki and Bourtzis, 2010; Schneider et al., 2011). Feminization converts genetic males into functional females, and occurs in the orders Hemiptera, Lepidoptera, and Isopoda. *Wolbachia*-induced parthenogenesis occurs in haplo-diploid hosts (e.g., Acari, Hymenoptera, and Thysanoptera), where unfertilized eggs, which would otherwise develop into males, develop into females. Male killing causes death of infected males to the presumed advantage of surviving infected female siblings, and occurs in Coleoptera, Diptera, Lepidoptera, and Pseudoscorpionida. Cytoplasmic incompatibility (CI) (Yen and Barr, 1971) prevents infected males from producing viable offspring upon mating with females lacking *Wolbachia* (or a compatible strain of *Wolbachia*; see below; **Figure 1**). CI is the most commonly reported *Wolbachia*-induced reproductive phenotype, and is found in Acari, Coleoptera, Diptera, Hemiptera, Hymenoptera, Isopoda, Lepidoptera, and Orthoptera.

Cytoplasmic incompatibility was discovered almost half a century ago (Yen and Barr, 1971), but its mechanism has not been fully elucidated. A useful conceptual model to understand the observed patterns of CI is "mod/resc" (Hurst, 1991; Werren, 1997). It postulates that *Wolbachia* has two functions: *mod* (for modification), which acts as a toxin or imprint of the male germline; and *resc* (for rescue), which acts as an antidote. The *mod* function acts on the nucleus in the male germline, before *Wolbachia* are shed from maturing sperm (Presgraves, 2000). When a sperm nucleus affected by *mod* enters the egg of an uninfected female, this nucleus encounters problems such as delays in DNA replication and cell-cycle progression, leading to embryo death. In contrast, if the appropriate *resc* ("the antidote") function is active in the egg, the defect caused by *mod* in the sperm is rescued, and the embryo proceeds through normal development. In the case of unidirectional CI (uni-CI), all or some of the eggs from uninfected females that are fertilized by sperm from *Wolbachia*-infected males (the "CI cross") fail to develop (**Figure 1A**). *Wolbachia*-infected females are compatible with uninfected males, and thus have a reproductive advantage over their *Wolbachia*-uninfected counterparts. Consequently, above a certain threshold of *Wolbachia* infection frequency in a host population, *Wolbachia* frequencies can rapidly increase to a stable equilibrium frequency. When CI is strong (e.g., all embryos from the CI cross fail), fitness costs of *Wolbachia* are low, and maternal (vertical) transmission is high, the threshold *Wolbachia* frequency to achieve invasion can be close to zero, and the stable equilibrium frequency can be close to 100% (Caspari and Watson, 1959; Turelli and Hoffmann, 1999; Rasgon, 2008). Bi-directional CI (bi-CI) results from crosses involving two different (incompatible) *Wolbachia* strains (**Figure 1B**). Crosses between females and males infected with the same or compatible *Wolbachia* strains are viable. Under bi-CI between two *Wolbachia* strains with equivalent fitness effects on a host, the infection

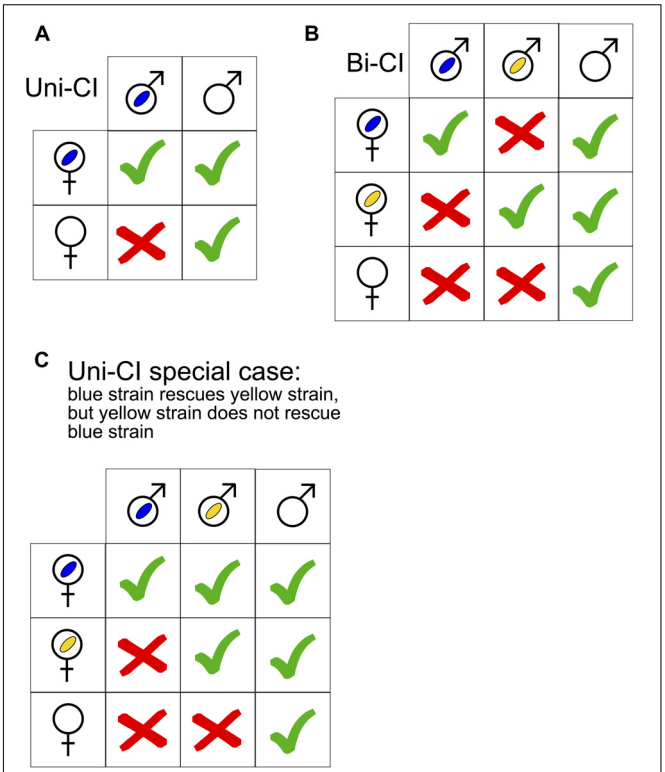

FIGURE 1 | (A,B) Qualitative illustration of uni- and bidirectional cytoplasmic incompatibility (CI) on the basis of the *Wolbachia* infection status of the parent generation. Empty male and female symbols signify absence of *Wolbachia*. Blue and yellow ovals represent distinct *Wolbachia* strains. Green tick marks = Successful offspring production. Red crosses = no offspring production. **(C)** A special case of unidirectional incompatibility in which one *Wolbachia* strain (see text) can rescue another strain (i.e., the yellow one), but not vice versa.

frequency of an introduced strain must exceed 50% to achieve invasion (Rousset et al., 1991; Dobson et al., 2002). Special cases of uni-CI and bi-CI patterns can occur. For example, a strain may not induce CI, but is able to rescue the defect caused by a different strain (**Figure 1C**) (Zabalou et al., 2004a).

Several recent breakthrough studies have collectively identified *Wolbachia*-encoded genes (of viral origin) that contribute to the induction and rescue of CI (Beckmann et al., 2017; LePage et al., 2017; Bonneau et al., 2018, 2019; Lindsey et al., 2018; Shropshire et al., 2018; Beckmann et al., 2019c; Chen et al., 2019; Shropshire and Bordenstein, 2019). *Wolbachia*-encoded genes that rescue CI are labeled as *cifA*, *cidA*, or *cindA*, depending on whether they rescue a defect caused by deubiquitylase (d), nuclease (n), both (nd); "f" is used by certain authors and/or when the nature of the defect is unknown (see Beckmann et al., 2019a,b; Shropshire et al., 2019). In CI-inducing *Wolbachia* strains, each of the above genes occurs upstream of a gene (its "cognate") similarly labeled, but with a "B" replacing the "A" (i.e., *cifB*, *cidB*, or *cindB*, respectively) that seems to function as a toxin. Certain *Wolbachia* strains have more than one "A–B" pair, and the combination of these is consistent with patterns of incompatibility in *Drosophila* (LePage et al., 2017) and *Culex*

(Bonneau et al., 2018). Knowledge accrued to date indicates that more than one *Wolbachia*-encoded mechanism of CI exists, and thus, information on the genes encoded by *Wolbachia* genomes can help predict expected patterns of incompatibility among strains that have not been experimentally characterized.

In addition to its reproductive phenotypes on arthropods, *Wolbachia* engages in obligate mutualistic interactions with filarial nematodes (Werren et al., 2008) and with members of five insect orders (reviewed in Zug and Hammerstein, 2015). As a facultative symbiont, *Wolbachia* can provide direct fitness benefits to its insect hosts by influencing development, nutrition, iron metabolism, lifespan, and fecundity (Dean, 2006; Aleksandrov et al., 2007; Weeks et al., 2007; Brownlie et al., 2009; Ikeya et al., 2009; Kremer et al., 2009; Newton and Rice, 2020), and most notably, by conferring resistance or tolerance to pathogens, particularly single-stranded RNA viruses (Hedges et al., 2008; Teixeira et al., 2008; Moreira et al., 2009). The interference of *Wolbachia* with the replication and transmission of certain viruses, along with its ability to spread in populations via CI, form the basis of several population replacement programs (reviewed in Ross et al., 2019b; Chrostek et al., 2020). *Wolbachia* in *Drosophila* appears to confer an additional fitness benefit in the form of increased recombination (Bryant and Newton, 2019; Singh, 2019).

Certain host-*Wolbachia* combinations incur fitness costs to the host, beyond reproductive parasitism, including reduced longevity, sperm competitive ability, and fecundity, as well as higher susceptibility to natural enemies (Hoffmann et al., 1990; Min and Benzer, 1997; Snook et al., 2000; Champion de Crespigny and Wedell, 2006; Fytrou et al., 2006; Vasquez et al., 2011; Suh et al., 2017; Sumida et al., 2017). Similarly, certain host-*Wolbachia* combinations may potentially enhance pathogen-vectoring capacities (Hughes et al., 2012b; Baton et al., 2013; Dodson et al., 2014; Murdock et al., 2014). *Wolbachia* has been reported to influence positively or negatively numerous aspects of their host's behavior including sleep, learning and memory capacity, mating, feeding, thermal preference, locomotion, and agression (reviewed by Bi and Wang, 2019; Wedell, 2019).

METHODS TO STUDY *Wolbachia*

Methods to Assess *Wolbachia* Infection Status

For purposes of this review, we consider a host species or population as "infected" with *Wolbachia*, even if the infection is transient or found at low titer. *Wolbachia*, and most cytoplasmically transmitted endosymbionts, are fastidious to culture outside host cells, such that their study typically relies on culture-independent methods. A recommended flow-chart of steps is depicted in **Figure 2**. The most utilized approach to date for identifying hosts infected with *Wolbachia* is through PCR screening of *Wolbachia* genes in DNA extracts of hosts. Different PCR primers have been used to perform such surveys, traditionally targeting a portion of the *16S ribosomal (r)RNA* gene or of a ubiquitous protein-coding gene (e.g., *wsp* or *ftsZ*). Simoes et al. (2011) evaluated the relative sensitivity and specificity of different primer pairs aimed at *Wolbachia* detection and identification, revealing that no single PCR protocol is capable of specific detection of all known *Wolbachia* strains. A related method known as "loop mediated isothermal amplification" (LAMP; not shown in **Figure 2**), which requires less infrastructure than PCR, has been successfully employed for *Wolbachia* detection in several insects (da Silva Gonçalves et al., 2014).

The two major shortcomings of utilizing solely PCR (or LAMP) to detect *Wolbachia* presence are the occurrence of false negatives and false positives. A false negative occurs when a specimen is infected by *Wolbachia*, yet the screening approach fails to detect its presence. The efficiency of the PCR can be affected by the presence of inhibitors (Marcon et al., 2011; Beckmann and Fallon, 2012), by low concentration/poor quality of the target DNA molecule, as well as type and concentration of the polymerase and other PCR reagents. At the very least, negative *Wolbachia* detection PCRs should be validated by evaluating the quality of the DNA extract, through positive amplification of a host-encoded gene (e.g., the mitochondrial Cytochrome Oxidase subunit I or single-copy nuclear genes). Several higher sensitivity approaches have been devised, particularly for low-titer infections, such as: long PCR (Jeyaprakash and Hoy, 2000); nested PCR (Arthofer et al., 2009a); quantitative PCR (Mee et al., 2015); or the design of alternative and/or more specific primers, including the use of *Wolbachia* multi-copy genes as PCR targets (Schneider et al., 2014). These methods, however, have not been widely implemented, likely due to the higher effort or cost involved.

False positives occur when a specimen not harboring *Wolbachia* is identified as *Wolbachia*-infected. Several instances have been reported where insect chromosomes carry *Wolbachia*-derived fragments, presumably from a horizontal gene transfer event that occurred at some point in the host lineage as the result of an active infection that was subsequently lost. The size of the horizontally transmitted fragment can range from ca. 500 bp to the equivalent of an entire *Wolbachia* chromosome (Dunning Hotopp et al., 2007). In some cases, entire *Wolbachia* chromosomes have been transferred more than once onto the same host genome (Brelsfoard et al., 2014; International Glossina Genome Initiative, 2014). The range of hosts carrying *Wolbachia*-derived genome fragments is broad and includes several dipterans (tephritids, *Glossina morsitans* Westwood; *Drosophila* spp., mosquitoes), other insects, as well as nematodes (Fenn et al., 2006; Dunning Hotopp et al., 2007; Nikoh et al., 2008; Brelsfoard et al., 2014; Morrow et al., 2015; Attardo et al., 2019). It is therefore desirable to corroborate PCR-based inferences with approaches that detect *Wolbachia* cells in host tissues. Such microscopy approaches can be based on nucleic acid hybridization (e.g., Chen et al., 2005) or antibody-based detection of *Wolbachia* proteins (e.g., *wsp*; Veneti et al., 2003; and *ftsZ;* Newton et al., 2015). A major drawback of these methods is that they require substantial investment in time and equipment compared to PCR-based approaches. False positives can also occur if the primers targeted at *Wolbachia* turn out to amplify a fragment of the genome of the host (not derived from *Wolbachia*) or of another symbiont of the host. Such false

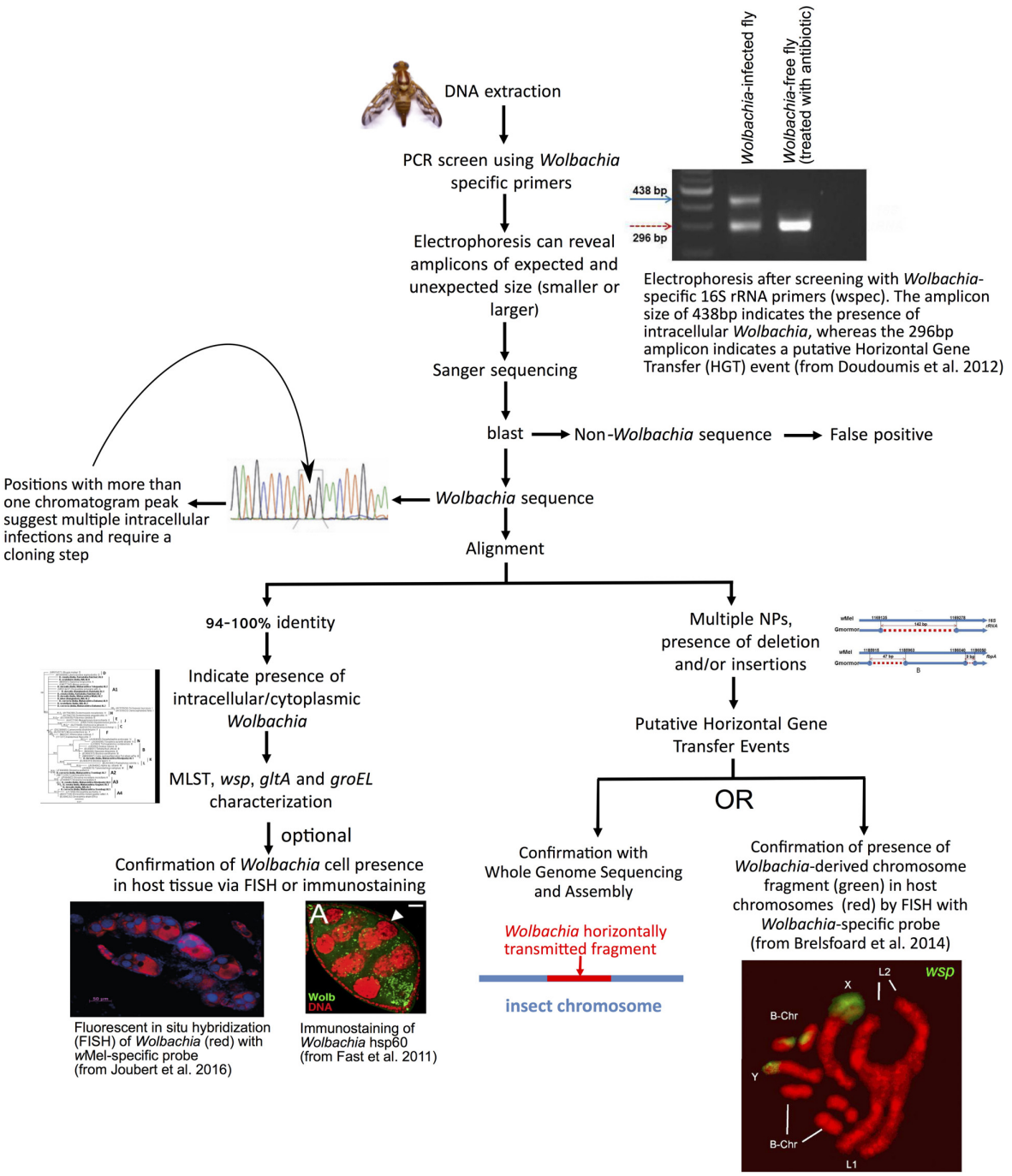

FIGURE 2 | Recommended steps to screen for *Wolbachia* infections in tephritids and other arthropods. A PCR is performed with *Wolbachia*-specific primers on DNA isolated from whole, or parts of (e.g., abdomens), insect. Agarose gel electrophoresis of the PCR products is used to determine whether the amplicon is of the expected size. Amplicons of expected size are directly sequenced (e.g., Sanger method). High sequence identity to other *Wolbachia* suggests *Wolbachia* infection. Clean chromatograms are consistent with a single *Wolbachia* strain. Otherwise, a cloning step to identify different *Wolbachia* alleles is required. Other genes are then amplified and sequenced for further genetic characterization of the strain. As an optional step, localization of *Wolbachia* cells within host tissues can be achieved by Fluorescent *In Situ* Hybridization (FISH) with *Wolbachia*-specific rRNA probe or immunolabeling with antibody specific to *Wolbachia* protein. An amplicon of an unexpected size might indicate the occurrence of a horizontally transmitted *Wolbachia* genome fragment to the insect chromosome, rather than a current infection. Similarly, multiple nucleotide polymorphisms (NP) or insertions/deletions, compared to known strains, are suggestive of *Wolbachia* pseudogenes (e.g., horizontally transferred to host genome). This can be further tested by *in situ* hybridization of *Wolbachia*-specific probe to host chromosomes, and/or by Whole Genome Sequencing of host. Photo of fly (*Anastrepha obliqua*) by Fabiola Roque (ECOSUR-UT). Image from Fast et al. (2011) freely available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4030408/bin/NIHMS391830-supplement-Supporting_Online_Material.pdf (accessed April 01, 2019). Original sources of other photographs are available in open access journals (Doudoumis et al., 2012; Brelsfoard et al., 2014; Joubert et al., 2016).

positives are relatively easy to rule out upon sequencing and analysis of the amplified product. Finally, as with any PCR work, false positives can result from contamination of the specimen, the DNA template, or the PCR reagents. Thus, it is important to implement adequate sterile practices and negative controls.

The above approaches require destruction of specimens for DNA isolation or for tissue fixation. As a rapid and non-destructive alternative, Near-Infrared Spectroscopy (NIR) has been developed for identification of specimens infected with *Wolbachia*, including the distinction of two different *Wolbachia* strains (Sikulu-Lord et al., 2016). This method, however, requires standardization according to species, sex, age, or any other condition that may affect absorbance, and is not 100% efficient. To our knowledge, this method has not been employed to assess *Wolbachia* infection in tephritids.

Methods to Taxonomically Characterize *Wolbachia* Strains

The main evolutionary lineages of *Wolbachia* are assigned to "supergroups" (Zhou et al., 1998). Sixteen supergroups have been recognized to date (Glowska et al., 2015; Bleidorn and Gerth, 2017). Supergroups A and B are widespread in arthropods and are common reproductive manipulators (Baldo et al., 2006; Werren et al., 2008). Supergroups C and D are obligate mutualists of filarial nematodes, whereas supergroup F is found in both arthropods and nematodes (Ros et al., 2009). Other supergroups have more restricted host distributions (Augustinos et al., 2011). *Wolbachia* are generally compared and classified on the basis of Multilocus Sequence Typing (MLST) systems (Baldo et al., 2006; Paraskevopoulos et al., 2006). The most commonly used MLST is based on the PCR amplification of fragments of five ubiquitous genes: *cox*A, *fbp*A, *fts*Z, *hcp*A, and *ga*tB. However, this MLST system has limitations, in that not all genes are readily amplified in all *Wolbachia* strains, and it fails to distinguish among very closely related strains (Augustinos et al., 2011; Bleidorn and Gerth, 2017). Several additional genes commonly amplified and reported are the 16S *rRNA*, *gro*EL, *glt*A, and the *wolbachia surface protein* (*wsp*) (O'Neill et al., 1992; Braig et al., 1998; Zhou et al., 1998; Augustinos et al., 2011). The *wsp* gene is highly variable and shows evidence of intragenic recombination (Werren and Bartos, 2001; Ros et al., 2012). An MLST database[1] is available to compare sequences of alleles for the five MLST loci and the *wsp* gene. Upon submission to the MLST database, new alleles for the *wsp* and for each of the MLST loci are assigned a unique number. A *Wolbachia* sequence type (ST) is defined on the basis of MLST allele combinations, with each allele combination assigned a unique ST number. Further characterization of each MLST-defined strain can be achieved by examination of four hypervariable regions (HVRs) of the *wsp* gene (Baldo et al., 2005).

Hosts can be infected by one or more distinct strains of *Wolbachia*. Traditionally, direct Sanger sequencing of PCR products that resulted in sequences with ambiguous base pairs would be subjected to cloning followed by sequencing. The allele intersection analysis method (AIA; Arthofer et al., 2011) can then be used to assign MLST alleles to *Wolbachia* strains, but it requires a priori knowledge on the number of strains present. AIA identifies pairs of multiply infected individuals that share *Wolbachia* and differ by only one strain. Alternative approaches to circumvent cloning include the use of strain-specific primers (e.g., for the *wsp* gene; Zhou et al., 1998; Arthofer et al., 2009b), or of high throughput sequencing approaches (e.g., Illumina HiSeq, MiSeq, and NovaSeq) to sequence MLST or other marker PCR amplicons (e.g., Gibson et al., 2014; Brandon-Mong et al., 2015). Primer bias, however, where the fragment of one *Wolbachia* strain is preferentially amplified over the other, has been reported (Arthofer et al., 2011), such that presence of certain *Wolbachia* strains might be missed.

Use of the MLST system alone has two major drawbacks. First, strains of *Wolbachia* sharing identical MLST or *wsp* alleles can differ from each other at other loci (Paraskevopoulos et al., 2006; Riegler et al., 2012). Secondly, the MLST, *16S rRNA*, and *wsp* loci contain limited phylogenetic signal for inferring relationships within *Wolbachia* supergroups (Bleidorn and Gerth, 2017). Therefore, to assess such intra-ST variation and to infer evolutionary relationships among closely related *Wolbachia* strains, additional (more variable) loci must be evaluated. The multiple locus variable number tandem repeat analysis developed by Riegler et al. (2012) allows distinction of closely related *Wolbachia* strains based on PCR and gel electrophoresis.

Whole genome sequencing represents a powerful approach to distinguish closely related *Wolbachia* strains, infer their evolutionary relationships, test for recombination, and identify genes of interest (e.g., Klasson et al., 2009; LePage et al., 2017). Due to its fastidious nature (but see Uribe-Alvarez et al., 2018 for a recent breakthrough) and occurrence of repetitive elements, genome sequencing and assembly of *Wolbachia* (and other host-associated uncultivable bacteria) has proven difficult. Recent advances, particularly those based on targeted hybrid enrichment (Lemmon and Lemmon, 2013) prior to high-throughput sequencing (Bleidorn, 2016; Goodwin et al., 2016) have been successfully applied to *Wolbachia* for short-read technologies (i.e., Illumina; Kent et al., 2011; Geniez et al., 2012). The combination of targeted hybrid capture and long-read technologies, such as Pacific Biosciences' Single Molecule Real-Time (e.g., Wang et al., 2015) or Oxford Nanopore Technologies' platforms is expected to greatly advance *Wolbachia* genomics research.

Methods to Functionally Characterize *Wolbachia* Strains

A major challenge to investigating the effects of *Wolbachia* on a host is to generate *Wolbachia*-present and *Wolbachia*-free treatments while controlling for host genetic background. The challenge stems from the difficulty of adding or removing *Wolbachia* to/from particular hosts. Addition of *Wolbachia* to a particular host background can be achieved by transinfection (reviewed in Hughes and Rasgon, 2014). Because the vertical transmission of *Wolbachia* appears to be dependent on its close association to the host germline, successful artificial transfer of *Wolbachia* typically relies on injection of cytoplasm from a donor egg (but see Frydman et al., 2006) or early embryo into

[1] https://pubmlst.org/wolbachia/

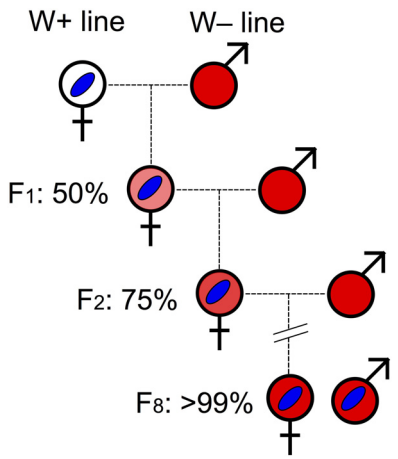

FIGURE 3 | Backcrossing procedure. *Wolbachia* infection is indicated by blue oval. Host nuclear backgrounds are indicated by colors: white represents the initial nuclear background of *Wolbachia*-infected host; red (darkest) indicates the host background of the *Wolbachia*-free line contributing males every generation. Different shades of red represent the increasing replacement of "white" nuclear background over backcrossing generations (F_1 to F_8) by "red" nuclear background.

a recipient embryo via microinjection (reviewed in Hughes and Rasgon, 2014). The success rate of the transinfection procedure is generally very low; in tephritids it is 0–0.09% (calculated as the proportion of injected embryos that emerged as *Wolbachia*-infected adult females that transmitted *Wolbachia* to offspring) (Zabalou et al., 2004b, 2009; Apostolaki et al., 2011; Martinez et al., 2016). The low success rate is generally a result of the low survival of injected embryos, the low proportion of *Wolbachia*-positive survivors, and the low/incomplete transmission of *Wolbachia* to their offspring.

Intra-species (or between sibling species) transfer of *Wolbachia* to a particular host nuclear background can also be achieved through introgression, whereby males of the desired background are repeatedly backcrossed with *Wolbachia*-infected females (e.g., Dobson et al., 1999; Jaenike, 2007). Under this scheme, after eight generations of consistent backcrossing, ∼99.6% of the host nuclear background is expected to have been replaced (**Figure 3**). The drawback of this approach is that the mitochondrial genome will not be replaced. Therefore, the effects of mitochondrial type and *Wolbachia* infection cannot be separated.

Due to less than perfect transmission, passive loss of *Wolbachia* in certain host individuals may be used to obtain *Wolbachia*-free and *Wolbachia*-infected hosts of equivalent genetic background. *Wolbachia* removal has also been achieved by "extreme" temperature treatment (e.g., 30°C; Ribeiro, 2009). The most common way of removing *Wolbachia*, however, is achieved via antibiotic treatment, but several potential biases must be addressed (reviewed by Li et al., 2014). Antibiotic treatment is likely to alter the microbiota, other than *Wolbachia*, associated with the host. In addition, antibiotics may affect the host in a microbe-independent manner. For instance, antibiotic treatment can affect host mitochondria (Ballard and Melvin, 2007), which in turn can reduce host fitness. A common practice to circumvent these problems is to wait several generations after antibiotic treatment, and to promote "restoration" of the host's pre-antibiotic microbiota, excluding *Wolbachia* (e.g., exposing the insects to the feces of non-treated individuals). *Wolbachia* does not appear to be efficiently transmitted via ingestion (e.g., Faria et al., 2016), but see discussion on horizontal transmission routes below. It is essential to monitor *Wolbachia* infection status of antibiotic-treated host strains, because antibiotics may not always fully remove infection. Instead, they may reduce *Wolbachia* densities to non-detectable levels in one or few generations (Li et al., 2014); this has been our experience in both *Anastrepha* (S.B. Lanzavecchia, C. Conte, and D.F. Segura, pers. obs.) and *Drosophila* (M. Mateos, pers. obs.).

Unidirectional CI is tested by comparing the embryo hatching rates of the CI cross (uninfected female X infected male) to that of one or more control crosses. For testing bidirectional CI, the reciprocal crosses of hosts infected by the different *Wolbachia* strains are assessed. A significantly lower embryo hatching rate of the CI cross(es) compared to that of the control cross(es) constitutes evidence of CI. CI can be partial or complete (100% embryo failure). As with any fitness assay, care must be taken to prevent potential biases, including crowding and age effects; which have been shown to influence CI (Hoffmann et al., 1990; Turelli and Hoffmann, 1995; Reynolds and Hoffmann, 2002). Adequate assessment of fertilization must be performed to ensure that failed embryos are not confused with unfertilized eggs. This may require testing for insemination of females that produce no larval progeny (e.g., Zabalou et al., 2009; Conte et al., 2019), or exclusion of females that predominantly lay unfertilized eggs, such as old *Drosophila melanogaster* virgin females (Menon et al., 2014).

Wolbachia IN TEPHRITIDS

Taxonomic Distribution of *Wolbachia*-Tephritid Associations

Based mostly on PCR and sequencing approaches, ∼66% of ∼87 tephritid species screened have at least one record of positive *Wolbachia* infection (excluding pseudogenes) in laboratory and natural populations (see **Supplementary File S1**; only supergroups A and B have been found in tephritids). For the genus *Anastrepha*, all but one species (*Anastrepha ludens*) of 17 screened to date harbor *Wolbachia* (Werren et al., 1995; and this study; Selivon et al., 2002; Coscrato et al., 2009; Martínez et al., 2012; Mascarenhas et al., 2016; Morán-Aceves, 2016; Prezotto et al., 2017; Conte et al., 2019; Devescovi et al., 2019). Most *Anastrepha* species harbor *Wolbachia* strains assigned to supergroup A. *Anastrepha striata* Schiner and *Anastrepha serpentina* (Wiedemann), however, harbor supergroup B in southern Mexico (Martínez et al., 2012; and H. Martinez and M. Mateos, pers. obs.; see **Supplementary File S1**) and supergroup A in Brazil (Coscrato et al., 2009). Up to three *Wolbachia* sequence types have been detected per locality within morphotypes of the *A. fraterculus* complex (Prezotto et al., 2017;

Conte et al., 2019), but co-infection of a single individual is generally not observed (except for one report in *A. fraterculus*; Cáceres et al., 2009).

Of the ∼49 species of *Bactrocera* that have been examined, ∼14 are reported to harbor *Wolbachia* (supergroup A and/or B) and three (*Bactrocera peninsularis* Drew & Hancock, *Bactrocera perkinsi* Drew & Hancock, and *Bactrocera nigrofemoralis* White & Tsuruta) carry what appear to be *Wolbachia*-derived pseudogenes, but not active infections (Kittayapong et al., 2000; Jamnongluk et al., 2002; Morrow et al., 2014, 2015). There is also the case of *Bactrocera zonata* Saunders, *B. dorsalis*, and *Bactrocera correcta* Bezzi that have been found to carry both active infections (cytoplasmic) and pseudogenized *Wolbachia* sequences (Asimakis et al., 2019). Up to five *Wolbachia* strains have been reported in a single individual of *Bactrocera ascita* Hardy (Jamnongluk et al., 2002), and double/multi infections have been reported in individuals of the following five *Bactrocera* species in Australia: *Bactrocera bryoniae* Tryon; *Bactrocera decurtans* May; *Bactrocera frauenfeldi* Schiner; *Bactrocera neohumeralis* Hardy; and *Bactrocera strigifinis* Walker (Morrow et al., 2014, 2015). Within the genus *Bactrocera*, polyphagous species are more likely to harbor *Wolbachia* compared to stenophagous or monophagous ones (Kittayapong et al., 2000).

For the genus *Ceratitis*, two species have been screened for *Wolbachia*. No evidence of *Wolbachia* was found in *Ceratitis fasciventris* Bezzi. Also, no evidence of infection was found in several populations of *C. capitata*, the Mediterranean fruit fly (medfly), in the early '1990s' (Bourtzis et al., 1994). PCR amplification and sequencing of the 16S rRNA gene in several field and lab specimens of *C. capitata* from Brazil suggested infection with *Wolbachia* supergroup A (Rocha et al., 2005). However, recent thorough surveys of wild populations and lab colonies indicate that *Wolbachia* is absent in *C. capitata* from numerous localities in different continents (**Supplementary File S1**).

Wolbachia is reported in the four species of *Rhagoletis* examined to date: *Rhagoletis cerasi* L.; *Rhagoletis pomonella* Walsh; *Rhagoletis cingulata* Loew; and *Rhagoletis completa* Cresson (Zabalou et al., 2004b; Arthofer et al., 2009b; Drosopoulou et al., 2011; Schuler et al., 2011, 2012, 2013, 2016b; Augustinos et al., 2014; Bakovic et al., 2018). Both A and B supergroups are found in *R. cerasi* and *R. completa*, including a putative A–B recombinant strain, and co-infections are common (e.g., *R. cerasi* and *R. pomonella*).

In *Zeugodacus* (formerly *Bactrocera*), both *Z. cucurbitae* and *Z. diversa* are reported to harbor *Wolbachia* or *Wolbachia* pseudogenes (Kittayapong et al., 2000; Jamnongluk et al., 2002; Asimakis et al., 2019). Two out of the six species of *Dacus* examined to date are reported to harbor *Wolbachia*: *Dacus axanus* Hering (Morrow et al., 2014); and *Dacus destillatoria* Bezzi (Jamnongluk et al., 2002). *Wolbachia* has not been detected in the monotypic genus *Dirioxa* (Morrow et al., 2015). *Wolbachia* (supergroup A) has been reported in *Carpomya vesuviana* (Karimi and Darsouei, 2014) and *Neoceratitis asiatica* (Wang et al., 2019).

Wolbachia Prevalence in Tephritids (in Time/Space)

Numerous studies report *Wolbachia* infection frequencies (or data from which this measure can be estimated) in natural populations of tephritids. Few of these studies, however, have adequate sample sizes for such inferences (e.g., many such studies are based on 10 or fewer individuals). Notwithstanding, inferred *Wolbachia* prevalence in tephritid populations is highly variable. In *Anastrepha*, ∼10 species harbor at least one population with prevalence ∼100%, whereas populations of three species reported lower frequencies (e.g., 88%, 51–60%, and 8.7%) (**Supplementary File S1**). In *Bactrocera*, one population of *B. caudata* had 100% prevalence, whereas all other species with positive *Wolbachia* results exhibited low prevalence.

The best studied tephritid system in terms of spatial and temporal variation in *Wolbachia* prevalence is that of *R. cerasi* in Europe, which was surveyed over a ∼15-year-period in 59 localities (Schuler et al., 2016b). Collectively, at least six strains *w*Cer1–6 have been identified from Europe and the Middle East. In an early (1998) survey, Riegler and Stauffer (2002) found all European *R. cerasi* individuals infected by one strain (*w*Cer1), most central and southern European populations harbored an additional strain *w*Cer2 (i.e., *w*Cer1 + *w*Cer2), and at least one Italian population harbored *w*Cer1 + *w*Cer4 (Zabalou et al., 2004b). A rapid spread of *w*Cer2 (a strain associated with cytoplasmic incompatibility) has been detected. Multiple infections, in various combinations of all five known *Wolbachia* strains from Europe, have been revealed recently. Samples from Poland, Italy, and Austria, are infected with strains *w*Cer1–5 those from Czech Republic (prior to 2009) and Portugal lacked *w*Cer2 only, while the Swiss samples lacked *w*Cer3 (Arthofer et al., 2009b). A more recent study of the Czech Republic (2015) and Hungary (2016) revealed that *w*Cer2 is spreading at a speed of 1.9 and 1 km/yr, respectively (Bakovic et al., 2018). Analysis of 15 Greek, two German and one Russian population confirm fixation for *w*Cer1 in all *R. cerasi* populations, and the presence of complex patterns of infections with four of the five known *w*Cer European strains (1, 2, 4, and 5) and the possible existence of new *Wolbachia* strains for the southernmost European *R. cerasi* population (i.e., Crete; Augustinos et al., 2014) and from Iran (*w*Cer6) (Karimi and Darsouei, 2014). Similarly, strain *w*Cin2 (which is identical to *w*Cer2 based on loci examined to date) is fixed in all populations of *R. cingulata*; a species native to the United States, but introduced into Europe at the end of the 20th century. Invasive populations in Europe harbor *w*Cin1 (identical to *w*Cer1 based on loci examined to date) at frequencies that vary over space and time (up to 61.5%), as a result of horizontal transfer (multiple events) from *R. cerasi* (Schuler et al., 2016b). The above studies indicate that the prevalence of *Wolbachia* types in *R. cerasi* and *R. cingulata* is highly dynamic.

Phenotypic Effects of *Wolbachia* in Tephritids

Despite the numerous reports of *Wolbachia* in tephritids, the fitness consequences of such associations remain mostly unknown. The studies reporting phenotypic effects of *Wolbachia*

have relied on transinfection and on antibiotic-curing; only two species of tephritids have been successfully transinfected with *Wolbachia* (**Table 1**). Evidence of *Wolbachia*-induced CI has been detected in four species of tephritids. Early studies (Boller and Bush, 1974; Boller et al., 1976) identified reproductive incompatibilities in *R. cerasi* that were later attributed to the *Wolbachia* strain *w*Cer2 (100% embryonic mortality in the CI cross; Riegler and Stauffer, 2002). Artificially transferred *Wolbachia* (strains *w*Cer2 and *w*Cer4) originally from *R. cerasi* to *C. capitata* also resulted in strong CI (100% embryonic mortality). *w*Cer2 in two genetic backgrounds of *B. oleae* resulted in strong CI as well (Apostolaki et al., 2011). In addition, *w*Cer2 and *w*Cer4 are bi-directionally incompatible in *C. capitata* (Zabalou et al., 2004b, 2009).

In addition to CI, *Wolbachia*-infected *C. capitata* females (Benakeio strain) exhibit higher embryonic mortality (17–32% in crosses with *Wolbachia*-free males and 65–67% in crosses with *Wolbachia*-infected males) than their *Wolbachia*-free counterparts crossed with *Wolbachia*-free males (12% embryonic mortality). Therefore, it appears that *w*Cer2 and *w*Cer4 have additional fertility effects on medfly females, other than CI. It is also possible that these *Wolbachia* strains can only partially rescue the modification that they induce in sperm (Zabalou et al., 2004b). A similar pattern is reported in the Vienna 8 genetic sexing strain (GSS) infected with *w*Cer2 (Zabalou et al., 2009). The *w*Cer2 strain also causes increased embryo death in non-CI crosses of *B. oleae* (Apostolaki et al., 2011). In *D. simulans*, *w*Cer2 causes fecundity costs, moderate levels of CI, and incomplete rescue of its own CI modification (Riegler et al., 2004). Interestingly, a recent study examined the genome of *w*Cer2 and revealed the presence of three pairs of Type I *cif* genes and one Type IV *cifB* gene without a *cifA* complement, which might explain its idiosyncratic expression of CI (Morrow et al., 2020).

Two studies conducted several years apart (Sarakatsanou et al., 2011; Kyritsis, 2016; Kyritsis et al., 2019) examined the effects of a single *Wolbachia* strain (*w*Cer2) on fitness components of two *C. capitata* genotypes (i.e., Benakeio and Vienna 8 GSS laboratory lines), as well as the effects of two different *Wolbachia* strains (*w*Cer2 and *w*Cer4) on a single medfly genotype (Benakeio). The following general patterns emerged (exceptions noted): (a) *Wolbachia* causes higher egg-to-larva mortality; (b) *Wolbachia* causes higher egg-to-adult mortality (exception: Vienna 8 GSS + wCer2 in Sarakatsanou et al., 2011); (c) *Wolbachia* shortens egg-to-adult development time (exception: Benakeio + wCer2 in Kyritsis, 2016; Kyritsis et al., 2019). In addition, Sarakatsanou et al. (2011) found that *Wolbachia* shortens both male and female adult lifespan (exception: males of Vienna 8 GSS and *w*Cer2), and reduces life time female net fecundity. However, Kyritsis (2016) and Kyritsis et al. (2019) reported no effects of *Wolbachia* infection on adult lifespan, and a reduced fecundity in the case of *w*Cer4 infection only. Even though *w*Cer2 and *w*Cer4 in general tended to have consistent effects on medfly, the magnitude of their effects differed. Collectively, the results from these studies indicate that the effect of *Wolbachia* infection on life history traits depends both on the *C. capitata* genetic background and on the *Wolbachia* strain. Furthermore, inconsistencies between the two studies might be indicative of evolution of the host and/or *Wolbachia* strain during that period. Evidence of *Wolbachia* evolving reduced fitness costs has been reported in *D. simulans* (Weeks et al., 2007). Adult flight ability and longevity under stress conditions also appear to be determined by the interaction of *Wolbachia* strain and medfly genotype (Kyritsis, 2016; Kyritsis et al., 2019). A more recent study (Dionysopoulou et al., 2020) demonstrated *Wolbachia* effects on medfly reared in natural host fruits and at different temperatures. Medlfies infected with *w*Cer4 had low survival rates in both apples and bitter oranges, whereas those infected with *w*Cer2 were less vulnerable in apples than in bitter oranges. In addition, *w*Cer4 infected flies were particularly susceptible to high temperatures.

A recent study by Conte et al. (2019) examined the phenotypic effects induced by two *Wolbachia* strains native to *A. fraterculus* (sp1). No evidence of bidirectional cytoplasmic incompatibility was detected in reciprocal crosses among singly infected laboratory strains. However, the same work described the presence of slightly detrimental effects on larval survival and a female-biased sex ratio, suggesting the induction of male-killing phenomena. Moreover, Devescovi et al. (2019) found that *Wolbachia* reduced the embryo hatching in crosses involving cured females and infected males (uni-directional CI) within two morphotypes of this cryptic species complex; stronger CI was detected within the Peruvian morphotype than the Brazilian-1 morphotype (also referred as to "*A. fraterculus* sp. 1"). No evidence of bidirectional CI was detected in the crosses between the two morphotypes, leading Devescovi et al. (2019) to conclude that *Wolbachia* is not directly involved in the speciation process of these morphotypes. Ribeiro (2009) reported evidence consistent with CI caused by *Wolbachia* in *A. obliqua* and in "*A. fraterculus* sp. 1," which according to *wsp* sequences, are identical. Nonetheless, confounding effects of the treatment to remove *Wolbachia* (removed by exposure of pupae to 30°C) or other potential biases cannot be ruled out, as all intraspecific crosses involving at least one cured parent resulted in much lower (<30%) embryo hatching than the intraspecific crosses involving both infected parents (66 and 81% embryo hatching).

Recent work demonstrates that *Wolbachia* infection can affect male sexual competitiveness of *C. capitata*. Different *Wolbachia* strains (*w*Cer2 and *w*Cer4) exerted differential impact on males mating competitiveness, and a single strain (*w*Cer2) had different impact on different medfly genotypes (Benakeio and Vienna 8 GSS laboratory lines) (Kyritsis, 2016; Kyritsis et al., 2019).

Modes of Horizontal Transmission of *Wolbachia* Between Tephritid Hosts

Considering the dynamics of *Wolbachia* associated with arthropods in general, at the population level *Wolbachia* appears to be predominantly maintained by vertical transmission. Above the species level, however, the lack of congruence between the host and symbiont phylogenetic trees implies that *Wolbachia* horizontal transfers and extinctions are common and underlie its widespread taxonomic and geographic distribution (Bailly-Bechet et al., 2017).

TABLE 1 | Successful and unsuccessful *Wolbachia* transfection attempts in tephritids.

ID of successfully transfected tephritid strain	Donor species/strain	Recipient species (and strain)	*Wolbachia* strain	References
C. capitata WolMed 88.6	*R. cerasi*	*C. capitata* Benakeio strain	wCer2	Zabalou et al., 2004b
C. capitata WolMed S10.3	*R. cerasi*	*C. capitata* Benakeio strain	*w*Cer4	Zabalou et al., 2004b
C. capitata VIENNA 8-E88	*C. capitata* WolMed 88.6	*C. capitata* VIENNA 8 Genetic Sexing Strain (GSS)	wCer2	Zabalou et al., 2009
	C. capitata VIENNA 8-E88	*Bactrocera oleae*	wCer2	Apostolaki et al., 2011
N/A (unsuccessful)	*A. striata*	*A. ludens*	*w*AstriB	Martinez et al., 2016

The possible routes by which *Wolbachia* may be horizontally acquired by a new host can generally be classified as via ingestion or via a vector. In both cases, to become established as a stable cytoplasmically inherited infection, *Wolbachia* must cross one or more cell types or tissues. For example, if *Wolbachia* invaded the host hemolymph directly as a result of a vector (e.g., parasitoid wasp or ectoparasitic mite), it would have to invade the egg during oogenesis. Similarly, if *Wolbachia* were acquired via ingestion (e.g., as a result of scavenging), it would have to cross the gut into the hemolymph, before it invaded the egg. Support for the above routes comes from studies reporting: (a) that *Wolbachia* can retain viability outside cells and infect mosquito cell lines, as well as ovaries and testes that are maintained *ex vivo* (Rasgon et al., 2006; Hughes et al., 2012a); (b) that *Wolbachia* cells injected into *Drosophila* hemolymph reach the germline after crossing multiple somatic tissues (Frydman et al., 2006); (c) that *Wolbachia* can move between parasitic wasp larvae (*Trichogramma*) sharing the same host egg, and achieve vertical transmission (Huigens et al., 2004); and (d) that parasitic wasps of the white fly, *Bemisia tabaci* (Gennadius), can transfer *Wolbachia* from an infected to a naïve host, as a result of non-lethal probing (i.e., probing without oviposition), whereby the parasitoid ovipositor or mouthparts function as a "dirty needle" (Ahmed et al., 2015).

No direct evidence of *Wolbachia* transmission via parasitoids exists in tephritids, but sharing of *Wolbachia* strains between a parasitoid and several sympatric tephritids (Morrow et al., 2014; Mascarenhas et al., 2016) is consistent with parasitoid-mediated transmission, or transmission from tephritid host to parasitoid (Johannesen, 2017). The potential for horizontal transfer of *Wolbachia* among tephritids via parasitoids is high, due to the multiple instances where a single parasitoid utilizes several different tephritid host species (Quilici and Rousse, 2012; Murillo et al., 2016; Schuler et al., 2016a), and the high frequency of superparasitism by some fruit fly parasitoids (Tormos et al., 2012; Devescovi et al., 2017).

Wolbachia may invade a new host species via introgressive hybridization between two host species. This mechanism would also transfer mitochondria from the infected to the uninfected species nuclear background, akin to the artificial backcrossing approach described above (**Figure 3**). Ability of tephritids to hybridize in the lab has been reported in numerous species (**Table 2**), and hybridization in nature has been documented in *B. dorsalis/B. carambolae* (Wee and Tan, 2005), members of the *Ceratitis* FAR complex (Virgilio et al., 2013), and *R. cingulata/R. cerasi* in Europe (Johannesen et al., 2013). Thus, there is potential for wild tephritid populations to acquire *Wolbachia* infections via hybridization.

CONSIDERATIONS FOR *Wolbachia*-BASED IIT IN TEPHRITIDS

There are two main approaches for implementing IIT, which depend on whether uni- or bi-directional CI will be used. If the target pest population lacks *Wolbachia*, such as the tephritids *C. capitata*, *B. oleae* (Gmelin), and *A. ludens* [and the mosquito *Aedes aegypti* (L.)], only unidirectional CI is feasible. In target populations that harbor one (or more) CI-inducing *Wolbachia* strain(s) (i.e., native strain; yellow in **Figure 4**), bi-directional CI can be achieved by releasing males that lack the native strain(s) and harbor one (or more) "foreign" *Wolbachia* strain(s) (blue in **Figure 4**) that is incompatible with the native strain. In contrast, if the released males are doubly infected with the native and foreign strains, the CI pattern employed for population suppresion is uni-directional (**Figure 4**).

In the case of Uni-CI patterns, the accidental release of *Wolbachia*-infected females, which would be reproductively compatible with wild and released males, may result in the replacement of the target pest population with a population harboring the *Wolbachia* infection of the released males, leading to failure of the IIT-based suppression program (Bourtzis, 2008). As described in Section "The Influence of *Wolbachia* on Host Ecology," under certain conditions, a *Wolbachia* infection with frequency close to zero might be able to rapidly spread through a host population. Thus, without efficient sex separation mechanisms (outlined in section below), it is desirable to ensure that accidentally released females are sterile. In several tephritid systems, female sterility is achieved at a lower irradiation dose than male sterility, such as *A. ludens*, *A. obliqua*, *Anastrepha suspensa*, *A. serpentina*, *B. tryoni*, and *Z. curcubitae* (Toledo et al., 2004; Bakri et al., 2005; Rull et al., 2007; Collins et al., 2009). Therefore, an IIT program that used radiation at doses to ensure female sterility without compromising male quality (e.g., male competitiveness) could be effective (e.g., *Drosophila suzukii* based on results to date; Nikolouli et al., 2020).

In an IIT program based on bi-CI pattern (e.g., the recent field study of *Aedes albopictus*; Caputo et al., 2019), accidental release of fertile transinfected females, which would only be

TABLE 2 | Representative tephritid genera where hybridization between one or more species has been reported.

Tephritid genera containing species that can hybridize	Reference(s)
Bactrocera	
B. tryoni* × *B. neohumeralis	Smith, 1979; Morrow et al., 2000; Meats et al., 2003; Pike et al., 2003
B. tryoni* × *B. jarvisi	Cruickshank et al., 2001
B. aquilonis × ***B. tryoni***	Drew and Lambert, 1986
B. jarvisi × ***B. neohumeralis***	Gilchrist et al., 2014
***B. dorsalis*^S × *B. philippinensis*^S**	Schutze et al., 2013
B. invadens^S × ***B. dorsalis*^S**	Bo et al., 2014
***B. dorsalis*^S × *B. papayae*^S**	Schutze et al., 2013
B. papayae^S × *B. philippinensis*^S	Schutze et al., 2013
B. papayae × *B. carambolae*	Ebina and Ohto, 2006
B. tryoni* × *B. jarvisi	Shearman et al., 2010
Ceratitis	
C. rosa × *C. fasciventris*	Erbout et al., 2008
Anastrepha	
Within ***A. fraterculus*** complex	Selivon et al., 1999, 2005; Cáceres et al., 2009; Segura et al., 2011; Roriz et al., 2017; Rull et al., 2018
A. fraterculus* × *A. obliqua	Dos Santos et al., 2001
A. sororcula* × *A. obliqua	Dos Santos et al., 2001
A. fraterculus* × *A. sororcula	Dos Santos et al., 2001
Rhagoletis	Schwarz and McPheron, 2007; Rull et al., 2010, 2012; Arcella et al., 2015; Tadeo et al., 2015
R. mendax × ***R. pomonella***	Bierbaum and Bush, 1990; Schwarz and McPheron, 2007
Within ***R. pomonella*** complex	Rull et al., 2010
R. completa* × *R. zoqui	Rull et al., 2012
R. pomonella × *R. zephyria*	Arcella et al., 2015
R. cingulata* × *R. indifferens	Doellman et al., 2019
Within ***R. cingulata***	Tadeo et al., 2015
R. cerasi* × *R. cingulata	Johannesen et al., 2013
Eurosta	
Within *Eurosta solidaginis*	Craig et al., 1997

[S]*B. papayae, B. philippinensis, and B. invadens are now considered junior synonyms of B. dorsalis (Drew and Romig, 2013; Schutze et al., 2015). Bold-face names are species where at least one report of Wolbachia infection exists (see* ***Supplementary File S1****).*

compatible with the released males, would not necessarily lead to population replacement and program failure. This is due to the generally higher threshold density required to achieve invasion (theoretically above 50% when the two incompatible *Wolbachia* strains exert equivalent fitness costs/benefits; see Section "The Influence of *Wolbachia* on Host Ecology"). Nonetheless, the actual outcome is strongly dependent in multiple factors (see Dobson et al., 2002; Moretti et al., 2018a). Therefore, for both uni-CI- and bi-CI-based IIT programs, as pointed out by Bourtzis et al. (2014), the outcome of accidental releases of infected females must be thoroughly evaluated via modeling and/or semi-field assays prior to field applications.

The Advantage of Genetic Sexing Strains (GSS)

In general, SIT and IIT are most effective when only males are produced and released (Kerremans and Franz, 1995; Rendón et al., 2004). The release of only males in a large-scale operation can be accomplished by either killing female zygotes during development or by selectively removing them from the mass-reared population prior to release (Robinson, 2002a; Gilles et al., 2014; Lutrat et al., 2019). Genetic sexing strains (GSS) are those in which individuals can be separated by sex prior to the adult stage on the basis of a sex-linked phenotype (Franz, 2005). The earlier in development the females are removed, the most cost-effective the mass rearing operation will be, as investment in growth of females would be null or minimized. In most tephritids, male sex is determined by the presence of the maleness factor on the Y chromosome (Pane et al., 2002). GSS based on male-linked [e.g., Y chromosome – autosome (Y;A)] translocations have been developed in a few species to produce conditional female lethality (e.g., temperature sensitive lethality during embryonic development) or a visual sex marker (e.g., pupal color). Examples of tephritid species for which GSS are available include *C. capitata* (Franz, 2005), *A. ludens* (Zepeda-Cisneros et al., 2014), *Z. cucurbitae* (McInnis et al., 2004), *B. dorsalis* (Isasawin et al., 2012), and *B. carambolae* (Isasawin et al., 2014). Unfortunately, despite substantial efforts, GSS are still lacking for most tephritid pests. The recent development of CRISPR/Cas9-mediated mutagenesis in tephritids, however, might enable a faster development of tephritid GSS (Reid and O'Brochta, 2016; Choo et al., 2017; see reviews by Ogaugwu and Durvasula, 2017).

Choice and Evaluation of *Wolbachia* Strains

The target population and the donor colony should be thoroughly screened for *Wolbachia*, ideally with the higher sensitivity methods described in Section "Methods to Assess *Wolbachia* Infection Status," to detect low-titer and multi-strain infections. The *Wolbachia* strain selected should cause strong uni-CI with a *Wolbachia*-free, or strong bi-CI with *Wolbachia*-infected, target population. The selected *Wolbachia* strain should be artificially transferred to one or more lab colonies, representative of the genetic background of the target pest population. Most cases of successful establishment of stable transinfected insect lines have relied on embryonic microinjection (Hughes and Rasgon, 2014). Introgressive backcrossing might be feasible in scenarios where geographically isolated populations of the same target species harbor distinct *Wolbachia* strains (e.g., *A. striata* in Mexico vs. *A. striata* in Brazil; **Supplementary File S1**).

A thorough biological characterization of the artificial host-*Wolbachia* association should be conducted, as both host background and *Wolbachia* strain are important determinants of CI expression and other relevant fitness parameters (Bourtzis and Robinson, 2006; reviewed in Bourtzis, 2008; see also Kyritsis et al., 2019). The main desired characteristics of the association are: strong induction of CI; no rescue by *Wolbachia* strain(s) present in the target population; small or no fitness cost for

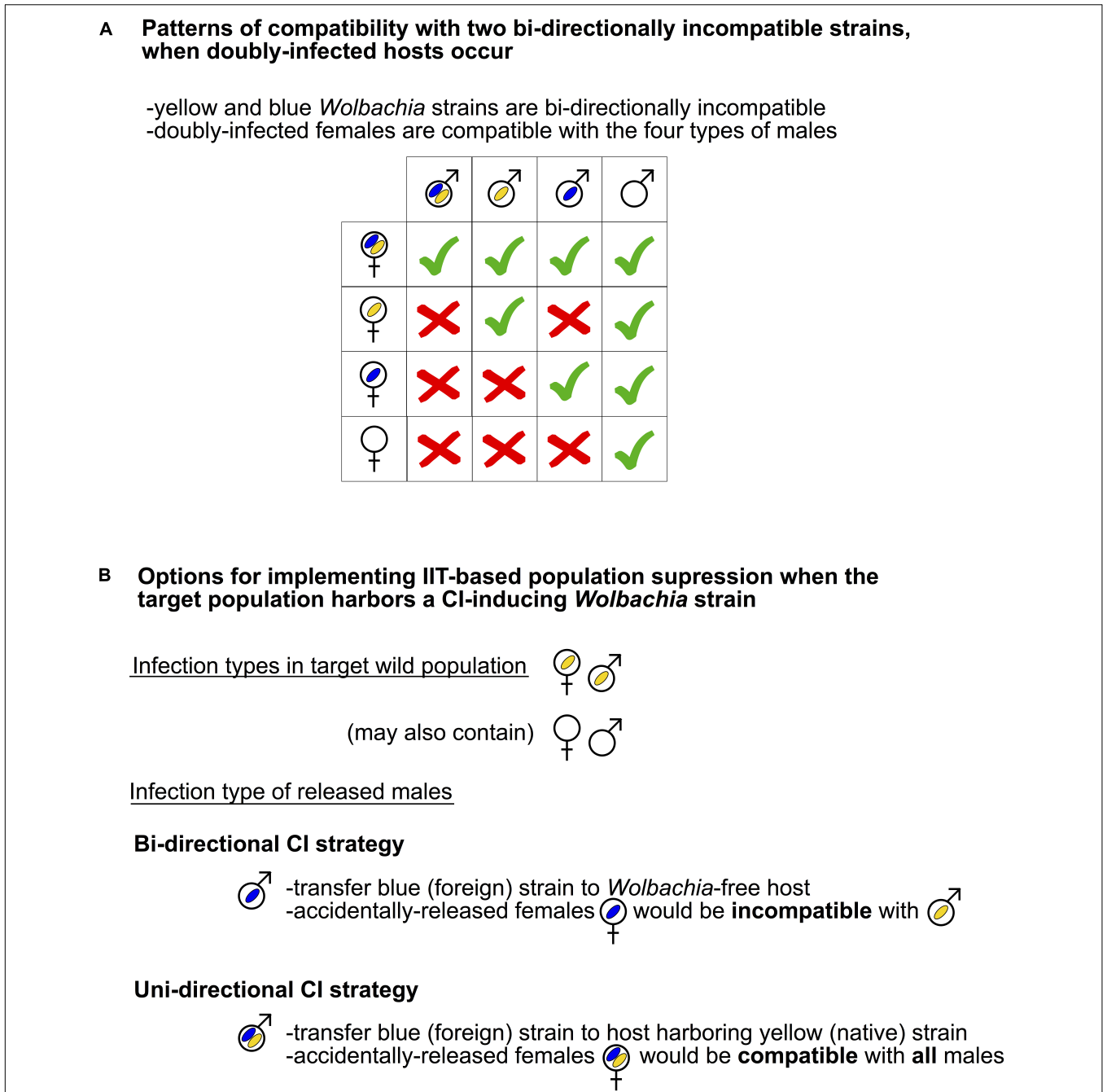

FIGURE 4 | Use of bi-directional CI in IIT-based population suppression programs. **(A)** Patterns of compatibility with two bi-directionally incompatible strains, when doubly infected hosts occur. Empty male and female symbols signify absence of *Wolbachia*. Blue and yellow ovals represent distinct (mutually incompatible) *Wolbachia* strains. Green tick marks = Successful offspring production. Red crosses = no offspring production. **(B)** Options for implementing IIT-based population suppression when the target wild population harbors a CI inducing strain (yellow = "native"), according to the patterns of compatibility depicted in panel a. Bi-directional CI is achieved when the released males only harbor a strain (blue = "foreign") that is incompatible with the native strain. Additional options exist, including double infections of both target and released insects with different *Wolbachia* strains (not shown), such as in *Aedes albopictus* (Moretti et al., 2018b).

parameters relevant to the program. These fitness parameters can be classified into those related to a cost-effective mass production (e.g., female fecundity including embryo hatching success) and those related to the success of released males (e.g., mating and sperm competitive ability, as well as dispersal/flight ability). Some host-*Wolbachia* combinations result in higher female fecundity, such as *D. simulans* after many generations (Weeks et al., 2007) and *Drosophila mauritiana* Tsacas and David (Fast et al., 2011). In contrast, other host-*Wolbachia* combinations result in lower fertility (e.g., low embryo success in

C. capitata and *B. oleae*; Zabalou et al., 2004b, 2009; Apostolaki et al., 2011). *Wolbachia* could affect male mating success by influencing assortative mating; a phenomenon detected in some studies of *Drosophila* (e.g., Koukou et al., 2006; Miller et al., 2010), but not others (e.g., Champion de Crespigny and Wedell, 2007; Arbuthnott et al., 2016; Cooper et al., 2017). Such influence of *Wolbachia* on mating preferences was questioned (Sharon et al., 2010) on the basis of evidence that gut microbiota influence assortative mating in *Drosophila* (Sharon et al., 2010; Ringo et al., 2011; Arbuthnott et al., 2016), a finding that itself has been questioned recently (Leftwich et al., 2017, 2018). In addition, at least one case has been reported where sperm from *Wolbachia*-infected males was less competitive (Champion de Crespigny and Wedell, 2006). Similarly, *Wolbachia*-infected *D. simulans* produce fewer sperm (Snook et al., 2000). All of the above parameters should be evaluated under relevant conditions known to interact with *Wolbachia*, such as temperature and nutrition (reviewed in Bourtzis and Robinson, 2006; e.g., Serbus et al., 2015; Corbin et al., 2017; Ross et al., 2019a), interaction with other microorganisms (e.g., Hughes et al., 2014; Ye et al., 2017), as well as male age, paternal grandmother age, and mating status (e.g., Karr et al., 1998; Awrahman et al., 2014; Layton et al., 2019).

Other Considerations

Species Recalcitrant to *Wolbachia*?

Certain species or clades appear to be "resistant" to *Wolbachia* infection, based on their lack of infection in nature and the failure to achieve stable transfections. The reasons are unknown, but could involve host and/or bacterial factors. For example, none of the members of the diverse *repleta* species group of *Drosophila*, comprised mostly of cactophilic flies (Markow and O'Grady, 2005), has ever been found to harbor *Wolbachia* (Mateos et al., 2006). Similarly, due to numerous failed transinfection attempts, and the lack of natural infection in wild *Anopheles* mosquitoes, this genus was regarded impervious to *Wolbachia* (reviewed in Hughes and Rasgon, 2014). This view has been challenged by the successful establishment of *Wolbachia*-transfected *Anopheles stephensi* Liston (Bian et al., 2013), and the recent discovery of a natural stable *Wolbachia* infection in *Anopheles coluzzii* Coetzee & Wilkerson (Shaw et al., 2016). Nonetheless, reports of *Wolbachia* in other species of *Anopheles* have been called into question (Sicard et al., 2019). The lack of natural infections and transinfection failure in *A. ludens* may reflect a general refractoriness to *Wolbachia*. Nonetheless, initial attempts to transinfect *C. capitata* also failed and transfection with *Wolbachia* was attained subsequently with different *Wolbachia* strains (Zabalou et al., 2004b). Hence, transinfection attempts with additional *Wolbachia* strains may result in successful and stable infection in *A. ludens* as well.

Potential for Target Populations to Become Resistant to Sterile Males

There are two ways in which a target population may become resistant to the effects of released *Wolbachia*-infected males. The first is endosymbiont-based, whereby the target population may acquire (e.g., via horizontal transmission) a *Wolbachia* strain that can rescue the modification (sterility) induced by the strain present in the released males. Generally, such acquisition of a *Wolbachia* strain during the relatively short lifespan of a release program seems unlikely. Nonetheless, knowledge on the *Wolbachia* infection status and strain identity of interacting species, such as other fruit flies sharing the same host plant and parasitoids, might aid in the selection of *Wolbachia* strains that are unlikely to be compatible with strains that can potentially be horizontally acquired by the target population. Permanent screening of wild flies from the target population could provide valuable information in order to foresee potential lack of effectiveness of the method. Laboratory experiments in which the conditions for horizontal transmission are favored (or even forced) might also help to determine the probability of such phenomena to occur in nature.

The second mechanism is host-based, whereby pre- or post-mating selection on wild females to avoid or reduce fertilization by incompatible sperm (reviewed by Wedell, 2013), acts on standing (or *de novo*) genetic variation. Evidence consistent with the influence of *Wolbachia* on premating mechanisms comes from the observation that females and males of *Drosophila paulistorum* Dobzhansky and Pavan exhibit assortative mating according to the *Wolbachia* strain they harbor (Miller et al., 2010; Schneider et al., 2019). In addition, treatment with antibiotic (which removed *Wolbachia*) decreases mate discrimination in *D. melanogaster* (Koukou et al., 2006). The evolution of resistance to mating with mass-reared males by wild females can be potentially minimized by frequently refreshing the genetic background of the mass-reared strain, with or without artificial selection (McInnis et al., 2002; Gilchrist et al., 2012; Zygouridis et al., 2014; Quintero-Fong et al., 2016; Sánchez-Rosario et al., 2017), which is a routine process in mass-rearing programs aimed at countering inbreeding and adaptation to mass rearing that is detrimental the success of released males (Robinson and Hendrichs, 2005). Nonetheless, if the basis for mate discrimination were solely determined by *Wolbachia* infection state (e.g., if females could distinguish *Wolbachia*-infected vs. *Wolbachia*-uninfected males solely on the basis of a *Wolbachia*-encoded factor), refreshing the fly genetic background of mass-reared strain is unlikely to slow down the evolution of resistance to released males in the target population.

Several lines of evidence are consistent with the influence of *Wolbachia* infection on post-mating mechanisms. The existence of genetic incompatibility is predicted to favor polyandry (multiple mating by females) as a female strategy to minimize the probability of her eggs being fertilized by sperm from incompatible males (Zeh and Zeh, 1996). Consistent with this prediction, uninfected *D. simulans* females remate sooner than *Wolbachia*-infected females (Champion de Crespigny et al., 2008). Furthermore, *Wolbachia* modifies the length of the spermathecal duct of females of the cricket *Allonemobius socius* Scudder (Marshall, 2007), which in turn may afford the female greater control on the outcome of sperm competition

(e.g., *D. melanogaster*; Miller and Pitnick, 2002). Finally, the fact that host background can influence the CI phenotype (reviewed by Bourtzis and Robinson, 2006), suggests that target populations may have genetic variants that are more resistant to CI, which could increase in frequency as a result of the strong selection exerted by the massive release of *Wolbachia*-infected males.

Potential Alternative Ways of Implementing *Wolbachia*-Based Approaches

The recent identification of *Wolbachia* "CI genes" offers potential alternative ways of harnessing reproductive incompatibility in control of pest tephritids. First, to identify strains with the desired characteristics, at least ability to induce CI, a productive endeavor might be to search for CI loci in the genomes of candidate strains being considered for IIT, prior to artificial transfer efforts. A candidate *Wolbachia* strain that lacks CI loci homologs, or that contains CI loci homologs that are highly similar to (and thus potentially compatible with) strains present in target population, should be avoided. Secondly, it may be possible in the future to genetically engineer *Wolbachia* strains with the desired characteristics (e.g., one or more specific CI operons) for IIT programs, or to replace strains used previously in a control program, as a means of addressing resistance phenomena (Sullivan and O'Neill, 2017). Finally, a thorough understanding of the CI mechanism might enable the development of IIT based on *Wolbachia* transgenes, rather than *Wolbachia* infection. This might be particularly helpful in the control of species that are resistant to *Wolbachia* infection. Nonetheless, the release of such genetically modified insects might not be feasible due to regulatory hurdles and lack of public acceptance.

It has recently been shown that some *Wolbachia* strains can provide protection against major pathogens and parasites of insects, including RNA viruses and bacteria (Hedges et al., 2008; Teixeira et al., 2008; Ye et al., 2013; Martinez et al., 2014). It is very common for pathogens to appear in rearing facilities. Thus, if a *Wolbachia* strain could simultaneously cause strong CI and protect against one or more pathogens (e.g., RNA virus), this would have multiple benefits in an operational *Wolbachia*-based population suppression program. Furthermore, a *Wolbachia* strain that does not induce (strong) CI, but protects against pathogens might be desirable in a program that does not rely on CI (e.g., SIT) for population suppression. *Wolbachia*-mediated pathogen protection would enable high production and quality levels, thereby contributing to a cost-effective and sustainable insect pest management program.

Potential Influence of Other Symbionts

Multiple studies have revealed that although *Wolbachia* appears to be the dominant facultative heritable symbiont of arthropods, numerous other diverse bacteria (e.g., *Spiroplasma*, *Arsenophonus*, *Rickettsia*, and *Cardinium*) form such associations with insects, causing a diversity of reproductive and non-reproductive phenotypes (reviewed in Zchori-Fein and Bourtzis, 2011; Hurst and Frost, 2015; McLean et al., 2018). Despite the long-standing recognition that "*Wolbachia* do not walk alone" (Duron et al., 2008), many studies of *Wolbachia* fail to rule out the association of their study organism with other facultative heritable symbionts. Even intensely studied groups in terms of heritable symbionts, such as tsetse flies (genus *Glossina*), can yield surprises of bacterial associates (e.g., the recent discovery of *Spiroplasma* in two species of *Glossina*; Doudoumis et al., 2017). With few exceptions (Martínez et al., 2012; Augustinos et al., 2015; Asimakis et al., 2019; Conte et al., 2019; Devescovi et al., 2019), research on tephritid facultative heritable bacteria has not examined the possibility of players other than *Wolbachia*. Therefore, we urge that such research include screens for other symbionts, including viruses, protozoans, and fungi.

Tephritids are hosts to non-heritable bacteria, generally harbored in their gut (for recent reviews see Noman et al., 2019; Raza et al., 2020). Whether *Wolbachia* influences tephritid interactions with other microbes, has not been evaluated, but evidence for such interactions exists for other systems (reviewed in Brinker et al., 2019). For example, in *Drosophila neotestacea* Grimaldi, James, and Jaenike, the presence of *Wolbachia* promotes the abundance of *Spiroplasma*, and is positively correlated with abundance of Bacteroidales and Lactobacillales (Fromont et al., 2019). Similarly, *Wolbachia* influences the microbiome of *D. melanogaster* (Simhadri et al., 2017) and *Armadillidium vulgare* (Latreille) (Dittmer and Bouchon, 2018). It is therefore important to evaluate interactions between *Wolbachia* and the microbiome that influence negatively or positively aspects of mass-reared tephritids used in IIT or SIT.

CONCLUSION

Given the widespread occurrence of *Wolbachia* in tephritids and its known fitness consequences in this group of dipterans and in other host taxa, *Wolbachia* is likely an influential component of tephritid ecology and evolution. Further exploration of *Wolbachia*-tephritid associations is expected to reveal a diversity of effects, including interactions with other microbial partners, as seen in more extensively studied systems such as *Drosophila* and mosquitoes. The recent exciting progress in understanding the basis of CI, and many other aspects of *Wolbachia* biology, should accelerate progress in the development of *Wolbachia*-based IIT for tephritid species, particularly with the aid of comparative *Wolbachia* genomics to identify potential CI patterns on the basis of CI gene composition. We consider that one of the major obstacles to effectively implementing IIT will be to avoid population replacement due to accidental release of *Wolbachia*-infected females. The threshold number of accidentally released females, which is generally much higher in systems that employ bidirectional-CI compared to unidirectional-CI, must be thoroughly investigated prior to any field implementation. Where an unacceptable risk of population replacement exists, we recommend that SIT be explored as a complementary strategy to support IIT.

AUTHOR'S NOTE

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MM led the drafting. HM, PL, JT, BM-A, KG, SL, DS, CC, AA, GT, EA, VD, NP, and GK edited multiple drafts.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01080/full#supplementary-material

FILE S1 | Compilation of published reports of screenings of *Wolbachia* (and other heritable bacteria) in pest Tephritidae. In our counts of species, *A. fraterculus* morphotypes (Hernández-Ortiz et al., 2015) are regarded as separate species. Additional references not cited in main text but cited in this table (Drosopoulou et al., 2010; Karimi and Darsouei, 2014; Yong et al., 2017; Gichuhi et al., 2019; Schebeck et al., 2019; Wang et al., 2019).

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REVIEW
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Dynamics of Insect–Microbiome Interaction Influence Host and Microbial Symbiont

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Insects share an intimate relationship with their gut microflora and this symbiotic association has developed into an essential evolutionary outcome intended for their survival through extreme environmental conditions. While it has been clearly established that insects, with very few exceptions, associate with several microbes during their life cycle, information regarding several aspects of these associations is yet to be fully unraveled. Acquisition of bacteria by insects marks the onset of microbial symbiosis, which is followed by the adaptation of these bacterial species to the gut environment for prolonged sustenance and successful transmission across generations. Although several insect–microbiome associations have been reported and each with their distinctive features, diversifications and specializations, it is still unclear as to what led to these diversifications. Recent studies have indicated the involvement of various evolutionary processes operating within an insect body that govern the transition of a free-living microbe to an obligate or facultative symbiont and eventually leading to the establishment and diversification of these symbiotic relationships. Data from various studies, summarized in this review, indicate that the symbiotic partners, i.e., the bacteria and the insect undergo several genetic, biochemical and physiological changes that have profound influence on their life cycle and biology. An interesting outcome of the insect-microbe interaction is the compliance of the microbial partner to its eventual genome reduction. Endosymbionts possess a smaller genome as compared to their free-living forms, and thus raising the question what is leading to reductive evolution in the microbial partner. This review attempts to highlight the fate of microbes within an insect body and its implications for both the bacteria and its insect host. While discussion on each specific association would be too voluminous and outside the scope of this review, we present an overview of some recent studies that contribute to a better understanding of the evolutionary trajectory and dynamics of the insect-microbe association and speculate that, in the future, a better understanding of the nature of this interaction could pave the path to a sustainable and environmentally safe way for controlling economically important pests of crop plants.

Keywords: plant–insect interaction, endosymbionts, bacterial genome size reduction, insect–microbiome co-evolution, microbial symbiosis, mutualists, plant–microbiome interaction, insect gut microflora

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INTRODUCTION

Insects represent one of the most diverse and ancient forms of life on Earth and can cause severe devastation if their population size exceeds a particular threshold. On the geological time scale, their existence dates back to the Paleozoic era when Orthopterans and Hemipterans first appeared on Earth (Misof et al., 2014). Since then, they have advanced and have successfully survived even various extreme climatic conditions. Though insects are both ecologically and economically important, in contrast, insect pests, largely a likely creation of man-manipulated habitats, and many of them an outcome of climate change, are involved in the destruction of crops to the extent of more than 20% annually (Deutsch et al., 2018). Further, changing climatic conditions are influencing the migration pattern of insects, timing of their life cycle and their population dynamics. While overcoming these challenges it has also enabled them to expand their host range, affected their behavior and biology, and thereby helping them invade and colonize different agro-climatic zones of the world (Shrestha, 2019). Their large population size combined with short reproductive cycles and high reproductive rates have enabled them to successfully combat all adverse conditions. Owing to their small body weight, light enough to be carried away by the wind currents, they have invaded various parts of the planet and currently they inhabit almost all the ecosystems on Earth.

Besides, the intricate relationship they share with the beneficial microbes has played a crucial part in their diversification and evolutionary success (Janson et al., 2008). Insects are known to be associated with microbes such as bacteria and fungi throughout their evolutionary history. Some bacterial species reside in specialized cells, within the insects, known as bacteriocytes and are referred to as 'endosymbionts,' whereas there are others, which are located on the body surface and are called 'ectosymbionts' (Thompson and Simpson, 2009). However, they are predominantly present in the digestive tract where they act as key modulators of the diverse lifestyles (both in terms of diet and ecological niches) of their insect host. The gut-microflora of an insect are known to (1) facilitate its feeding even on recalcitrant food; (2) provide immunity and protection against various predators, pathogens and parasites; (3) compensate the nutrient-poor diet (e.g., in the case of sap-sucking insects); (4) mediate inter- and intra-specific communication; (5) control mating and reproductive success; (6) aid digestion and, (7) supply essential amino acids, metabolic compounds and nutrients (Russell et al., 2014; Douglas, 2015; Arbuthnott et al., 2016; Wielkopolan and Obrêpalska-Stêplowska, 2016; Engl and Kaltenpoth, 2018). In fact, Jing et al. (2020) have shown that essential nutrient provisioning is the primary task of symbionts followed by digestion and detoxification. Thus, it implies that insects are highly dependent on their gut microbiome for survival and normal transactions related to their life cycle. Furthermore, based on the degree of dependence, their association can be classified as obligate (or primary) and facultative (or secondary) (Baumann, 2005; Moran et al., 2008). However, there is no clear demarcation between these two categories as facultative bacteria can become obligate under special circumstances (Ferrari and Vavre, 2011).

Together, the endosymbionts and its insect host have formed a very intricate and intriguing relationship, various aspects of which are yet to be explored and understood. Some researchers consider these symbionts as 'intracellular parasites' that have hijacked the insect body and thereafter evolved various mechanisms to ensure their survival while providing benefits to their host. However, it is equally probable that the insect initiated this relationship with its microbiome for its survival. Whichever the case, they have now adapted themselves to each other. Bacterial species present within an insect gut can exhibit mutualism, commensalism or could even be pathogenic (Dillon and Dillon, 2004).

Usually, insects initiate an immune response against the pathogenic bacteria but can selectively maintain the beneficial microbes (Mikonranta et al., 2014). Specific functions of symbiotic anti-microbial peptides (AMPs) have been studied experimentally, revealing that they regulate symbiotic interactions by limiting the reproduction of symbiotic bacteria, sometimes transforming them into a differentiated form, and eliminating undesirable, sensitive bacteria (Mergaert, 2018). Several structural families of AMPs are reported from insects that include apidaecin, hymenoptaecin, defensins, cecropins, drosocins, attacins, diptericins, ponericins, metchnikowins, and melittin (Kwong et al., 2017; Wu et al., 2018). In bees, the expression of the AMPs, apidaecin and hymenoptaecin, is up-regulated in gut tissue, upon microbial acquisition (Kwong et al., 2017). However, it is shown that the endosymbiotic bacteria do not induce the antibacterial responses, such as expression of genes coding for AMPs, in insects (Eleftherianos et al., 2013). For instance, *Wolbachia* when present as a facultative symbiont in *Aedes albopictus* does not trigger the synthesis of AMPs (Bourtzis et al., 2000); however, it induces an immune response in *Anopheles gambiae*, which is not its natural host (Hughes et al., 2011). Likewise, the presence of *Serratia symbiotica* in aphids does not alter the expression of defense-related genes (Burke and Moran, 2011). Additionally, it is observed that different symbionts interact differently with the insect immune system; while some can successfully bypass the insect's cellular immune response others affect the melanization response (a defense mechanism present in insects) (Thomas et al., 2011). Similarly, in this context, recent data seem to indicate several means by which the symbionts are capable of evading insect immunity. However, the processes by which endosymbionts acquired this ability to circumvent the insect immune system or how the insect is able to differentiate between the 'beneficial symbionts' and potentially pathogenic ones is still unclear. But what is clear is that upon initiation of symbiotic association, both the participating partners (the bacteria and the insect) undergo several changes, mediated by the action of various evolutionary forces, which possibly endow the bacterial symbionts with the capability to bypass or evade the insect immune system.

Microbial symbiosis involves acquisition, colonization and transmission. While insects readily acquire several bacteria during their life cycle, others are vertically transmitted or inherited. Post acquisition, successful colonization is crucial for their survival and persistence, which, in turn, is highly influenced by the physical and physiological conditions

of the insect gut. Thereafter, and especially for obligate endosymbionts that are completely dependent on their host for survival, transmission across generations becomes critical. Therefore, to ensure transmission, microbes have evolved several fascinating mechanisms that will be discussed elsewhere in this communication.

Previous studies have unraveled mechanisms involved in the acquisition, maintenance and transmission of endosymbionts. However, we have limited information regarding the mechanisms that drive this entire transition, i.e., the transition of a free-living microbe to an obligate symbiont residing within an insect. Interestingly, the bacterial genome is known to undergo changes to acclimatize itself to the gut environment. It not only modifies itself but some microbes are even capable of manipulating its insect host for their survival (Yuval, 2017). Because of the increased focus on microbiome in general and microbiome of insects in particular in the past decade, our understanding of insect-microbe interactions has also increased. Researchers have now demonstrated that both the insects and their symbionts are tightly inter-connected at almost every level of their evolution. The insect host is known to play a major role in shaping its microbiome (Engel and Moran, 2013), and these endosymbiotic bacteria have now become such an integral part of the insect's body, that they co-evolve with their host. This review is an attempt to summarize our current understanding of the fate of microbes inside an insect's gut, and highlight physical, physiological and functional implications on their insect host. We believe that dissecting the mechanisms directing co-evolution of insect–microbial symbiosis would not only provide us with a better understanding of this association, but information thus obtained could further be applied toward devising sustainable pest management strategies.

DIVERSE FORMS OF INSECT–MICROBE ASSOCIATIONS – FROM INITIATION TO THEIR DIVERSIFICATION

Acquisition of microbes by the insect from the environment is usually the foundation of insect–microbe symbiosis and subsequently, after acquisition, these bacteria undergo a gradual transition from free-living organisms to being intracellular parasites. Once inside the insect's body, their persistence depends largely on the host's life cycle. Upon acquiring bacterial species not only do insects change their feeding habits but they also create specialized niches and gut compartments for housing these microbes that enable and promote microbial persistence (Engel and Moran, 2013). Microbial colonization is heavily affected by the physiochemical conditions of the gut, particularly its pH. Insects housing huge microbial communities provide a favorable environment to their bacterial symbionts by providing them with the optimal pH within the gut (Engel and Moran, 2013). Varied types of bacterial endosymbionts present within the insect body have been reported and each with their distinctive features (**Table 1**).

The obligate mutualists upon entering the insect's body, localize themselves inside bacteriocytes, provide benefits and fitness advantage to the host, and are transmitted maternally across generations. They establish a very stable mutualistic association with their host. They supplement the nutritional requirement of their host by synthesizing essential amino acids and rare vitamins especially for the hemimetabolous sap-sucking insects that feed on nutrient-poor diets. For instance, in *Buchnera aphidicola*, an obligate symbiont of aphids, and probably the most studied model, it has been shown that the bacteria fulfills the nutritional requirement of the insect host to an extent that its removal dramatically affects aphid survival and fecundity (Feng et al., 2019). Similarly, symbiosis between the sap-feeding insect *Megacopta cribraria* and its primary bacterial symbiont, *Candidatus* Ishikawaella capsulate, is essential for host survival to adulthood (Couret et al., 2019). And it is just not the insect that is dependent upon these obligate symbionts but also many long-term obligate symbionts over time have become highly dependent on their insect host. For example, *Buchnera* that lives in a metabolic collaboration within the pea aphid (*Acyrthosiphon pisum)* has lost genes for the synthesis of various branched-chain amino acids (such as isoleucine, valine, and leucine). *Buchnera* is, therefore, entirely reliant on its insect host for the supply of these amino acids, which are crucial in vitamin biosynthesis pathways (Wilson et al., 2010; Hansen and Moran, 2011; Russell et al., 2013).

In contrast, the facultative microbes exhibit an entirely different scenario as some of them are vertically transmitted e.g., *Wolbachia, Spiroplasma,* and *Cardinium*, whereas others are acquired afresh after every generation such as *Burkholderia* and *Serratia*. Further, in several cases, e.g., in the Dipteran pest of rice, the Asian rice gall midge, it has been shown that the community structure of several facultative microbes is highly influenced by the host's developmental stage and diet (Ojha et al., 2017). Besides, the facultative symbionts pre-dominantly assist their insect hosts in digestion and xenobiotic detoxification e.g., some species of *Pseudomonas*, a gram-negative Gamma-proteobacteria found in *Spodoptera frugiperda*, are involved in providing pesticide resistance to their hosts (de Almeida et al., 2017); *Serratia grimesii*, in nematodes, possesses genes involved in the degradation of phytotoxins such as terpenes; and *Candidatus* Ishikawaella capsulate is known to metabolize alkaloids in stinkbugs (Itoh et al., 2018). Moreover, several groups of vertically transmitted facultative endosymbionts such as *Wolbachia, Rickettsia, Arsenophonus, Spiroplasma,* and *Cardinium* are involved in sex determination and are known to induce sexual aberrations across various insect orders (Kageyama et al., 2012). Therefore, some facultative microbes are beneficial for the host (at least under certain circumstances) whereas some are commensals and others even pathogenic. They are known to inhabit various parts of an insect's body and represent the dynamic component of the insect's microbiome. While some are localized to the hemocoel and are present ubiquitously (scattered pattern), others are restricted to the bacteriocytes (confined pattern) (Marubayashi et al., 2014). Unlike obligate symbionts that mostly exhibit transovarial transmission, the facultative symbionts have evolved various fascinating mechanisms to ensure their transmission and propagation inside an insect's body. For instance, *Sodalis glossinidius,* a facultative symbiont of the

TABLE 1 | Types of microbial symbionts of insects and their attributes.

Features	Types of microbial symbionts			References
	Obligate	**Facultative**	**Phytopathogenic**	
Acquisition	[a]Maternal	[a]Environmental	[d]Plants via feeding	[a]Baumann, 2005
Localization	[a]Bacteriocytes	[b]Ubiquitously (Hemocoel)/confined (Bacteriocytes)	[c]Salivary glands	[c]Kwon et al., 1999; [b]Marubayashi et al., 2014
Transmission	[a]Vertical	[a]Horizontal	[d]Horizontal	[d]Chrostek et al., 2017
Key functions	[a]Nutrition provisioning	[e]Digestion and detoxification	[d]Enhances virulence, facilitate feeding	[e]Moran et al., 2005
Genome size	Highly reduced	Normal	Normal	Nakabachi et al., 2013
Host dependency	Mutualists	Mutualists, Commensals or pathogenic	Mutualists, Commensals or pathogenic	Moran et al., 2008

The reference citations and references are indicated with superscript letters when the provided information is derived from more than one reference.

tsetse fly has evolved the capacity to be transmitted through transovarial transmission via haemolymph (Cheng and Aksoy, 1999), or vertically to the intrauterine larvae via milk gland secretions and in some instances, horizontal transmission during mating (De Vooght et al., 2015) was also observed. Apart from this, there are substantial number of interesting studies indicating the nature of various bacterial symbionts across different insect orders, and these are summarized in **Table 2**.

As a result of the feeding process, besides obligate and facultative symbionts, several phytopathogenic microbes are also found in insect bodies. However, plants, being immobile, become a major obstacle in the transmission of these phytopathogenic bacteria. This led to the dependency of these microbes on vectors, which are usually insects, for their dispersal and propagation. And consequently, initiating an association of these microbes with their insect vector and in turn shaping this complicated relationship that is currently observed between them. These bacteria not only actively interact with their insect host but also modify it for their own benefit. Some microbes can multiply within its insect vector (propagative) while some cannot (non-propagative). This implies that besides serving as the vector, the insect also serves as an alternate host for these bacteria (Nadarasah and Stavrinides, 2011). Some microbes once acquired by the vector are readily transmitted to the host plant (semi-persistent, non-circulative) whereas others circulate through the body of the insect and are transmitted only after a latent period (persistent, circulative transmission) (Perilla-Henao and Casteel, 2016). Upon entering the insect's body they migrate to the midgut or hindgut epithelium and are subsequently released into the haemolymph. From the haemolymph, they enter the salivary gland and are transmitted to the plant during the feeding process (Kwon et al., 1999). In turn, insects have also evolved mechanisms that enable them to tackle these pathogens and derive certain advantages out of this insect-microbe interaction. Though the mechanisms behind the co-evolution of these microbes and their insect vector are both fascinating and crucial for the understanding of microbes–insect–plant interactions, it will not be discussed further as it is beyond the scope of this review.

As discussed in earlier sections and evident from the data summarized in **Table 2**, these endosymbionts have, over time, evolved various mechanisms that are critical for sustenance within an insect body. The occurrence of these varied types of associations raises the question, what led to this diversification? Why and how did some bacteria become an obligate intracellular symbiont in some insects while it remained facultative in others? What determines the nature of association of any microbe for a particular insect? It could be the likely outcome of its (bacterial) functional capacities (the capacity to fulfill the host's requirement) and capabilities (an important aspect being the capability to evade insect's immune system). Furthermore, it could also be determined by the insect host based on the extent of its dependency on that bacterium. However, the possibility that these associations are made under selective influence, where an insect found in a particularly harsh environment is forced to form an association with the microbe to overcome the immediate biotic and abiotic stresses, cannot be negated. Despite several studies, we still have very limited information regarding mechanisms that led to the evolution and eventually diversification of these associations.

FATE OF MICROBES WITHIN AN INSECT BODY – THEIR JOURNEY OF TRANSITION FROM FREE-LIVING TO AN OBLIGATE SYMBIONT

The fitness of an organism and its success at any given point of time depends upon its genome flexibility that provides it with the capability to adapt and adjust as and when required depending on the environment. However, it is ironic to note that while organisms strive toward achieving genome stability, this drive could also become a reason for its demise or extinction. Although genome stability allows maintenance of adapted phenotypes, it is also a major obstacle in the evolution of novel and superior traits that enable an organism to tolerate change (Schubert and Vu, 2016). Evolutionary data suggest that several species have become extinct because of their inability to cope well with the changing environmental conditions. However, insects, due to their genome flexibility, can rapidly adapt by undergoing modifications in their genome size, composition and its architecture (Robertson, 2005) and thereby helping it overcome/survive the adverse conditions. Moreover, owing to the large population size, any beneficial variation, induced by the evolutionary forces, gets fixed in an insect population rapidly.

TABLE 2 | List* of bacterial species and type of their associations with insects; their known mode of acquisition, localization and transmission.

Bacterial symbiont	Nature of association	Insect host(s)	Mode of acquisition	Localization within the host	Mode of transmission	References
Buchnera aphidicola	Obligate Mutualism	Aphids	Inherited	Bacteriocytes	Transovarial	Baumann, 2005
Carsonella ruddii	[a]Obligate mutualism	[a]Psyllids	[b]Inherited	[b]Bacteriocytes	[b]Transovarial	[a]Thao et al., 2000; [b]Thao et al., 2001
Blochmannia floridanus	Obligate mutualism	Carpenter ants	Inherited	Somatic cells surrounding ovarioles	Transovarial	Kupper et al., 2016
Wigglesworthia glossinidia	Obligate mutualism	Tsetse flies	Inherited	Bacteriocytes	Transovarial	Bing et al., 2017
Serratia symbiotica	Facultative commensalism	Aphids	Environmentally acquired	NA	Horizontal transmission	Pons et al., 2019
Regiella insecticola	Facultative commensalism	Aphids	Inherited	Bacteriocytes, Haemolymph	Transovarial	Vorburger et al., 2010
Hamiltonella defensa	Facultative Commensalism	Aphids, Whiteflies	Acquired and Inherited	Sheath Cells, Secondary Myocetocytes, Haemolymph	Horizontal and Maternal	Marubayashi et al., 2014
Portiera aleyrodidarum	Obligate mutualism	Whiteflies	Inherited	Bacteriocytes	Transovarial	Santos-Garcia et al., 2015
Tremblaya princeps	Obligate mutualism	Mealy bugs	Inherited	Bacteriome	Transovarial	López-Madrigal et al., 2013
Sodalis glossinidius	Secondary facultative	Tsetse flies	Inherited and Acquired	Numerous tissues	[a]Milk gland, [b]Transovarial, and [a]Mating	[a]De Vooght et al., 2015 [b]Cheng and Aksoy, 1999
Baumannia cicadellinicola	Obligate mutualism	Sharpshooters	Inherited	Bacteriocytes	Transovarial	Wu et al., 2006
Sulcia muelleri	Obligate mutualism	Sharpshooters	Inherited	Bacteriocytes	Transovarial	Moran et al., 2005
Nardonella sp.	Obligate mutualism	Weevils, Beetles	Inherited	Bacteriocytes	Transovarial	Kuriwada et al., 2010
Candidatus Arsenophonus arthopodicus	Facultative commensalism	Louse flies	Inherited	Intestine wall (bacteriocytes), Lumen of milk glands	Transovarial	Nováková et al., 2015
Wolbachia sp.	Facultative parasite	Various insects	Inherited	Bacteriocytes, extracellularly scattered	Transovarial	Miller, 2013
Rickettsia sp.	Facultative parasite	Various insects	[b]Inherited	Extracellularly Scattered, Bacteriocytes	Transovarial	[a]Behar et al., 2010 [b]Gottlieb et al., 2006
Spiroplasma sp.	[a]Facultative parasite	Various insects	Inherited	Haemolymph, Endocellularly localized	Transovarial	Bové, 1997
Cardinium sp.	Facultative parasite	Planthoppers	Inherited	Gut, testicles, oocytes, glands	Transovarial	Gonella et al., 2011
Ishikawaella capsulata	Obligate mutualism	Plataspid stinkbugs	Inherited	Extracellular (midgut)	Capsule	Nikoh et al., 2011
Rosenkranzia clausaccus	Obligate mutualism	Stinkbugs	Inherited	Midgut crypts	Egg smearing	Hayashi et al., 2015
Rhodococcus rhodnii	Facultative mutualism	Assassin bugs	Environmentally acquired	NA	Coplophagy	Kikuchi, 2009
Serratia marcescens	Pathogenic	Grassland locusts	Soil	Fat bodies	Insecticidal properties	Tao et al., 2006
Burkholderia sp.	NA	Bean bugs, stinkbugs	Environmentally acquired	Crypts at posterior midgut region	Horizontal transmission	Kikuchi and Yumoto, 2013
[a]*Rickettsia* sp., [b]*Cardinium* sp., [a]*Wolbachia* sp.	Facultative parasites	Leafhoppers	[a]Inherited, acquired	[a]Intracellular and Scattered	Transmitted to plants	Nakamura et al., 2009; Gonella et al., 2015
Candidatus liberibacter psyllaurous	Facultative	Tomato psyllids	Acquired during feeding	Extracellular	Vector	Hansen et al., 2008

(Continued)

TABLE 2 | Continued

Bacterial symbiont	Nature of association	Insect host(s)	Mode of acquisition	Localization within the host	Mode of transmission	References
Moranella endobia	Obligate mutualism	[b]Mealy bugs	[b]Inherited	[a]Inside Tremblaya *princeps* cells	Maternal	[a]López-Madrigal et al., 2013 [b]Husnik and McCutcheon, 2016
Blochmennia floridanus	Obligate commensalism	Carpenter ants	Inherited	Oocytes, Enterocytes of midgut tissue (Bacteriocytes)	Maternal	Zientz et al., 2006
Xenorhabdus nematophilus	Pathogenic	Wax moths	Acquired	Haemolymph	Toxic	Mahar et al., 2005
Photorhabdus luminescens	Pathogenic	Tobacco hornworm	Acquired	Haemocoel	Toxic	Münch et al., 2008
Serratia marcescens	Opportunistic pathogen	Fruit flies	Acquired	Gut, body cavity, intestinal epithelial cells	Toxic	Nehme et al., 2007
Serratia entomophila	Pathogenic	Grass grubs	Feeding	Digestive tract	Toxic	Hurst et al., 2004
Enterobacter aerogenes, Bacillus cereus, Bacillus sphaericus, Serratia sp., *Klebsiella* sp., *Morganella* sp.	Temporal association	Neuropterans (Antlions)	NA	NA	Toxic	Nishiwaki et al., 2007
Erwinia chrysanthemi	Specific pathogen	Aphids	Septic injury, oral injection	NA	Toxic	Costechareyre et al., 2012

[a]Though most of the bacterial species can be found to be associated with more than one insect species, the predominant host is listed here. NA, data not available. The reference citations and references are indicated with superscript letters when the provided information is derived from more than one reference.

In addition, it harbors these endosymbiotic bacteria that it has remodeled for its benefit. It has been shown that the several bacterial species that are present within an insect body differ remarkably from their free-living counterparts (Kikuchi, 2009). Studies have revealed that the genomes of endosymbiotic bacteria carry signatures not only signifying its phylogenetic position, but also revealing the kind of lifestyle to which it has adapted. Various genome-specific signatures such as base composition, GC-skew, purine-pyrimidine ratio, dinucleotide abundance, codon bias, oligonucleotide composition etc. have been identified from the endosymbiotic bacterial genomes (Dutta and Paul, 2012). Besides, the bacterial species present in an insect gut have a highly reduced genome (i.e., they have small, gene-dense genome) as a result of sequential gene loss (**Figure 1**; Wernegreen, 2002). It has been demonstrated experimentally by several research groups that endosymbiosis involves massive genomic rearrangements brought about primarily by mobile element proliferation and pseudogenization of non-essential genes (Van Ham et al., 2003; Pérez-Brocal et al., 2006; Moran et al., 2009). In aphids, it is shown that the recently incorporated *Serratia symbiotica* (genome size ∼2.79 Mb) is at the pseudogene proliferation stage with ∼550 pseudogenes as opposed to ∼12 pseudogenes found in its free-living relative, *S. proteamaculans*; whereas the other co-residing ancient endosymbiont, *Buchnera aphidicola* (genome size ∼0.652 Mb) has undergone pseudogenization of several non-essential genes (Nicks and Rahn-Lee, 2017). However, an exception to this is *Sodalis glossinidius,* a facultative bacterial symbiont of tsetse flies, whose genome analysis revealed large-scale and significant expression of pseudogenes and thereby suggesting that it is a recent acquisition by these insects (Goodhead et al., 2020). Furthermore, *Carsonella,* an obligate symbiont of psyllids, and one of the smallest known symbionts in terms of its genome size (i.e., ∼173 kb) has undergone extensive gene loss making it entirely reliant on its host for survival (Thao et al., 2000). These findings have been further corroborated by correlation analysis, carried out by Fisher et al. (2017) on 58 obligate bacterial symbionts found in 89 host species including plants, fungi, insects, and other arthropods, that suggested a negative correlation between host dependence and symbiont genome size. Thus indicating that genome reduction due to gene losses lead to complementation and functional redundancy, which reinforces the inter-dependency of microbes on one another and their host. And this is one of the widely accepted phenomena that are known to occur within an insect gut.

Moreover, it appears that the extent of genome reduction depends upon the nature of association. It is generally observed that the bacteria under obligate symbiotic association have a comparatively smaller genome than when it occurs as a facultative symbiont. For instance, *Arsenophonus* sp. when found as an obligate symbiont in *Riesia pediculicola,* has a genome of ∼570 kb while the one that is associated with *Nasonia* spp. (as a facultative symbiont) has a genome size of approximately 3500 kb (Nováková et al., 2016). This suggests that the smaller the genome size higher is its dependency on the host. And, it also indicates that gene loss is probably one of the primary reasons for the transition of any facultative symbiont to an obligate symbiont

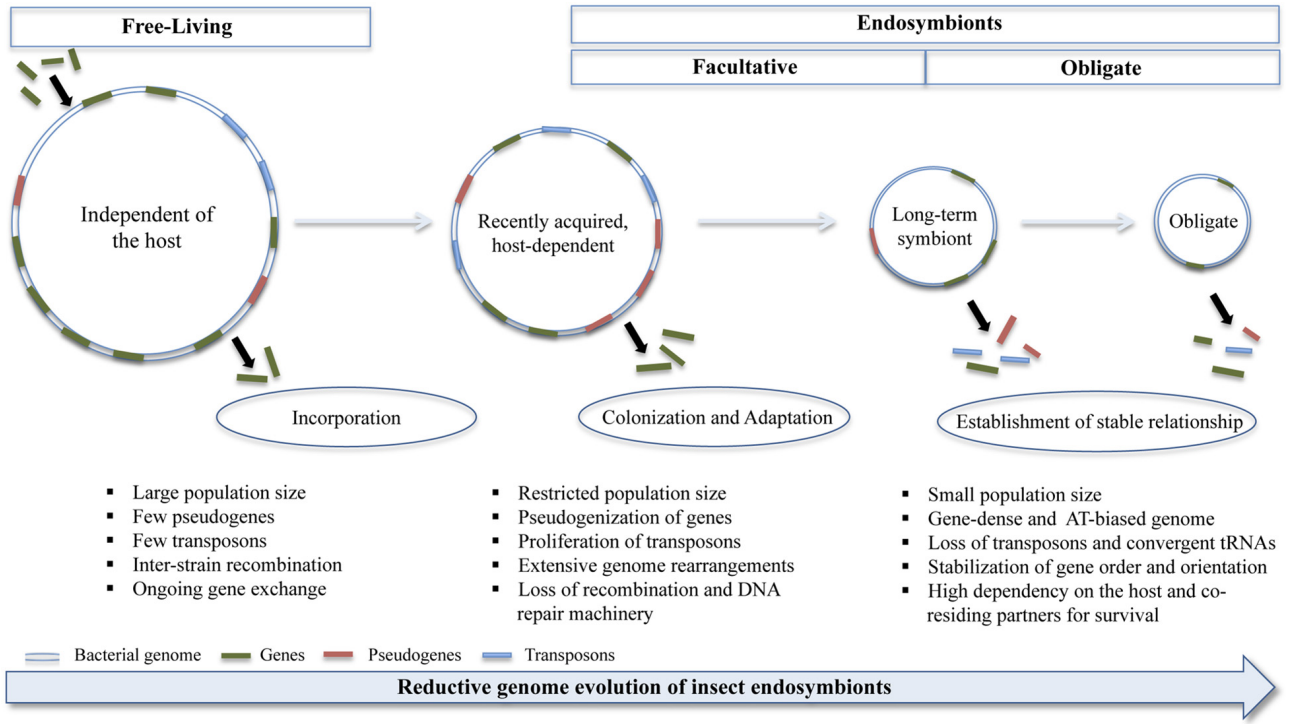

FIGURE 1 | Diagrammatic representation of steps involved in the transition of free-living bacteria to obligate insect endosymbionts. The host-restricted bacteria undergo changes in its genomic size, composition and architecture; and these are brought about by excessive gene loss, chromosomal rearrangements, proliferation of transposons and pseudogenization of several non-essential genes during the adaptation stage and these processes are attenuated on transformation to obligate symbionts (see text for details). Objects in the figure are not drawn to scale.

inside the host. This phenomenon is comparable to evolution of mitochondrial and chloroplast DNA within a eukaryotic cell, which represents a classical case of genome reduction during symbiosis. Mitochondria and chloroplasts represent the ultimate outcome of 'reductive evolution' as they have undergone up to 95% reduction in their genome upon transition from free-living to obligatory intracellular parasite (Gray, 2012). Obligate and facultative symbionts possess one of the smallest genomes when compared to their free-living forms (**Figure 2** and **Supplementary Table T1**).

Besides, these studies also indicate that the symbionts of the recently formed associations with hosts are slightly reduced in their genome size whereas the ones that represent an older association have a highly reduced genome. For instance, *Serratia* that is known to be associated with *A. pisum* for over 100 million years has one of the highly eroded genomes ranging from 0.65–1.76 Mb as opposed to 5.11–5.45 Mb genome of its free-living counterpart (Richards et al., 2010). Likewise, *Buchnera aphidicola*–aphid symbiosis originated about 200 million years ago, and over time *Buchnera* genome has been drastically reduced to less than 0.7 Mb inside its insect host (Gil et al., 2002). Furthermore and in congruence, the last decade has witnessed several studies in this regard (**Supplementary Table T1**) and results from such studies point toward the fact that the genome size of a bacterial endosymbiont is inversely proportional to the time for which it has been associated with the host.

Researchers have shown that the endosymbiotic bacteria have undergone drastic genetic, phenotypic and biochemical changes as observed when compared with their free-living counterparts (**Figure 1**). And the gene-loss observed in the bacterial symbiont, while in association with its host, appears to be a non-random and a continuous phenomenon (Sabater-Muñoz et al., 2017). It has been observed that the gradual decrease in the genome size upon transition from free-living to obligate form, is accompanied by several other changes in genome characteristics including decrease in its GC-content and number of coding genes (Van Leuven and McCutcheon, 2012); reduction in the number of copies of *rRNA*, *tRNA* and other non-coding genes (Manzano-Marín and Latorre, 2016); proliferation of mobile elements at first (during facultative association) and their subsequent loss upon transition to an obligate symbiont (Belda et al., 2010). Genome reduction is also known to influence translation in endosymbionts where convergent tRNA loss has been observed in microbes that have undergone severe genome reduction. For instance, loss of modified nucleoside pathways, introduction of high AT-bias that resulted in reduced tRNA thermostability and various alternations in tRNA features crucial for translation, have been reported for *Buchnera* symbiont of aphids (Hansen and Moran, 2012).

In addition, it is often noticed that the bacterial partner usually retains the genes involved in symbiotic functions (McCutcheon, 2016). For example, *Portiera* has retained genes

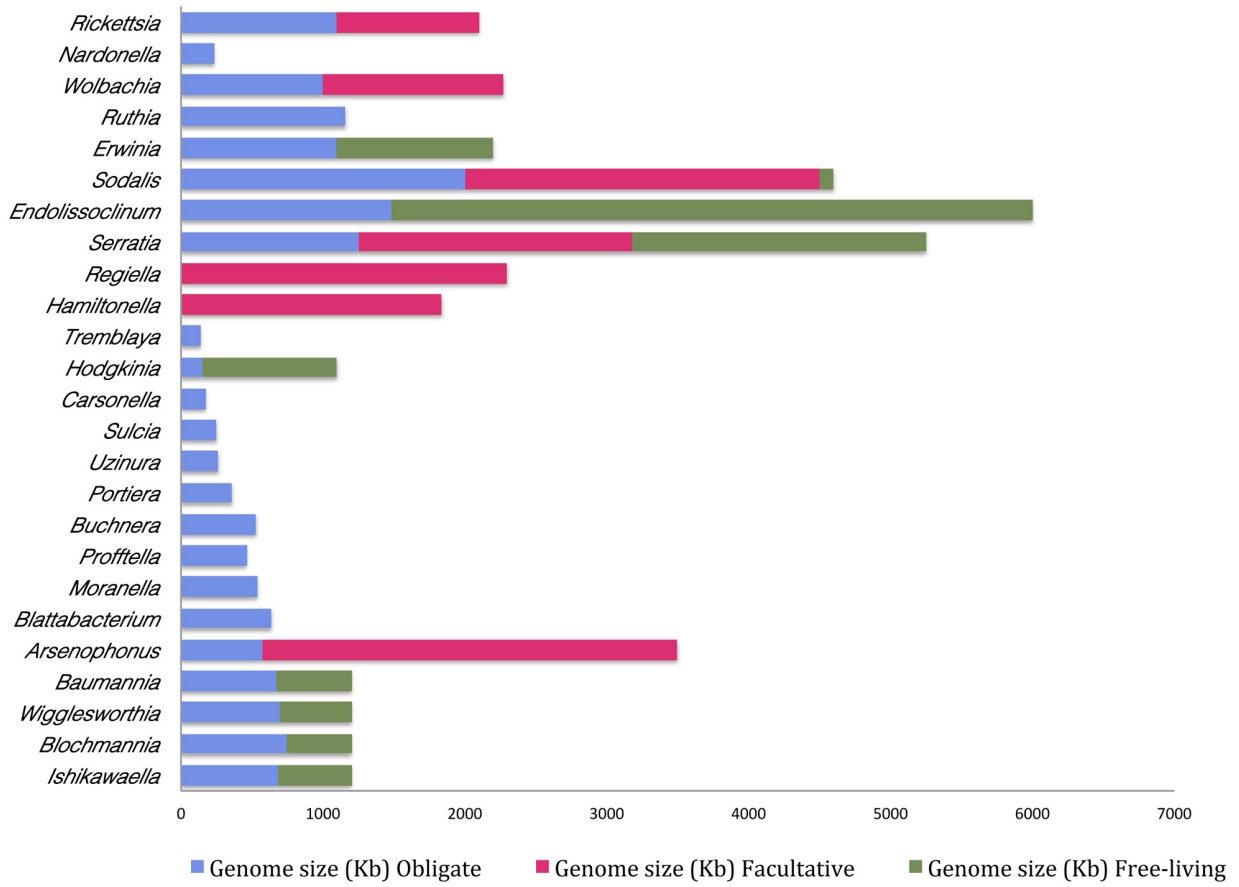

FIGURE 2 | Differences in genome sizes of the free-living bacteria and their cognates in a facultative or obligate symbiotic relationship with an insect host. Names on the *Y*-axis are the bacterial species and genome sizes (Kb) are indicated on the *X*-axis. (This figure is based on published information and the relevant references are provided in **Supplementary Table T1**).

encoding essential amino acids and carotenoids but lacks several vitamin and co-factors producing genes which are compensated by the other symbiont, *Hamiltonella,* that is known to co-occur with *Portiera* within their common insect host, *Bemisia tabaci* (Rao et al., 2015). Together they also fulfill the nutritional requirements of their host. Similarly, to obtain nitrogen from the uric acid stored in the fat bodies, the cockroach (*Blattella germanica*) utilizes urease produced by its primary endosymbiont *Blattabacterium cuenoti* (López-Sánchez et al., 2009; Patiño-Navarrete et al., 2013); *Candidatus* Portiera aleyrodidarum supplements the diet of their phloem-feeding hosts by supplying essential amino acids and vitamins (Zientz et al., 2004; McCutcheon and von Dohlen, 2011); *Sulcia muelleri* and its co-resident *Hodgkinia cicadicola* synthesize essential amino acids in cicadas (McCutcheon et al., 2009). Interestingly *Cardinium,* in spite of its reduced genome size with several genes coding for various metabolites lost, encodes the complete biosynthetic pathways for biotin and lipoate, which are crucial for its host's nutrition (Zeng et al., 2018). And not only are there examples of host-endosymbiotic metabolic collaboration but the endosymbiotic bacteria are also known to complement each other in several insects. For instance, *Moranella endobia* and *Tremblaya princeps* are known to complement each other within mealybugs (López-Madrigal et al., 2013); *Serratia* complements *Buchnera* in aphids (von Dohlen and Moran, 2000); *Carsonella eucalyptia* (primary symbiont) and *Heteropsylla cubana* (secondary symbiont) exhibits strict complementarity in the biosynthesis of tryptophan in psyllids (Sloan and Moran, 2012); *S. muelleri* supplies amino acids to various co-residing symbionts (Rao et al., 2015).

It is also interesting to note that the bacterial species that complement each other the most, co-occur inside the same bacteriocyte. For instance, *Portiera* and *Hamiltonella* have undergone genome reduction, exhibit metabolic complementation and are mostly present inside a single bacteriocyte in their host, *Bemisia tabaci* (Rao et al., 2015). Similarly, Alonso-Pernas et al. (2017) studied the bacterial communities localized to the hindgut wall in the forest cockchafer, *Melolontha hippocastani*, and have shown that the composition of bacterial community depends on the insect's life stage. Further, their data revealed the occurrence of specialized bacterial niches ('pockets') attached and connected to both sides of the distal part of the hindgut wall. In addition, they have reported that the Poly-β-hydroxybutyrate (PHB) accumulating

bacteria *Achromobacter* sp., was co-localized within these 'pockets' and, therefore, it was speculated that the presence of this polymer might play a role in the colonization of these specialized niches. These studies indicate the possibility that the microbes within an insect are compartmentalized into separate bacteriocytes based on their functional capabilities and complementation. However, due to the lack of sufficient and reliable experimental evidences, this is merely a conjecture.

Currently, there is enough information available regarding the nutritional and metabolic collaboration among bacterial endosymbionts of insects. However, it is still uncertain how these intricately intertwined metabolic networks have evolved. Is it a random phenomenon driven by evolutionary forces such as mutation and genetic drift or is guided by selection? The last decade has witnessed several studies in this regard that indicate the complexity and intricacy of the evolutionary mechanisms that are responsible for shaping an insect's microbiome and will be discussed in the following section.

MECHANISMS DRIVING THE TRANSITIONAL EVOLUTION OF INSECT ENDOSYMBIONTS

It is observed that the parasites and symbionts undergo 'simplification' rather than evolving complex metabolic pathways inside their insect host and 'genome reduction' is considered a dominant mode of evolution of endosymbionts (Wolf and Koonin, 2013). Taken together, studies strongly indicate the likelihood of 'community-level selection' being imposed on these bacterial species residing within their insect host. The famous 'black-queen hypothesis (BQH)' proposed by Morris et al. (2012) appears to hold true in several cases of insect–microbe symbiosis. According to BQH, insect–microbiome dependencies and collaborations are a consequence of selection-driven reductive genome evolution of endosymbiotic bacterial species. Lee and Marx (2012) have already shown the prevalence of selection-driven genome reduction in experimental populations of *Methylobacterium extorquens*. They observed parallel deletions (resulting in ∼10% reduction in genome size) in a megaplasmid present in this bacterium when cultured for ∼1500 generations under constant selection under laboratory conditions. Their data, therefore, provide the ideal evidence to corroborate the concept of selection-driven reductive evolution of gut endosymbionts.

Moreover, the gene loss in these symbionts confers a selective advantage by conserving energy and resources where gene function is dispensable. The bacterial species within an insect host are functionally synchronized and thereby reducing the pressure on individual bacterial species to maintain its complete metabolic network. This is achieved when the co-residing bacterial species become part of a diversified metabolic network working in partnership with each other while not subjecting their insect hosts to additional metabolic load. Therefore, this 'adaptive genome streamlining' of bacterial endosymbionts could prove to be highly beneficial especially for the insect host. In addition, these changes that occur in the bacterial genome, likely endow the bacteria with structural and functional stability. The bacterial genome, upon losing non-coding DNA and genes not critical for symbiont function, becomes highly stable with regard to gene order and orientation (Sabater-Muñoz et al., 2017). This implies that these changes are non-random and adaptive and are, therefore, likely to be driven by selection.

In contrast, this could also be brought about by the action of genetic drift. According to "Muller's ratchet hypothesis" the endosymbionts evolve under the influence of drift, as they are believed to experience a relaxed selection imposed by small population bottlenecks within an insect gut (Pettersson and Berg, 2007). The effect of drift and bottlenecks is profoundly exaggerated in the symbionts that solely rely on vertical transmission and there is no horizontal transmission for compensation (Mira and Moran, 2002). In addition, the asexual mode of reproduction in symbiotic bacterial species results in their isolation from the recombination processes resulting in rapid genome degradation (Moran and Plague, 2004). Therefore, the degenerative trajectory of the bacterial genomes present inside insects could be very well explained by the reduced efficiency of natural selection. Also, it has been observed that the endosymbionts have genomes that are highly AT-rich. Usually, selection force and recombination events eliminate the AT-rich sequences and favor GC-rich coding gene sequences (Bobay and Ochman, 2017). But the fact that the genomes of endosymbionts are AT-rich, supports the hypothesis that the evolution of endosymbionts is under weak-selection combined with the absence of genetic recombination.

However, it is also worth considering that the early events involving gene-inactivating mutations and replication slippage are biased toward the GC-rich component of the bacterial genome (i.e., mostly in the genic regions) (Clayton et al., 2016). However, once it has adapted to the insect gut, these events could be deleterious. Therefore, possessing an AT-rich genome could also be an adaptive trait, conferring stability to the bacterial genome.

Additionally, another interesting observation is that symbionts often lose DNA replication and repair mechanisms along with the recombination system quite early in their association with the host (Moran et al., 2008). And this gene loss is not random but is both pre-determined and adaptive. Loss of DNA repair pathway leads to increased deleterious mutations, and in turn, gene inactivation. This also increases the scope for introducing variation in the genome especially when the organism is struggling to adapt to a certain lifestyle within an insect host. A study by Giraud et al. (2001) showed that the mutated phenotypes that arise in the natural and laboratory-based experimental populations of bacteria are highly similar to the mutated phenotypes present within an insect gut. This means that these mutations are advantageous as they facilitate symbiont adaption to the insect gut environment. Moreover, it does correlate with the loss of genes that encode products targeted by an insect's immune system as a part of the adaption of bacteria to the insect's body (Ratzka et al., 2012). Similarly and in congruence, data obtained by Toft and Fares (2008) while studying the evolution of flagellar assembly pathway in genomes of endosymbiotic bacteria, suggest that flagellar genes in endosymbiotic bacteria, of insects, belonging

to Gamma-proteobacteria, have functionally diverged to adapt to the new environment and become specialized in exporting proteins from the bacterium to the host. These processes are too intricately organized to occur randomly and it is hard to comprehend that merely random processes such as mutations and drift are sufficient for driving transition of any free-living bacteria to an obligate endosymbiont. However, owing to limited information, it is still uncertain whether the reductive evolution of endosymbionts is driven by genetic drift or guided by selection or is a consequence of combination of both the evolutionary forces acting upon it.

Furthermore, although it seems reasonable to believe that the bacterial community within the insect gut experience relaxed selection but what about the selection pressures experienced by the host? Importantly, it is worth noting that changes occurring in the bacterial genome mostly benefits the host. Moreover, now there are indications that various environmental constraints, dietary shifts, change in ecological niches due to colonization and invasion of new habitats, could dramatically affect the bacterial community within the insect (Ng et al., 2018). In fact, insects adapt to various environmental fluctuations by modifying their microbiome. For instance, *Drosophila melanogaster* survivability under extreme conditions is determined by its microbiome composition. Moghadam et al. (2018) showed that reshaping the gut microbiota of *D. melanogaster* immensely affected its thermotolerance capacity. Therefore, the role of insect host in shaping its microbiome cannot be negated. The insect and its bacterial symbiont are so tightly coupled that the selection pressures experienced by the insect host could also play a major role in channeling the direction of evolution of its endosymbiotic bacteria. Rennison et al. (2019) demonstrated that changes in the gut microbial communities take place in conjunction with their host colonization, adaptation and speciation. They studied the impact of host speciation and divergence on the evolution of its gut bacteria. And their results indicate that the gut microbial communities have shifted by undergoing parallel divergence and speciation to be in synchrony with their stickleback hosts. Thus suggesting the involvement of insect-microbe interactions in driving the evolution of microbial endosymbionts.

While it is true that bacterial composition and structure inside an insect gut is primarily determined by the action of various evolutionary forces acting upon the residing endosymbiotic bacteria and its host, the fact that bacterial populations within an insect body live as a community where they have to share limited resources, cannot be overlooked. Under such a scenario, where the resources are limited, the constant battle for resources amongst the co-residing microbes is bound to occur and as a consequence, inter- and intra-specific competition is inevitable. Though we could not find any direct evidence of such phenomenon occurring inside an insect gut, there are indications from studies on the human-microbe symbiotic association and phytopathogenic bacteria vectored by insects corroborating the above conclusion. Several studies on the human microbiome reveal temporary shifts in the microbiome composition depending upon the dietary intake (Leeming et al., 2019). Recently, it has also been reported that *Gardnerella* subgroups (component of human vaginal microbiome) compete with each other and that in turn affects their population dynamics (Khan et al., 2019). Studies by Jones et al. (2019) indicate that the host plant directly influences the composition of gut microbiota in *Helicoverpa zea*. They have shown that the bacterial communities differ between populations of *H. zea* feeding on different host plants distantly located at different feeding sites. Additionally, a study conducted on the phytopathogenic symbionts that are transmitted by leafhoppers also supports this hypothesis. Rashidi et al. (2014) have shown that though two *Phytoplasmas* are acquired by the leafhopper, *Euscelidius variegates,* during the feeding process, i.e., *Candidatus* Phytoplasma vitis, that causes Flavescence dorée (FDP), and *Candidatus* Phytoplasma asteris, which is the causal agent of Chrysanthemum yellows (CYP), only CYP was efficiently transmitted by the doubly infected leafhoppers. Additionally, it was shown that *P. vitis* was outcompeted by *P. asteris* and hence, was seldom detected in the salivary glands. They conclude that the competition between the two *Phytoplasmas* affected salivary gland colonization by *P. vitis* and during the course of their association with the leafhoppers; *P. asteris* had acquired the greater ability to colonize the insect body and thus ensuring its transmission. Based on these studies, it is plausible to state that microbes compete with each other for survival even within an insect body. And as nutrient accessibility is a major limiting factor, therefore, it is reasonable to believe that the predominance of microbes within an insect body could also be determined by the availability of nutrients and their rate of consumption. Additionally, this could also account for microbiome fluctuations observed in insects when they feed on resistant and susceptible plants or on recalcitrant food sources. Here, it is speculated that under certain conditions, microbes can co-exist (e.g., nutrient-rich conditions) while under other conditions (e.g., nutrient-poor conditions) the specific taxa are outcompeted due to acute nutritional limitations. This would imply that the "resource ratio" competition model, which was first proposed by Tilman (1977) based on the work on plankton algae and later on was reported to hold true for various bacterial communities thriving together, is likely to hold true for insect gut endosymbionts as well. However, additional experimental verification would be required to prove this hypothesis.

IMPLICATIONS OF GENOME SIZE REDUCTION FOR THE ENDOSYMBIOTIC BACTERIA

Bacterial population that is continuously experiencing genome degradation (either due to the selection pressure or as a consequence of genetic drift) cannot escape extinction. Ultimately, a critical stage of genome erosion is achieved; wherein obligate endosymbionts start suffering from 'genome reduction syndrome' (Latorre and Manzano-Marín, 2017) that symbolizes their evolutionary "dead-end." Extreme gene losses lead to complete dependency of these bacteria on one another and/or their host, as they are incapable of surviving on their own (Husnik and Keeling, 2019). While reducing the metabolic versatility of these endosymbionts, on a long-term evolutionary

scale the bacteria with reduced genome have less flexibility and thus lower chances of survival (as compared to their wild-type forms), especially during a sudden environmental change. Although gene loss increases the dependency of the symbiont on the host while reducing the cost associated with symbiosis, excessive genome decay leads to a point where the bacteria is unable to maintain "healthy" association with its host, i.e., it becomes incapable of fulfilling host requirement (Latorre and Manzano-Marín, 2017). Under such a scenario, the bacterial population either suffers a collapse or is marked for replacement. Vogel and Moran (2013) have already shown the replacement of *Buchnera aphidicola* by yeast-like endosymbionts in *Cerataphis brasiliensis*. However, recently the second case of loss of this ancient endosymbiont *Buchnera* from the members of aphid genus *Geopemphigus* has been documented. Here, *Buchnera* was found replaced by another symbiont from the bacterial phylum Bacteroidetes (Chong and Moran, 2018).

So how do symbionts avoid such an evolutionary scenario? One possible strategy to escape extinction is to replace the inefficient bacterium with its free-living counterpart every now and then. This is possible for facultative symbionts and the ones that are environmentally acquired or horizontally transmitted. But strict vertical transmission of some bacterial species becomes a major obstacle for many obligate symbionts. Until recently, it was a puzzle as to how these obligate symbionts managed to survive for long periods in an insect gut. However, it has been now shown that these ancient symbionts establish a di-symbiotic relationship with newly acquired bacterial species. Manzano-Marín et al. (2020) have reported that *Erwinia,* (which is a newly acquired symbiont of aphids) complements *Buchnera* (an ancient symbiont) by serial horizontal transfer of several vitamin biosynthesis genes and thus, compensating for the massive gene loss undergone by *Buchnera* during the long period of its association with its insect host. Similarly, a horizontal gene transfer event was observed between *Cardinium* and its donor organisms, *Wolbachia* and *Rickettsia*, which counterbalance the significant gene loss undergone by *Cardinium* to adapt to the gut environment of its host (Zeng et al., 2018). In addition, recently it has been demonstrated that genome reduction in bacterial species is usually preceded by the acquisition of genes, essential for host survival, from other co-residing microbes via horizontal gene transfer. This is evident from the study conducted by Waterworth et al. (2020) where it has been shown that *Burkholderia gladioli,* present in the beetle, *Lagria villosa,* has undergone extensive genome reduction over time. However, to sustain the symbiotic relationship and avoid extinction, it has acquired the lagriamide *lga* biosynthetic gene cluster, required to augment the metabolic pathway of the host, from other associated symbionts. Furthermore, in some cases, the obligate symbionts are highly reliant on the facultative symbionts for their survival, especially, under extreme conditions. Recently, it has been shown that aphid populations upon exposure to high temperature have reduced lifetime, fecundity and population densities of both obligate and facultative symbionts. However, this reduction is significantly less in aphids that are infected with either of the two facultative symbionts *Regiella insecticola* or *Fukatsuia symbiotica*. Moreover, it was observed that the reduced population density of the obligate symbiont, *Buchnera*, as a result of heat shock, could be successfully recovered in aphids infected with *Regiella* or *Fukatsuia*, but not in uninfected insects (Heyworth et al., 2020). Thus implying that sensitivity of *Buchnera* to heat shock, as a consequence of extreme gene loss, is compensated by the co-residing facultative symbionts.

Could These Changes Lead to Speciation of Bacterial Species Within Insect Gut?

Considering that genome re-arrangements and major genome deletions are known to occur in the microbial genomes within an insect, an obvious question that arises is do new species of microorganism originate within insects? Recent studies have hinted toward the incidence of sympatric speciation of bacterial species occurring within an insect gut. For instance, *Candidatus Hodgkinia cicadicola* has a highly reduced genome and is reported to have split into two interdependent bacterial species in some species of cicadas. However, it is interesting to note that in some cicadas the ancestral type is found to co-exist with its newly evolved form (Van Leuven et al., 2014). As discussed above, the endosymbiotic bacteria undergo massive changes in its genome and experiences high evolutionary pressures within an insect gut, and therefore, it is plausible to believe that these changes are manifested in the form of evolution of new species, i.e., leads to speciation.

In summary, although possessing a dynamic genome facilitates bacterial adaptations to insect gut, it also has certain disadvantages. Though recent studies have unraveled some of the mechanisms evolved by these endosymbiotic bacteria to cope with the repercussions of having an unstable genome, there likely exist several other mechanisms that are yet to be discovered.

CONSEQUENCES OF INSECT–MICROBIOME INTERACTIONS ON INSECT HOSTS

Insect populations are exposed to various types of environmental fluctuations and stresses periodically. And the only way for any organism to survive the extreme conditions is 'adaptation.' Though insects are capable of accommodating variations in its genome brought about by changing environmental conditions, these variations could sometimes be deleterious. Under such eventualities, insects can utilize its microbiome as an alternative for ensuring its adaptation, without compromising or putting its survival at stake. Also, it has been observed that the insect's microbiome is highly dynamic in terms of its structure, function and composition as it experiences high evolutionary pressures within an insect gut. Though there are cases where the primary symbionts, despite possessing a highly reduced genome, are extremely stable in terms of their gene content, still there is always enough scope for rapid sequence evolution between closely related bacterial species. And with the knowledge that changes in microbiome dramatically influence the host physiology, it

is reasonable to believe that insects could exploit this genetic variation, present in its symbiotic species, for its own benefit. A direct evidence of such an occurrence comes from the study carried out on polymorphic *Buchnera* populations present in its insect host, *A. pisum*. The *Buchnera* populations displayed polymorphism in the promoter of a heat shock gene, *ibpA*, which affects the thermotolerance of its insect host (Dunbar et al., 2007). Therefore, it appears that the evolutionary changes in the endosymbiotic bacteria have profound implications on host biology. Infection of *Rickettsiella viridis* in the pea aphid, *A. pisum*, is known to remarkably alter the host phenotype. Aphid populations have red and green colored genetic morphs and it is reported that upon infection with *R. viridis*, red aphids become green due to increased production of green polycyclic quinone pigments (Nikoh et al., 2018). This suggests that gut microbiome can drastically influence the phenotype of their insect host.

Interestingly, endosymbionts also modulate the gene expression of their insect hosts for ensuring their survival and persistence within an insect body. *Candidatus* Liberibacter asiaticus alters the energy metabolism of its psyllid vector, *Diaphornia. citri*, in order to secure its own needs. Genome analysis of *L. asiaticus* revealed the presence of an ATP translocase, which is involved in the uptake of ATP and other nucleotides from the medium for its growth and multiplication. To meet its energy requirements, *D. citri* produces ATP and other energetic nucleotides; however, their utilization by the insect is competitively inhibited by *L. asiaticus* (Killiny et al., 2017). This suggests that the symbiotic bacteria likely influence the biochemical processes within their insect hosts.

In fact, several changes in the insect genome have also been reported that are crucial to insect-microbe symbiotic relationship. Usually, to combat microbial infections, insects have evolved the Toll-like receptor (TLR) and Immune Deficiency (IMD)-like pathways that are responsible for the immune response that functions through the production of antimicrobial peptides (AMPs). For instance, in weevils, the IMD-like pathways are usually involved in secluding the endosymbionts within the bacteriocytes and mediating the systemic and local immune responses to exogenous challenges faced by insects as reported by Maire et al. (2018). Likewise, in the red flour beetle, *Tribolium castaneum*, the IMD pathway was proposed to confer resistance against the Gram-negative and Gram-positive pathogens *Enterobacter cloacae* and *Bacillus subtilis*, respectively (Yokoi et al., 2012); IMD homolog (*Tm*IMD) cloned and functionally characterized from the mealworm beetle, *Tenebrio molitor*, is involved in the expression of nine *AMPs,* which confer resistance against Gram-negative bacteria (Jo et al., 2019). However, in several cases of insect–microbe symbiosis, it is shown that the IMD pathway has been disrupted in insects, and these disruptions likely ensure the survival of its bacterial partners. For instance, the non-functional IMD signaling pathway and absence of several antimicrobial peptides in aphid has probably facilitated the *Buchnera aphidicola*-aphid symbiosis (Gerardo et al., 2010) which originated ∼200 million years ago (Baumann, 2005). Dependence of insects on their beneficial endosymbionts is believed to act as a selective force, which has led to reduction in their immune capabilities. Similarly, *Rhodnius prolixus* has lost several steps critical in the IMD pathway rendering it inactive (Salcedo-Porras et al., 2019); the bedbug, *Cimex lectularius* has a non-functional IMD pathway, an adaptation to prevent elimination of beneficial symbiotic gut microbes (Benoit et al., 2016). This implies that insect hosts too have undergone biochemical and genetic changes to accommodate these beneficial microbes and thereby indicating co-evolution of insect host with its bacterial partner.

In recent years, researchers have also proposed a role for epigenetics in promoting microbial persistence in insects. It is reported that the alternation of DNA methylation patterns by microbes attenuates immune responses in insects and thereby, ensuring the survival of bacterial symbionts (Kim et al., 2016). Additionally, bacterial symbionts with highly reduced genomes have evolved various small RNAs that help them modulate the expression of essential symbiotic genes and regulate core housekeeping processes in their insect hosts (Hansen and Degnan, 2014).

In addition to the biochemical and genetic changes, several behavioral changes in insects could also be attributed to their microbiome (Lewis and Lizé, 2015). In *Drosophila*, it has been demonstrated that gut microbes play a crucial role in determining its behavior and development, as they are involved in the identification of suitable feeding and egg-laying locations. Furthermore, the results of the oviposition assays showed that while exposing *Drosophila* to *Saccharomyces cerevisiae, Lactobacillus plantarum,* and *Acetobacter malorum* promoted its development, exposure to only *S. cerevisiae* and *A. malorum* resulted in the development of larger ovaries and increased egg numbers (Qiao et al., 2019). Further, the microbiome not only influences the host feeding preferences but also determines the insect's feeding capabilities. For instance, in *Megachile punctatissima* and *M. cribraria,* during egg-laying the females deposit a symbiont-containing capsule that is ingested by the offspring upon emergence. They have evolved this mechanism as a means to exchange bacterial species amongst them. *M. punctatissima* normally feeds on pea while *M. cribraria* is unable to do so. However, when there is an exchange of bacterial species the inability of *M. cribraria* to feed on pea is reversed (Hosokawa et al., 2007). Thus, evidence points toward the role of microbiome in widening host's niche by allowing its survival on a particular food resource.

Endosymbionts are also known to determine the viable temperature ranges, modulate desiccation tolerance and detoxify xenobiotics for their insect hosts (Lemoine et al., 2020). For instance, it was reported that the microbiome infection frequencies determine the geographic distribution of the chestnut weevil, *Curculio sikkimensis*. It was shown that higher titers of *Sodalis*, *Wolbachia* and *Rickettsia* were present in weevils found at the localities of higher temperature; lower numbers of *Wolbachia* and *Rickettsia* were detected in the population found in the regions with higher snowfall; and higher *Curculioniphilus*, *Sodalis*, *Serratia*, *Wolbachia,* and *Rickettsia* infections were characteristically present in weevils feeding on acorns than on chestnuts (Toju and Fukatsu, 2011) and thus indicating the involvement of symbionts in expanding or limiting the insects' abiotic niches. Based on the above examples, it would

be reasonable to state that the microbiome impacts the insect's ability to colonize and invade varied ecosystems on Earth.

INDICATIONS FOR EXPLOITING MICROBIOME – A PROMISING APPROACH TOWARD SUSTAINABLE PEST CONTROL

With the recent advances in science and technology, we have made significant progress in the transformation of agricultural and horticultural industry and thus ensuring self-sufficiency in food production in several parts of the world. However, with rapidly increasing population coupled with rising demand for food, feed, fodder along with a gradual decline in the area under cultivation, have brought out new challenges that are threatening food, nutritional and livelihood security, globally. Though we have made remarkable progress in increasing food production, it is ironic and unacceptable that malnutrition is still widespread in various parts of the world especially in the under-developed and the developing countries. According to the recent World Resources Institute [WRI] (2019) report, food demand is expected to increase anywhere between 60–90% by 2050 due to exponentially increasing human population. Therefore, one of the major global challenges is to be able to meet the rising food requirements of a rapidly growing population. Although crop production is adversely affected by numerous biotic and abiotic factors, agriculture suffers an annual yield loss of ∼20–40% due to insect pests alone (FAO, 2019).

Several pestilent outbreaks of insect pests of agricultural importance can be prevented if such occurrences can be predicted. However, lack of proper forewarning systems and coupled with indiscriminate use of pesticides and excessive use of nitrogenous fertilizers (facets that have become an integral part of the modern agricultural practices), further compounds the problems faced by farmers. To develop an alternative to conventional pesticides, various companies have introduced low dosage molecules in the market but they are neither cost-effective nor easily accessible and moreover, conventional pest-management strategies are proving ineffective. Additionally, invasive pests are one of major problems faced by farmers, globally. Biological control of these 'alien' pests is often not possible as the natural enemies that would keep their population size under control are normally left behind in their aboriginal home or at their native place. In spite of the availability of modern agricultural techniques and practices for controlling such pests, which are effective up to a certain extent, they often have many ecological and environmental repercussions. Therefore, devising a pest-management strategy without compromising the sustainability of agro-ecosystems is a major challenge. Researchers have shown that extensive genome degradation makes the obligate symbionts more sensitive to environmental fluctuations than the host itself. The southern green stinkbug, *Nezara viridula,* depends on a specific Gammaproteobacterial symbiont with a highly reduced genome for its normal growth and survival. Severe gene loss has made this symbiont highly sensitive to temperature fluctuations and even small shifts in temperature would kill these symbionts and, eventually, their hosts as well (Kikuchi et al., 2016). Thus, imposing restrictions on the insect in its ability to colonize inhospitable niches. In such cases, limitations imposed by obligate symbionts may help counter the spread of invasive pests and restrict the geographic reach of invertebrate species.

As indicated by their rapidly changing population structure, the insects are evolving at a much faster rate than their host. And, changing climatic and environmental conditions act as a trigger for inducing these changes in insect pests. Based on literature, it would be appropriate to state that throughout its history, microbes have played a very crucial role in insect survival. Furthermore, as gut bacteria experience high evolutionary pressures within an insect body, it seems a likely candidate that facilitates quick adaptations of the insect host to the ever-changing environment. Moreover, owing to the relatively shorter life cycle, the symbiotic bacteria can adapt more readily than the invasive insects to the new environments (Lu et al., 2016). Therefore, merely focusing on plant-insect interaction would be insufficient as insects share an intimate association with its gut microflora that influences the colonizing capabilities of insects. Moreover, understanding the evolutionary trajectory of insects would enable us to determine their population structure and predict their likelihood of invading a particular area.

Indications that the microbiome could be exploited for insect control also comes from various studies conducted on insect that pose serious risks to human health. Insects like wasps, hornets and bees can cause a severe, and sometimes lethal, allergic reaction in humans. Moreover, mosquitoes are known to vector several deadly viruses such as the Zika virus, the Dengue virus, and the West Nile virus. Therefore, significant efforts have been made to control their spread and manipulation of microbiota is emerging as a novel and promising approach to vector control (Scolari et al., 2019). For example, it has been recently shown that wMel strain of *Wolbachia* induces cytoplasmic incompatibility and when introduced into *Ae. aegypti,* it negatively impacts its ability to act as a vector for the Dengue virus (Thomas et al., 2018; Ross et al., 2019). Additional strategies have been developed for identifying and disrupting natural symbionts of mosquitoes such as *A. gambiae* or alter them genetically to express anti-pathogen effectors (Wang and Jacobs-Lorena, 2013). Fisher et al. (2017) demonstrated that removal of the vertically transmitted obligate symbionts from insects results in reduced fitness and this reduction is twice as large as that observed with horizontally transmitted symbionts. Moreover, this increases to three times if the symbiont is involved in providing nutritional benefits to the host. Therefore, understanding the nature of insect-microbial symbiosis and targeting the primary symbionts could prove to be an efficient strategy to control the spread of harmful pests.

Some phytopathogenic bacteria, especially those belonging to the family Enterobacteriaceae, were initially insect commensals (i.e., non-harmful associates) but now have evolved into plant pathogens following repeated inoculations into the phloem by their insect hosts during feeding. Therefore, unraveling the interactions established between phytopathogenic bacteria and insect symbionts could also offer a promising tool to impair and therefore, control the transmission of phloem limited plant pathogens in a sustainable and environment-friendly manner

(Gonella et al., 2019). Moreover, it has been shown that the tripartite interactions between insects, microbes and plants contribute to the success of various coleopterans such as the Colorado potato beetle (*Leptinotarsa decemlineata*), cereal leaf beetle (*Oulema melanopus*), western corn rootworm (*Diabrotica virgifera virgifera*), red flour beetle (*Tribolium castaneum*), the rice weevil (*Sitophilus oryzae*) and several others (Wielkopolan and Obrêpalska-Stêplowska, 2016). Therefore, understanding and acquiring knowledge regarding the role of insect-associated microbes would be extremely useful in the development of effective control strategies for crop protection against these economically important agricultural pests.

While significant efforts have been made to develop elite plant varieties of crop plants that can tolerate or resist insect attacks, it is well documented that resistance is often not durable. Within a few generations, the insects are able to successfully overcome host defenses and ultimately the plant succumbs to the insects. Despite the progress that we have made in the area of insect–plant interactions, the mechanisms operating in insects, that endow them with the trait of adaptation under stress, are still unclear. Therefore, under the circumstances, it is pertinent to study and unravel and eventually exploit these mechanisms to devise a long-term pest control strategy. As it would be clear from the evidences presented here, the gut microbiome can dramatically influence the physiology, behavior, and genetics of its insect host, and therefore, targeting the microbiome could be counted as an effective approach for developing an integrated, environment-friendly and a sustainable pest-management strategy.

AUTHOR CONTRIBUTIONS

AG and SN contributed to the conception, wrote the manuscript, participated in the writing and analysis, and read and approved the final manuscript. Both authors contributed to the article and approved the submitted version.

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Insights in the Global Genetics and Gut Microbiome of Black Soldier Fly, *Hermetia illucens*: Implications for Animal Feed Safety Control

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The utilization of the black soldier fly (BSF) *Hermetia illucens* L. for recycling organic waste into high-quality protein and fat biomass for animal feeds has gained momentum worldwide. However, information on the genetic diversity and environmental implications on safety of the larvae is limited. This study delineates genetic variability and unravels gut microbiome complex of wild-collected and domesticated BSF populations from six continents using mitochondrial *COI* gene and 16S metagenomics. All sequences generated from the study linked to *H. illucens* accessions KM967419.1, FJ794355.1, FJ794361.1, FJ794367.1, KC192965.1, and KY817115.1 from GenBank. Phylogenetic analyses of the sequences generated from the study and rooted by GenBank accessions of *Hermetia albitarsis* Fabricius and *Hermetia sexmaculata* Macquart separated all samples into three branches, with *H. illucens* and *H. sexmaculata* being closely related. Genetic distances between *H. illucens* samples from the study and GenBank accessions of *H. illucens* ranged between 0.0091 and 0.0407 while *H. sexmaculata* and *H. albitarsis* samples clearly separated from all *H. illucens* by distances of 0.1745 and 0.1903, respectively. Genetic distance matrix was used to generate a principal coordinate plot that further confirmed the phylogenetic clustering. Haplotype network map demonstrated that Australia, United States 1 (Rhode Island), United States 2 (Colorado), Kenya, and China shared a haplotype, while Uganda shared a haplotype with GenBank accession KC192965 BSF from United States. All other samples analyzed had individual haplotypes. Out of 481,695 reads analyzed from 16S metagenomics, four bacterial families (Enterobactereaceae, Dysgonomonadaceae, Wohlfahrtiimonadaceae, and Enterococcaceae) were most abundant in the BSF samples. Alpha-diversity, as assessed by Shannon index, showed that the Kenyan and Thailand populations had the highest and lowest microbe diversity, respectively; while microbial diversity assessed through Bray Curtis distance showed United States 3 (Maysville) and Netherlands populations to be the most dissimilar. Our findings on

genetic diversity revealed slight phylogeographic variation between BSF populations across the globe. The 16S data depicted larval gut bacterial families with economically important genera that might pose health risks to both animals and humans. This study recommends pre-treatment of feedstocks and postharvest measures of the harvested BSF larvae to minimize risk of pathogen contamination along the insect-based feed value chain.

Keywords: genetic diversity, gut microbiome, *Hermetia illucens*, mitochondrial *COI* gene, 16S-metagenomics

INTRODUCTION

The black soldier fly (BSF) *Hermetia illucens* (Linnaeus, 1758; Diptera: Stratiomiydae) is a highly adaptable saprophagous cosmopolitan insect species (Carles-Tolra and Andersen, 2002). Its distribution has widely expanded over time to the warmer parts of the world (Marshall et al., 2015). Global records of *H. illucens* indicate an increased frequency of encounters in Europe from 1950–1960, although this is not a true indication of their abundance. It was first recorded in southern Europe in 1926. Preceding these recordings, the first record of BSF in South Africa, Kenya, and Ghana were in 1915, 2015, and 2018, respectively (Picker et al., 2004; Marshall et al., 2015; Stamer, 2015; Chia et al., 2018). The exact original distribution of *H. illucens* is not well known. *H. illucens* was recorded in the Southeastern United States as far back as the 1800s (Marshall et al., 2015), reflecting a northward spread from a native range in Central America and the northern parts of South America in historical times. Several questions remain unanswered about the origin, invasion history and current distribution status of *H. illucens* because available data are scant. However, there is consensus that the spread of *H. illucens* was dependent on maritime transport that likely played a role in repeated, accidental introductions through trade of fruits and vegetables along coastlines and islands (Picker et al., 2004). Currently, molecular evidence supporting the biogeography of *H. illucens* and its colonization of Africa and the world at large is lacking.

Black soldier fly larvae are among the most efficient waste decomposers, recycling a wide range of organic waste into high-quality edible biomass of 38.5–62.7% crude protein and 14.0–39.2% fat content that is rich in energy (5282 kcal/kg gross energy; Sheppard et al., 1994; Tomberlin et al., 2005, 2009; Caruso et al., 2013; Banks, 2014; Myers et al., 2014; Lalander et al., 2015). The larvae are also a rich source of micronutrients (iron and zinc) and all essential amino acids including relatively high amounts of cereal-limiting amino acids such as lysine, threonine, and methionine. Processed larvae are therefore used as a high-quality protein valuable for feed ingredient for various monogastric animal species, including poultry, pigs and fish (Bondari and Sheppard, 1987; St-Hilaire et al., 2007). In addition, BSF is neither a pest nor a disease vector, and does not constitute a nuisance like other flies (Diener, 2010). Frass produced by BSF larvae is an excellent fertilizer able to increase crop yields. Finally, insect-based feed protein technologies, which can be implemented at low-cost, have the potential to provide employment opportunities and livelihood improvement for both farmers and urban entrepreneurs (Diener et al., 2015).

Despite the economic importance of *H. illucens*, currently no data is available on worldwide population genetics, including genetic variability within and between geographic populations. Knowledge of the genetic structure of BSF populations would provide a sound framework for gaining insight into their dispersion and mating compatibilities, and for identifying their actual and potential routes of gene flow. Also, lack of knowledge of the genetic structure of BSF populations have prevented identification of its areas of origin in newly colonized parts of the world, including Africa, tracing the route of its colonization process both within and outside North America, and assessment of colonization effects on population differentiation. The introduction or invasion of *H. illucens* has been reported in many countries with chances of the species undergoing rapid evolutionary events.

Bacteria are an essential component of decomposing organic waste (Burkepile et al., 2006; Barnes et al., 2010; Miki et al., 2010) and are always associated with insects such as BSF that use these resources. Many insect species including BSF largely depend on obligate bacterial mutualism for their survival, viability and reproduction (Werren et al., 1995). Recent efforts have demonstrated that BSF larvae reduce pathogenic bacteria within animal wastes (Erickson et al., 2004; Liu et al., 2008). Bacteria isolated from BSF larvae have been extensively used as probiotic to enhance manure reduction and subsequent larval development (Yu et al., 2011). Many of these beneficial bacteria could be natural constituents of the larval environment or potentially vertically transmitted. Although BSF might suppress potential pathogens, it is not clear if other opportunistic pathogens might proliferate in their presence and present potential health and environmental risks. In this study, genetic variability and microbial diversity among BSF populations from different geographic locations in the world were investigated using the barcode region of the mitochondrial cytochrome oxidase I (*mtCOI*) gene and microbiome through 16 S metagenomics.

MATERIALS AND METHODS

Sampling

Larvae of BSF were collected from different indoor rearing facilities in various countries across the globe namely: Australia, China, Costa Rica, Ghana, Kenya, Nigeria, South Africa,

Thailand, Netherlands, Uganda, and United States. The samples from each location were preserved in 95% ethanol and brought to the Arthropod Pathology Unit at the International Center of Insect Physiology and Ecology (*icipe*, Nairobi, Kenya) for further processing.

DNA Extraction, Polymerase Chain Reaction (PCR) and Sequencing of the Insect Larvae

Each individual insect larva was surface sterilized using 3% NaOCl and rinsed with distilled water. Genomic DNA was extracted using the Isolate II Genomic DNA Kit (Bioline, London, and United Kingdom) following the manufacturer's instructions. The purity and concentration of the resultant DNA was determined using a Nanodrop 2,000/2,000 c spectrophotometer (Thermo Fischer Scientific, Wilmington, United States). PCR was performed to amplify the *COI* barcode region of the mitochondrial DNA region in a total reaction volume of 20 μL containing 5X My *Taq* reaction buffer (5 mM dNTPs, 15 mM $MgCl_2$, stabilizers, and enhancers; Bioline), 10 pmol/μl of primers [LepF1 5′ ATTCAACCAATCATAAAGATATTGG 3′, LepR1 5′ TAAACTTCTGGATGTCCAAAAAATCA 3′ (Hajibabaei et al., 2006)], 0.5 mM $MgCl_2$, 0.0625 U μl^{-1} My *Taq* DNA polymerase (Bioline), and 15 ng/μl of DNA template in a Nexus Mastercycler gradient machine (Eppendorf, Hamburg, Germany). The following cycling conditions were used: initial denaturation for 2 min at 95°C, followed by 40 cycles of 30 s at 95°C, 45 s annealing at 52°C, extension for 1 min at 72°C, and a final elongation step of 10 min at 72°C. The amplified PCR products were resolved through a 1.2% agarose gel. DNA bands on the gel were analyzed and documented using KETA GL Imaging System *Trans*-Illuminator (Wealtec Corp, Meadowvale Way Sparks, United States). Successfully amplified products were excised and purified using Isolate II PCR and Gel Kit (Bioline) following the manufacturer's instructions. Purified samples were shipped to Macrogen Europe BV (Meibergdreef, Amsterdam, Netherlands) for bi-directional sequencing.

Next Generation Sequencing

Insect samples from each locality were surface sterilized in 3% NaOCl then washed thrice in sterile water. The cuticle was excised using a sterile scalpel and then the whole gut contents were transferred into 1.5 ml Eppendorf tubes from which genomic DNA was extracted as described above. The resultant DNA were lyophilized into 1.5 ml DNAstable tubes (Biomatrica, San Diego, United States) then sent to Macrogen Europe BV for 16 S metagenomics [Illumina 16 S amplicon (V3–V4 region) library preparation + Illumina MiSeq 2 × 300 bp sequencing, 100,000 reads per sample].

Data Analyses

Mitochondrial DNA Data Analysis

The sequences obtained were assembled and edited using Chromas Lite Version 2.1.1[1] and Geneious Version 8[2] (Kearse et al., 2012). The primer sequences were identified and removed from the consensus sequences generated from both the forward and reverse reads. Pairwise and multiple alignments were performed in Clustal X software (version 2.1; Thompson et al., 1997). The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model (Kimura, 1980) using MEGA X (Kumar et al., 2018). The tree with the highest log-likelihood (–1906.90) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value. The reliability of the clustering pattern in the tree was evaluated using a bootstrap analysis with 1,000 replicates and involving 76 nucleotide sequences. Evolutionary divergence over sequence pairs between groups were calculated using the Kimura 2-parameter distance model (Kimura, 1980) in MEGA X and principal coordinate plots constructed from the genetic distances using GenAlEx 6.41 (Peakall and Smouse, 2006).

Bayesian analysis was carried out with MrBayes V.3.2 (Ronquist et al., 2012). The GTR + I + G model (general time reversible model incorporating variant sites and a gamma distribution) model was selected for Bayesian analysis as determined by MrModelTest V.2.3 (Nylander, 2008). In Bayesian analysis, the Markov chain Monte Carlo process was generated at four chains and 4,000,000 generations resulting in 40,000 trees. The sampling frequency was 100 generations. Analyses ran until the average standard deviation of split frequencies were below 0.01. The trees were checked for convergence of parameters (standard deviation of split frequencies and potential scale reduction factor) in MrBayes V.3.2. Effective sample size (ESS) was also checked using Tracer V1.5 (Rambaut and Drummond, 2007). The first 1,000,000 generations (10,000 trees) were excluded as the burn-in step, corresponding to the standard deviation of split frequencies below 0.01, potential scale reduction factor equal to 1.0 and ESS value above 200, indicating a good posterior probability distribution sample. The remaining trees (30,000 trees) were used to evaluate the posterior probabilities. The Bayesian topology was visualized using the FigTree V.1.4 program (Rambaut, 2012).

For conclusive identification of the species, similarity searches, phylogenetic analyses and genetic divergence of the *COI* barcoding gene were conducted. Similarity searches were carried out by querying the consensus sequences via the basic local alignment search tool (BLAST). The BLAST algorithm finds regions of local similarity between sequences, in which consensus sequences were compared to reference sequences in the GenBank database. All the sequences generated in the study were submitted to GenBank and assigned accession numbers (**Table 1**).

BSF 16S Metagenome Data Analysis

Sample data consisted of pooled samples per location, comprising of 5 individuals from each of the 13 different geographical regions and 2,819 taxa. Illumina-sequenced paired-end fastq sequences were checked for quality using FastQC v 0.11.28 (Andrews, 2010) and pre-processed to remove adapters and sequencing primers

[1]https://technelysium.com.au/wp/chromas/

[2]http://www.geneious.com

TABLE 1 | Sample collection details and identification and GenBank accessions of *Hermetia illucens* from different regions.

Country of collection	Collection points	Sample name	GenBank similarity and accession	*E*-value	GenBank accession numbers assigned
Australia (Tucki)	28° 46′ 13.79″ S, 153° 18′ 29.89″ E, 129 m.a.s.l	Aus 49–54	*Hermetia illucens,* FJ794367.1	0	MT483920 – MT483925
China (Pudang)	26° 07′ 48.4″ N, 119° 19′ 49.0″ E, 13 m.a.s.l	Chi 56–57, 59–60	*Hermetia illucens,* FJ794367.1	0	MT483914 – MT483917
Costa Rica (Escazu)	09° 55′ 15.06″ N, 84° 08′ 45.86″ W, 1087 m.a.s.l	CosR 1	*Hermetia illucens,* KC192965.1	0	MT483918
Ghana (Greater Accra)	05° 41′ 38.06″ S, 00° 01′ 58.93″ W, 298 m.a.s.l	Ghana 7–12	*Hermetia illucens,* FJ794361.1	0	MT483926 – MT483931
Kenya (Nairobi)	01° 13′ 14.6′′ S, 036° 53′ 44.5′′ E, 1612 m.a.s.l	Ken 31–36	*Hermetia illucens,* FJ794367.1	0	MT483937 – MT483942
Netherlands (Wageningen)	52° 08′ 28.15″ N, 05° 35′ 42.91″ E, 7 m.a.s.l	Neth 1–2, 4, 6–7	*Hermetia illucens,* KY817115.1	0	MT483932 – MT483936
Nigeria (Awka)	06° 22′ 18.60″ N, 07° 04′ 16.51″ E, 39 m.a.s.l	Nig 25–30	*Hermetia illucens,* FJ794361.1	0	MT520657 – MT520662
South Africa (Cape Town)	33° 55′ 14.79″ S, 18° 25′ 27.17″ E, 30 m.a.s.l	SA 1–6	*Hermetia illucens,* KY817115.1	0	MT520651 – MT520656
Thailand (Chiang Mai)	18° 56′ 04.63″ N, 98° 57′ 39.09″ E, 833 m.a.s.l	Thai 1–6	*Hermetia illucens,* KM967419.1 and FJ794355.1	0	MT520663 – MT520666; MT520687
Uganda (Kampala)	00° 21′ 08.99″ N, 32° 34′ 55.09″ E, 1186 m.a.s.l	Uga 19–24	*Hermetia illucens,* KC192965.1 and FJ794367.1	0	MT520675 – MT520680
United States (Rhode Island; Boulder; Maysville for United States 1, 2, and 3, respectively)	41° 34′ 48.34″ N, 71° 28′ 38.74″ W, 22 m.a.s.l; 39° 56′ 20.92″ N, 105° 10′ 37.67″ W, 2235 m.a.s.l; 38° 35′ 41.69″ N, 83° 47′ 05.69″ W, 254 m.a.s.l	United States _1: 37–42; United States _2: 15–18; United States _3: 44–46, 48	*Hermetia illucens,* FJ794367.1 and FJ794355.1	0	MT520681 – MT520686; MT520671 – MT520674; MT520667 – MT520670

using Cutadapt v1.18 (Martin, 2011). Illumina-sequenced paired-end fastq sequences were imported and assembled in QIIME2-2018.11 (Bolyen et al., 2019). The DADA2 pipeline (Callahan et al., 2016) was used to denoise the reads based on per base quality scores and merge the paired-end-reads the sequences into amplicon sequence variants (ASVs). Subsequently, the denoised representative sequences was checked for chimeric sequences using Qiime-Vsearch and the chimeric sequences filtered using Uchime and these were excluded from the downstream analyses. The resulting representative sequence set was aligned and given a taxonomic classification using SILVA 32 database[3]. Additional analyses, such as rarefaction curves and Good's coverage, were carried out with QIIME2. During data filtering, a total of 77 low abundance features were removed based on prevalence. Data were normalized as described by Weiss et al. (2017). The bacterial reads were binned into OTUs using an open OTU-picking strategy with 97% similarity and taxonomic assignment against the SILVA 32 database, which uses a bacterial and archaeal classification based on Bergey's Taxonomic Outlines (Boone and Mah, 2001; Garrity et al., 2005; De Vos et al., 2009; Krieg et al., 2012). Taxonomic composition was performed using stacked bar/area plot and a pie charts, a minimum abundance cut-off of 0.1% was used to select the most abundant taxa in each sample. Taxa with cumulative read counts below the 0.1% cut-off were collapsed into the "Others" category. Both alpha and beta diversity analyses were performed using the phyloseq package (McMurdie and Holmes, 2013) while Hierarchical Ward's linkage clustering based on the Pearson's correlation coefficient of the microbial taxa abundance was performed with the hclust function in the package stat generated with R version 3.4.3 (RStudio Team, 2015). In our study, the haplotype networks of the closely related species were constructed in R version 3.5.1 (RStudio Team, 2015).

[3]https://www.arb-silva.de/

RESULTS

BSF Identification and Phylogeny

Both similarity and phylogenetic analyses were conducted for identification of species (**Table 1**). BLAST search linked all the sequences generated in the study to *H. illucens* accessions KM967419.1, FJ794355.1, FJ794361.1, FJ794367.1, KC192965.1, and KY817115.1 with a percentage IDs ranging from 97 to 100%. The tree separated all the samples into three branches, with a paraphyletic relationship between the *H. illucens* and *Hermetia sexmaculata* clusters. Samples from this study formed a monophyletic clade with *H. illucens* from GenBank. Within the *H. illucens* clade, the samples from West Africa (Nigeria and Ghana) formed a distinct cluster, samples from Thailand and United States 3 were closely related, while some samples from Uganda clustered separately. All samples from Australia, Netherlands, South Africa, Kenya, United States 1, United States 2, and

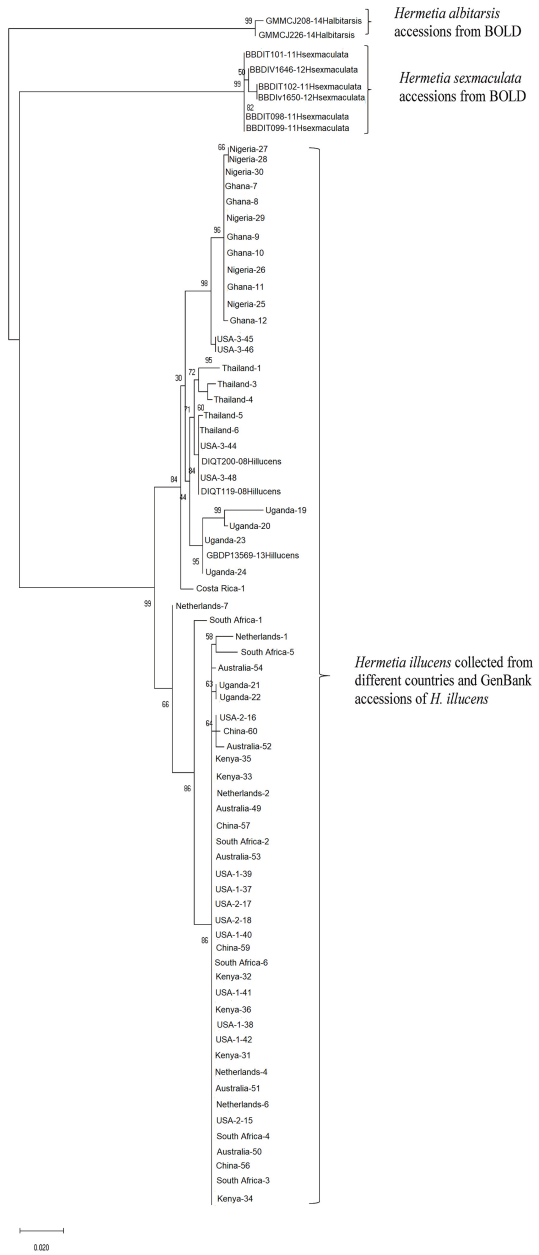

FIGURE 1 | Phylogenetic relationship between for the *Hermetia illucens* samples collected from different countries and other GenBank accessions of closely related species inferred using Maximum Likelihood method by MEGA X (Kumar et al., 2018).

China, clustered together (**Figure 1**). These results were further substantiated with the Bayesian analysis (**Supplementary Figure 1**). Estimates of evolutionary divergence over sequence pairs between groups were successfully generated from all sequenced samples and GenBank accessions of *H. illucens*, *H. sexmaculata* and *Hermetia albitarsis*. Numbers of base substitutions are presented as a genetic distance matrix (**Table 2**). The intraspecific genetic distance between *H. illucens* samples from the study and the GenBank accessions of *H. illucens* ranged between 0.91% and 4.07% which falls within the acceptable range of species limit. The *H. sexmaculata* and *H. albitarsis* samples clearly separated from all *H. illucens* by distances of 17.45% and 19.03%, respectively, confirming the phylogenetic analyses. The distance matrix was used to generate a Principal Coordinate Analysis (PCoA) where the first two axes in the PCoA

TABLE 2 | Estimates of Evolutionary divergence over sequence pairs between groups generated by MEGA X (Kumar et al., 2018).

	Hsexmaculata	Thailand	United States 3	Hillucens	Costa_ Rica	Uganda	Nigeria	Ghana	United States 2	SA	Australia	China	Kenya	Holland	United States 1	Halbitarsis
Hsexmaculata	0.00%															
Thailand	17.74%	0.00%														
United States 3	17.37%	1.44%	0.00%													
Hillucens	17.33%	0.91%	1.07%	0.00%												
Costa_Rica	16.75%	1.88%	1.80%	1.46%	0.00%											
Uganda	17.89%	3.24%	3.16%	2.49%	3.32%	0.00%										
Nigeria	17.59%	2.73%	1.47%	2.28%	2.70%	3.97%	0.00%									
Ghana	17.55%	2.69%	1.43%	2.25%	2.66%	3.91%	0.10%	0.00%								
United States 2	17.47%	4.57%	4.12%	3.97%	4.54%	3.50%	4.83%	4.77%	0.00%							
SA	17.60%	4.66%	4.10%	4.07%	4.64%	3.69%	4.78%	4.74%	0.48%	0.00%						
Australia	17.45%	4.60%	4.17%	4.03%	4.59%	3.56%	4.88%	4.83%	0.16%	0.56%	0.00%					
China	17.41%	4.63%	4.17%	4.03%	4.60%	3.51%	4.88%	4.83%	0.12%	0.53%	0.21%	0.00%				
Kenya	17.41%	4.52%	4.06%	3.92%	4.49%	3.48%	4.78%	4.74%	0.05%	0.43%	0.13%	0.10%	0.00%			
Holland	17.33%	4.31%	3.94%	3.73%	4.16%	3.47%	4.69%	4.66%	0.61%	0.92%	0.68%	0.66%	0.56%	0.00%		
United States 1	17.41%	4.52%	4.06%	3.92%	4.49%	3.48%	4.78%	4.74%	0.05%	0.43%	0.13%	0.10%	0.00%	0.56%	0.00%	
Halbitarsis	21.34%	19.69%	19.11%	19.15%	17.70%	19.28%	19.86%	19.81%	18.79%	18.95%	18.73%	18.86%	18.73%	18.99%	18.73%	0.00%

Distances were calculated as percentage of pairwise distances (p-distances) under the Kimura 2-parameter model.

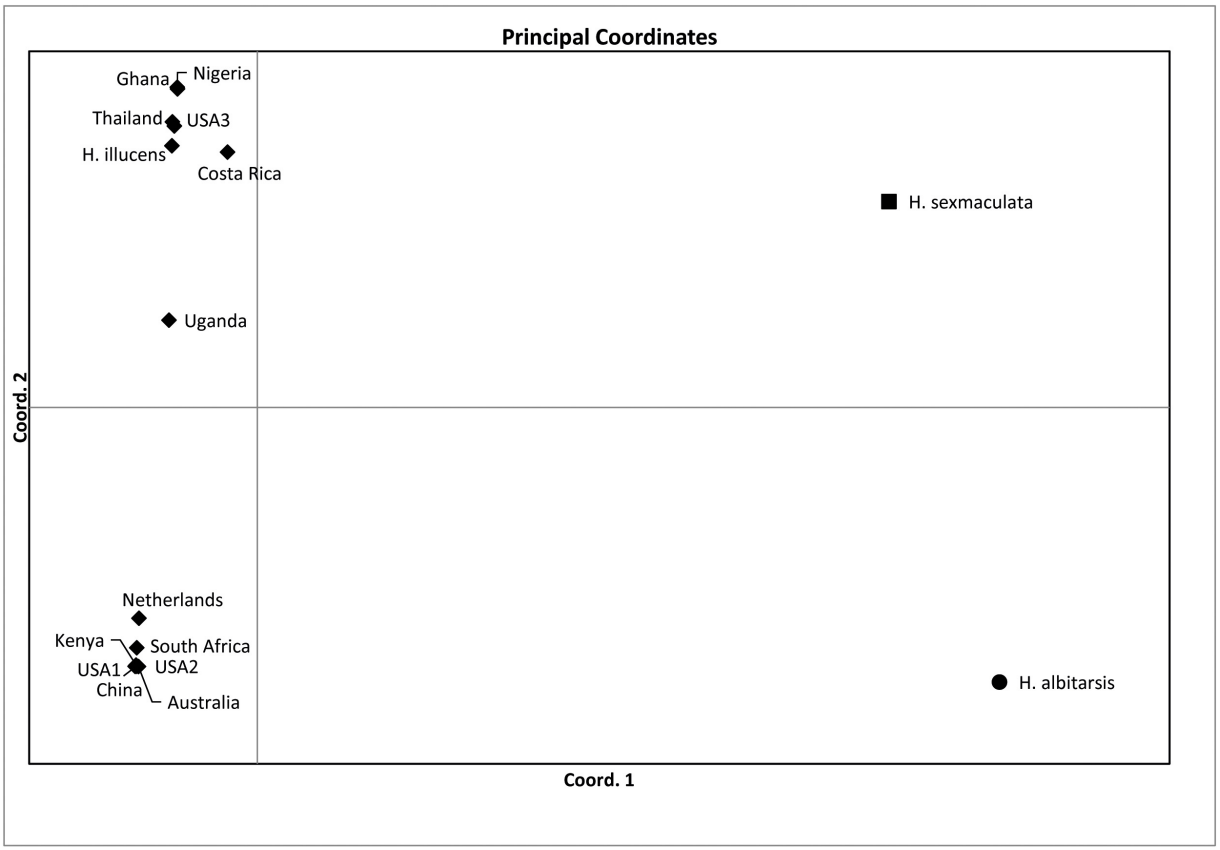

FIGURE 2 | Plots of the principal coordinate analysis (PCoA) for the *Hermetia illucens* samples collected from different countries and other GenBank accessions of closely related species calculated using GenAlEx 6.4.

plot explained 66.88% of the variation (the first axis 42.56% and the second axis 24.32%) between all the *Hermetia* samples analyzed from the study and GenBank accessions (**Figure 2**). The PCA clustered all *H. illucens* samples in the study with the GenBank accession of *H. illucens* in one axis though in specific clusters. However, that of two other species, *H. albitarsis* and *H. sexmaculata* occurred in the other axis, which further confirmed the phylogenetic analyses. The haplotype network map demonstrated that samples from Australia, United States 1, United States 2, Kenya, and China had a shared haplotype, while samples from Uganda shared a haplotype with a GenBank accession (KC192965) of *H. illucens* (**Figure 3**). The other samples each occupied an individual haplotype (**Figure 3**).

BSF Bacterial Microbiota Diversity

The bacterial microbiota analysis was based on a total of 188,863 sequences. The final dataset per sample was; Australia (16,840), China (14,766), Costa Rica (27,383), Ghana (10,376), Kenya (11,444), Netherlands (6,915), Nigeria (15,278), South Africa (12,658), Thailand (18,589), Uganda (8,973), United States 1 (12,199), United States 2 (19,263), and United States 3 (14,179). The direct quantitative comparison of the abundance of the microbiota showed the most abundant families were Wohlfahrtiimonadaceae (18.22%), Enterobacteriaceae (16.85%), Enterococcaceae (16.05%), and Dysgonomonadaceae (10.03%; **Figure 4A**). The comparison at genus level showed Ignatzschineria (22%), Enterococcus (20%), and Dysgonomonas (11%) to be the most abundant genera (**Figure 4B**). The cumulative abundance of the bacterial genera in the BSF populations showed that Enterobacteriaceae was the most abundant family in samples from Australia, China, Nigeria, Thailand and United States 3 while Dysgonomonadaceae was the most abundant in samples from Kenya, United States 1, and United States 2. Wohlfahrtiimonadaceae was the most abundant family in Ghana, South Africa and Uganda samples while Enterococcaceae was the most abundant in Costa Rica and Netherlands samples (**Figure 5A**). The most abundant genus in the samples from Australia, Costa Rica, and Netherlands was *Enterococcus*. Ghana, South Africa and Uganda had *Ignatzschineria* as the most abundant genus, Nigeria, and Thailand had *Providencia*, United States 1, and United States 2 had *Dysgonomonas*, China had *Morganella*, Kenya had *Moheibacter,* and United States 3 had *Lactobacillus* as the most abundant genus (**Figure 5B**). The intra population diversity (alpha diversity), as assessed by Shannon index, showed that the Kenyan population was the most diverse with a Shannon index of 3.5 while the Thailand population with a Shannon

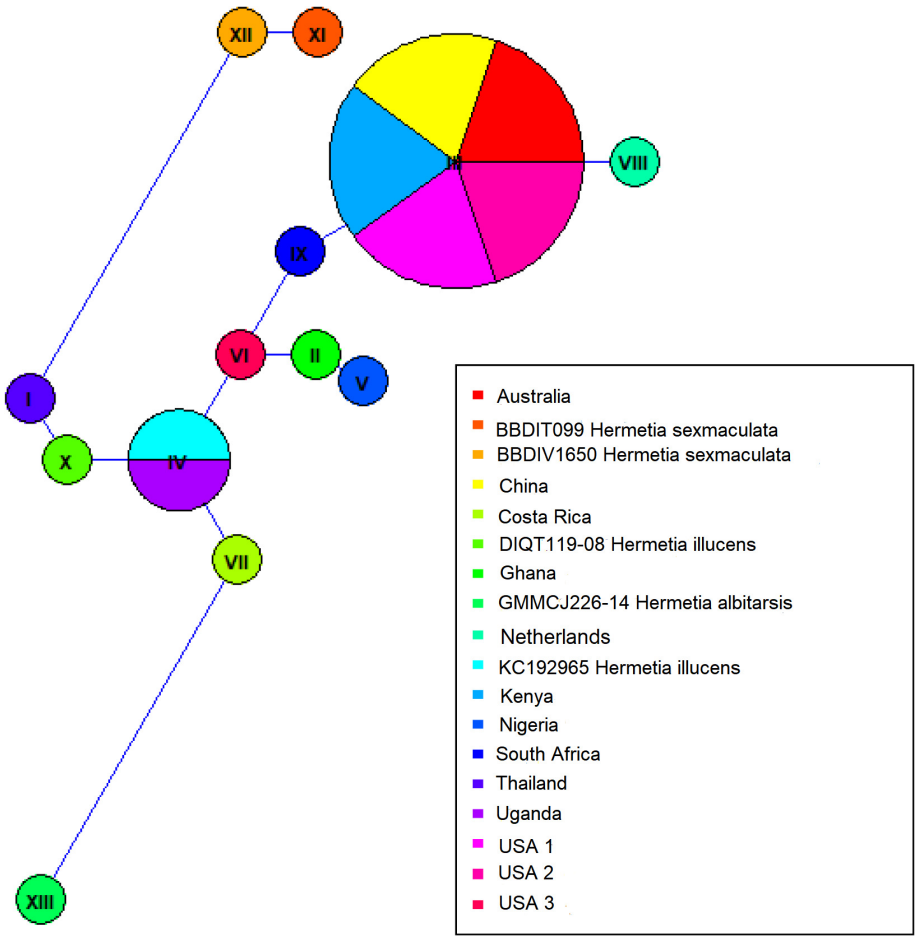

FIGURE 3 | Haplotype network map of the BSF samples.

index of 2.1 had the least microbiome diversity (**Figure 6**). The microbial diversity between the populations as assessed through Bray Curtis distance showed the populations from United States 3 and Netherlands were the most dissimilar populations. Principal component analysis showed two main clusters: Australia, China, Ghana, Kenya, Nigeria, Thailand, Uganda, United States 1, and United States 2 in one cluster and Netherlands, South Africa, and Costa Rica in a second cluster (**Figure 7**). The hierarchical clustering as seen in the heatmap, showed the relative abundance of bacterial taxa within the populations of BSF analyzed in the study. The hierarchical linkage clustering based on Pearson's correlation coefficient of the microbial taxa abundance showed three main clusters with Ghana, Uganda and South Africa similarly clustered, Australia, China, Costa Rica, Kenya, Nigeria, and Thailand similarly clustered while Netherlands, United States 1, United States 2, and United States 3 were similarly clustered. The correlation between the bacterial abundance and the source countries as shown in the quantitative comparison of the microbiota, indicated that the most abundant genus in each country was positively correlated to the source country in all the samples analyzed (**Figure 8**). All the 16S metagenomic data generated in this study has been submitted to GenBank and available through BioProject PRJNA625868.

DISCUSSION

It is believed that BSF has a common ancestry, and the phylogenetic analysis has shown very limited variation between the different populations based on mitochondrial *COI* barcode region. Common trade routes and similar feedstocks in these regions could be one of the reasons for this phenomenon. Furthermore, several studies have shown that other gene regions like Cytochrome b (*CytB*) gene (Chen et al., 2019) and NADH dehydrogenase 4 (*ND4*) gene (Tang et al., 1995) may be more polymorphic and thus give better resolution of intraspecific divergence of certain arthropod species. Therefore, an evaluation of the complete mitochondrial genome as well as other regions such as the nuclear microsatellites can be employed to resolve the population genetic structure of these closely related populations. The maximum likelihood and Bayesian analysis trees resulted in two branches of the BSF samples with clear separation of the two outgroups that were included in the analyses, *H. sexmaculata*

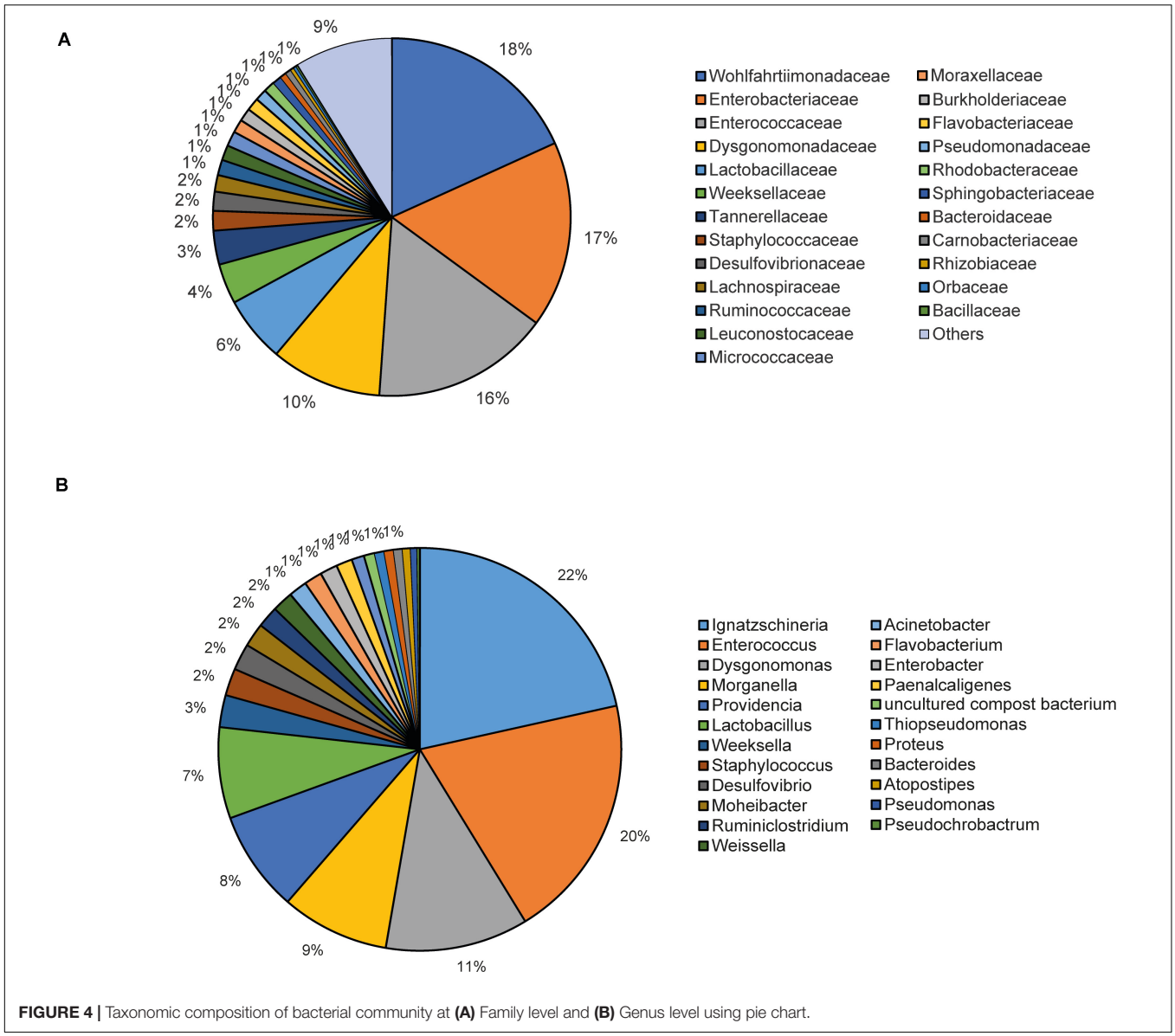

FIGURE 4 | Taxonomic composition of bacterial community at **(A)** Family level and **(B)** Genus level using pie chart.

and *H. albitarsis*. Despite the low genetic differences amongst the BSF populations in this study, five samples from four geographic regions (Australia, China, Kenya, and United States) shared a haplotype indicating a single origin for these populations. The Ugandan samples also shared a haplotype with GenBank accession of *H. illucens* from United States indicating the probable origin of the Uganda *H. illucens* sample. Two haplotypes were identified from the samples from West Africa (Nigeria and Ghana), but the variation between them was low and the phylogeny showed that they both also linked closely to a sample from the United States 3. The haplotype network also showed that the United States 3 haplotype was a centroid to the other haplotypes. Furthermore, the *H. illucens* samples from the study are closely related to samples from the United States as observed in the phylogeny and the haplotype network. Further research work comparing BSF samples from the native range in Northern South America countries such as Colombia, Venezuela, Guyana, Suriname, French Guiana, and Ecuador is crucial to establish the dispersal pattern from its native range to other parts of the world.

Based on the 16S rRNA sequencing and from the 481,695 reads analyzed, Enterobacteriaceae, Dysgonomonadaceae, Wohlfahrtiimonadaceae and Enterococcaceae were the most abundant families across the different countries. In addition, through taxonomic profiling, alpha-diversity (within-sample diversity) showed that the Kenyan and Thailand populations had the highest and the lowest microbiome diversity, respectively. Enterobacteriaceae, the most abundant family in Australia, China, Thailand, and United States 3, consists of bacterial genera that are ubiquitous in nature with many species free-living in diverse ecological niches, while some being associated with animals, plants or insects. Some of the species belonging to Enterobacteriaceae are significant human, animal, and plant

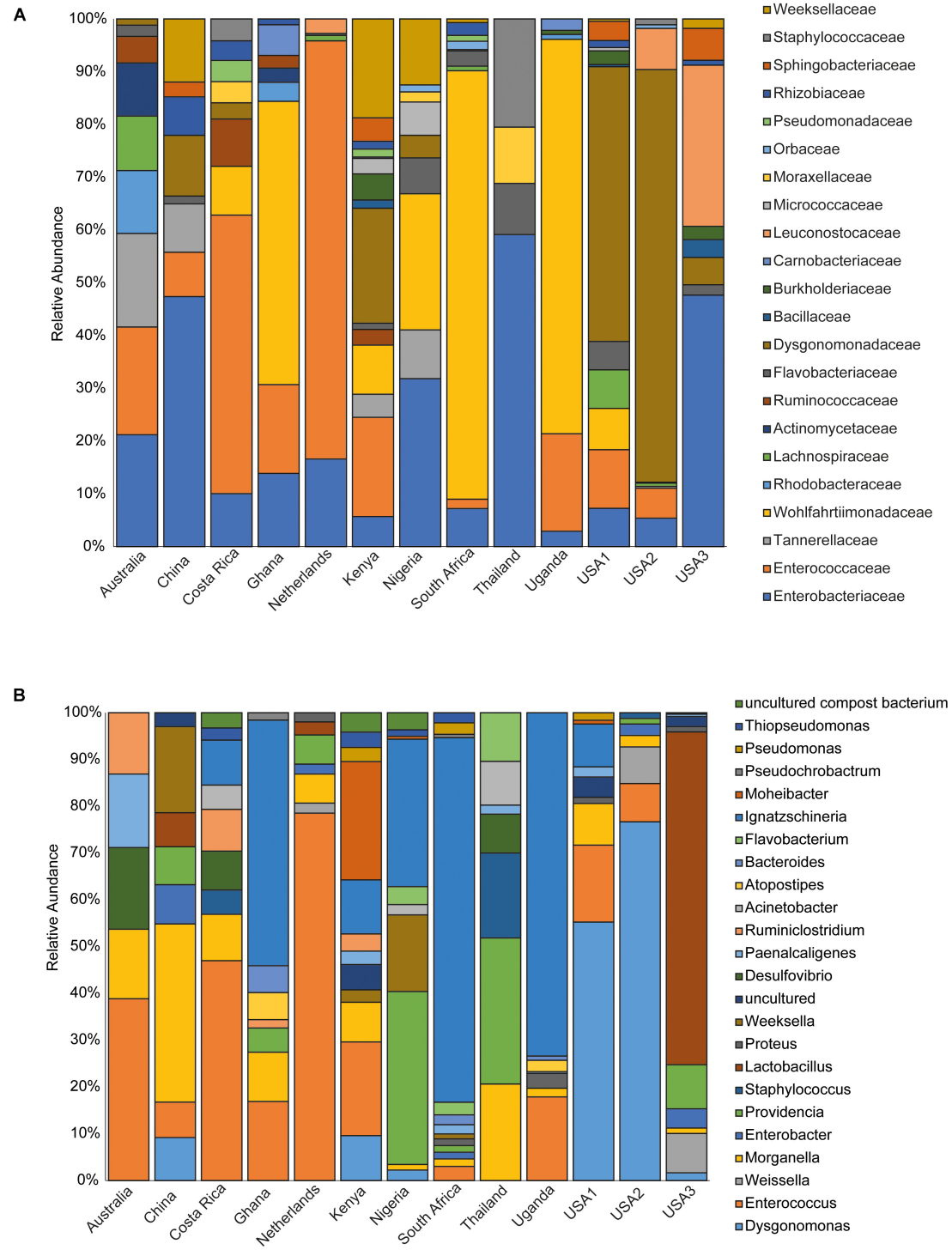

FIGURE 5 | Incidence of the major bacterial taxonomic groups. The stacked bar chart shows the relative abundances of bacterial **(A)** Family and **(B)** genera identified in *Hermetia illucens* larvae analyzed from different countries.

pathogens causing a range of infections, hence the emphasis on sterile conditions for the rearing of the BSF. However, most of the species are not pathogenic and are utilized in several processes such as production of various recombinant proteins and non-protein products, control of infection diseases, anticancer agents, and biowaste recycling and bioremediation (Octavia and Lan, 2014). The family Dysgonomonadaceae was predominant in samples from Kenya, United States 1, and

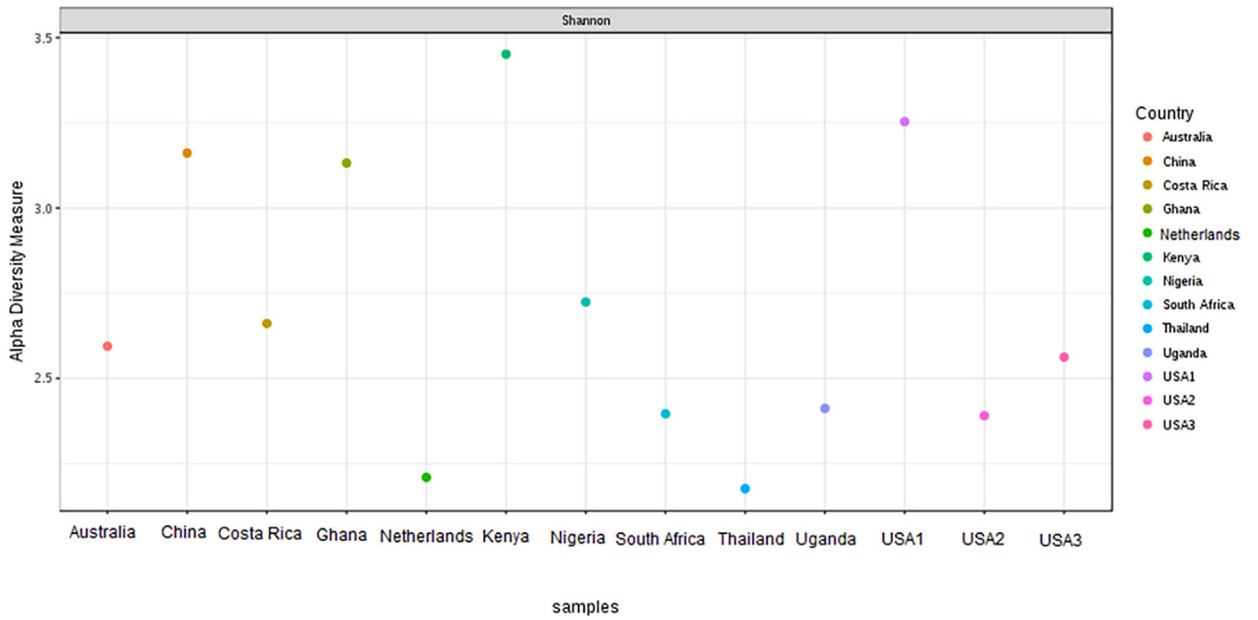

FIGURE 6 | Alpha-diversity measure using Shannon at OTU level across all the samples. The samples are represented on *X*-axis and their estimated diversity on *Y*-axis. Each sample is colored based on country class.

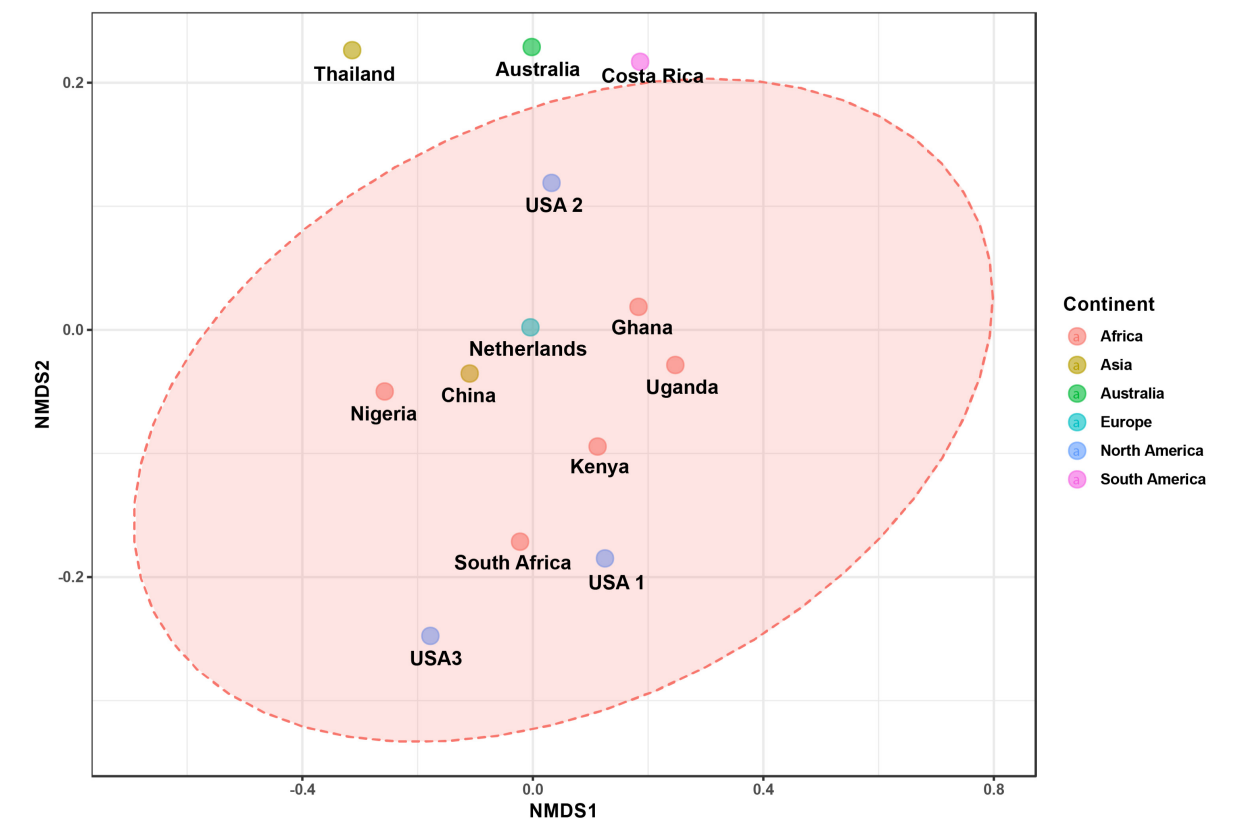

FIGURE 7 | The ordination plot represented in 2-D NMDS plot using bray distance. Statistical significance is found out using (ANOSIM) *R*: 0.5558; *p*-value < 0.004.

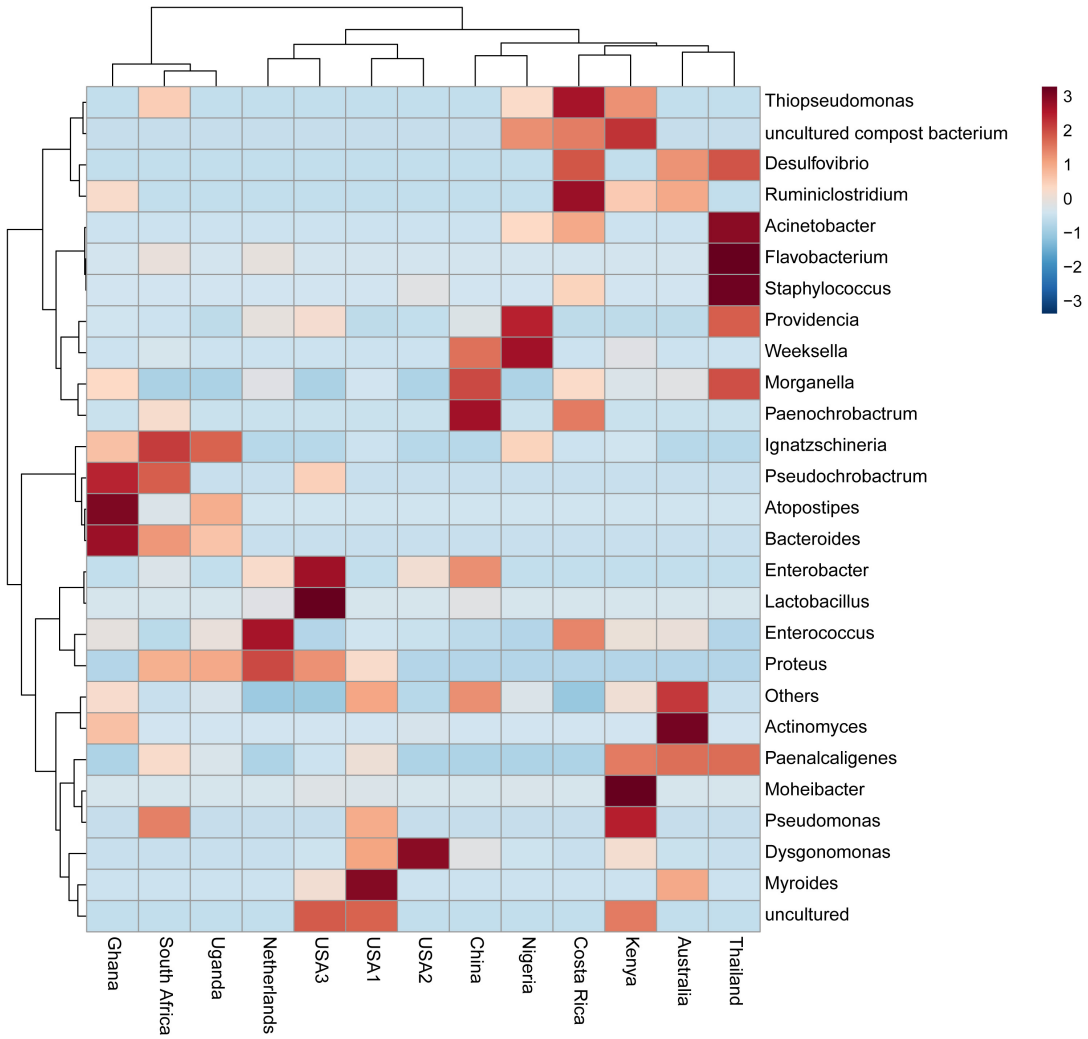

FIGURE 8 | Heatmap based on microbiota composition at genus level. Hierarchical Ward's linkage clustering based on the Pearson's correlation coefficient of the microbial taxa abundance. Blue and red colors represent positive and negative correlations, respectively. The color scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance, and blue indicating low abundance.

United States 2. Bacteria belonging to this family are capable of degrading various polysaccharides derived from host-ingested food, such as algae (Murakami et al., 2018) and could be an integral part in the role of BSF larvae as bioremediation organisms. Wohlfahrtiimonadaceae was the most abundant family in samples from Ghana, Nigeria and South Africa. This family consists of the genus *Wohlfahrtiimonas* which was described by Tóth et al. (2008) and consist of only one species, *Wohlfahrtiimonas chitiniclastica* $S5^T$. This species has been associated with myiasis (Campisi et al., 2015) and so far, two cases of sepsis have been reported (Rebaudet et al., 2009; Almuzara et al., 2011). The most abundant family detected in the samples collected from Costa Rica was Enterococcaceae. This family is comprised of Gram-positive, facultatively anaerobic, anaerobic, or microaerophilic bacteria with some species being carboxyphilic or halophilic. They are associated with a wide range of ecological sources including; plants, humans, animals, the gastrointestinal tract of insects, fermented foods, drinking water, surface water, and seawater (Ludwig et al., 2009). The diverse bacterial genera identified from the different countries in this study could be indicative of the different diets in addition to the key BSF microbiota in the sampled countries with varying implications on the biology of the BSF as well as the industrial applications of BSF. For example, *Providencia*, which is vertically transmitted through the insect life cycle (De Smet et al., 2018) but can also be a pathogen in humans (Galac and Lazzaro, 2011) was most abundant in Nigeria and Thailand. Furthermore, BSF fed on fish diet has been shown to be exposed to gut dysbiosis because of a microbiota severely dominated by *Providencia* species (Bruno et al., 2019). *Ignatzschineria* which was the most abundant genus, in Ghana, South Africa and Uganda, has been shown to trigger repellency in *H. illucens* and thus reduce egg deposition (De Smet et al., 2018). *Dysgonomonas* which has been reported to degrade complex polysaccharides, was most

abundant in two populations from United States (Bruno et al., 2019), and the high abundance of *Lactobacillus* in one population from United States has been shown to enhance biodegradation of food waste by *H. illucens* (Jiang et al., 2019).

Although the 16S data provides poor resolution at the species level, it has been able to unravel the families with genera that might pose risks to both animals and human health. As such the introduction of insects such as BSF as a high-quality protein ingredient in animal feed should be accompanied by proper safety measures. This postharvest treatment measures such as processing of the BSF larvae into dried products, defatting and proper storage in hermetic bags (Moreno-Martinez et al., 2000; Murdock et al., 2012) would be essential to minimize microbial spoilage and reduce the risk of pathogen contaminations along the insect-based feed value chain. Also, the choice of rearing substrate is crucial because of the putative transmission of microbiota to BSF larva with possible clinical implications. Although pre-treatment or sterilization of organic waste substrates before usage in all rearing systems across the world is a possible option, it is usually time-consuming and expensive. Therefore, we strongly recommend that harvested larvae of BSF from various waste substrates should be carefully sterilized during processing to eliminate potential microbial contaminants. Unfortunately, there are no published reports which provide evidence for the role of hygienic design, cooling facilities, sanitation programs and personal hygiene as measures to prevent microbial feed safety hazards for insect-based feed value chain. Therefore, potential preventive measures and intervention strategies as described above become crucial at all stages of the supply chain with thorough investigations in the insect-based protein feed enterprises globally.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

CT and FK conceptualized the study. CT, FK, SE, FO, MD, KA, WS, and JL contributed to data curation. FK, CT, and FO did the formal analysis. MD, JL, SE, and CT acquired the funding. FK, CT, FO, KA, MD, JL, SE, KF, TD, SM, and SS carried out the investigation. FK, CT, and FO provided the methodology. MD, JL, SE, and CT contributed to project administration. MD, JL, CT, FK, and SE provided the resources. FK, FO, and CT helped with the software. CT, MD, JL, FK, SE, SS, and KF supervised the study. FK, CT, and FO validated the study and worked on the visualization. FK, FO, and CT wrote the original draft. FK, FO, KA, SS, SM, KF, WS, JL, CT, TD, MD, and SE reviewed, edited, and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01538/full#supplementary-material

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A Shift Pattern of Bacterial Communities Across the Life Stages of the Citrus Red Mite, *Panonychus citri*

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As one of the most detrimental citrus pests worldwide, the citrus red mite, *Panonychus citri* (McGregor), shows extraordinary fecundity, polyphagia, and acaricide resistance, which may be influenced by microbes as other arthropod pests. However, the community structure and physiological function of microbes in *P. citri* are still largely unknown. Here, the high-throughput sequencing of 16S rDNA amplicons was employed to identify and compare the profile of bacterial communities across the larva, protonymph, deutonymph, and adult stages of *P. citri*. We observed a dominance of phylums Proteobacteria and Firmicutes, and classes α-, γ-, β-Proteobacteria and Bacilli in the bacterial communities across the host lifespan. Based on the dynamic analysis of the bacterial community structure, a significant shift pattern between the immature (larva, protonymph, and deutonymph) and adult stages was observed. Accordingly, among the major families (and corresponding genera), although the relative abundances of Pseudomonadaceae (*Pseudomonas*), Moraxellaceae (*Acinetobacter*), and Sphingobacteriaceae (*Sphingobacterium*) were consistent in larva to deutonymph stages, they were significantly increased to 30.18 ± 8.76% (30.16 ± 8.75%), 20.78 ± 10.86% (18.80 ± 10.84%), and 11.71 ± 5.49% (11.68 ± 5.48%), respectively, in adult stage, which implied the important function of these bacteria on the adults' physiology. Actually, the functional prediction of bacterial communities and Spearman correlation analysis further confirm that these bacteria had positively correlations with the pathway of "lipid metabolism" (including eight sublevel pathways) and "metabolism of cofactors and vitamins" (including five sublevel pathways), which all only increased in adult stages. In addition, the bacterial communities were eliminated by using broad-spectrum antibiotics, streptomycin, which significantly suppressed the survival and oviposition of *P. citri*. Overall, we not only confirmed the physiological effects of bacteria community on the vitality and fecundity of adult hosts, but also revealed the shift pattern of bacterial community structures across the life stages and demonstrated

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the co-enhancements of specific bacterial groups and bacterial functions in nutritional metabolism in *P. citri*. This study sheds light on basic information about the mutualism between spider mites and bacteria, which may be useful in shaping the next generation of control strategies for spider mite pests, especially *P. citri*.

Keywords: *Panonychus citri* (McGregor), spider mites, developmental stages, 16S rDNA amplicon pyrosequencing, dynamics of bacterial community, bacterial metabolism

INTRODUCTION

Many arthropods harbor diverse microbial communities in their digestive systems or intracellular/intercellular niches for symbiotic systems (Dillon and Dillon, 2004). Many of these microbiota play important roles in interactions with their hosts for improved physiology, life history traits, environmental adaptability, reproduction, and are even essential for the host's survival (Engel and Moran, 2013; Kwong and Moran, 2016). In addition to the beneficial effect on host nutritional digestion of varied diets, especially the diets with poor or unbalanced nutrition or recalcitrant components (Hongoh et al., 2008; Huang et al., 2010), microbiota may (1) increase arthropod host fitness through protecting the host from parasites and pathogens or improving the host's tolerance to heat stress (Dillon and Dillon, 2004; Hartman et al., 2020), (2) influence the host's lifespan (Storelli et al., 2011; Noman et al., 2020), (3) improve the insect's social communication (Dillon et al., 2002), and (4) govern mating and reproductive systems (Sharon et al., 2010). In some cases, microbiota facilitated the pest host's survival under traditional pest control by enhancing pesticide resistance (Kikuchi et al., 2012; Zhou and Yao, 2020). The microbiota are even involved in the pest status of the stinkbug species, *Megacopta punctatissima* (Hosokawa et al., 2007). The diversity and structure of microbial communities in some important pests have been identified (Engel and Moran, 2013). Based on the advantage of microbial relationships with host, some new pest management strategies have been developed. For instance, commensal bacteria were genetically modified as novel biocides by expressing toxic substances, such as *Bacillus thuringiensis* toxin protein (Cyt1A) and dsRNA of critical genes for pest population persistence or expansion (Kuzina et al., 2002; Whitten et al., 2016). In some insect vectors of specific diseases (e.g., mosquito borne disease), *Wolbachia* have been successfully used in the biological control of these diseases (Pan et al., 2012).

Despite the recent massive increase in studies of the microorganisms living in arthropod guts, the mechanism of microbial biofunction is just beginning to emerge. In addition, the arthropods are the most diverse and abundant animal clade, so the diversity and function of microbial communities in the arthropods should be extremely diverse, and need more investigation in more species groups, especially in pests. The spider mites, including *Tetranychus urticae*, are one of serious agricultural pest groups in agro-ecosystems, especially for horticultural crops (Hoy and Jeyaprakash, 2005). The life cycle of spider mites consists of five stages, the egg, larva (with three pairs of legs), protonymph, deutonymph, and adult stages (with four pairs of legs in the last three stages) (Kasap, 2009), which is quite different from that of other arthropods, such as insects. Although the distributions, abundances, and bio-functions of some important genera or species of microorganisms, including bacteria, rickettsiae, fungi, and viruses, have been investigated in several spider mite species (mainly in *Tetranychus* species) (Poinar and Poinar, 1998; Hoy and Jeyaprakash, 2005; Niu et al., 2019; Ribeiro et al., 2020), only a few reports have mentioned the biofunctions and profiles of microbial communities across the life stages in spider mites (Zhu et al., 2019b), thus their biofunctions and profiles are still largely unknown. In addition, similar with other arthropods (Meng et al., 2019; Yao et al., 2019), the compositions and structures of the bacterial communities may alter across the lifespan of spider mites to adapt to the nutritional and/or physiological requirements of their hosts' developments, which deserves investigations.

The citrus red mite, *Panonychus citri* (McGregor) (Acari: Tetranychidae), is a spider mite with worldwide distribution and is regarded as one of the most important citrus pests in many countries (Takafuji and Fujimoto, 1986). Consistent with other spider mites, it performs a lifespan consisting of egg, larva, deutonymph, protonymph and adult stages, and feeds on leaves, fruits and/or green twigs by piercing-sucking across the immature and adult stages (Zanardi et al., 2015). *P. citri* possess several critical characteristics for its environmental adaptation and plant damage, including (1) strong fertility (Ali et al., 2017), (2) a wide range of host plants (111 species of citrus and wood plants) (Gerson, 2003), and (3) high levels of resistance to various acaricides (Ran et al., 2009; Pan et al., 2020). All these characteristics facilitate this important fruit pest to live on diverse host plants with high fecundity, and also survive fatal acaricides, which may be associated with the inner-bacterial community. However, it is still unidentified the role of inner-bacterial community in the physiology and vitality of *P. citri*. Although very few researches on the symbiont bacteria have been reported, which indicated no existence of *Wolbachia* and *Cardinium* in the tested *P. citri* population (Chen et al., 2009), the structure and diversity of the microbial community across the lifespan of *P. citri* remain largely unknown. As understanding the characteristics of microbial community is the first critical step for developing the novel symbiont-based strategy of controlling pests (Mereghetti et al., 2017), the physiological function, profile and dynamics of microbial community in citrus red mite deserve investigations.

In the current study, the bacterial community structures across immature (including larva, protonymph, and deutonymph) and adult stages of citrus red mite were determined and compared by pyrosequencing of the 16S rDNA V3–V4 region, which is the next-generation DNA sequencing approach with low-cost, high throughput, and high

accuracy developed in recent years (Meng et al., 2019; Yao et al., 2019). Our results indicated a significant shift pattern of bacterial communities between the immatures and adults, which may influence the predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of "lipid metabolism" and "metabolism of cofactors and vitamins." Finally, the effects of bacteria on survival and oviposition were confirmed by treatment with broad-antibiotic, streptomycin.

MATERIALS AND METHODS

Cultivation of *P. citri*

To control the significant variation of microbiota associated with the outdoor environment, *P. citri* were collected from the sweet orange orchard (N 30° 28′ 26″, E 114° 21′ 5″), Huazhong Agricultural University, Hubei Wuhan, China, and reared for at least 15 populations in indoor condition as follow: the citrus red mites were kept on fresh leaves of *Citrus maxima* (Burm.) Merr., which were placed ventral-side up and surrounded by wet cotton sliver, and placed on 5 mm layer of distilled water-saturated sponge; leaf disks were renewed weekly; the temperature and moisture were controlled at 26 ± 1°C and 60 ± 5% Relative Humidity (RH), respectively and the photoperiod was 14 h (light):10 h (dark).

P. citri Sample Preparation

Samples of *P. citri* at four stages, including larvae, protonymphs, deutonymphs and female adults, were collected separately in three repetitions (each repetition containing at least 300 mites). All these mites were collected from the *P. citri* cultivation originating from eggs laid in the same day (called one-day cultivations) in the same generation reared in parallel. To remove surface contaminants, each repetition of pooled mite individuals was surface-sterilized with 75% ethanol for 3 min and rinsed three times in sterile water. Then, the pool of mite individuals was used for subsequent DNA extraction for high throughput sequencing.

DNA Extraction

The total DNA for the high-throughput sequencing was extracted by Fast DNA SPIN extraction kit (MP Biomedicals, Santa Ana, CA, United States) according to the manufacturer's instruction, and stored at −20°C. The quality and quantity of DNA sample was determined by the agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), respectively.

16S rDNA Amplicon Pyrosequencing

The V3–V4 region in bacterial 16S rDNA was amplified by PCR with the primers of 338F (5′-ACTCCTACGGGAGGCA GCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Xu et al., 2016). The PCR system consisted of Q5 reaction buffer (5×, 5 μL), Q5 high-fidelity GC buffer (5×, 5 μL), Q5 high-fidelity DNA polymerase (5 U/μL, 0.25 μL), dNTPs (2.5 mM, 2 μL), each primer (10 μM, 1 μL), a total DNA template (2 μL), and ddH_2O (8.75 μL). The PCR reaction was performed as initial denaturation (98°C for 2 min), 25 reaction cycles (each at 98°C for 15 s, 55°C for 30 s, and 72°C for 30 s), and a final extension (72°C for 5 min). PCR products were purified and quantified using Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN, United States) and PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, United States), respectively. Then, PCR amplicons were sequenced (paired-end 2 × 300 bp) using the Illlumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

Sequence Analysis

The Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) pipeline were employed to analyze the raw sequencing data as previously described (Caporaso et al., 2010). Briefly, the low-quality sequences that had average Phred scores < 20 bp, had length < 150 bp, contained ambiguous bases, and contained mononucleotide repeats > 8 bp were first filtered. Then the remained paired-end reads were assembled using Fast Length Adjustment of SHort Reads (FLASH) (Magoc and Salzberg, 2011). After chimera detection, the high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by clustering algorithm UCLUST (Edgar, 2010), which followed by selecting a representative sequence for each OTU using default parameters. To classify the OTU taxonomy, the OTU sequences were aligned by Basic Local Alignment Search Tool (BLAST) to search the representative sequences set against the Greengenes Database using the best hit (DeSantis et al., 2006). The extremely rare OTUs (<0.001% of total sequences across all samples) were discarded. Finally, an averaged, rounded rarefied OTU table was constructed by averaging 100 evenly resampled OTU subsets under the 90% of the minimum sequencing depth to minimize the differences in sequencing depths across all samples.

Bioinformatics Analysis

The bioinformatics analysis of sequence data were mainly performed using QIIME and R packages (v3.2.0). Alpha diversity indices (OUT level), including the Shannon diversity index, Simpson index, Berger-Parker dominance index, observed richness (Sobs), Abundance-based Coverage Estimator (ACE) metric, and Chao richness estimator, were calculated using the OTU table in QIIME. To investigate the structural variation of microbial communities across the life stage samples, beta diversity analysis was performed using weighted UniFrac distance metrics (Lozupone and Knight, 2005) and then visualized via the heatmap at class level, unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering at genus level, and principal coordinate analysis (PCoA) at OTU level (Ramette, 2007). Analysis of similarities (ANOSIM) using R package "vegan" was employed to assess the significance of differentiation of microbiota structure among groups. Based on the occurrence of OTUs across samples regardless of their relative abundance, R package "VennDiagram" was employed to generate Venn diagram for visualizing the shared and unique OTUs among different life stages. The functions of bacterial community were predicted by PICRUSt (Langille et al., 2013).

Antibiotic Treatment via Feeding Assays

To identify the physiological function of bacterial communities on *P. citri* performance, the bacteria were eliminated by streptomycin. For antibiotic treatment, we followed a protocol that was previously established for spider mites with minor modification (Zhu et al., 2019a): selected fresh leaves were prewashed with double-distilled water and cut into small pieces of equal sizes (3 cm in diameter), then dried at a constant temperature of 45°C for 15 min. This was followed by dipping the leaf pieces in the antibiotic solutions (3 mg/ml of penicillin or streptomycin) for 1.5 h. After being dried for 3 min with a laminar flow of air, the antibiotic-treated leaves were surrounded by a wet cotton sliver and kept on sponges saturated with 1% of the antibiotic solutions. Thirty days young female adults (1–2 days after last molting), which had been starved for 3 h before use, were placed on antibiotic-treated leaves. The antibiotic-treated leaves and the antibiotic solutions for saturating sponges were changed every 3 days. All experiments were repeated three times. At each of 1, 3, 5, 7, and 9 days after the beginning of antibiotic treatment, the mite survival status (the numbers of survived and dead mites) and the number of laid eggs between the measured and the previous time points were detected. The number of laid eggs per female at each time point was calculated as (the number of laid eggs between the measured and the previous time points)/(survived female number at the measured time point)/duration. The cumulative number of laid eggs per female in 9 days was calculated by accumulating all of (the number of laid eggs per female at each time point) × duration. In addition, the total contents of nitrogen, phosphorus, potassium, and soluble sugar were determined as described in **Supplementary Methods**.

Real-Time Quantitative PCR for Bacterial Counting

The mite samples (three repetitions for each time point; about 50 individuals per repetition) with or without antibiotic treatments were first surface-sterilized with 75% ethanol for 3 min and rinsed three times in sterile water. Then, the total DNA containing both bacterial genomic DNA and host DNA was extracted as mentioned above for subsequent real-time quantitative PCR.

The amounts of total bacteria were quantified by real-time quantitative PCR of partial 16S rDNA with the universal primers (Eub338F, 5′-ACTCCTACGGGAGGCAGCAG-3′; Eub518R, 5′-ATTACCGCGGCTGCTGG-3′) (Yao et al., 2019) and normalized by real-time quantitative PCR of alpha tubulin as internal control with the specific primers (TUBA-F, 5′-CGAA TCCATTTCCCCTTAGT-3′; TUBA-R, 5′-CAACGTCTCCTCG GTAAAGA-3′) (Niu et al., 2012). Each PCR mixture consisted of 10 μL of SYBR Green Mix (Bio-Rad, Hercules, CA, United States), 100 nM of each primer and 2 μL of total DNA. The amplification program consisted of preincubation at 95°C for 2 min, and 40 cycles at 95°C for 5 s and annealing at 60°C for 30 s.

Statistical Analysis

The variations among the different stages of *P. citri* were statistically analyzed by one-way analysis of variance (ANOVA), which was followed by the *post hoc* Duncan's Multiple Range Test (DMRT). The difference in cumulative mortality, daily or cumulative laid egg numbers between the adults with and without antibiotic-treatment was statistically analyzed by Student's t-test. The significant correlations between bacterial species and KEGG pathways were analyzed by Spearman's correlations and illuminated by heatmaps by GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, United States). P-value < 0.05 was representative of statistical significance. Results are presented as the means ± standard error of mean (SEM).

RESULTS

16S rDNA -Sequencing Data of Bacterial Communities Across the Life Stages of *P. citri*

The bacterial composition in the larvae, protonymphs, deutonymphs, and adults of *P. citri* were quantified by Illumina MiSeq platform of 16S rDNA gene amplicons. The sequencing data yielded 588,357 high-quality pyrosequencing reads with an average read length of 452.7 bp of the 16S rDNA spanning the variable regions V3–V4 from mite samples, each of which possessed 36,023–47,660 valid sequences with high quality (**Table 1**). Based on the 97% similarity threshold, these sequences yielded 357–724 OTUs of each sample with over 99% coverage, which indicated that most bacterial taxa (>99%) in each sample was covered (**Table 1**). According to the rank-abundance curves (**Supplementary Figure S1A**), most of the OTUs in the bacterial communities belonged to rare species. The rarefaction curves showed that the accumulation of OTUs tended toward saturation in the numbers of reads, which indicated that the samples were sufficient to reveal the bacterial communities (**Supplementary Figure S1B**). These results indicated that our sequencing captured most of the bacterial diversity associated with *P. citri*.

Varied Diversities of Bacterial Communities Across the Life Stages of *P. citri*

To identify the diversity of bacteria in the larvae, protonymphs, deutonymphs, and adults of *P. citri*, six indexes were detected, including Shannon, Simpson and Berger-Parker for bacterial diversity, and observed species (Sobs), ACE and Chao for species richness. The diversity indexes were highest in larvae (Simpson and Berger-Parker) or protonymphs (Shannon), then decreased in the following life stages, especially in the adult stage with significant differences for Shannon and Berger-Parker indexes (all $P < 0.05$, *post hoc* DMRT) (**Figures 1A–C**). When considering the species richness indexes (i.e., Sobs, ACE, and Chao1), there was no significant difference between any stage (**Figures 1D–F**).

Varied Compositions of the Bacterial Communities Across the Life Stages of *P. citri*

A Venn diagram analysis showed that subsets of 342 bacterial OTUs were shared across the lifespan of *P. citri*

TABLE 1 | Summary of the 16S rRNA read counts of endo-bacteria across the life stages of *P. citri**.

Sample ID	Read NO.	Mean length (bp)	Phylum	Class	Order	Family	Genus	Species	OUT	Coverage
L4	48717	452.9	24	53	81	152	245	275	724	0.996
L5	49976	450.8	21	39	63	126	199	219	574	0.997
L7	54413	449.7	24	49	76	142	222	244	619	0.997
PN4	49231	453.3	23	51	82	152	242	267	572	0.997
PN5	49068	453.2	22	53	85	160	243	274	589	0.997
PN6	46153	445.4	22	53	79	150	226	252	497	0.997
DN4	52141	450.3	21	43	73	138	218	247	631	0.998
DN5	51455	450.3	20	42	63	120	189	214	357	0.999
DN7	49628	452	23	48	73	135	210	240	445	0.999
A4	39288	463.4	20	42	72	134	204	228	452	0.997
A6	43734	459.3	21	45	78	144	227	252	560	0.999
A7	54553	454.3	18	41	64	120	173	194	432	0.997

**L, PN, DN, and A indicated the larvae, protonymphs, deutonymphs, and adults, respectively.*

(**Supplementary Figure S2A**). According to the OTU classification based on the Greengenes Database, 24 bacterial phyla were detected in the bacterial communities of larvae, protonymphs, deutonymphs, and adults. Proteobacteria was the dominant and most diverse phylum in all samples (69.48 ± 1.57% of average relative abundance), followed by Firmicutes (11.76 ± 1.20%), Actinobacteria (7.42 ± 1.14%), Bacteroidetes (5.93 ± 1.65%), and Cyanobacteria (1.90 ± 0.53%) (**Supplementary Figure S2B**; see detailed phylum lists in **Supplementary Table S2**). Accordingly, at the class taxa level, the major classes were α-, γ-, and β-Proteobacteria (45.22 ± 4.36%, 16.96 ± 5.18%, and 7.00 ± 0.65% of relative abundances, respectively) in phylum Proteobacteria, Bacilli and Clostridia (7.09 ± 0.98% and 4.24 ± 0.44%, respectively) in Firmicutes, Actinobacteria (6.99 ± 1.12%) in Actinobacteria, and Sphingobacteria and Chitinophagia (2.96 ± 1.92% and 1.51 ± 0.36%, respectively) in Bacteroidetes (**Supplementary Figure S2C**; see detailed class lists in **Supplementary Table S3**).

Furthermore, the relative abundances of the major families (and corresponding genera) varied across the lifespan of *P. citri* in six patterns (**Figures 2**, **3**; see detailed family and genus lists in **Supplementary Tables S3, S4**, respectively): (1) the relative abundances of families Pseudomonadaceae (genus *Pseudomonas*), Moraxellaceae (*Acinetobacter*), and Sphingobacteriaceae (*Sphingobacterium*) were consistent in larvae, protonymphs and deutonymphs, but only significantly increased to 30.18 ± 8.76% (30.16 ± 8.75%), 20.78 ± 10.86% (18.80 ± 10.84%), and 11.71 ± 5.49% (11.68 ± 5.48%), respectively, in adults in comparison to all three immature stages; (2) the relative abundances of Brucellaceae (*Ochrobactrum*), Xanthomonadaceae (*Stenotrophomonas*), and Streptococcaceae were consistent in three immature stages, but only significantly decreased to 17.96 ± 5.21% (17.95 ± 5.19%), 1.00 ± 0.29% (0.86 ± 0.22%), and 0.59 ± 0.22%, respectively, in adults in comparison to the highest level in one or three immature stage(s); (3) the relative abundances of Phyllobacteriaceae (*Mesorhizobium*) and Chitinophagaceae (*Sediminibacterium*) were consistent in larvae and protonymphs, but significantly decreased in both deutonymphs and adults in comparison to the highest level in the larva or protonymph stage; (4) in comparison to that of larvae, the relative abundances of Enterobacteriaceae (*Serratia*), Comamonadaceae (*Delftia*), Burkholderiaceae (*Burkholderia*), and genus *Lactococcus* were all significantly increased in protonymphs, then dropped in deutonymph and adult stages to the levels similar to that of larvae stage, except for significantly lower levels of *Serratia* in deutonymph and adult stages than that of larva stage; (5) the relative abundances of Alcaligenaceae (*Achromobacter*) were highest in larvae, and significantly decreased in the followed three stages; (6) the relative abundances of Bacillaceae (*Bacillus*), unclassified family in Actinomycetales (unclassified genus in Actinomycetales), and other major classes (genera) were consistent across the life stages ($P < 0.05$ for all significant differences, *post hoc* DMRT).

Varied Structures of the Bacterial Communities Across the Life Stages of *P. citri*

Based on the compositions of the bacterial communities across the life stages, the community dissimilarities in class, genus, and OTU taxa levels were investigated further. According to weighted UniFrac metrics, the heatmap analysis at the class level (**Figure 4A**), the analysis using the UPGMA based on hierarchical clustering at genus level (**Figure 4B**), and PCoA analysis at the OTU level (**Figure 4C**) were constructed. All analyses indicated that community structures of bacteria in the larvae, protonymphs, and deutonymphs were similar and clustered into one group, and that in the adults were different and clustered into another group (except that adult sample A2 was more similar to immature samples than the other two adult samples, according to hierarchical clustering analysis at the genus level). Moreover, the analysis of similarities (ANOSIM) results indicated a significant difference in the bacterial community of *P. citri* across the lifespan ($R = 0.4506$ and $P = 0.001$; **Figure 4C**), which supported the clustering of the bacterial communities over the four life stages into two groups.

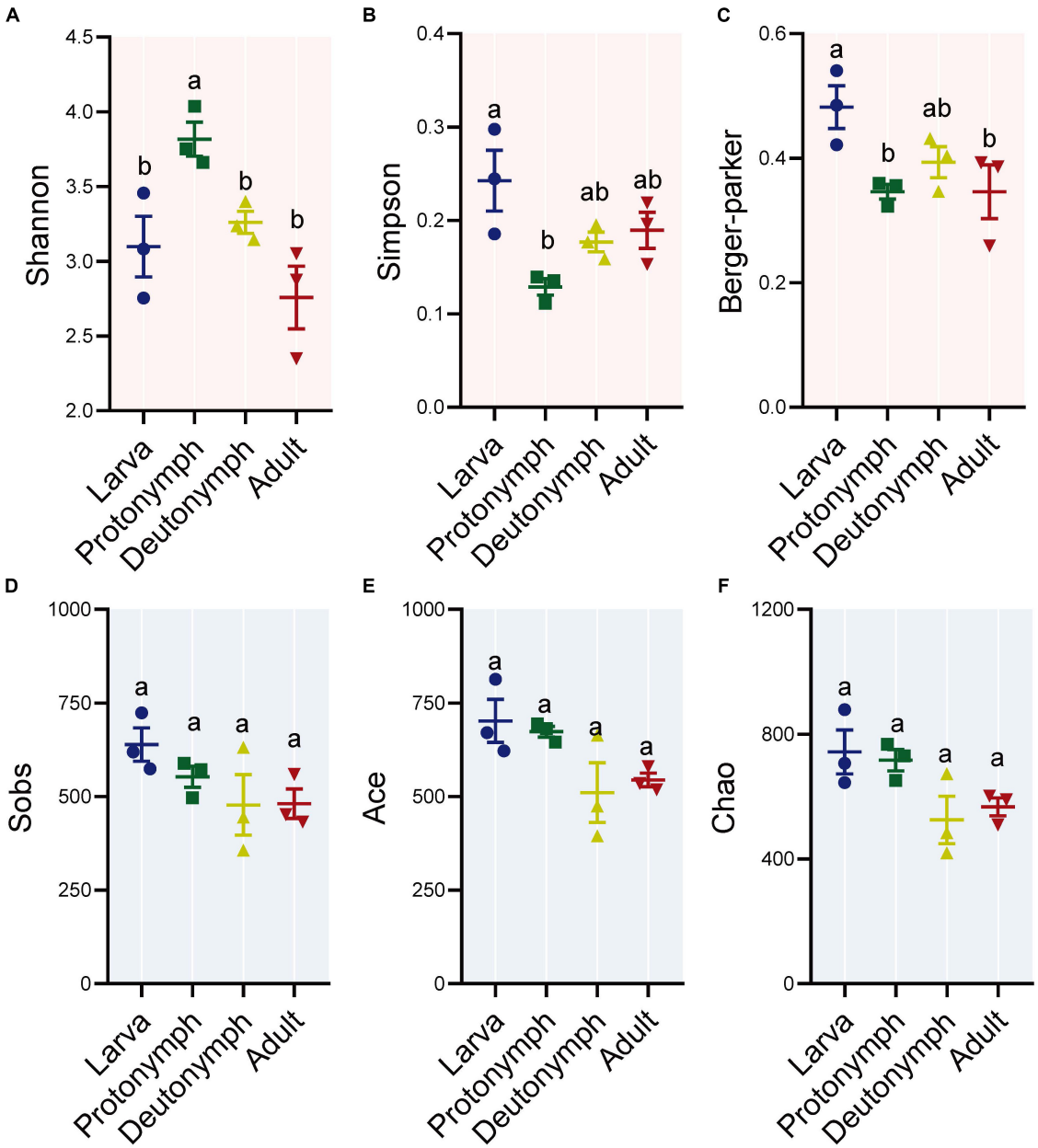

FIGURE 1 | Alpha diversity of bacterial communities across the lifespan of *P. citri*. The Shannon **(A)**, Simpson **(B)**, and Berger-Parker **(C)** indexes for bacterial diversity, and observed species (Sobs, **D**), ACE **(E)** and, Chao **(F)** indexes for species richness were applied. All $n = 3$. Different lowercase letters denote significant differences between different life stages ($P < 0.05$, *post hoc* Duncan's Multiple Range Test [DMRT]). Plots showed means ± standard error of mean (SEM).

Varied Functions of the Bacterial Community Across the Life Stages of *P. citri* Based on PICRUSt Metagenomic Prediction

Using PICRUSt metagenomic prediction, 7, 37, and 290 KEGG pathways were obtained at level 1, 2, and 3, respectively (see detailed lists in **Supplementary Tables S5–S7**, respectively). Among the major KEGG pathways at level 2 (relative abundance > 1%), the relative abundances of "lipid metabolism" (**Figure 5A**) and "metabolism of cofactors and vitamins" (**Figure 6A**) were consistent within larvae, protonymphs, and deutonymphs, but significantly increased in adults in comparison to that of immatures ($P < 0.05$ for all significant differences, *post hoc* DMRT). Meanwhile, similar patterns were observed in other six pathways, including "folding, sorting and degradation," "replication and repair," "transcription," "translation," "glycan biosynthesis and metabolism," and "metabolism of terpenoids and polyketides" (**Supplementary Figure S3**).

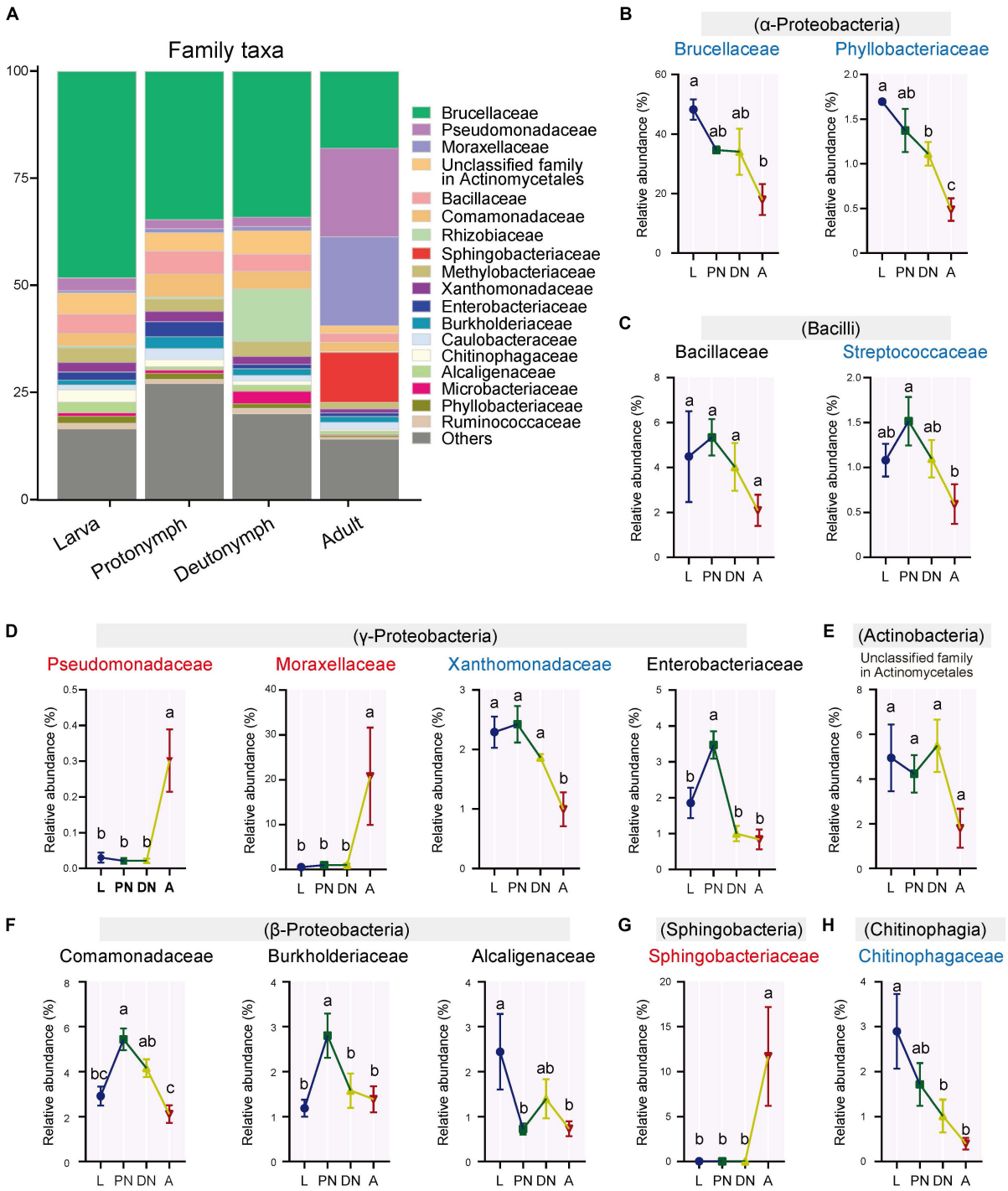

FIGURE 2 | Variations of the relative abundances of bacterial families across the lifespan of *P. citri*. **(A)** Whole profiles of the relative abundances of the families in each life stage; only taxa with a relative abundance > 1% in at least one sample were analyzed. **(B–H)** Comparisons of the relative abundances of 14 major families across the larva (L), protonymph (PN), deutonymph (DN), and adult (A) stages; all $n = 3$; different lowercase letters denote significant differences between different life stages ($P < 0.05$, *post hoc* DMRT); the family names in red or blue color indicated the relative abundances of corresponding families significantly increased or decreased in the adult stage, respectively; the corresponding classes were presented in parentheses; plots showed means ± SEM.

To investigate the KEGG pathways related to the physiology of host adults, the "lipid metabolism" and "metabolism of cofactors and vitamins," which have been identified to be important for the vitality and fecundity of host (Douglas, 2015; Ali et al., 2017; Zhang X. et al., 2018; Canfora et al., 2019), were selected for further analysis. In the "lipid metabolism," the

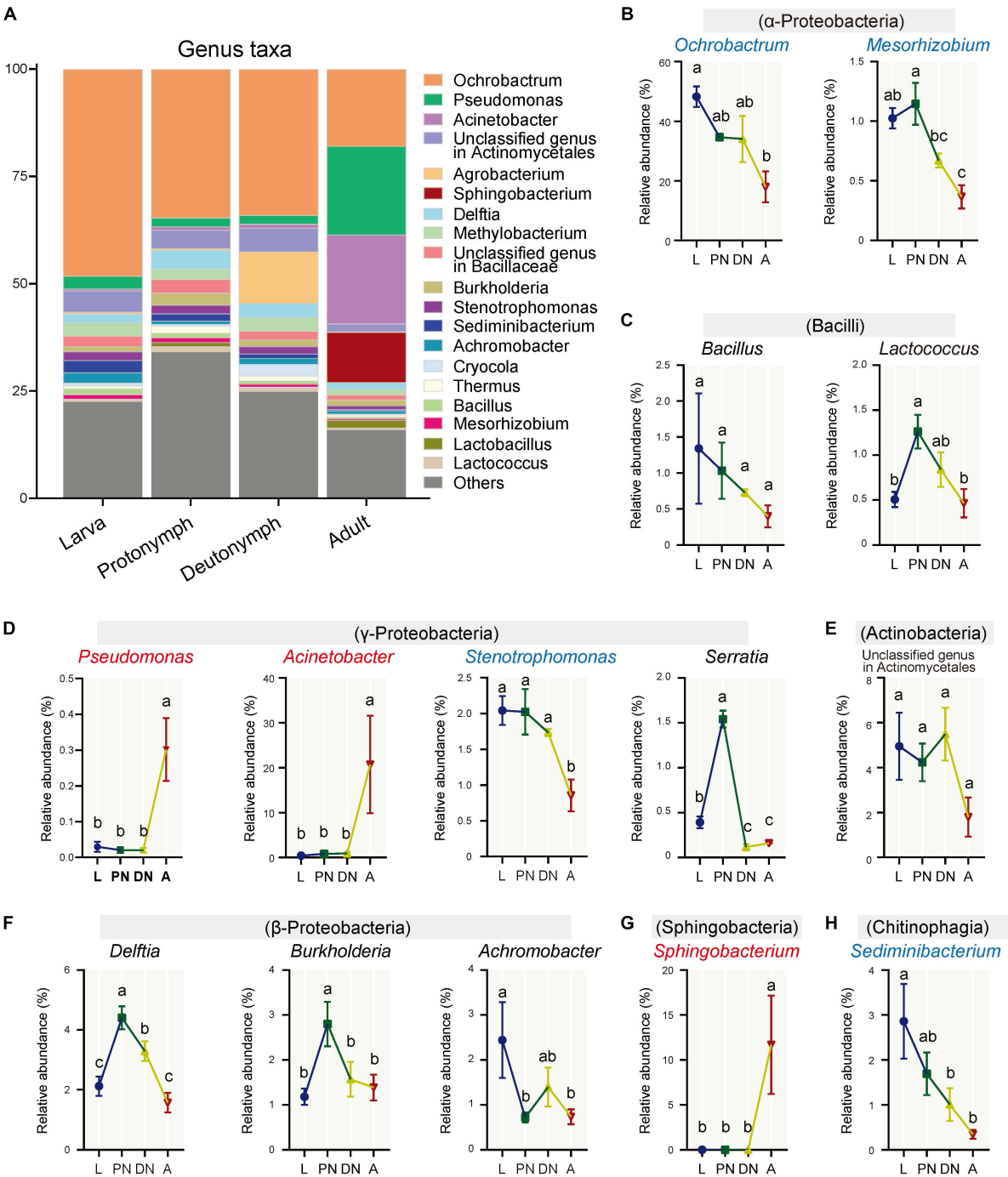

FIGURE 3 | Variations of the relative abundances of bacterial genera across the lifespan of *P. citri*. **(A)** Whole profiles of the relative abundances of the genera in each life stage; only taxa with a relative abundance > 1% in at least one sample were analyzed. **(B–H)** Comparisons of the relative abundances of 14 major genera across the larva (L), protonymph (PN), deutonymph (DN), and adult (A) stages; all $n = 3$; different lowercase letters denote significant differences between different life stages ($P < 0.05$, *post hoc* DMRT); the genus names in red or blue color indicated the relative abundances of corresponding genera significantly increased or decreased in the adult stage, respectively; the corresponding classes were presented in parentheses; plots showed means ± SEM.

relative abundances of eight pathways at level 3, including "lipid biosynthesis proteins," "biosynthesis of unsaturated fatty acids," "sphingolipid metabolism," "α-linolenic acid metabolism," "arachidonic acid metabolism," "glycerophospholipid metabolism," "steroid hormone biosynthesis," and "synthesis and degradation of ketone bodies," were consistent in larvae, protonymphs and deutonymphs, but significantly increased in adults; and other pathways were consistent across the

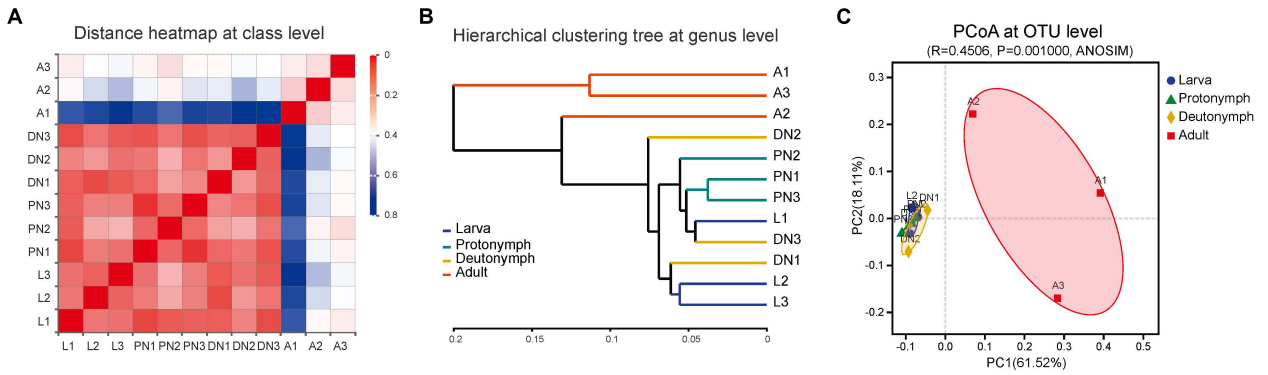

FIGURE 4 | Bacterial community dissimilarity across the lifespan of *P. citri* based on weighted UniFrac metrics. **(A)** Sample distance heatmap analysis at class level. **(B)** Analysis of unweighted pair-group method with arithmetic mean (UPGMA) based on hierarchical clustering at genus level. **(C)** Principal coordinated (PCoA) analysis at OTU level; the significance of variation in communities across four life stages was analyzed by analysis of similarities (ANOSIM) test with 999 permutations. L, PN, DN, and A indicated the larva, protonymph, deutonymph, and adult stages, respectively.

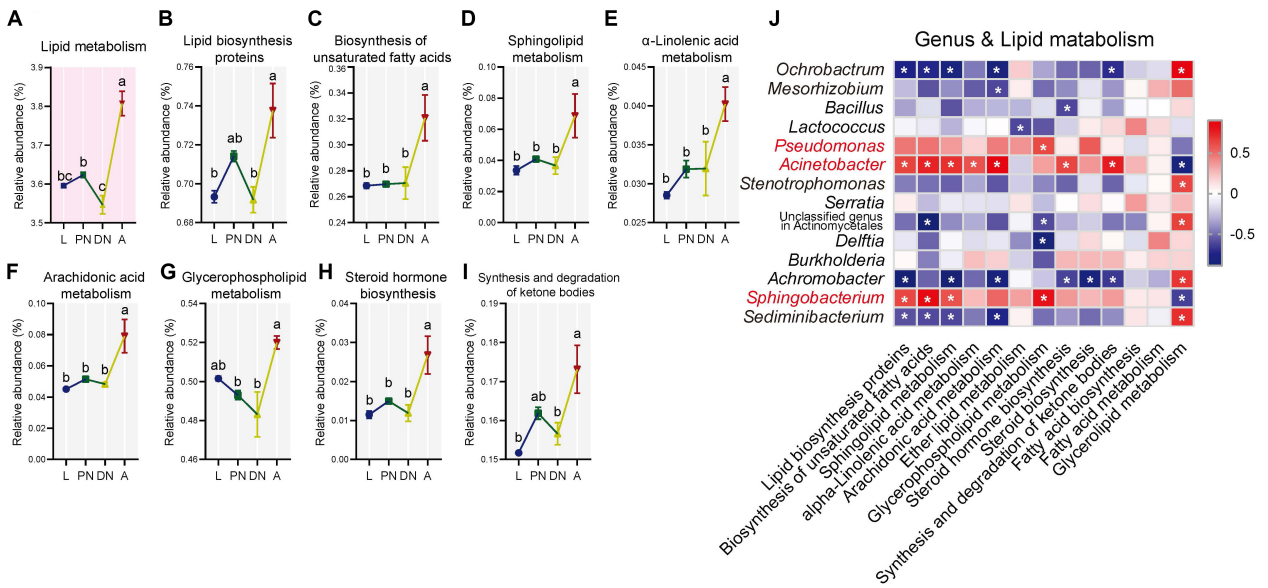

FIGURE 5 | Dynamics of predicted KEGG pathways of "lipid metabolism" and sublevel pathways across the lifespan of *P. citri*. **(A–I)** The "lipid metabolism" pathway at level two (A) and eight sublevel pathways at level three **(B–I)** significantly varied across the larva (L), protonymph (PN), deutonymph (DN), and adult (A) stages; all $n = 3$; different lowercase letters denote significant differences between different life stages ($P < 0.05$, *post hoc* DMRT); plots showed means ± SEM. **(J)** Heatmaps based on Spearman's correlation analysis between the relative abundances of "lipid metabolism" pathways and major genera; the red and blue represent positive and negative correlations between the relative abundances of pathways and bacterial genera, respectively; *indicated $P < 0.05$, Spearman's correlation.

lifespan (**Figures 5B–I**; $P < 0.05$ for all significant differences, *post hoc* DMRT). In terms of the "metabolism of cofactors and vitamins," the relative abundances of five pathways at level 3, including "thiamine metabolism," "one carbon pool by folate," "folate biosynthesis," "biotin metabolism," and "nicotinate and nicotinamide metabolism," were consistent in the three immature stages, but significantly increased in adults; the "porphyrin and chlorophyll metabolism" was consistent in the three immature stages, but significantly decreased in adults; and other pathways were consistent across the lifespan (**Figures 6B–G**; $P < 0.05$ for all significant differences, *post hoc* DMRT). The detailed information of dynamics of other six KEGG pathways mentioned above was presented in the **Supplementary Table S8**.

To clarify the association between the alteration of the composition and predicted functionality of the bacterial communities, Spearman's correlation analysis was performed. The results showed that relative abundances of genera *Pseudomonas*, *Acinetobacter*, and *Sphingobacterium* had positive correlations with almost all the predicted pathways for "lipid metabolism," which with one, seven, and four significant positive correlations, respectively. In addition, the same genera had positive correlations with half of the predicted pathways for "metabolism of cofactors and vitamins"

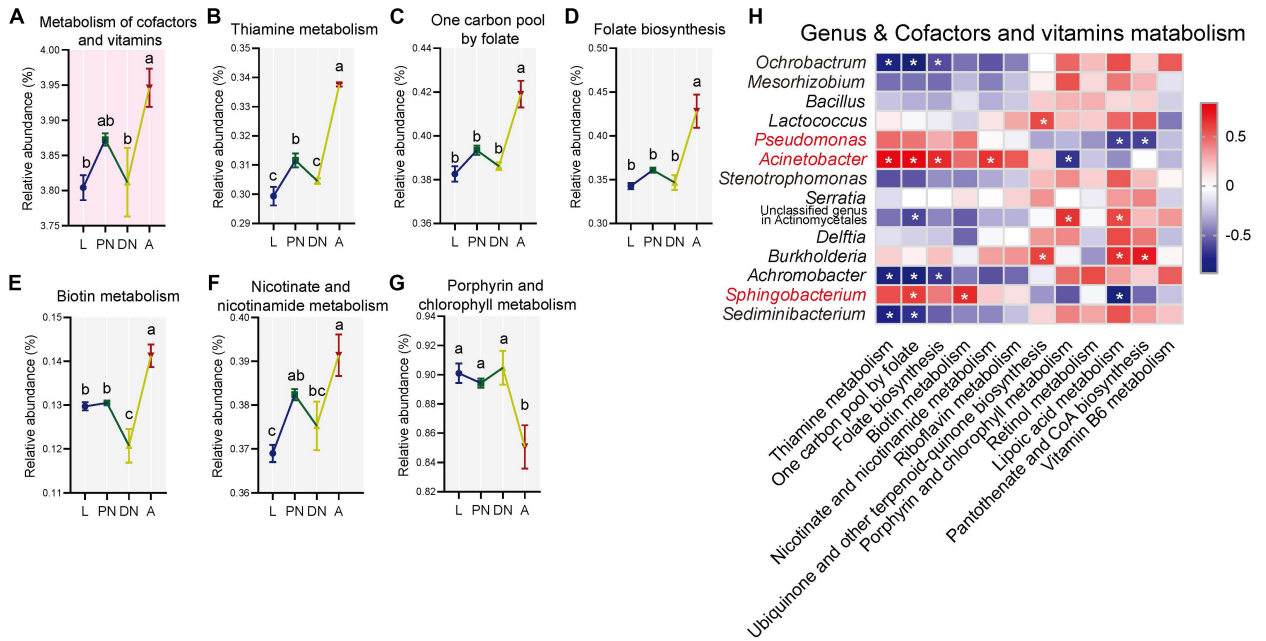

FIGURE 6 | Dynamics of predicted KEGG pathways of "metabolism of cofactors and vitamins" and sublevel pathways across the lifespan of *P. citri*. **(A–G)** The "metabolism of cofactors and vitamins" pathway at level two (A) and five sublevel pathways at level three **(B–G)** significantly varied across the larva (L), protonymph (PN), deutonymph (DN), and adult (A) stages; all $n = 3$; different lowercase letters denote significant differences between different life stages ($P < 0.05$, *post hoc* DMRT); plots showed means ± SEM. **(H)** Heatmaps based on Spearman's correlation analysis between the relative abundances of "metabolism of cofactors and vitamins" pathways and major genera; the red and blue represent positive and negative correlations between the relative abundances of pathways and bacterial genera, respectively; *indicated $P < 0.05$, Student's *t*-test.

(including all pathways performed with significant increases in the adult stage), which with zero, four, and two significant positive correlations, respectively (**Figures 5J**, **6H**). In contrast, the relative abundances of *Ochrobactrum*, *Mesorhizobium*, *Stenotrophomonas*, *Sediminibacterium,* and *Achromobacter* had negative correlations with almost all the predicted pathways for "lipid metabolism," which with five, one, zero, four, and six significant positive correlations, respectively. In addition, these genera had negative correlations with half of the predicted pathways for "metabolism of cofactors and vitamins" (including all pathways demonstrating with significant increases in the adult stage), which with three, zero, zero, two, and three significant negative correlations, respectively (**Figures 5J**, **6H**).

Abundance of Symbionts Across the Life Stages of *P. citri*

According to the symbionts reported in other spider mites (such as the *Tetranychus* species) and sap-feeding insects (such as aphids and psyllids) (Meyer and Hoy, 2008; Zhao et al., 2016; Staudacher et al., 2017; Meng et al., 2019), the sequencing reads of *Wolbachia*, *Carsonella*, *Arsenophonus*, *Profftella*, *Oxalobacter*, *Herbaspirillum*, *Rickettsia*, *Cardinium*, *Spiroplasma*, *Buchnera*, *Hamiltonella*, *Regiella*, *Serratia*, and *Spiroplasma* were screened in the sequencing data. No or almost no read for each symbiont was detected across the life stages, except that *Serratia* had very low relative abundances (0.0061–1.263%) across the life stages, which was significantly highest in protonymphs (**Table 2**).

Bacterial Elimination by Antibiotic Treatment Suppress the Survival and Oviposition of *P. citri*

Finally, to identify the physiological function of bacterial communities on adult performance of *P. citri*, the bacteria in host adults were eliminated by broad-spectrum antibiotics, streptomycin, which has been used in eliminating the bacteria in some insect pests and stored-product mites (Kopecky et al., 2014; Paramasiva et al., 2014; Lin et al., 2015). Under the antibiotic treatment, the relative bacterial counts were significantly decreased to 9.13 ± 0.38% of control (without antibiotic treatment) at 1 day, then kept at 13.37 ± 3.14%, 10.84 ± 1.75%, and 34.09 ± 13.67% at 3, 5, and 9 days, respectively (**Figure 7A**; all $P < 0.05$, Student's *t*-test). In comparison to control, although the daily mortalities were not significantly different under antibiotic treatments at each time point of 1, 3, 5, 7, and 9 days (**Supplementary Figure S4**), the cumulative mortality at 9 days was significantly increased by 54.7% in streptomycin-treatment groups (**Figure 7B**; $P < 0.05$, Student's *t*-test). In addition, the daily numbers of laid eggs were significantly reduced at 1 and 9 days (**Figure 7C**; all $P < 0.05$, Student's *t*-test); therefore the cumulative number of laid eggs was significantly decreased by 35.7% at 9 days after streptomycin treatments (**Figure 7D**; $P < 0.05$, Student's *t*-test). To exclude the possibility of the adverse effect of antibiotics on the nutritional condition of citrus leaves to influence the mite performance, the appearances and major nutrition contents

TABLE 2 | Sequencing reads of symbionts across the life stages of *P. citri**.

Species	Larva	Protonymph	Deutonymph	Adult	References
Wolbachia	0	<0.0001	0	0	Zhao et al., 2016; Staudacher et al., 2017; Meng et al., 2019
Carsonella	0	0	0	0	Meyer and Hoy, 2008; Meng et al., 2019
Arsenophonus	0	0	0	0	Zhao et al., 2016
Profftella	0	0	0	0	Meyer and Hoy, 2008; Meng et al., 2019
Oxalobacter	0	0	0	0	Meyer and Hoy, 2008
Herbaspirillum	<0.0001	0	<0.0001	<0.0001	Meyer and Hoy, 2008
Rickettsia	0	0	0	0	Zhao et al., 2016; Staudacher et al., 2017
Cardinium	0	0	0	0	Staudacher et al., 2017
Spiroplasma	0	0	0	0	Staudacher et al., 2017
Buchnera	0	0	0	0	Zhao et al., 2016
Hamiltonella	0	0	0	0	Zhao et al., 2016
Regiella	0	0	0	0	Zhao et al., 2016
Serratia	$0.00276 \pm 0.00038^{(b)}$	$0.01161 \pm 0.00082^{(a)}$	$0.00083 \pm 0.00021^{(c)}$	$0.00127 \pm 0.00015^{(bc)}$	Zhao et al., 2016
Spiroplasma	0	0	0	0	Zhao et al., 2016

**L, PN, DN, and A indicated the larvae, protonymphs, deutonymphs, and adults, respectively. Different lowercase letters in parentheses denote significant differences between different life stages ($P < 0.05$, post-Duncan's test).*

(including the total contents of nitrogen, phosphorus, potassium, and soluble sugar) of citrus leaves were determined (see the detailed methods in **Supplementary Methods**), which indicated no significant differences in appearances and major nutrition contents after antibiotic-treatments (**Supplementary Figure S5** and **Supplementary Table S9**). These results indicated that the survival and fertility of *P. citri* were reduced by eliminating bacteria, rather than by aggravating the nutritional status of citrus leaves, under broad-spectrum antibiotic treatments.

DISCUSSION

In contrast to insect pests, little is known about the physiological effects of the microbial community on the spider mite performance and its composition across different development stages (Poinar and Poinar, 1998; Hoy and Jeyaprakash, 2005; Ribeiro et al., 2020), especially in the detrimental pest, citrus red mites. Here, the compositions and structures of the bacterial communities were revealed by high-throughput sequencing of 16S rDNA and compared across the development stages, which indicated a significant shift pattern between the immature and adult stages of *P. citri*. Accordingly, the relative abundances of family Pseudomonadaceae (genus *Pseudomonas*), Moraxellaceae (*Acinetobacter*), and Sphingobacteriaceae (*Sphingobacterium*) were consistent in larva to deutonymph stages, but significantly increased in adult stage. Furthermore, the bacterial functions of the "lipid metabolism" and "metabolism of cofactors and vitamins" pathways were predicted to be enhanced in the adult stage, and significantly positively correlated with the coenhancements of Pseudomonadaceae (*Pseudomonas*), Moraxellaceae (*Acinetobacter*), and Sphingobacteriaceae (*Sphingobacterium*), which implies that the enhancements of these bacteria may contribute to adapt to the physiological requirement of host adults by strengthening the metabolisms of lipid, cofactors and vitamins. To preliminarily test this implication, the broad-spectrum antibiotics, streptomycin, was used to eliminate the bacterial community in adult stage of *P. citri*. The results indicated the significant effects of bacterial community on the physiology of *P. citri*, such as survival and fertility. Our results not only confirmed the physiological effects of bacteria in *P. citri*, but also elucidated the profiles of bacterial communities and the shift pattern of community structures. In addition, the abundance enhancements of several specific bacteria, including Pseudomonadaceae (genus *Pseudomonas*), Moraxellaceae (*Acinetobacter*), and Sphingobacteriaceae (*Sphingobacterium*), were correlated with the physiological requirement of host adult and predicted to contribute to the adult vitality and fecundity of *P. citri*. This facilitates our understanding of the mutualism between host and bacteria, and may contribute to shaping potential biocontrol approaches against *P. citri* and other spider mite pests.

In *P. citri*, the phylum Proteobacteria (with α- and γ-Proteobacteria as the major classes) and Firmicutes (with Bacilli and Clostridia) were dominant and diverse across the developmental stages, which is consistent with other spider mites and many insect species in Lepidoptera, Diptera, Coleoptera, Hymenoptera and Hemipter (Yun et al., 2014; Zhang Z.Y. et al., 2018; Zhao et al., 2018; Meng et al., 2019; Yao et al., 2019), but quite different from some sap-feeding insects, such as aphids (Gauthier et al., 2015; Zhao et al., 2016). Proteobacteria species are important in nitrogen fixation, metabolisms of critical nutritional components (including sugars and proteins), insecticide resistance, and protection against parasites and pathogens in fruit flies, aphids, and moths (Oliver et al., 2003; Behar et al., 2005; Xia et al.,

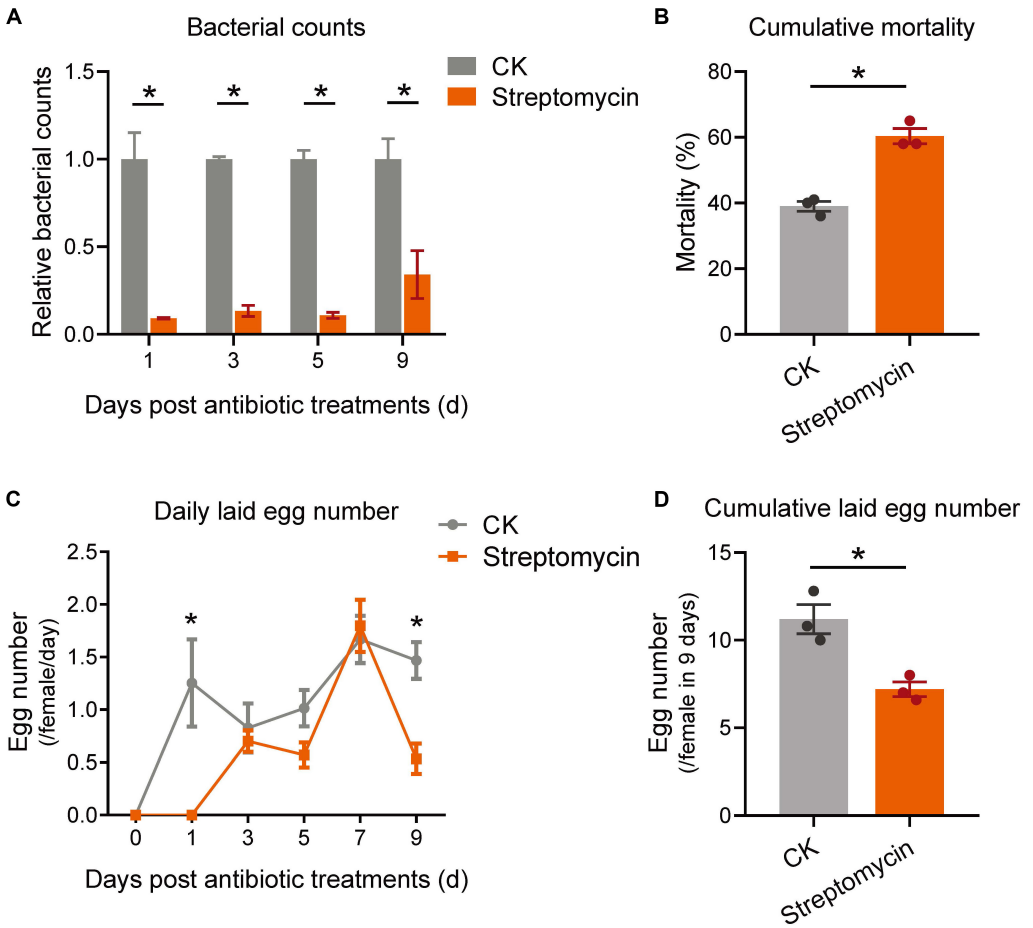

FIGURE 7 | The effects of antibiotic-treatments on the survival and reproduction of *P. citri*. After treatment of streptomycin (3 mg/ml), the relative bacterial counts **(A)**, cumulative mortalities **(B)**, daily **(C)**, and cumulative laid egg number **(D)** were detected and compared to that of control (CK). All $n = 3$. The bacterial counts at each time point in (A) were normalized to the mean of CK to calculate the relative bacterial counts. *Indicated the significant difference between streptomycin treatment and CK. Plots showed means ± SEM.

2013; Zhao et al., 2018; Meng et al., 2019; Yao et al., 2019). Thus, the α- and γ-Proteobacteria species in *P. citri* were predicted to be important in the degradation and use of plant materials for nutritional supply and anti-pathogens for its hosts. Meanwhile, the Bacilli and Clostridia in Firmicutes may also play important roles in host development, detoxification of plant toxic compounds, and anti-pathogens, which has been identified in *Drosophila*, moths, and bees (Shin et al., 2011; Storelli et al., 2011; Xia et al., 2013; Mereghetti et al., 2017; Shao et al., 2017). These results imply that broad-spectrum antibiotic treatment may influence the vitality and fecundity of *P. citri* by eliminating the dominant bacteria, such as Proteobacteria and Firmicutes species. However, some major families and genera discovered in *P. citri* in our study rarely exist in other spider mites and insects. For instance, the most dominant family Brucellaceae (genus *Ochrobactrum*) consisted of 33.71 ± 3.87% of the average relative abundance in the bacterial community of *P. citri*. The Brucellaceae family was considered as environmental bacteria and only reported in mammals as opportunistic pathogens and gut bacterial species in dipteran *Lutzomyia longipalpis*, which is the vector of *Leishmania infantum* (the leishmaniasis pathogen) (Kelly et al., 2017; Zheng et al., 2019). These uncommon bacteria species indicate that the bacterial community in this spider mite pest is distinct from that in other spider mites or insects, and may be determined by the host's diets and/or physiological requirements for host development and reproduction (Engel and Moran, 2013; Mereghetti et al., 2017).

Across the life stages, the bacterial diversity showed a declining trend in *P. citri* (**Figures 1A–C**), which is consistent with that in some insects, such as psyllids, fruit flies, planthoppers, and ladybirds (Yun et al., 2014; Meng et al., 2019; Wang et al., 2019; Yao et al., 2019). The higher bacterial diversity in the immature stages of insect has been associated with the diverse environmental microbes originating from the eggs and/or diets; and during the development, some exogenous bacteria may be unable to adjust the hosts' digestive tracts in the adult stage. Thus, these bacteria perform as transient microbes, which may induce the decrease of bacterial diversity (Meng et al., 2019; Yao et al., 2019). This implies that some major bacterial families and genera in *P. citri*, which significantly reduced in the adult

stage (**Figures 2**, **3**), originated from eggs and/or plant diets and colonized in the internal niches (such as digestive tracts) during immature stages, but reduced in adult stage because of the declined adaptability to the internal niches in this spider mite. To clarify this phenomenon in *P. citri*, a comparison of microbe communities from all stages (including egg stage) and diets (such as the citrus leaves) deserves further investigation.

Cluster analysis showed a clear separation of the adults' bacterial community from immatures (including larvae, protonymphs and deutonymphs) of *P. citri* (**Figure 4**). This shift pattern of bacterial community structures between immature and adult stages was discovered in acarid species for the first time, and is similar to many insects, including both holometabolous and hemimetabolous species (Zhao et al., 2018; Meng et al., 2019; Nobles and Jackson, 2020). In holometabolous insects, the diets and habitats are usually extremely varied across the larvae, pupa to adults, and are considered as critical factors for inducing extreme transmission between immatures and adults (Zhao et al., 2018; Yao et al., 2019). However, the diets and habitats were consistent across the life stages of *P. citri* (Zanardi et al., 2015). Therefore, other factors, such as alterations in physiology, immune system, and/or phylogeny across the developmental stages (Yun et al., 2014; Yao et al., 2016), probably play important roles in the shift pattern of bacterial community in *P. citri*, which has been identified in hemimetabolous insects, such as psyllids, planthoppers, and whiteflies (Indiragandhi et al., 2010; Meng et al., 2019; Wang et al., 2019).

Consistent with the alteration of the bacterial community structure, some major families/genera and predicted bacterial metabolic pathways including "lipid metabolism" and "metabolism of cofactors and vitamins" were co-enhanced in the adult stages in comparison to other immature stages, and performed with significant positive correlations (**Figures 5**, **6**). These major families/genera include families Pseudomonadaceae (genus *Pseudomonas*), Moraxellaceae (*Acinetobacter*), and Sphingobacteriaceae (*Sphingobacterium*) (**Figures 2**, **3**, **5**, **6**). Considering the critical role of lipid accumulation in the fecundity of *P. citri* (Ali et al., 2017), enhanced bacterial function in lipid metabolism should facilitate host lipid production, which has been supported by the significant influence of bacteria-produced short chain fatty acids on the host lipid metabolism or glucose-lipid homeostasis as previous reports (Zhang X. et al., 2018; Canfora et al., 2019). As well, the bacterial metabolisms of cofactors and vitamins are important for insect survival and reproduction (Douglas, 2015), which reveals the potential role of the bacterial metabolism of cofactors and vitamins in the vitality and fecundity of *P. citri*. Furthermore, the significantly positive correlations between the bacterial predicted functions and these families (and genera) implies that abundance enhancements of Pseudomonadaceae (*Pseudomonas*), Moraxellaceae (*Acinetobacter*), and Sphingobacteriaceae (*Sphingobacterium*) may contribute to the enhancements of bacterial "lipid metabolism" and "metabolism of cofactors and vitamins," therefore facilitate the survival and reproduction in adult host. Consistently, the elimination of bacteria by antibiotic-treatment suppressed the vitality and fertility of *P. citri*, which preliminarily supports this implication. Previous reports have discovered that the Sphingobacteriales abundance in compost may be influenced by fatty acid content (Reddy et al., 2018), *Pseudomonas* isolates consisted of nutritionally versatile chemoorganotrophs (such as lipase) to metabolize a very wide range of organic compounds (such as lipids) *in vitro* (Gilbert, 1993; Litthauer et al., 2002), and *Acinetobacter calcoaceticus* isolate could use short chain monocarboxylic acids *in vitro* (Du Preez et al., 1985). However, it is the first study to establish the positive correlation between specific species of Proteobacteria/Sphingobacteria and the metabolisms of lipid, cofactors and vitamins, which may be critical for the survival and reproduction of spider mites and deserves further investigation. In contrast, the relative abundances of some major families and genera decreased in the adult stage (including family Brucellaceae [genus *Ochrobactrum*], Xanthomonadaceae [*Stenotrophomonas*], Streptococcaceae, Phyllobacteriaceae [*Mesorhizobium*], and Chitinophagaceae [*Sediminibacterium*]) or fluctuated across the lifespan (including Enterobacteriaceae [*Serratia*], Comamonadaceae [*Delftia*], Burkholderiaceae [*Burkholderia*], Alcaligenaceae [*Achromobacter*], and genus *Lactococcus*) (**Figures 3**, **4**). This implies that these bacteria are less important for the survival and fertility of adult mites. These bacteria may originate from the eggs and/or native plant diet, and perform as transient microbes with specific functions at immature stages (Mereghetti et al., 2017; Yao et al., 2019).

Unexpectedly, no or almost no obligate and facultative symbiont was detected in all stages, except for *Serratia* with very low abundance across the life stages of *P. citri*. This result is consistent with previous reports on other *P. citri* populations (Gotoh et al., 2003; Chen et al., 2009), but differs from some other spider mites (such as several *Tetranychus* species) and sap-feeding insects (such as psyllids and aphids) (Gauthier et al., 2015; Zhao et al., 2016; Meng et al., 2019; Ribeiro et al., 2020). In fact, different from persistence of symbionts in sap-feeding insects which are essential for the host's development and survival (Zhao et al., 2016; Meng et al., 2019), the symbiont is absent in some spider mites (Gotoh et al., 2003), including *P. citri* in our study, which indicates the unnecessity of these symbionts for the life history traits of these arthropod hosts. Therefore, in *P. citri*, we predicted that the specific bacteria species in Pseudomonadaceae (*Pseudomonas*), Moraxellaceae (*Acinetobacter*), and Sphingobacteriaceae (*Sphingobacterium*) identified in our study, rather than any known bacterial symbionts, play important roles in the survival and reproduction of *P. citri*, which deserves further investigation.

Our results indicated the physiological functions of bacteria community on the vitality and fecundity in *P. citri* adults by eliminating the bacteria using broad-spectrum antibiotics. Dissimilar from using tetracycline to eliminate specific symbionts (such as *Wolbachia*) in *Tetranychus* species (Staudacher et al., 2017; Zhu et al., 2019a), the current study used streptomycin to reduce the total bacterial accounts because of the absence of the symbionts which have been reported in other spider mites and sap-feeding insects (Meyer and Hoy, 2008; Zhao et al., 2016; Staudacher et al., 2017; Meng et al., 2019). Therefore, we just inferred that the

abundance enhancements of Pseudomonadaceae (*Pseudomonas*), Moraxellaceae (*Acinetobacter*), and Sphingobacteriaceae (*Sphingobacterium*) may contribute to the adult vitality and fecundity of *P. citri*, which needs further clarification. In many cases, the bacteria in the insect hosts were eliminated by bactericidal antibiotic treatments to clarify the bacterial biofunction on the hosts (Lin et al., 2015; Staudacher et al., 2017), which is consistent with our study. However, we still cannot exclude the adverse effects of bactericidal antibiotics on the host physiology by inducing mitochondrial dysfunction and oxidative damage to cell, which has been observed in studies of mammals (Kalghatgi et al., 2013). To avoid the adverse influence of antibiotic treatment and confirm the bacterial physiological functions on *P. citri*, generation of germ-free mites by rearing in a sterile environment can be employed (Bing et al., 2018), which deserves further investigation.

CONCLUSION

The present study not only confirmed the physiological function of bacteria on spider mite's vitality and fertility, but also revealed the profiles of bacterial communities, which greatly differed between the immature and adult stages in *P. citri*. Additionally, the abundance enhancements of families Pseudomonadaceae (genus *Pseudomonas*), Moraxellaceae (*Acinetobacter*), and Sphingobacteriaceae (*Sphingobacterium*) in adult stage were identified and positively correlated to the enhancement of bacterial KEGG pathways of lipid, cofactor and vitamin metabolism, which implies that a novel mechanism potentially exists for commensalism of mites and bacteria to facilitate the hosts' survival and reproduction. Reaching similar determinations using various populations collected from different habitats (including ourdoor/natural environments) and plants will facilitate in better understanding the characteristics of core microbes and the dynamics of the bacterial community that are affected by environmental factors, which are worth investigating further.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA608605.

AUTHOR CONTRIBUTIONS

ZZ contributed to the experimental design and implementation, data analysis, and manuscript preparation and submission. HZ conceived and designed the laboratory experiments. ZZ conducted the main experiments and collected, analyzed, and interpreted the data. MA, HS, SL, XY, and QL assisted in parts of the experiments. ZZ, MA, and HZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01620/full#supplementary-material

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Attractiveness and Sexual Competitiveness of *Anastrepha obliqua* Males (Diptera: Tephritidae) Fed on a Diet Enriched With *Providencia rettgeri*

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The West Indian fruit fly, *Anastrepha obliqua* (Macquart), is the second most important tephritid fruit fly in Mexico, infesting mango, hog plum and guava fruits. To control this pest, the Mexican government has implemented the use of the sterile insect technique (SIT), which involves the mass production, sterilization and release of flies. However, the *A. obliqua* laboratory males used in SIT are selected to a lesser extent by the wild females during competitiveness tests. The objective of this study was to compare the effects of males fed on fruit fly food enriched with *Providencia rettgeri* to those in males fed on food alone, assessing male mating competitiveness, capture of females using traps baited with males fed with the enriched diet and sex pheromone components. The results indicated that males fed with the diet enriched with *P. rettgeri* had increased mating competitiveness and captured more females in the field cage tests. However, no difference was observed in the proportion of volatile sex pheromone components identified during the calling of *A. obliqua* males. The results suggest the value of incorporating bacteria into the mass rearing technique of *A. obliqua* adults in order to improve the sexual competitiveness of males from the laboratory compared to wild males.

Keywords: sterile insect technique, sexual competitiveness, field cages, pheromone components, diet

INTRODUCTION

An important aspect of insect biology that allows these organisms to increase their diversity and abundance is the symbiotic relationships they share with microorganisms that impact directly on their life history traits. Although many of these microorganisms may be commensalist or parasitic (Poveda-Arias, 2019), others play an important role in the nutrition, metabolism and immune protection of their hosts (Dillon and Dillon, 2004; Brune, 2010; Koch and Schmid-Hempel, 2011; Engel and Moran, 2013). Early studies in the Mexican fruit fly, *Anastrepha ludens* (Loew) were carried out to evaluate resistance or sensitivity to a variety of antibiotics in bacteria isolated from

this same species (Kuzina et al., 2001) and to test the attractive effect of metabolites produced by bacteria on *A. ludens* (Martínez et al., 1994). In addition, studies have determined the presence of *Wolbachia* in *Anastrepha* species (Mateos et al., 2020), including guava fruit fly, *A. striata* (Martínez et al., 2012), West Indian fruit fly, *A. obliqua* (Mascarenhas et al., 2016), South American fruit fly, *A. fraterculus* (Conte et al., 2019) and sapote fruit fruit fly, *A. serpentina* (Coscrato et al., 2009). On the other hand, studies have revealed that the gut bacterial community in flies consists mainly of species of the Enterobacteriaceae family (Noman et al., 2020), and that these microorganisms may be involved in nitrogen fixation, nutrition and insect fitness, including mating behavior and sexual competitiveness, reproductive success, longevity, improve the males fitnnes (Gavriel et al., 2011; Hamden et al., 2013; Andongma et al., 2015; Khaeso et al., 2017; Yuval, 2017; Juárez et al., 2019), protection against pathogens and detoxification (Ben-Yosef et al., 2015; Guo et al., 2017).

Some of the species of the Enterobacteriaceae family correspond to *Klebsiella*, *Enterobacter*, *Providencia*, *Pectobacterium*, *Pantoea*, *Morganella* and *Citrobacter* (Ben-Ami et al., 2010; Aharon et al., 2013). *Providencia rettgeri* is a gram negative, opportunistic pathogenic bacteria for humans (O'Hara et al., 2000), which has also been reported as pathogenic for *Drosophila melanogaster* (Galac and Lazzaro, 2011). However, the pathogenicity of *P. rettgeri* in Mediterranean fruit fly, *Ceratitis capitata* (Wied.) depends on the concentration supplied (Msaad-Guerfali et al., 2018), and previous experiments indicate that *A. obliqua* males can actually increase their sexual competitiveness under laboratory conditions when fed on *P. rettgeri* (Gómez-Alonso, 2013).

The West Indian fruit fly, *A. obliqua* (Macquart), is considered one of the most important tephritid fruit flies affecting the fruit production industry in Mexico and many tropical countries of America because it infests mangoes (*Mangifera indica* L.), hog plum fruits (*Spondias* spp.), sapodilla (*Achras zapota* L.), carambola (*Averrhoa carambola* L., Oxalidaceae) and guava (*Psidium guajava* L.) (Norrbom and Kim, 1988; Hernández-Ortiz, 1992; Birke et al., 2013; Aluja et al., 2014). To avoid or minimize the harmful effects of *A. obliqua*, mango growers must comply with the health and safety standards required by the market, applying an area-wide management approach involving chemical, biological, cultural and sterile insect (SIT) techniques (Reyes et al., 2000). The SIT involves the mass production of 70 million of bisexual pupae of the target species per week, followed by their sterilization and release (Reyes et al., 2000; Domínguez et al., 2010). However, the *A. obliqua* males used in the SIT are selected for mating by the wild females with a lower frequency (Rull et al., 2012) and, according to that observed for *Ceratitis capitata* by Lance et al. (2000), could therefore be considered less competitive than the wild males.

The sexual competitiveness of sterile male fruit flies is increased by using different strategies: 1) increasing the protein content in the diet provided during sexual maturation (Yuval et al., 2007); 2) adding the juvenile hormone analog (methoprene) to the adult diets (Pereira et al., 2011; Teal et al., 2013; Muñoz-Barrios et al., 2016); 3) applying aromatherapy by exposing the males to volatiles of ginger (*Zingiber* officinale Roscoe) (Shelly, 2006; Flores et al., 2011) and orange (*Citrus sinensis* L.) (Charalampos et al., 2012; Segura et al., 2018) oil; and 4) using enriched foods containing Enterobacteriaceae (Ben-Ami et al., 2010; Gómez-Alonso, 2013; Yuval et al., 2013; Augustinos et al., 2015). In this way, although *A. obliqua* shows facultative autogeny (Polloni and Da Costa-Telles, 1987), foods with nitrogenous compounds are key for the *A. obliqua* males to complete sexual maturation and increase their sexual competitiveness. However, insects cannot synthesize some of the protein-forming amino acids. Many insects that feed on plant material rely on symbiotic association with microorganisms for some aspect of their nutrition (Fitt and O'Brien, 1985). Particularly in Dipterans, it has been documented that bacteria in the digestive tract can mitigate this metabolic limitation, providing their host with essential amino acids (Boush and Matsumura, 1967; Douglas, 2009). Gut bacteria facilitate the absorption of some nutrients by providing digestive enzymes, e.g., microbial hydrolases (Ben-Yosef et al., 2008). Previous studies under laboratory conditions indicate that the main benefit of food enriched with bacteria is an increase in the incidence of mating in *A. obliqua* when males are fed with *P. rettgeri* (Gómez-Alonso, 2013). The objective of this study was to evaluate under field cage conditions the effect of an diet enriched with *P. rettgeri* on males mating competitiveness, male attractiveness and pheromone components of *A. obliqua*.

MATERIALS AND METHODS

This research was performed in the Methods Development laboratory of the Moscafrut Program (SADER-SENASICA) in Metapa de Dominguez, Chiapas.

Obtaining Insects

Pupae were obtained from the Moscafrut (SADER-SENASICA) facility, located in Metapa de Dominguez, Chiapas, Mexico, from a colony that had been mass-reared for more of 150 generations (Orozco-Dávila et al., 2014). All of the pupae used in this study were irradiated for 48 h pre-emergence with 80 Gy of gamma radiation using a Cobalt 60 source (Toledo et al., 2004). The mass-rearing procedures and conditions followed those described by Artiaga-López et al. (2004). The adults were separated by sex at 2 d of age. In all experiments, we used 100 male flies per treatment, which were placed in separate acrylic cages (30 × 30 × 30 cm). To replace the dead flies, 500 females were placed in acrylic cages (30 × 30 × 40 cm) for the mating tests. Both males and females were kept at a density of 1 fly per cm^3, at 26 ± 1°C and 70–80% RH under a photoperiod of 12:12 h (L:D). The photophase began at 07:00 h and ended at 19:00 h (FAO, 2007).

Obtaining Bacteria

The bacteria strain of *P. rettgeri* used in this experiment was previously isolated by Gómez-Alonso (2013) from the guts of wild larvae and adult flies obtained from infested hog plum fruits collected in Metapa de Dominguez, Chiapas and indentified in Laboratory of Microbiology of the Instituto Politécnico Nacional

(IPN) in Mexico City. For the purpose of this study, *P. rettgeri* was isolated from adults caught in traps loca ted around of Metapa de Dominguez Chiapas (14°50′N, 92°11′W). A sample of 30 larvae were kept for pupation and adult emergence. Prior to dissection, the insects were superficially disinfected by repeated immersion in solutions of 10% sodium hypochlorite, 70% ethanol (v/v), with a final wash in sterile distilled water. Each of these steps was carried out for 1-min. The larvae were then dissected in sterile conditions under a stereoscopic microscope to obtain the intestines, which were placed in phosphate buffered saline (PBS) and ground using sterile rods. The homogeneous solution of intestinal tracts was then used to make serial dilutions and spread on duplicate plates of nutritive agar (DIBICO, Cuautitlán Izcalli, Mexico City). These plates were incubated at 28°C for 1 to 2 days, and each colony type was then categorized and quantified. Pure cultures were obtained and stored at −70°C for further analysis.

The presumptive identification of the isolates was performed by the Analytical Profile Index (API) (BioMerieux, Hazelwood, Mo) and the results had a >98% accuracy for *P. rettgeri* (Code API 20E: 027431157). Final identification of the strains was performed by sequencing the 16S *rRNA* gene (1500 bp), for which *DNA* was obtained from each isolate using the modified technique described by Hoffman and Winston (1987). The isolated bacteria were grown in 20 ml of nutrient broth (DIBICO) and incubated at 28°C for 24 – 48 h with shaking (120 rpm). The suspension was centrifuged at 8000 rpm, and the resulting cell pellet was subjected to chemical breakdown by adding 500 μl of lysis solution (Tris–HCl 10 mM, pH 8.0; 1 Mm EDTA; NaCl 10 mM; SDS 1% Triton X-100), and mechanical breakdown using glass beads (0.5 g). Next, 200 μL of phenol-chloroform-isoamyl alcohol (25: 24: 1) was added and the mixture agitated for 3 min using a vortex and incubated at −70°C for 20 min before undergoing heat shock at 65°C for 30 min. After the incubation, the sample was centrifuged at 14,000 rpm for 5 min, and the supernatant placed in a new tube. To precipitate the DNA, 1 ml of isopropanol was added, and the sample was incubated at −20°C for 20 min and centrifuged at 14,000 rpm for 10 min. The supernatant was removed and evaporated on a hub (A160 Speedvac) at 45°C, and the resulting pellet suspended in 30 μl of injectable water. DNA integrity was visualized by gel electrophoresis using a 1% agarose gel.

The PCR amplification of the 16S *rRNA* gene was conducted using a fragment of the 16S r*DNA* gene of approximately 1500 bp in length, which was obtained from the *DNA* amplified using the universal primers 27F (5′-AGA GTT TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT ACG ACT T-3′) (Lane, 1991). The reaction mixture had a final volume of 25 μL, and included 2.5 μL of Buffer (10X) containing $MgCl_2$ (50 mM), 1.0 μL of dNTPs (10 mM), 1.0 μL of initiator 27 F (0.1 nm/μL), 1.0 initiator 1492R (0.1 nm/μL), 1.0 μL of DNA (>80 ng/μL), and 0.2 μL of Taq DNA polymerase (Invitrogen) (5 U/μL). The amplification conditions were an initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 5 min. The amplified product was purified using the GFX PCR DNA and Gel Band Purification kit (GE). DNA integrity was verified by electrophoresis using a 1% agarose gel. The amplified sequence was obtained using the ABI Prism 3100 (Applied Biosystems) DNA sequencer.

The isolated 16S *rDNA* gene sequence was compared to the reference sequences deposited in the databases of the National Center for Biotechology Information (NCBI). Alignment of the reference sequences and the query sequence was performed using the ClustalX program v.1.83. Editing and removal of ambiguous sites of the query sequence was performed with the Seaview v.2.01 program. Taxonomic identification of the bacteria was conducted by alignment of the isolated and reference sequences according to the criteria of Rossello-Mora and Amann (2001) and using a 97% similarity of the 16S *rRNA* genes based on the similarity matrix following the Myers and Miller (1988) method, implemented in the program MatGAT v.2.1 (Campanella et al., 2003).

Kinetics of *Providencia rettgeri* Growth

The viability of the *P. rettgeri* used in our experiments was ensured obtaining bacteria in the logarithmic growth phase. For this, *P. rettgeri* was grown in 100 ml of nutrient broth (DIBICO) and incubated at 35 ± 2°C for 24–48 h with shaking (120 rpm). The growth kinetics were performed in a culture medium of 100 mL and the absorbance of inoculum of 0.2. The growth of *P. rettgeri* was determined by spectrophotometry at 600 nm (Jenway brand, model 6715, Stone Bibby Scientific Ltd., China) and count by mass dilution. In addition, the absorbance and the quantity of colony forming units (CFU) per ml were correlated. The bacterial concentration was determined by taking 0.2 ml samples of the pure culture to make serial dilutions, of which the dilutions 10^{-4}–10^{-6} were seeded in triplicate using the standard plate agar method. The colony forming units (CFU) in the plates with nutrient agar were quantified after 48 h at 36°C. The bacterial growth curve was then calculated and the bacterial concentration estimated at different times of growth.

Incorporation of *P. rettgeri* Into the Fly Diet

The logarithmic phase bacterial culture (Abs = 0.8 nm, 720 min) was separated from the liquid medium by centrifugation at 8000 rpm for 10 min. The concentration of microorganisms used was 5×10^4 CFU per gram of food (Gómez-Alonso, 2013). The resulting microorganism pellet was then suspended in 1 ml of sterile distilled water, and this suspension was immediately added to the Mb® food and mixed until completely homogenized.

The Mb® food consisted of a mixture of amaranth flour (*Amaranthus cruentus* L.), refined sugar and peanuts (*Arachis hipogaea* L.). It contained 10.19% protein, had a pH of 6.15 UI (Hernández et al., 2010), and was developed to feed the adults of *Anastrepha* spp. during sexual maturation under confined conditions in the facility, for subsequent release in application of the SIT.

Feeding the Flies

Males were fed with diet corresponding to each treatment: Mb® food or Mb® food enriched with *P. rettgeri*. The laboratory females were fed with Mb® enriched with the bacteria. The Mb® food enriched with the bacterium *P. rettgeri* was renewed every two days, since this is the length of time for which the >50%

bacterial cells remain viable (living cells capable of reproducing) in the food (Gómez-Alonso, 2013).

Experimental Design

The evaluation under field cage conditions of the effect of diet enriched with *P. rettgeri* on males mating competitiveness, attractiveness capture and pheromone components of *A. obliqua* was performed according to 3-factors experimental desing for sexual competitiveness and attractiveness. Factor 1: Diet with and without bacteria. Factor 2: Age (8–10 days old) of the males,. Factor 3: Cohort, 1, 2, 3, and 4. The variables reponse corresponded to the percentage of matings to determine the effect of the treatments on the sexual competitiveness, the percentage of flies caught in traps in a field cage to evaluate attractiveness. Three replicates were performed for each treatment. The effect on the pheromone compounds was evaluated in a design in blocks, the factor corresponded to the diet with and without bacteria and the age (8 and 9 days old) of the males was considered as blocks. The area under each peak was determined, and this was the variable analyzed in order to assess the effect of diet on the sex pheromone components. Four replicates were performed for each treatment.

Sexual Competitiveness Tests in Field Cages

The tests of sexual competitiveness, capture and analysis of volatiles were carried out after the flies were 8 days old, in order to ensure sexual maturity, which was determined by observing the characteristics of this phase: vigorous fluttering and dilatation of the pleural glands (males) and the presence of eggs (females) (Aluja, 1993).

This test was performed in a field cage of 3 m in diameter and 2 m in height, supported by a metal structure and covered with a mesh cloth. An orange tree was placed in the center of the field cage to simulate a natural environment. The test was performed with sexually mature flies of four cohorts, flies of thre differents ages (8–10 days old) (all flies emerged on the same date) of each cohort, three replicates each ($n = 3$), for a total of 36 experimental units for each treatment.

Mating of the males fed with the Mb® enriched with *P. rettgeri* was compared to that of the males fed on the Mb® alone. The males were marked in the upper thorax with non-toxic vinyl paint (Vinci de Mexico, S.A. de C.V., Mexico City), with different colors denoting the different treatments (Meza-Hernández and Díaz-Fleischer, 2006). For each cage, 25 males from each group (treatment and control) were introduced into the field ca ges, with 30 females introduced 15 min later. Thus, there was a total of 80 insects per cage every day. The observation time lasted from 6:30 am to 10:00 am, which corresponds to the period of maximum sexual activity for *A. obliqua* (Aluja et al., 2009), and mating pairs were removed from the field cage for scoring the treatments.

Attractiveness Capture Test in Field Cage

This test was performed in field cages, as described above, using sexually mature flies of 8–10 days of age. Three Multilure® traps were distributed randomly in the branches of the tree within the field cage. The traps were adapted into the inside of the lid with a basket made of tulle mesh and, within these baskets, 10 males from each treatment were confined, including a trap containing males feeding on the Mb® enriched with *P. rettgeri*, another containing males feeding on Mb® food alone and an empty third trap included as a control. At the base of the traps was a diluted solution of water and neutral soap in which to capture the visiting females. After 15 min, 30 females were released into the cage field in order to capture the sexually mature females attracted by the call of the males in the Multilure® traps. The observation time was from 6:30 am to 10:00 am, and the number of females captured by each treatment was scored. The test was performed with sexually mature flies of four cohorts, flies of three differents ages (8–10 days old) (all flies emerged on the same date) of each cohort, three replicates each ($n = 3$), for a total of 36 experimental units for each treatment.

Identification of the Pheromone Components

The sex pheromone was collected for two consecutive days using sexually mature males of 8 and 9 days of age after emerging from the same batch. Four batches of flies were used. Collection was performed at room temperature (25°C) in the Chemical Ecology Laboratory of ECOSUR. We used 10 males from each treatment, which were placed in 300 ml flasks that were completely sealed to prevent escape of the volatile of interest. The conditioned) needle of the Solid-phase microextraction (SPME) syringe was introduced into the flask to expose the polydimethylsiloxane/divinylbenzene (PDMS/DVB, Supelco, Bellefonte, PA, United States) fiber in order to collect the volatiles emitted by the males. Collection was conducted from 7:00 am to 10:00 am, covering the period of calling in the *A. obliqua* male. A total of 11 replicates were performed for each age and treatment.

Analysis of the volatiles was performed on a gas chromatograph coupled to a mass spectrometer (GC-MS) (CP-3800 Varian, Palo Alto, CA, United States) using a fused silica capillary column VF-5MS (30 m × 0.25 mm ID Varian, United States). The samples were injected into "splitless" mode. The carrier gas, high-purity helium, was used at a constant flow of 1 ml min^{-1}. The temperature was programmed from 50°C for 1 min, increasing at 15°C min^{-1} up to 280°C, which was maintained for 10 min. The mass spectrometer was operated by electron impact (EI) at 70 eV. The transfer line was kept at a constant temperature of 280°C, while the ionization source was at 180°C. Exploration of the mass spectra was performed over a range of m/z 40–350 in SIM mode. The collected volatiles were identified by comparing the retention time and NIST-92 and NIST-98 mass spectrum databases. The area under each peak was determined, and this was the variable analyzed in order to assess the effect of diet on the sex pheromone components.

Statistical Analysis

Statistical analysis of all data was performed using R software (R version 3.0.3) (R Core Team, 2018). Shapiro-Wilk normality test, Bartlett test of homogeneity of variances indicated that

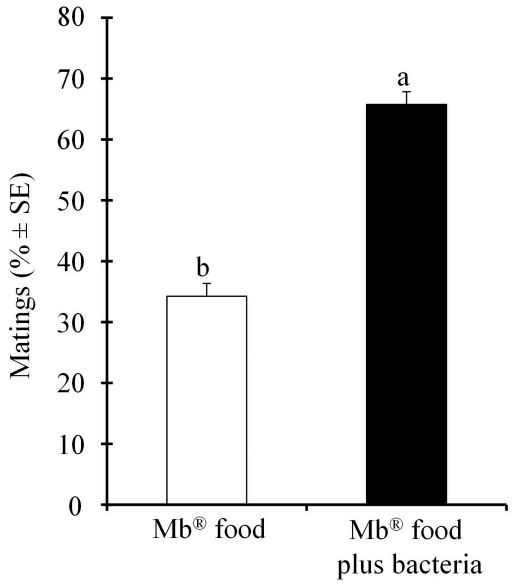

FIGURE 1 | Mean percentages (± SE) of the number of mating *Anastrepha obliqua* males fed on Mb® enriched with *Providencia rettgeri*, compared to the males fed on the Mb® diet alone, in field cages.

percentages of matings showed normality distribution and homogeneous variances. The data of matings and caught flies were analyzed by the Mixed-Effect model to determine the effect of the cohort and age as random factors, later the same data were analñyzed by a Generalized Linear Model (GLMs) considering a Poison (random) and log distribution. The data for volatile sex pheromone components were subjected to a *t*-test for unequal variances ($\alpha = 0.05$).

RESULTS

Bacteria Identification

One isolated colony, which presented 99.6% 16S *rRNA* gene sequence similarity with *P. rettgeri* (access number: NR042413) from the National Center for Biotechology Information (NCBI) gene bank, was selected. The bacterial identification with API (BioMerieux, Hazelwood, Mo) produced a >98% accuracy for *P. rettgeri* (Code API 20E: 027431157). This coincides with the comparison of the isolated 16S *rRNA* gene with the reference sequences deposited in NCBI.

Sexual Competitiveness Tests in the Field Cage

We found that the number of mating *A. obliqua* males that were fed with Mb® food enriched with *P. rettgeri* increased significantly ($\chi^2 = 367.36$; df = 1; $P < 0.001$) compared to those fed on the Mb® food alone (**Figure 1**).

Attractiveness Capture Test in the Field Cage

We found that the Multilure traps baited with the males feeding on the Mb® enriched with *P. rettgeri* captured significantly ($\chi^2 = 2423.32$; df = 1; $P < 0.001$) more females than the Multilure traps baited with the Mb® food only (**Figure 2**). The empty (unbaited) Multilure traps captured the lowest number of females (**Figure 2**).

Identification of the Pheromone Components

Analysis of the sex pheromone components by GC-MS showed that the *A. obliqua* males fed on the Mb® food enriched with *P. rettgeri* and the males fed only on Mb® released seven compounds that were identified as (*Z*)-3-nonenol ($t = 0.63$; df = 14; $P = 0.55$), nonadienol ($t = 0.36$; df = 14; $P = 0.73$), sesquiterpene ($t = 0.97$; df = 14; $P = 0.35$), β-farnesene ($t = 0.38$; df = 14; $P = 0.71$), (E-Z)-α-farnesene ($t = 0.56$; df = 14; $P = 0.58$), (E,E)-α-farnesene ($t = 0.07$; df = 14; $P = 0.94$) and a farnesene isomer ($t = 0.18$; df = 14; $P = 0.84$) (**Figure 3**), which showed no significant difference in the proportion of pheromone components released between the two treatments.

DISCUSSION

This study produced three important findings: The first indicated that *A. obliqua* males from the mass-rearing colony fed on the Mb® food enriched with *P. rettgeri* under field cage conditions presented increased mating compared to the males fed on the Mb® food alone. The second finding showed that a higher number of females were captured with the Multilure traps baited with males fed on the Mb® plus *P. rettgeri* diet. The third finding demonstrates that the *A. obliqua* males that were fed on the Mb® and Mb® enriched with *P. rettgeri* diets released the same compounds during their sexual calling and there was no significant difference between the quantities of each compound released by the males treated with the bacterium and by the control males. These results suggest that the Mb® food enriched with *P. rettgeri* served to improve the sexual competitiveness of the *A. obliqua* males from the laboratory under field cage conditions. Similar results under laboratory conditions have been reported by Gómez-Alonso (2013), who determined the mating competitiveness of sterile *A. obliqua* and *A. serpentina* males fed on diets enriched with autogenous bacteria, using cages of 30 × 40 × 30 cm. The autogenous bacteria are present in the gut of the wild fruit flies and belong to the *Enterobacteriaceae* family (Behar et al., 2008; Ben-Yosef et al., 2008, 2010; Lauzon et al., 2009; Ben-Ami et al., 2010; Yuval et al., 2013).

Some of the functions attributed to these microorganisms include biosynthesis of nutrients that are minimally available or unavailable in the food (Cohen, 2004), such as essential amino acids, sugars and vitamins (Girolami, 1983). A few microorganisms have the capacity to fix the atmospheric nitrogen used by the organism *in vivo* in order to construct proteins involved in reproductive activity, i.e., *Citrobacter freundii,*

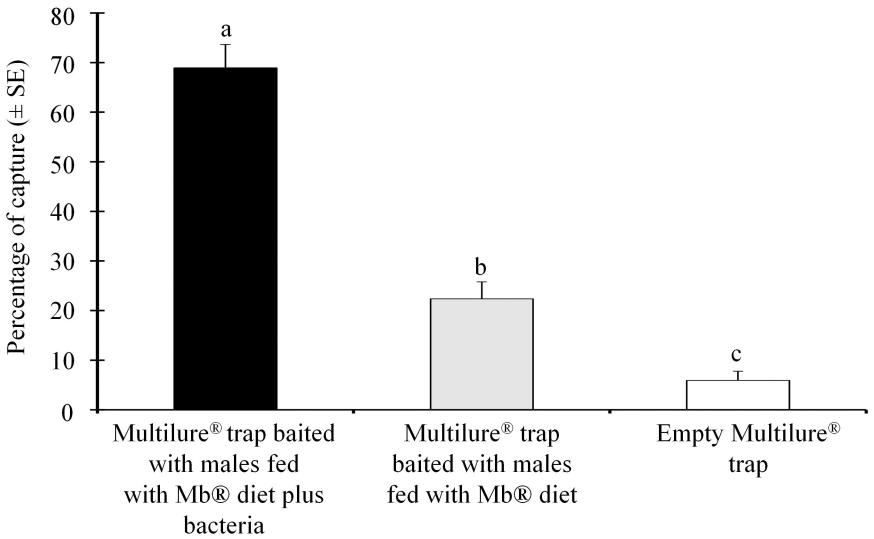

FIGURE 2 | Mean percentages (± SE) of the number of female *Anastrepha obliqua* captured in Multilure traps baited with males fed on the Mb® enriched with *Providencia rettgeri*, compared with the those on the Mb® diet alone as a control.

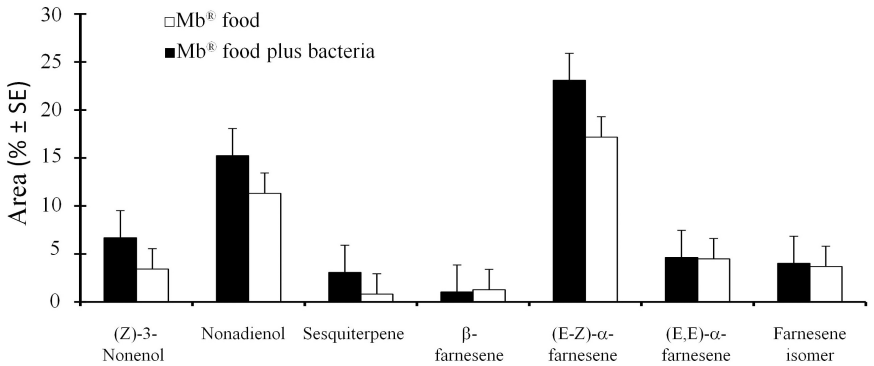

FIGURE 3 | Mean percentages (± SE) of the quantity of volatiles released by the *A. obliqua* males fed on the Mb® enriched with *Providencia rettgeri*, and by the males fed on the Mb® diet alone.

Enterobacter agglomerans, Desulfovibrio spp., *Klebsiella* spp. and *Enterobacter* spp. (Nardi et al., 2002; Behar et al., 2005). *Providencia rettgeri* is a bacterium that is found in the gut of *A. ludens* (Kuzina et al., 2001) and was recently found in the gut of wild *A. obliqua* (Gómez-Alonso, 2013). The effects on the number of mating males and the capture of females in *A. obliqua* adults could be attributed to the essential amino acids, sugar or vitamins biosynthesized by the bacteria that are un available in the diet, or perhaps the *P. rettgeri* provided in the diet did not successfully establish in the gut and its contribution was merely as a nutrient source.

In this study, the volatile compounds found during sexual calling in *A. obliqua* coincide with those reported by López-Guillén et al. (2008, 2011) and Chacón-Benavente et al. (2013). Although a significant difference was not found, we observed that the release of (Z)-3-nonenol, nonadienol, sesquiterpene and (E-Z)-α-farnesene consistently increased when the males were fed with food enriched with *P. rettgeri*. In this way, Juárez et al. (2019) reported ten volatile compounds identified in *Anastrepha fraterculus* males, and they were significantly affected by the diet. They found that sugar fed males were significantly affected in all compounds released with exception of limonene, suspensolide, E, β-ocimene and unknown compound. In addition, in sugar + protein fed males, the authors not found differences (Juárez et al., 2019). Our results are different with those of Juárez et al. (2019), possibly by the lower number of replicates used in the volatile collection.

The hypothesis that food affects attraction, and therefore capture, is in accordance with Sharon et al. (2010), who affirmed that the symbiotic bacteria present in the gut of *Drosophila melanogaster* influence the production of cuticle hydrocarbons, which in turn modify the mating choice. This could explain the observed preference for mating with their counterparts that present the same type of gut bacteria, but does not explain why the females of A. obliqua that were fed with the food enriched with *P. rettgeri* were captured in greater numbers in

the traps baited with males that had been fed the same diet. The diet determine the gut bacteria, and this in turn affects major cuticular hydrocarbons (Sharon et al., 2010), that according with Silk et al. (2017) enhance the responses to sex pheromone, as observed for the spruce budworm, *Choristoneura fumiferana*. The analysis of chemical signals depends on understanding the relationship between social and/or environmental context and the expression of different chemical combinations (Gershman et al., 2014). Although CHCs are not volatile, they can be broken into volatile fragments by reacting with environmental agents (Hatano et al., 2020), which could be used to produce a wide range of compounds as by-products of physiological processes and its regulation during the communication (Gershman et al., 2014).

In summary, in this study, we found that male *A. obliqua* adults that had been fed with the Mb® food enriched with *P. rettgeri* presented significantly increased mating and captured more females in the field cages, suggesting that this diet acts to improve the sexual competitiveness of the males and can be used in the SIT.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

EH and MA-M designed the experiment. LR-R, EH, and MA-M performed the experimental work. CV performed molecular identification of the bacteria. JT and EM designed the experimental, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01777/full#supplementary-material

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Comparative Microbiomics of Tephritid Frugivorous Pests (Diptera: Tephritidae) From the Field: A Tale of High Variability Across and Within Species

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The family Tephritidae includes some of the most notorious insect pests of agricultural and horticultural crops in tropical and sub-tropical regions. Despite the interest in the study of their gut microbiome, our present knowledge is largely based on the analysis of laboratory strains. In this study, we present a first comparative analysis of the gut microbiome profiles of field populations of ten African and Mediterranean tephritid pests. For each species, third instar larvae were sampled from different locations and host fruits and compared using 16S rRNA amplicon sequencing and a multi-factorial sampling design. We observed considerable variation in gut microbiome diversity and composition both between and within fruit fly species. A "core" microbiome, shared across all targeted species, could only be identified at most at family level (Enterobacteriaceae). At genus level only a few bacterial genera (*Klebsiella*, *Enterobacter,* and *Bacillus*) were present in most, but not all, samples, with high variability in their relative abundance. Higher relative abundances were found for seven bacterial genera in five of the fruit fly species considered. These were *Erwinia* in *Bactrocera oleae*, *Lactococcus* in *B. zonata*, *Providencia* in *Ceratitis flexuosa*, *Klebsiella,* and *Rahnella* in *C. podocarpi* and *Acetobacter* and *Serratia* in *C. rosa*. With the possible exception of *C. capitata* and *B. dorsalis* (the two most polyphagous species considered) we could not detect obvious relationships between fruit fly dietary breadth and microbiome diversity or abundance patterns. Similarly, our results did not suggest straightforward differences between the microbiome profiles of species belonging to *Ceratitis* and the closely related *Bactrocera/Zeugodacus*. These results provide a first comparative analysis of the gut microbiomes of field populations of multiple economically relevant tephritids and provide base line information for future studies that will further investigate the possible functional role of the observed associations.

Keywords: gut microbiomics, insects, *Bactrocera*, *Zeugodacus*, *Ceratitis*

INTRODUCTION

Plants are able to produce a wide variety of allelochemicals that act as deterrents against phytophagy. The capability of phytophagous insects to overcome these toxic compounds is strictly associated with the insect feeding preferences and host plant range. This is thought to represent an important evolutionary process promoting insect speciation and, ultimately insect-plant co-evolution (Aluja and Norrbom, 1999; Després et al., 2007; Winkler and Mitter, 2016; Chen et al., 2017). The family of the Tephritidae (Diptera), commonly referred to as fruit flies, consists worldwide of more than 4500 species distributed over 500 genera (White and Elson-Harris, 1992; Uchôa, 2012). Multiple species are found on all continents, excluding Antarctica, but they mainly thrive in tropical and sub-tropical environments. Although the majority infests the seed-bearing organs of plants, about half of the 4500 fruit fly species use the actual fruits for their own reproduction. Eggs are laid in ripening fruits and the different stages of larval development take place within the fruit. Larvae leave the fruit before pupation, pupate in the soil in order to emerge and become adult fruit flies (Christenson and Foote, 1960; Aluja and Norrbom, 1999). This larval development causes damage to the fruit, both directly by damaging the fruit tissue, and indirectly by accelerating the rotting process and increasing infestation by other insects, fungi and bacteria (Pierre, 2007; Badii et al., 2015; Qin et al., 2015; Alvarez et al., 2016). Fruit flies are found in both wild and commercial fruits and because of this, infestations by fruit flies can have huge economic impacts on the agricultural sector.

As many other phytophagous insects, tephritids can differ widely in their degree of host plant specialization and attack only one host plant species (monophagous flies), only one genus of host plant species (stenophagous), different genera within the same family (oligophagous) or a wide range of hosts belonging to several unrelated plant families (polyphagous). However, the functional classification based on feeding preferences is sometimes ambiguous as flies are also sporadically recorded not only on their "natural" host plants (*sensu* Aluja and Mangan, 2008) but also, and sporadically, on "unconventional" hosts (De Meyer et al., 2015; Hafsi et al., 2016). Previous phylogenetic research suggested that the evolutionary relationships observed in fruit flies might be related to their feeding preferences and host plant specialization (Virgilio et al., 2009). In particular, strong specialization on host plant species (i.e., monophagy and stenophagy) seems to be associated with the capacity to metabolize toxic secondary compounds of the host plant enabling fruit flies to exploit hosts inaccessible to polyphagous flies (Erbout et al., 2011; Pavlidi et al., 2013, 2017; Ben-Yosef et al., 2015). Because of the overall importance of microbial symbionts, it has been hypothesized that microbes might play a crucial role in shaping the dietary range and host plant specialization of herbivorous insects (microbial facilitation hypothesis (Janson et al., 2008; Brucker and Bordenstein, 2012; Douglas, 2013; Hansen and Moran, 2014; Hammer and Bowers, 2015). However, it is not entirely clear how important the relative contribution of microbial symbionts is in facilitating host plant shifts and host plant specialization compared to other processes, including the capacity of insects to produce plastic metabolic responses when changing host plant (Pfennig et al., 2010).

In recent years, an increasing number of studies have focused on the gut microbiome of tephritid fruit flies (Lauzon et al., 2000; Bourtzis and Miller, 2003; van den Bosch and Welte, 2016; Cheng et al., 2017; Ras et al., 2017; Cáceres et al., 2019). Largely thanks to the emergence of high throughput sequencing (HTS) techniques which facilitated the analysis of complex assemblages generally including thousands of Amplicon Sequence Variants (ASVs) (Wang A. et al., 2014; Wang H. et al., 2014; Andongma et al., 2015). As observed in other insects, evidence has emerged that bacteria help to overcome pesticides (Cheng et al., 2017) and boost host defenses (Ben-Yosef et al., 2015) or generally increase longevity of fruit flies (Niyazi et al., 2004; Behar et al., 2008a; Hamden et al., 2013; Sacchetti et al., 2013). Complex relationships may exist between the feeding strategy and the gut microbiome with the general expectation that monophagous flies might harbor a more specialized gut microbiome, while polyphagous species should harbor a more diverse and less specialized gut microbiome. Precedence for this kind of relationship was found in *Bactrocera oleae,* a strict monophagous species. Studies have unveiled a close evolutionary relationship between *B. oleae* and the bacterial species *"Candidatus* Erwinia dacicola" (Capuzzo et al., 2005; Estes et al., 2009). It has been shown that this bacterial species has an important role in facilitating the digestion of olives, and that its absence may strongly reduce survival rate of *B. oleae* in the field (Ben-Yosef et al., 2008, 2015).

Most of the currently available research on tephritid gut microbiomics focuses on fruit fly laboratory populations (i.e., fed with artificial diets) and often aims at investigating the optimal rearing conditions for species of interest for the sterile insect technique (SIT) (Augustinos et al., 2015, 2019; Kyritsis et al., 2017, 2019; Asimakis et al., 2019) while the composition and levels of variability of microbiome profiles of wild tephritid flies are far less known.

A number of studies have targeted one (Wang H. et al., 2014; Deutscher et al., 2018; Malacrinò et al., 2018; De Cock et al., 2019) or a few (Morrow et al., 2015) fruit fly species and compared the microbiomes of wild and laboratory populations. Other studies investigated relationships between the microbiome composition of a single fruit fly species and the host plant attacked (Zaada et al., 2019) or the geographic origin of larvae (Hadapad et al., 2015; Koskinioti et al., 2019). Regardless of that, there is still the need for a better understanding of patterns of variability of microbiome profiles in wild flies, and studies providing wide inter- and intra-specific comparisons in field conditions are, to our knowledge, currently missing.

The present study aimed at providing a first wide-range comparative analysis of the microbiome profiles of tephritid flies as observed under field conditions (i.e., from larvae sampled while feeding on their natural host plants). In this respect, we characterized the microbiome profiles of representative monophagous, stenophagous, oligophagous and polyphagous species from three economically important genera in the Mediterranean region and Sub-Saharan Africa.

Due to the relatively high heterogeneity previously observed for the microbiome of both laboratory and field populations of

Ceratitis capitata (De Cock et al., 2019) we decided to characterize the intra-specific variability of microbiome assemblages by considering field populations from replicated sampling sites and host plants. This approach aimed at verifying the presence of particular groups of gut symbionts consistently associated to the targeted fruit fly species while disentangling the effects of geographic variability and host plant choice.

MATERIALS AND METHODS

Sample Collection and Experimental Setup

We targeted three tephritid genera of economic relevance (*Bactrocera, Zeugodacus,* and *Ceratitis*) including ten representative fruit fly species [*B. dorsalis* (Hendel), *B. oleae* (Rossi), *B. zonata* (Saunders), *Z. cucurbitae* (Coquillett), *C. capitata* (Wiedemann), *C. cosyra* (Walker), *C. flexuosa* (Walker), *C. podocarpi* (Bezzi), *C. quilicii,* De Meyer, Mwatawala & Virgilio, and *C. rosa,* Karsch]. The species selection covered a range of feeding strategies including monophagy (*B. oleae, C. flexuosa*), stenophagy (*C. podocarpi*), oligophagy (*Z. cucurbitae*), and polyphagy (at increasing levels of polyphagy: *C. cosyra, B. zonata, C. quilicii, C. rosa, C. capitata,* and *B. dorsalis*).

A first part of this study was based on a balanced sampling design (**Supplementary Table S1**), as required for ANOVA/PERMANOVA (see below). Here we considered five fruit fly species (*B. dorsalis*, *Z. cucurbitae, B. oleae*, *C. capitata,* and *C. quilicii*) and, for each of them, the geographic variability of microbiome assemblages was estimated by collecting samples from two arbitrarily chosen locations in two different African or European countries. Similarly, intraspecific variability associated to host-plant choice was estimated by collecting three replicate samples in fruits from two randomly chosen host plant species at each location (see **Supplementary Tables S1**, **S2**). As in De Cock et al. (2019), we tried to reduce inter-individual variability by pooling, for each sample, the dissected guts of five third instar larvae. This first balanced experiment (dataset A) included a total of 60 samples as obtained from 300 dissected guts.

This dataset was then expanded with 33 additional samples from the ten fruit fly species listed above, collected from additional host plants and sampling locations (see details in **Supplementary Tables S1**, **S2**). This allowed considering a larger dataset (dataset B) including a total of 93 samples obtained from the dissection of 465 larval guts which was used for a wider range of statistics (see below).

Laboratory Procedures

After collection in the field or in the rearing facilities of partner Institutions (see Acknowledgments), larvae were immediately stored in 70% ethanol before being transferred to the Royal Museum for Central Africa (Tervuren, Belgium). There, individual larvae were rinsed again in 70% ethanol for 30 s and washed in sterile phosphate buffed saline (PBS) water. The complete gut was dissected under sterile conditions as detailed in De Cock et al. (2019). Although we acknowledge that the use of diluted rather than absolute ethanol as a killing and preserving agent is suboptimal and might have affected the gut microbial community, contributing to the variability of microbiome profiles across experimental replicates (see De Cock et al., 2019). We eventually considered this was the only suitable methodological approach to keep the larval tissues soft and allow dissections. From each gut, DNA was extracted using the Qiagen DNAeasy kit, as per manufacturer's instructions. After DNA extraction the identity of each larva was confirmed via DNA barcoding as described in Virgilio et al. (2012) and DNA concentrations were quantified using a Qubit fluorometer (Thermo Fisher Scientific). We only selected DNA extracts from larvae having a correct identification and DNA concentrations higher than 1 ng/μl. For each sample, three replicates were prepared, each consisting of the pooled DNA extracts from five individual larvae (normalized DNA concentrations). This way about 951 larvae were processed from which 358 DNA extracts needed to be rejected (199 wrong identification, 82 failed identification and 77 DNA concentration to low). From the remaining 593 DNA extract, 465 extracts were selected to create our pooled samples. A mock community was composed consisting of the DNAs of 18 pure bacterial strains (see **Supplementary Table S3**) obtained from the BCCM/LMG Bacteria Collection[1]. The species were selected based on literature reports of their occurrence in fruit fly guts. Bacterial strains were individually grown following the BCCM/LMG catalog instructions. DNA was extracted using the Qiagen DNAeasy kit, and mixed in equal concentrations (DNA concentration: 10 ng/μl). This mock sample and a blank sample were also included in sample preparation and sequencing protocol as, positive and negative control.

Genomic library preparation for 16S rDNA metagenomics relied on the Nextera XT kit (Illumina, 2016). In a first step, the primers 341F and 806R (insert size 465 bp), targeting the V3–V4 region of the 16S ribosomal RNA (Takahashi et al., 2014), were used to amplify the targeted region of the bacterial 16S rRNA, simultaneously two Illumina sequencing adapters were attached to the target DNA fragment. In a second step, dual-index barcodes were attached to the Illumina sequencing adapters. If needed, this second step was repeated to increase DNA yield. A final check of quality and fragment size was performed via an Agilent 2100 Bioanalyzer. Libraries were sequenced on an Illumina MiqSeq platform (300 bp paired end sequencing) by Macrogen (Amsterdam).

Data Analysis

Read quality was evaluated using FastQC (Andrews, 2014). The pipeline DADA2 (Callahan et al., 2016), implemented in R, was used for data filtering. This pipeline is based in a self-learning algorithm, which sets up a parametric error model that fits the raw data. This model is then used to infer sequencing error. In DADA2, raw reads were trimmed, demultiplexed, filtered and paired (Callahan et al., 2016). Processed reads were assigned to Amplicon Sequence Variants (ASVs) according to the Bayesian classifier method implemented by DADA2 (Wang

[1]http://bccm.belspo.be/

et al., 2007) (percentage of identity = 97% similarity, *p*-min-consensus = 0.51). Taxonomic assignment of ASV relied on the Silva v132 (26) database. The robustness of the assignment was double-checked against the RDP (Cole et al., 2014) and Greengenes databases (DeSantis et al., 2006, data not shown). The full analytical pipeline is detailed in **Supplementary Table S4**. As in De Cock et al. (2019), before analyses, single- and doubletons reads were filtered out to reduce possible biases due to sequencing error. For comparative analysis, normalized data, based on the median sample number of reads, was used (de Cárcer et al., 2011).

The data were processed in both univariate and multivariate frameworks. The effects of Fruit Fly Species (FFSp), Location (Lo), and Host plant (Ho) on univariate patterns of alpha diversity, as estimated by the Simpson index D (Sagar and Sharma, 2012), were tested via Analysis of Variance (ANOVA) (Underwood, 1997). Comparisons of multivariate patterns were done by using Permutational Multivariate Analysis of Variance (PERMANOVA, Anderson, 2017) and Permutational Multivariate Analysis of Dispersion (PERMDISP, Anderson, 2001). We used PERMANOVA to test differences in the relative abundance of ASVs (2749 in total, see section "Results"), while, as the PERMDISP routine of Anderson (2001) can only be implemented on a maximum of 500 variables, this analysis was implemented on the relative abundance of genera (401 in total, see section "Results"). In order to reduce differences in scale among variables while preserving information about taxa proportions, we transformed the multivariate data following Clarke (1993). This approach allowed reducing the importance of dominant, compared to the less abundant, taxa and to better identify more subtle changes in the abundance of non-dominant species. We compared the possible impact of data transformation by implementing both (1) presence-absence transformation (as an example of extreme transformation severely affecting abundance proportions) and (2) fourth-root transformation (as an example of less aggressive transformation, of common use in community ecology. For both ANOVA and PERMANOVA a three-way factorial setup was adopted with fruit fly Species (FFSp) as a fixed, orthogonal factor and Location [Lo(FFSp)] and Host Plant [Ho(FFSpxLo)] as random, nested factors. For PERMDISP, that only allows two-way designs (Anderson, 2006), we tested the effects of FFSp, and either [Lo(FFSp)] or [Ho(FFSp)]. *A posteriori* pairwise comparisons of significant factors were implemented via Tukey's Honestly Significant Difference (HSD) test (Abdi and Williams, 2010) for ANOVA and permutational t-statistics for PERMANOVA and PERMDISP (Anderson, 2001, 2017). Probability values of repeated *a posteriori* tests were corrected for Type I errors using the False Discovery Rate procedure (Benjamini and Hochberg, 1995) with experiment-wise probability $p = 0.05$. In order to increase the power of the multivariate *a posteriori* test (Underwood, 1997), we increased the number of permutable units (Anderson, 2017) by pooling together the replicates of non-significant terms. Following de Cárcer et al. (2011), we repeated multivariate tests on both data fourth-root transformed to the median and presence/absence data. The analysis of presence/absence data allowed stressing the possible effects of less abundant taxa.

Further investigation of the gut microbiome composition was done using the packages Phyloseq (McMurdie and Holmes, 2013), Vegan (Oksanen et al., 2019), and ggplot2 (Wickham, 2009), as implemented in R version 3.1.0. Principal Coordinates Analyses (PCoAs) based on Bray-Curtis distance (Bray and Curtis, 1957) were calculated for both fourth-root transformed data and presence/absence data. PCoAs for separated species were not incorporated due to the relatively small proportion of variance represented in PCoAs and to the relatively small sample size of samples available for each host and location. ASVs were pooled based on the bacterial genera and the percentage contribution of each of these genera to the average Bray-Curtis dissimilarity between fruit fly species was calculated using SIMPER (Clarke, 1993) on standardized, untransformed data. A permutational test based on 10,000 iterations was used to identify bacterial genera significantly differing between fruit fly species. Repeated permutational tests were corrected using FDR (Benjamini and Hochberg, 1995) at an experiment-wise $p < 0.01$. The results of SIMPER pairwise tests were summarized by considering only those bacterial genera (a) significantly differing between fruit fly species and (b) with an average contribution to dissimilarity higher than 5%.

RESULTS

The MiSeq Illumina run produced more than 19×10^6 paired-end (PE) reads (average per sample = 213185.07; *SD* = 72270.58). Following quality assessment in FastQC (Andrews, 2014), the forward and reverse reads were trimmed at respectively 240 and 210 bp. Based on read quality, a strict error rate (max N's = 0, max error rate = 1, see **Supplementary Table S4**) was applied in DADA2. After filtering, demultiplexing and merging about 5.4×10^6 reads, 2749 unique ASVs were identified. The analysis of reads from the positive control did not suggest relevant biases while reads corresponding to 11 ASVs detected in the negative control (see **Supplementary Table S5**) were eliminated from the datasets to avoid possible biases.

The 2749 ASVs were assigned to 401 genera belonging to 142 different families and 22 phyla (**Supplementary Figure S1**). Of these phyla, Proteobacteria was by far the most dominant, representing 89.25% of all reads, followed by Firmicutes (8.43%), Bacteroidetes (0.95%), Actinobacteria (0.83%), Epsilonbacteraeota (0.22%), and Tenericutes (0.18%). The remaining phyla represented only about 0.01% of total reads. The phylum Proteobacteria consisted of 62 bacterial families, mainly represented by Enterobacteriaceae (65.60% of all reads), Acetobacteraceae (16.72%), Rhizobiaceae (3.37%), and Burkholderiaceae (0.69%) (**Supplementary Figure S2**). The phylum Firmicutes consisted of 27 bacterial families, mainly represented by Leuconostocaceae (4.16%), Streptococcaceae (2.60%), and Lactobacillaceae (0.52%) (**Supplementary Figure S2**). The phylum Bacteroidetes consisted of 23 bacterial families, mainly represented by Weeksellaceae (0.57%), Dysgonomonadaceae (0.14%), and Flavobacteriaceae

(0.10%) (**Supplementary Figure S2**). The phylum Actinobacteria consisted of 33 bacterial families, mainly represented by Microbacteriaceae (0.29%) and Corynebacteriaceae (0.27%) (**Supplementary Figure S2**). The remaining phyla are all represented by only one or a few bacterial families. Of the above-mentioned phyla, only Proteobacteria was present in every sample. The phylum Firmicutes was present in almost all samples (> 90%) but had a very low abundance in some samples. The phyla Bacteroidetes and Actinobacteria were present in most samples, respectively, 64 and 73%. All remaining phyla were present in less than 25% of the samples. At bacterial family level, only the family of Enterobacteriaceae was present in all samples. Of the remaining bacterial families only Moraxellaceae, Burkholderiaceae, Streptococcaceae, Acetobacteraceae, Bacillaceae, Corynebacteriaceae, Leuconostocaceae and Staphylococcaceae were present in more than half of the samples. At bacterial genus level there were no genera present in every sample and only a few genera were present in the majority of the samples, including *Klebsiella* (96.43% of samples), *Bacillus* (96.43%), *Enterobacter* (92.86%), and *Acinetobacter* (89.29%). However, high variability between samples, and replicates could be observed with no bacterial genera dominant across all samples. A detailed overview of the most abundant bacterial genera for each fruit fly species can be found in **Supplementary Table S11**.PERMANOVA on fourth-root transformed data (Dataset A, **Table 1**, and **Supplementary Table S6**) showed that the gut microbiome composition significantly differs between fruit fly species ($p < 0.01$) and host plants ($p < 0.001$). PERMANOVA on presence/absence data (**Table 1** and **Supplementary Table S7**) could also detect a significant effect of location, suggesting that the gut microbiome of conspecific samples from different locations differs with respect to the less abundant ASVs. The *post hoc* tests on fourth-root transformed data (pooled for location) showed significant differences in all pairwise comparisons with *B. oleae* as well as between *B. dorsalis* and all other species but *C. capitata*, between *Z. cucurbitae* and all other species but *C. capitata*, and between *C. quilicii* and all other species but *C. capitata* (**Supplementary Table S4**). The *post hoc* comparison also provided indications on variability of the gut microbiome composition of the same species when feeding on different host plants. While the microbiome profiles of *Z. cucurbitae* and *C. quilicii* did not show significant variation across host plants, in both *B. dorsalis* and *C. capitata,* we found differences in most (all but one) pairwise comparisons (**Supplementary Table S6**). *Post hoc* comparison on presence/absence data did not reveal any significant effect (**Supplementary Table S7**).

Pooling the taxonomically assigned ASVs for the balanced experiment (Dataset A), by genus resulted in a dataset of 401 distinct bacterial genera. On both fourth-root transformed and presence/absence data, PERMDISP revealed significant effects of fruit fly species ($p < 0.01$) and host ($p < 0.001$) on multivariate dispersion (**Table 2** and **Supplementary Tables S8**, **S9**). Although the average dissimilarity between replicates in *B. oleae* (as calculated from fourth-root transformed data) was lower than in all other species, we did not observe significant differences in the *post hoc* comparisons between species (**Table 2**, **Supplementary Tables S8**, **S9**).

The PCOAs of the five species included in the balanced experiments (Dataset A, **Figure 1**) only accounted for a relatively limited amount of variation, explaining in total 27.9 and 23.7% of variability (PC1 + PC2, 4th root transformed data and presence-absence, respectively). The 95% confidence ellipses allowed resolving *B. oleae* from all other species. Adding the additional species to the PCOA (data not shown), allowed

TABLE 1 | PERMANOVA (fourth-root transformed and presence/absence data; dataset A) testing differences in the microbiome profiles (2,749 ASVs considered) of five fruit fly species (FFSp, *B. dorsalis, Z. cucurbitae, B. oleae, C. capitata, C. quilicii)* sampled in two locations [Lo(FFSp)] from two host plants within each location [Ho(FFSpxLo)].

	df	*MS*	*F*	*p*-value	
Fourth-root transformed data					
FSp	4	23502.690	3.521	0.001	**
Lo(FFSp)	5	6675.461	1.220	0.189	n.s.
Ho(FFSpxLo)	10	5472.781	2.548	0.000	***
Residual	40	2148.190			
Total	59				
Presence/Absence data					
FFSp	4	18961.396	2.557	0.000	***
Lo(FFSp)	5	7414.919	1.345	0.041	*
Ho(FFSpxLo)	10	5514.679	2.733	0.000	***
Residual	40	2018.200			
Total	59				

$^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, n.s.$p > 0.05$.

TABLE 2 | PERMDISP (fourth-root transformed and presence/absence data; dataset A) testing differences in the microbiome profiles (401 bacterial genera considered) of five fruit fly species (*B. dorsalis*, *Z. cucurbitae*, *B. oleae*, *C. capitata*, *C. quilicii*) sampled from four different host plants.

	df	*MS*	*F*	*p*	
Fourth-root transformed data					
FFSp	4	1555.209	6.668	0.004	**
Ho(FFSp)	15	233.240	6.179	0.000	***
Residual	40	37.745			
Total	59				
Presence/Absence-data					
FFSp	4	749.530	7.637	0.003	**
Ho(FFSp)	15	98.143	1.822	0.045	**
Residual	40	53.860			
Total	59				

Average within-group dissimilarities	**Fourth-root transformed data**	**Presence/ Absence-data**
B. dorsalis	63.213	65.326
Z. cucurbitae	79.797	51.782
B. oleae	14.186	66.527
C. capitata	83.920	65.283
C. quilicii	82.305	69.623

$^{***}p < 0.001$, $^{**}p < 0.01$, n.s. $p > 0.05$.

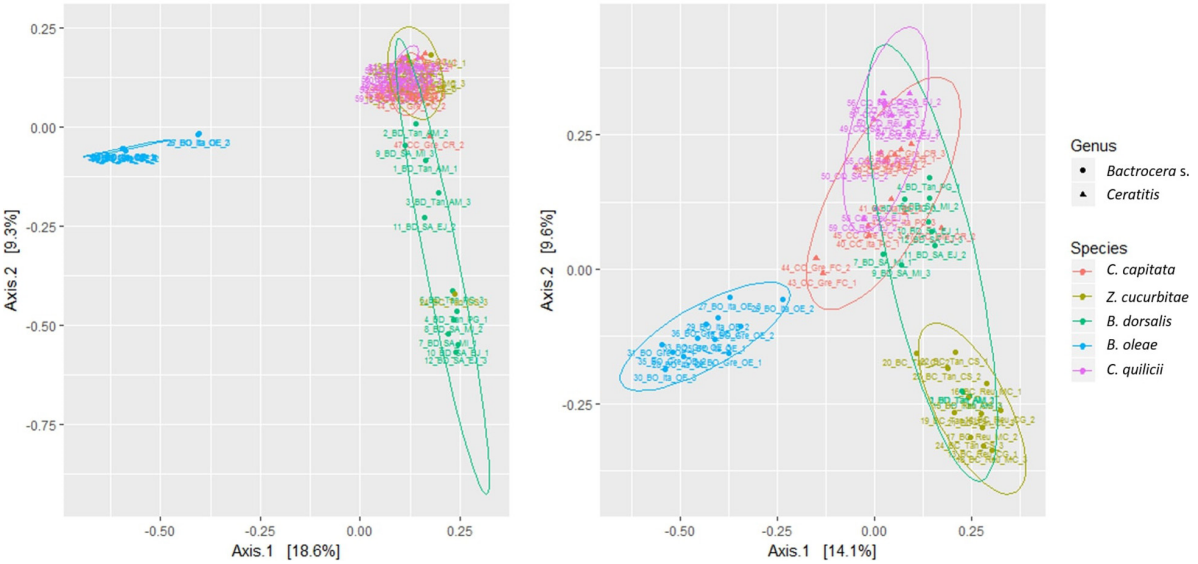

FIGURE 1 | Multivariate ordination (PCOA) of gut microbial assemblages in five target fruit fly species (*B. dorsalis, Z. cucurbitae, B. oleae, C. capitata, C. quilicii;* dataset A); Shape = Fruit fly genus, Color = Fruit fly species; Left: Abundance data; Right: Presence-absence data.

accounting for 25.0 and 18.8% of variability (PC1 + PC2, 4th root transformed data and presence-absence, respectively). Again, inspection of the 95% confidence ellipses showed that only *B. oleae* clustered separately from all other species. Even when removing *B. oleae* from the PCOA an extensive overlap between the different species was still observed. The preliminary analysis of separate PCoAs for each of the fruit fly species targeted in this study did not provide additional suggestions on possible patterns related to location or host-plant (data not shown).

The mean alpha diversity, as estimated by the Simpson index, across all samples (Dataset A) was $D = 0.62$ (median: 0.72, SD: 0.28). ANOVA revealed a significant effect of both fruit fly species ($p < 0.05$) and host plant ($p < 0.01$) on gut microbiome diversity (**Table 3**, **Supplementary Table S10**, and **Figure 2**). *Post hoc* tests revealed significantly lower alpha diversity in *B. oleae* ($D = 0.234$; SD: 0.27) (**Supplementary Table S10**) compared to all other species except *C. capitata*. Effects of host plants on gut microbiome diversity were found for *B. oleae* between two varieties of *Olea europaea* and for *C. quilicii* between *Harpephyllum caffrum* and *Eriobotrya japonica* (**Supplementary Table S10**).

TABLE 3 | ANOVA testing differences in alpha diversity (as estimated by the Simpson index, D; dataset A) of microbiome profiles of five fruit fly species (FFSp, *B. dorsalis, Z. cucurbitae, B. oleae, C. capitata, C. quilicii)* sampled in two locations [Lo(FFSp)] from two host plants within each location [Ho(FFSpxLo)].

	df	Mean Sq	*F*-value	*p*	
Abundance-data					
FFSp	4	0.602	5.651	0.043	*
Lo(FFSp)	5	0.107	1.008	0.461	n.s.
Ho(FFSpxLo)	10	0.106	4.072	0.001	***
Residual	40	0.026			

****p* < 0.001, **p* < 0.05, n.s.*p* > 0.05.

The permutational similarity percentage (SIMPER) analysis (Clarke, 1993) (Dataset B, **Table 4**, **Figure 3**, and **Supplementary Table S12**) suggested that five of the 10 investigated fruit fly species had characteristic associations with one or more bacterial genera. These putative associations were observed in all (9 out of 9) pairwise comparisons involving (a) *C. flexuosa*, which showed comparably higher abundances of reads from the genus *Providencia* (average abundance = 31.73%, *SD* = 24.45%) (b) *C. podocarpi,* with higher abundances of *Klebsiella* (average abundance = 52.83%, *SD* = 62.55%) and *Rahnella* (average abundance = 17.70%, *SD* = 25.03%)*;* (c) *C. rosa* with higher abundances of *Acetobacter* (average abundance = 55.30%, *SD* = 11.45%) and *Serratia* (average abundance = 0.06%, *SD* = 0.10%); (d) *B. oleae,* with higher abundances of *Erwinia* (average abundance = 93.28%, *SD* = 19.98%) and (e) *B. zonata*, with significantly higher abundances of *Lactococcus* (average abundance = 22.63%, *SD* = 38.71%). Other bacterial genera significantly contributed to the dissimilarity in most of the pairwise comparisons, such as *Morganella* and *Pantoea* in *C. capitata*, *Enterobacter* in *B. dorsalis* and *Gluconobacter* in *C. quilicii.* In *C. cosyra* none of the bacterial genera significantly contributed to more of 5% to the dissimilarity in at least five out of nine pairwise tests.

DISCUSSION

One of the main difficulties in the analysis of relationships between gut microbiome profiles and life history traits, including host plant choice, is represented by the high intra-

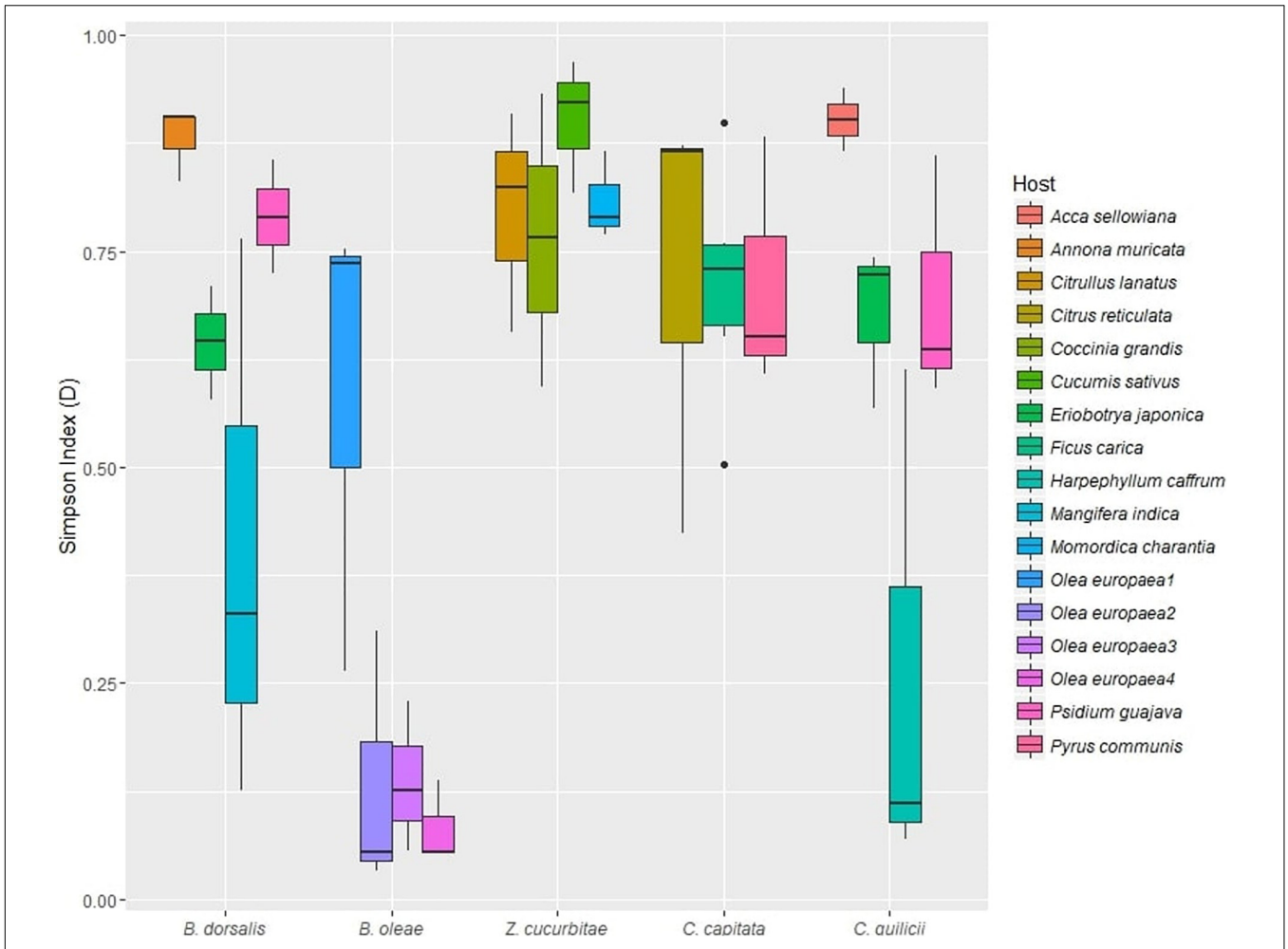

FIGURE 2 | Alpha diversity (Simpson index, D) of gut microbial assemblages in five target fruit fly species (*B. dorsalis, Z. cucurbitae, B. oleae, C. capitata, C. quilicii*; dataset B) sampled in two locations, from two host plants at each location.

and -interspecific variability of gut microbiomes that include thousands of ASVs (e.g., see De Cock et al., 2019). Differences between microbiome profiles can be related to life history traits and environmental factors including life stage (Lauzon et al., 2009; Andongma et al., 2015), diet (Santo Domingo et al., 1998; De Vries et al., 2004; Colman et al., 2012; Morrow et al., 2015), or technical artifacts (De Cock et al., 2019). Consistent with what has been reported in other studies on frugivorous tephritids (Thaochan et al., 2010; Wang et al., 2011; Andongma et al., 2015; Morrow et al., 2015; Augustinos et al., 2019), the gut microbiome profiles of third instar larvae of the ten fruit fly species targeted by the present study were mainly composed of Proteobacteria and Firmicutes which together represented more than 98.49% of reads in all tephritid species targeted. Andongma et al. (2015) suggested that Proteobacteria might be the most abundant phylum in earlier developmental stages of *Bactrocera*, while Firmicutes the most abundant in adult stages, possibly as a result of changes in habitat and diet. The dominance of Proteobacteria in larval stages is consistent with what is observed in the present work not only for *Bactrocera* and *Zeugodacus* but also for *Ceratitis* and it further confirms variation in microbiome profiles across developmental stages, as also described in *C. capitata* (De Cock et al., 2019).

Previous studies reported contrasting results on the most abundant gut bacterial families in tephritid fruit flies. While most of these studies report Enterobacteriaceae (Proteobacteria) as a major, dominant component of fruit fly gut microbiomes (Kuzina et al., 2001; Behar et al., 2008b; Wang et al., 2011; Wang H. et al., 2014) there are also notable exceptions such in Andongma et al. (2015) where Comamonadaceae are shown to represent a dominant taxon in immature stages of *B. dorsalis*. The dominance of Enterobacteriaceae, as the major component of the gut microbiome of most of the targeted species was confirmed by the results of the present study, with the notable exception of *C. quilicii* and *C. rosa* for which Acetobacteraceae (Proteobacteria) was the bacterial family with the highest abundance. At genus level and ASV level, we observed a high variability both between fruit fly species and within species. Only a few bacterial genera (*Klebsiella*, *Enterobacter,* and *Bacillus*) were present in a large proportion of samples, albeit with

TABLE 4 | Pairwise SIMPER permutational tests (10,000 iterations) between fruit fly species (*C. capitata, C. flexuosa, C. podocarpi, C. quilicii, C. rosa, C. cosyra, B. dorsalis, B. oleae, B. zonata, Z. cucurbitae*; dataset B).

	Bacterial genera significantly contributing to > 5% dissimilarity	*C. capitata*	*C. flexuosa*	*C. podocarpi*	*C. quilicii*	*C. rosa*	*C. cosyra*	*B. dorsalis*	*B. oleae*	*B. zonata*	*Z. cucurbitae*	Proportion of significant pairwise tests (FDR $p < 0.05$)
C. capitata	*Morganella*		*		*		*	*	*		*	6/9
	Pantoea				*		*	*	*		*	5/9
C. flexuosa	*Providencia*	*		*	*	*	*	*	*	*	*	9/9
C. podocarpi	*Klebsiella*	*	*		*	*	*	*	*	*	*	9/9
	Rahnella	*	*		*	*	*	*	*	*	*	9/9
C. quilicii	*Gluconobacter*	*	*	*			*	*	*	*	*	8/9
C. rosa	*Acetobacter*	*	*	*	*		*	*	*	*	*	9/9
	Serratia	*	*	*	*		*	*	*	*	*	9/9
C. cosyra	–											
B. dorsalis	*Enterobacter*	*	*		*	*	*		*		*	7/9
B. oleae	*Erwinia*	*	*	*	*	*	*	*		*	*	9/9
B. zonata	*Lactococcus*	*	*	*	*	*	*	*	*		*	9/9
Z. cucurbitae	*Lactococcus*	*			*			*	*	*		5/9
	Ochrobactrum	*			*		*	*	*			5/9

Results are reported for bacterial genera producing significant differences in at least 5 pairwise tests out of 9 (: FDR-corrected $p < 0.01$) and contributing to > 5% of dissimilarity between groups. The complete results are available in* ***Supplementary Table 13****.*

high variability in their relative abundance. Patterns observed for *C. capitata* were generally in line with what previously observed in laboratory populations (De Cock et al., 2019), with Proteobacteria and Firmicutes as the most abundant phyla and Enterobacteriaceae representing the most abundant family.

Regardless the relatively high number of individual guts used for this screening, the microbiome profiles of larvae collected in the field from their natural host plants showed highly variable patterns both between and within species, with intraspecific variation often, but not always, showing significant changes according to the host plant attacked. Interspecific variation of microbiome profiles was significantly affected by larval diet only in the two most polyphagous fruit fly species, *B. dorsalis* and *C. capitata*. Similarly, both the polyphagous *C. quilicii* and the monophagous *B. oleae* seemed also affected by host plant, even if to a lesser extent (i.e., they showed differences in univariate patterns of diversity but not in their multivariate patterns) while the oligophagous *Z. cucurbitae* did not seem significantly affected by host-plant choice. Regardless of that, the geographic variability of microbiome profiles from fruit fly populations thousands of km distant was relatively limited (relatively, as significant effects could only be observed from the analysis of presence/absence data). This suggests that the variable patterns observed across fruit fly species and host plants (particularly for *B. dorsalis* and *C. capitata*), are geographically consistent, even at large spatial scales (i.e., across different countries in the same continent). More focused experimental designs (i.e., based on a larger number of replicated samples and hosts) are now needed for a more detailed characterization of changes in the microbiome profiles of *B. dorsalis* and *C. capitata* across different host fruits. It would also be of interest to include the microbiome of the fruit that the larvae are sampled from in these studies.

Similarly, we did not find indications of obvious relationships either between microbiome profile diversity and fruit fly dietary breadth or between the microbiome profiles of the three different genera targeted in this study (*Ceratitis* on one hand and the closely related *Bactrocera/Zeugodacus* on the other).

From our observation, a core microbiome for the targeted fruit fly species could be defined only at family level, where the family Enterobacteriaceae was the single recurrent element in all samples. At genus or ASV level, however, we could not identify universal core microbiome elements shared by all fruit fly species tested. For individual fruit fly species, however, we could identify a set of key bacterial genera whose abundance was significantly higher in particular fruit fly species, irrespective of the host plant or sampling location considered. Major differences (i.e., significantly higher in all pairwise comparisons implemented) were found in the abundance patterns of seven bacterial genera in five fruit fly species considered. These were *Erwinia* in *B. oleae*, *Lactococcus* in *B. zonata*, *Providencia* in *C. flexuosa*, *Klebsiella,* and *Rahnella* in *C. podocarpi* and *Acetobacter* and *Serratia* in *C. rosa*. Other but less pronounced differences (as significant in a large proportion of pairwise comparison but not in all) were found for genera such as *Ochrobactrum* in *Z. cucurbitae*, *Gluconobacter* in *C. quilicii* and *Enterobacter* in *B. dorsalis*. Further experimental validation is now needed to verify the generality of these patterns and to test the occurrence of stable associations between larval dietary niche and the presence of the above-mentioned gut symbionts.

In herbivorous insects, gut microbes can aid with the breakdown of complex polysaccharides that make up the plant cell wall, or supplement the nutritionally poor plant diet with nitrogen, vitamins and sterols (Douglas, 2009; Ben-Yosef et al., 2010, 2014). There is also evidence that they take part in the

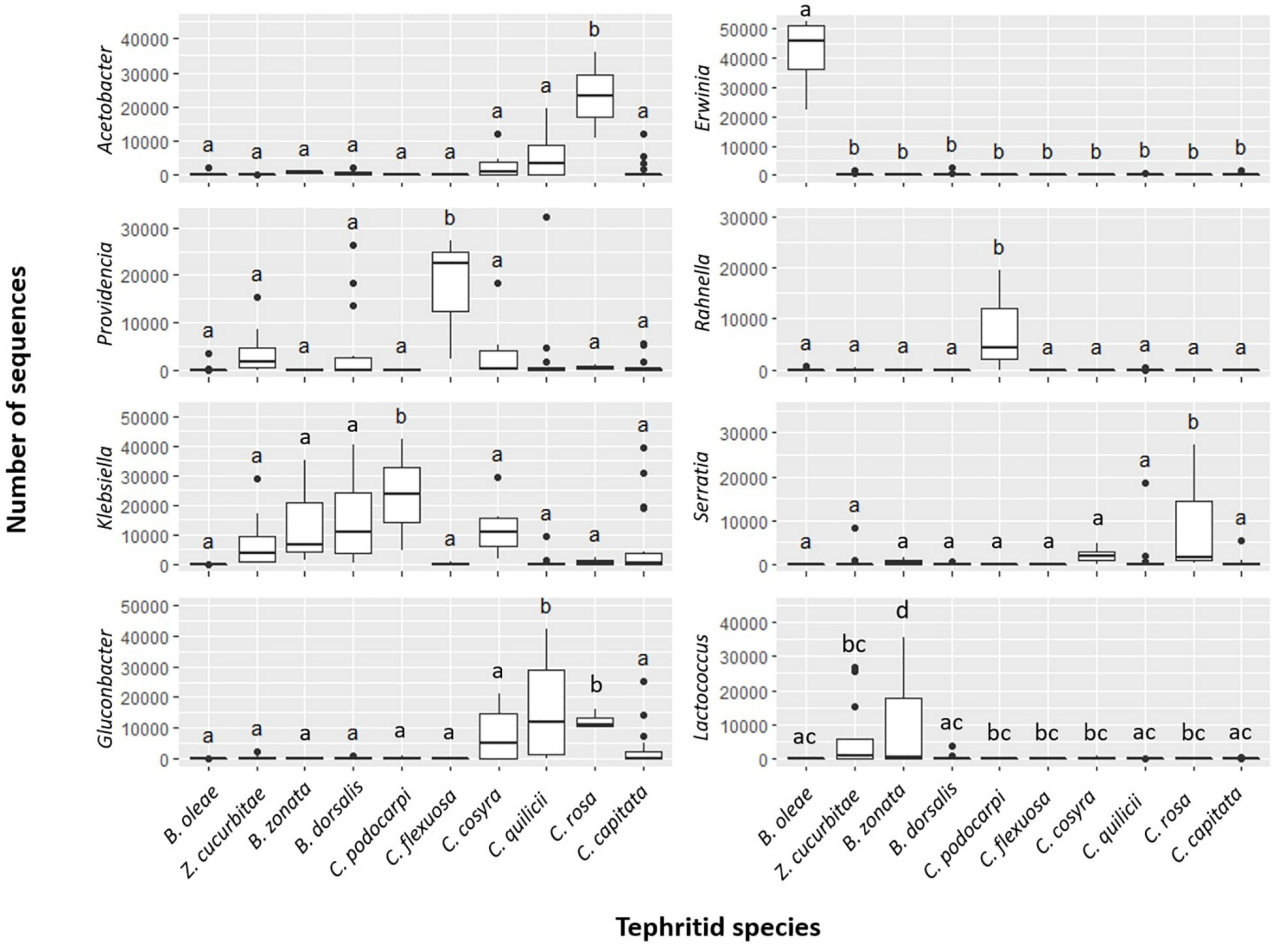

FIGURE 3 | Abundances (as estimated in number of reads; dataset B) of most representative bacterial genera in ten targeted fruit fly species. Results are reported for bacterial genera producing significant differences in at least 8 pairwise tests out of 9 (FDR-corrected $p < 0.01$) and contributing to > 5% of dissimilarity between groups. For each species, significance letters for pairwise tests are indicated (see also **Table 4** and **Supplementary Table 12**).

detoxification of plant allelochemicals (Hammer and Bowers, 2015). The relationship between the genus *Erwinia* and *B. oleae* has been studied extensively as it is a prime example of the coevolution between an insect and its gut microbiome (Capuzzo et al., 2005; Ben-Yosef et al., 2014, 2015; Estes et al., 2014; Blow et al., 2016; Pavlidi et al., 2017). It is hypothesized that the close relationship with this bacterium allows *B. oleae* to exploit olives as a food source by detoxifying plant defense compounds (Ben-Yosef et al., 2015) and providing additional nutrition (Ben-Yosef et al., 2014). "*Candidatus* Erwinia dacicola" allows larvae of *B. oleae* to develop in unripe olives, which contain high concentrations of the toxin oleuropein (Ben-Yosef et al., 2015; Pavlidi et al., 2017). As such, host-associated microbial communities seem to play an important role in the evolution and possibly speciation of the host (Zilber-Rosenberg and Rosenberg, 2008), in particular in fruit flies (Behar et al., 2008a; Ben-Yosef et al., 2010, 2014, 2015).

The bacterial genus *Ochrobactrum*, which in our study showed higher abundance in *Z. cucurbitae* in a number of interspecific pairwise comparisons, has often been reported as a plant endophyte of Cucurbitaceae (Weller et al., 2006; Akbaba and Ozaktan, 2018) and described in a number of cucurbit feeder fruit flies including *Z. cucurbitae* (Mishra and Sharma, 2018), *Z. tau* (pumpkin fly) (Khan et al., 2014; Prabhakar et al., 2013; Luo et al., 2018), and in the polyphagous *B. tryoni* (Jessup and Mccarthy, 1993). Similarly, the bacterial genus *Rahnella* which we consistently found in higher abundances in the gut microbiome of *C. podocarpi* has been reported in different species of bark beetles (Lacey et al., 2007; Vasanthakumar et al., 2009; Brady et al., 2014; Hernández-García et al., 2017), many of which feed on bark of coniferous trees. While these beetles and *C. podocarpi* do not share a taxonomic link, they do share a similar host: *C. podocarpi* exclusively targets members of the family Podocarpaceae, which also belong to the group of conifer trees. Even more so, the fruits of *Afrocarpus falcatus* (syn. *Podocarpus falcatus*) are known to be edible, but very resinous (source ICRAF Agroforestree Database; Oduol et al., 1988). The presence of *Rahnella* in the gut of these insects could be linked to the presence of this resin, which is also found plentiful in other coniferous trees.

CONCLUSION

Consistent with literature, we found that the gut microbiome of all fruit fly species included in the present study, was composed mainly of members of the bacterial phyla Proteobacteria and Firmicutes. At family level, we found that the family of Enterobacteriaceae was the dominant component in most species, except in *C. quilicii* and *C. rosa* where Acetobacteraceae was the dominant bacterial family. Despite heterogeneous abundances, we consistently observed Enterobacteriaceae across all samples, making it the single bacterial family that could be considered a part of the "core" gut microbiome. At genus level and ASV level, we observed a high variability both between fruit fly species (regardless of fruit fly genus) and within species. As such, we could not identify "core" gut microbiome members at genus or ASV levels that were shared across the targeted fruit flies. The few bacterial genera (*Klebsiella*, *Enterobacter* and *Bacillus*) that were present in most samples, showed a high variability in their relative abundance. Interestingly, we observed that interspecific variation of microbiome profiles was significantly affected by larval diet only in a part of the targeted fruit fly species (i.e., the most polyphagous ones, *B. dorsalis* and *C. capitata*), and that the observed patterns were geographically consistent. Finally, we could identify a number of bacterial genera (such as *Erwinia*, *Ochrobactrum* and *Rahnella*) that were consistently associated with particular fruit fly species (respectively *B. oleae*, *Z. cucurbitae,* and *C. podocarpi*). With these results, the present study provides a first comparative analysis of the gut microbiome of major fruit fly pests as well as, new base line information for future studies that will further investigate the functional role of the described associations.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, SRR8741994 to SRR8742034.

AUTHOR CONTRIBUTIONS

MDM, AW, MV, KB, and PV designed the research and secured the funding. MDC and MV designed and performed the experiments. MDC and MV analyzed the data with input from all other authors. MDC drafted the manuscript. All authors proofread, edited, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01890/full#supplementary-material

FIGURE S1 | Bubble plot representing the bacterial phylum composition per sample. Bubble color: number of bacterial genera per phylum; Bubble size: Log of reads per phylum; X-axis: Sample labels build with a consistent structure: XX_YY_ZZ in which XX is fruit fly species, YY is sample location and ZZ is host plant.

FIGURE S2 | Relative abundances (%, as estimated from number of reads) of the dominant bacterial families per fruit fly species. Error bars (SD) as calculated from averaged three replicates per species are indicated.

TABLE S1 | Overview of sample design implemented in this study. Blue: balanced experiment including five species (*B. dorsalis, Z. cucurbitae, B. oleae, C. capitata, C. quilicii*) sampled in two locations from two host plants within each location (three replicate samples for each host plant species); Gray: additional samples complementing the balanced experiment and including additional fruit fly species (and including *B. zonata, C. cosyra, C. rosa, C. flexuosa, C. podocarpi*), locations and host plants. Blue = dataset A; Blue + Gray = dataset B.

TABLE S2 | Collection data of samples considered in this study.

TABLE S3 | Detailed list ofthe strain composition of the MOCK community.

TABLE S4 | Detailed analytical pipeline implemented for genomic data filtering and taxon assignment.

TABLE S5 | Identification and number of reads for ASVs that were detected in the negative control.

TABLE S6 | *A posteriori* pairwise comparisons (permutational t-statistics) for the significant effects detected by the PERMANOVA test reported in **Table 1** (fourth root transformed data; dataset A). '***' = $p < 0.001$, '**' = $p < 0.01$, '**' = $p < 0.05$, 'n.s.' = $p > 0.05$.

TABLE S7 | *A posteriori* pairwise comparisons (permutational t-statistics) for the significant effects detected by the PERMANOVA test reported in **Table 1** (presence-absence data; dataset A). '***' = $p < 0.001$, '**' = $p < 0.01$ '*' = $p < 0.05$, 'n.s.' = $p > 0.05$.

TABLE S8 | *A posteriori* pairwise comparisons (permutational t-statistics) for the significant effects detected by the PERMDISP test reported in **Table 2** (fourth root transformed data; dataset A). '***' = $p < 0.001$, '**' = $p < 0.01$, '*' = $p < 0.05$, 'n.s.' = $p > 0.05$.

TABLE S9 | *A posteriori* pairwise comparisons (permutational t-statistics) for the significant effects detected by the PERMDISP test reported in **Table 2** (presence-absence data; dataset A). '***' = $p < 0.001$, '**' = $p < 0.01$, '*' = $p < 0.05$, 'n.s.' = $p > 0.05$.

TABLE S10 | *A posteriori* pairwise comparisons (Student-Newman-Keuls test) for the significant effects detected by the ANOVA test reported in **Table 3**. '***' = $p < 0.001$, '**' = $p < 0.01$, '*' = $p < 0.05$, 'n.s.' = $p > 0.05$ (Dataset A).

TABLE S11 | Most abundant bacterial genera observed in each fruit fly species (% as estimated from number of reads).

TABLE S12 | Permutational SIMPER analysis (10,000 iterations; dataset B) of the microbiome assemblages of 10 fruit fly species (*C. capitata, C. flexuosa, C. podocarpi, C. quilicii, C. rosa, C.cosyra, B. dorsalis, B. oleae, B. zonata, Z. cucurbitae*). sd: standard deviation of contribution to dissimilarity between groups; ratio: average to sd ratio; FDR p: probability value after False Discovery Rate correction (Benjamini and Hochberg, 1995); *: FDR p < 0.05, **: FDR p < 0.01. Results are reported for bacterial genera contributing up to 95% of dissimilarity between fruit fly species. Bacterial genera with contribution to average group dissimilarity >5% and significant FDR p are highlighted in blue.

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frontiers in Microbiology

ORIGINAL RESEARCH
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Hydrolytic Profile of the Culturable Gut Bacterial Community Associated With *Hermetia illucens*

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Larvae of the black soldier fly (BSF) *Hermetia illucens* (L.) convert organic waste into high valuable insect biomass that can be used as alternative protein source for animal nutrition or as feedstock for biodiesel production. Since insect biology and physiology are influenced by the gut microbiome, knowledge about the functional role of BSF-associated microorganisms could be exploited to enhance the insect performance and growth. Although an increasing number of culture-independent studies are unveiling the microbiota structure and composition of the BSF gut microbiota, a knowledge gap remains on the experimental validation of the contribution of the microorganisms to the insect growth and development. We aimed at assessing if BSF gut-associated bacteria potentially involved in the breakdown of diet components are able to improve host nutrition. A total of 193 bacterial strains were obtained from guts of BSF larvae reared on a nutritious diet using selective and enrichment media. Most of the bacterial isolates are typically found in the insect gut, with major representatives belonging to the Gammaproteobacteria and Bacilli classes. The hydrolytic profile of the bacterial collection was assessed on compounds typically present in the diet. Finally, we tested the hypothesis that the addition to a nutritionally poor diet of the two isolates *Bacillus licheniformis* HI169 and *Stenotrophomonas maltophilia* HI121, selected for their complementary metabolic activities, could enhance BSF growth. *B. licheniformis* HI169 positively influenced the larval final weight and growth rate when compared to the control. Conversely, the addition of *S. maltophilia* HI121 to the nutritionally poor diet did not result in a growth enhancement in terms of larval weight and pupal weight and length in comparison to the control, whereas the combination of the two strains positively affected the larval final weight and the pupal weight and length. In conclusion, we isolated BSF-associated bacterial strains with potential positive properties for the host nutrition and we showed that selected isolates may enhance BSF growth, suggesting the importance to evaluate the effect of the bacterial administration on the insect performance.

Keywords: black soldier fly, waste valorization, nutrient recycling, larval weight, pupal weight, bacterial isolation, probiotics

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INTRODUCTION

A growing body of evidence is supporting the gut microbiota as a component of paramount importance for the host physiology, development and health (as reviewed, among others, by Sommer and Bäckhed, 2013, and Schwarzer et al., 2018). Metataxonomic sequencing (primarily targeting the bacterial 16S rRNA gene or the fungal Internal Transcribed Spacer, ITS) is the most commonly used culture-independent method to characterize the host-associated microbial communities at the taxonomic, structural and network levels. On the other hand, functional profiles of the host microbial partners are typically investigated by metagenomic sequencing, which requires a deep sequencing coverage to precisely assign functions to different host-associated strains. Nevertheless, these approaches cannot disentangle the contributions of closely related but functionally different strains (Forster et al., 2019), with few exceptions reported on honeybees which show a simple and conserved microbial community composition (Engel et al., 2012; Ellegard and Engel, 2016, 2019). Functional properties of closely related strains in the same species can differ substantially (Barbato et al., 2019) and their metabolic potential may not be easily revealed by culture-independent methods (Prakash et al., 2013). Microbial cultures remain the gold standard to experimentally validate the microbial role and to obtain reference genome sequences that help in elucidating the functional roles of microorganisms (Forster et al., 2019), highlighting the importance of integrated "culturomics" approaches (Lagier et al., 2016). Additionally, microbial cultivation is pivotal for selecting and validating beneficial probiotic strains capable, when administered to the host, to promote food digestion, enhance growth or protect from pathogens (Prakash et al., 2013). However, culturable strains represent only a small part of the microbiome associated to a specific host or habitat: it has been generally estimated that only 1–10% of the bacterial diversity has been cultivated (Prakash et al., 2013) and that this value depends on the considered environment (Lagkouvardos et al., 2017). Thus, in regard to host-microbe interactions, it is, however, noteworthy to point out that also the unculturable fraction may contain important symbionts that cannot be exploited just because they are not culturable (Hosokawa et al., 2016; Ankrah et al., 2018).

The black soldier fly (BSF) *Hermetia illucens* (L.) (Diptera: Stratiomyidae) is a promising candidate for waste management, feed production or biodiesel conversion (van Huis, 2013; Makkar et al., 2014). It is native of the tropical and warm temperate zone of America and it is currently present in many countries around the world. Adults are considered non-pest insects that can survive without feeding, but their lifespan can be significantly extended in the presence of a sucrose solution or water (Lupi et al., 2019). Saprophagous larvae can consume a wide range of organic materials, ranging from food waste to animal remains and manures, converting them into high value biomass rich in proteins and fats (Sheppard et al., 1994; Makkar et al., 2014; Nguyen et al., 2015; Bava et al., 2019; Jucker et al., 2020). BSF has been shown to decrease *Escherichia coli* and *Salmonella* spp. population loads in manures (Liu et al., 2008; Lalander et al., 2015), probably due to the abundance of different antimicrobial peptides (AMPs) that it can produce (Vogel et al., 2018). Since it has been estimated that the food wastage (a term that includes both food waste and food lost) accounts approximately for one third of all the food produced for human consumption (FAO, 2013), BSF could be used for organic waste reduction and valorization, converting it into biomass with a final added value as feed, food or fuel (van Huis, 2013; Nyakeri et al., 2017; Chia et al., 2018; Onsongo et al., 2018; Shumo et al., 2018; Cappellozza et al., 2019). Under this perspective and considering the increasing exploitation of *H. illucens* for bio-waste disposal and its conversion in animal feed, elimination of toxic and anti-nutritional compounds (i.e., that can reduce the availability of nutrients such as phytate) present in the insect diet source could be also an important aspect to take into account in order to improve the insect growth.

In recent years, BSF microbiota has been investigated considering different host developmental stages and feeding conditions. The studies have highlighted that both the diet source and life stage directly influenced the microbiota diversity (Jeon et al., 2011; Zheng et al., 2013b; Varotto Boccazzi et al., 2017; De Smet et al., 2018; Jiang et al., 2019; Wynants et al., 2019) and it has been recently shown that the bacterial communities varied in density and phylogenetic composition along the anterior, middle and posterior portions of the midgut (Bruno et al., 2019).

As potential probiotics BSF-associated microorganisms could be exploited to sustain the insect performance (Crotti et al., 2012). For instance, larval development on poultry manure has been shown to benefit by supplementation with *Bacillus subtilis* isolated from BSF both in term of larval weight increase and developmental time reduction (Yu et al., 2011). The strategy to assess the role of specific isolates as beneficial probiotics characterizes their phenotypic properties *in vitro* to select potentially suitable strains and tests their effectiveness in promoting larval weight, growth rate and adult survival along the insect cycle, upon supplementation in the host diet (Prosdocimi et al., 2015). In this work we implemented such strategy on a large collection of bacterial isolates from the gut of BSF larvae reared on a nutritionally complete diet (standard diet, SD). The isolates were identified and functionally screened *in vitro* for potentially valuable nutritional properties. Two selected isolates with high probiotic potential were then tested *in vivo* for their potential contribution to BSF development, by their administration to larvae reared on a nutritionally poor diet (fruit diet, FD). Specifically, we have screened the isolates for the ability to degrade organic waste polymers (such as cellulose, starch and pectin), produce exopolysaccharides (EPS) potentially useful for adhesion to the host epithelium and for the potential to contribute to the host nitrogen metabolism and phosphorous recycling.

MATERIALS AND METHODS

Insect Colony

Hermetia illucens was reared at the entomological facility of the University of Milan, Italy (Jucker et al., 2017). BSF larvae were fed *ad libitum* on a nutritionally complete standard diet

(SD), composed of wheat germ 50%, alfalfa 30%, corn flour 20%, to which is added an equal volume of water according to Hogsette (1992), under controlled conditions of 25°C and 60–65% relative humidity (RH).

Larval Gut Dissection and Bacterial Isolation

Guts from four larvae (with a weight between 0.14 and 0.16 g), reared on standard diet (SD), were used for bacterial isolation. All the isolation procedures were performed in aerobic conditions. Insects were dissected close to the flame of a Bunsen burner using sterile forceps and needles, after a step of external surface sterilization that was done to exclude the epibiont microorganisms. Larvae were first washed in 50 ml tubes according to the following protocol: sodium dodecyl sulphate (SDS) 0.1% for 5 min, sodium hypochlorite 1% for 3 min, ethanol 70% for 1 min three times and finally rinsed five times in sterilized distilled water. The water from the last rinse was plated on agarized Nutrient Broth (NB) and incubated at 30°C for 72 h in order to evaluate the sterilization efficiency. Once dissected in sterile phosphate-buffered saline (PBS), guts were homogenized using sterile plastic pestles in 900 μl of 0.9% NaCl, which were then used to inoculate liquid enrichment media in order to select uricolytic and cellulolytic strains. Specifically, we used 3 different enrichment media: (i) enrichment uric acid medium [0.8% uric acid; 0.05% KH_2PO_4; 0.2% K_2HPO_4; 0.01% NaCl; 0.01% $MgSO_4{\cdot}7H_2O$; 0.01% $CaCl_2{\cdot}2H_2O$; pH 7.0 (Ghosh and Sarkar, 2014)]; (ii) enrichment FP medium (0.25%$NaNO_3$, 0.2% KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.02% NaCl, 0.01% $CaCl_2{\cdot}6H_2O$, and Filter paper (FP) - Whatman no. 1, two disks of 2.00 cm^2 per 30 ml); and (iii) enrichment CMC (carboxymethylcellulose) medium [0.25% $NaNO_3$, 0.2% KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.02% NaCl, 0.01% $CaCl_2{\cdot}6H_2O$, and 0.2% CMC (Gupta et al., 2012)]. Liquid media were then incubated at 30°C in shaking conditions. Subcultures were made every 7 days, for three times, and finally plated onto Basal-Trace (BT) solid medium (0.3% uric acid, 0.2% K_2HPO_4, 0.05% KH_2PO_4, 0.01% $MgSO_4{\cdot}7H_2O$, 0.01% NaCl, 0.01% $CaCl_2$, 1% (v/v) trace element solution (5.0% $FeSO_4{\cdot}7H_2O$, 5% $CuSO_4{\cdot}7H_2O$), pH 7.0, agar 1.5%) in case of cultures from Enrichment Uric Acid medium (Ghosh and Sarkar, 2014), whereas FP and CMC liquid media were plated on CMC agar plates (Gupta et al., 2012). In addition, serial dilutions (0.1 ml) of the gut homogenates were spread on the surface of different types of plates: (i) basal medium (0.1% $[NH_4]NO_3$; 0.1% yeast extract; 50 ml standard salt solution; 1 ml trace element solution and 1.5% agar and final pH 7.0) added with 0.5% Avicel (Sigma-Aldrich) or 0.5% CMC with 0.1% Congo-red (Sigma-Aldrich) for differential isolation of exo- or endo-cellulolytic, respectively (Ventorino et al., 2015); (ii) casein agar (1.5% peptone from casein; 0.5% soy peptone; 0.5% NaCl; 1.5% agar; pH 7.3 ± 0.2); (iii) pectin agar (0.4% $[NH_4]SO_4$, 0.01% NaCl; 0.01% $MgSO_4{\cdot}7H_2O$, 0.01% $CaCl_2{\cdot}2H_2O$, 0.05% yeast extract, 0.0052% Fe(III)-citrate, 500 ml potassium phosphate buffer (50% K_2HPO_4 1M + 50% KH_2PO_4 1M), 0.5% pectin, agar 1.5%, pH adjusted to 7.0); (iv) chitin agar (0.25% $NaNO_3$; 0.2% K_2HPO_4; 0.02% $MgSO_4{\cdot}7H_2O$; 0.02% NaCl; 0.01% $CaCl_2{\cdot}2H_2O$; 0.5% chitin from crab shells; 1.5% agar pH 6.8–7.2); (v) nutrient agar-uric acid (NA-UA) medium [0.5% gelatin peptone, 0.3% beef extract, 0.2% NaCl, 0.3% uric acid, pH 7.0, and agar 1.5% (Ghosh and Sarkar, 2014)]. When colonies appeared on the agar plates, they were picked up and streaked on the same media used for the isolation three times to ensure purity. The bacterial collection was then conserved in 25% glycerol solution at −80°C.

Bacterial Identification

Total DNA from each isolate was extracted by boiling lysis (Ferjani et al., 2015) or using a phenol-chloroform DNA extraction based protocol (Sambrook et al., 1989) in case of 16S rRNA PCR amplification failure on bacterial DNA extracted according to boiling lysis. The bacterial collection was dereplicated by ITS-PCR fingerprinting using the primer pair ITS-F (5′-GTC GTA ACA AGG TAG CCG TA-3′) and ITS-Reub (5′-GCC AAG GCA TCC ACC-3′) (Mapelli et al., 2013; Soldan et al., 2019). For each ITS group one/two candidates were selected and the 16S rRNA gene was amplified using the primers 27F (5′-AGA GTT TGA TCM TGG CTC AG -3′) and 1492R (5′-CTA CGG CTA CCT TGT TAC GA -3′) (Mapelli et al., 2013; Soldan et al., 2019). PCR fragments were partially sequenced at Macrogen (South Korea) and sequences were then aligned against the EzBioCloud database (Yoon et al., 2017). Sequences were deposited at the European Nucleotide Archive under the accession number PRJEB30516.

Screening of Metabolic Activities of Bacterial Isolates

For amylase-screening, bacterial cultures were spotted onto NB agar plate supplemented with starch (1%) and then incubated at 30°C for 48 h. After incubation, plates were flooded with 1% Lugol's iodine solution (Jacob and Gerstein, 1960) to identify extra-cellular amylase activity.

Cellulase-screening was performed as described by Ventorino et al. (2015) using a medium containing 0.1% $[NH_4]NO_3$, 0.1% yeast extract, 50 ml standard salt solution, 1 ml trace element solution (0.01% H_3BO_3, 0.012% $MnSO_4$ H_2O, 0.125% $ZnSO_4$ $7H_2O$, 0.078% $CuSO_4$ $5H_2O$, 0.01% MoO_3), 0.5% CMC, 0.1% Congo red (Sigma-Aldrich), and 1.5% agar at pH 7. Five microliters of liquid bacterial cultures grown overnight in shaking conditions at 30°C were spotted on plates and incubated at 30°C for 4 days. After incubation, strains with cellulolytic activity showed clear halo zones around the colonies.

Pectinase-screening medium contained 0.67% Yeast Nitrogen Base, 1.0% pectin, and 1.5% agar at pH 7.0 ± 0.2 (Park et al., 2007). The plates were then treated with 1% n-hexadecyltrimethylammonium bromide solution (CTAB) and pectin degradation was assessed through the observation of a clear halo around the colonies.

Esterase activity was evaluated using a medium composed of 1.0% peptone, 0.5% NaCl, 0.01% $CaCl_2{\cdot}2H_2O$, 1 ml tween 80, and 2.0% agar at pH 7.4 ± 0.2 (modified from Mazzucotelli et al., 2013). A white precipitate formation around the colonies, resulting from the deposition of crystals of calcium salt, indicated the solubilization of fatty acids due to the esterase activity.

Esterase/lipase activity was detected on tributyrin agar medium which contained 0.8% NB, 10 ml tributyrin, 4 ml tween 20 and 1.5% agar at pH 7.5 ± 0.2. Tributyrin agar plates with spotted isolates were incubated at 30°C for 72 h. The clear zone of hydrolysis is indicative of either esterase and/or lipase activity according to Gupta et al. (2003).

True lipase activity was screened by rhodamine oil agar (ROA) medium containing 0.8% NB, 0.4% NaCl, 3.125% olive oil, 10 ml rhodamine B (1 mg/mL solution), and 2% agar at pH 7, following the protocol described by Kumar et al. (2012). After incubation at 37°C for 48 h, positive strains were identified by the formation of orange fluorescent halos around bacterial colonies under UV light.

Extracellular protease activity was assessed using milk agar medium composed of 0.5% pancreatic digest of casein, 0.1% glucose, 0.25% yeast extract, 3.5% skim powder milk and 1.5% agar (modified from Jeon et al., 2011). Plates were examined after 72 h of incubation at 30°C. The appearance of a clear zone around spotted isolates indicated the production of extracellular protease.

Ammonia production was evaluated as described by Cappuccino and Sherman (1992). Briefly, after an overnight incubation at 30°C in TSB in shaking condition, 500 μl of bacterial culture were inoculated in 10 ml of peptone water and incubated at 30°C for 4 days. Then, 1 ml of Nessler reagent was added to each culture: the development of orange color indicated the strain ability to produce ammonia. Uninoculated medium developed a green color, as well as bacteria without ammonia production ability.

To detect urea degradation, isolates were inoculated in tryptic soy broth (TSB) liquid medium and incubated overnight at 30°C in shacking condition; 0.5 ml of cultures were then transferred in 1.5 ml tubes and washed twice with 0.9% NaCl (5 min, 4500 rpm, room temperature) to remove the residual growing medium. Pellets were resuspended with 470 μl of solution B (0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.5% NaCl, 0.013% $NiCl_2$, 1 mL phenol red 0.2%, and 100 mL dH_2O) and 30 μl of solution A (2 g urea, 2 mL ethanol and 4 mL dH_2O) and incubated at 30°C for 1–2 h. Color was then checked: positive strains showed a color change from yellow to bright pink (modified from Mora et al., 2002).

Uric acid breakdown was screened observing the formation of clear haloes around the isolates spotted onto NB-UA plates (0.8% NB, 0.5% uric acid, and 1.5% agar), incubated for 48 h at 30°C (modified from Morales-Jiménez et al., 2013).

Phytase-screening medium (PSM) contained 1% glucose, 0.4% Na-phytate, 0.2% $CaCl_2$, 0.5% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$, 0.001% $MnSO_4{\cdot}H_2O$, and 1.5% agar at pH 7. Degradation of Na-phytate was evaluated after incubation at 30°C for 4 days. The presence of clear zones around the isolates spotted on plates was considered as indication of phytate degradation (Jorquera et al., 2008).

Exopolysaccharides (EPS) production was estimated according to Santaella et al. (2008) using a RCV medium modified with the addition of sucrose (2%). Bacterial strains were streaked on agar plates of RCV medium and, after 5 days of incubation at 30°C, colonies showing translucent and mucoid growth were considered positive for EPS production.

All the bacterial screenings were performed in aerobic conditions.

Bacterial Administration to the Insect Diet

Bacillus licheniformis HI169 and *Stenotrophomonas maltophilia* HI121 cells were administered to *H. illucens*, singularly or in mix. The laboratory strain *Escherichia coli* DH5α pKan(DsRed) was used in the trials as a control strain, outsider of the BSF commensal community (Crotti et al., 2009). Strains HI169 and HI121 were inoculated in tryptic soy broth medium (TSB medium) and cultured overnight at 30°C, whereas *E. coli* DH5α pKan(DsRed) was inoculated in Luria Bertani medium (LB medium) and cultured overnight at 37°C. The following day, 5 ml of the cultures were inoculated in 100 ml of the proper growth media and incubated for 24 h. After growth, cells were centrifuged at 3000 rpm for 15 min at 4°C, the supernatants were discarded and the pellets were washed three times with saline (NaCl 0.9%) in order to remove the spent medium. The collected cells were properly diluted to a final concentration of 10^8 cfu/ml in saline.

In order to evaluate any possible antagonistic interaction between the strains, *B. licheniformis* HI169 and *S. maltophilia* HI121 were co-cultured on the same plate. Briefly, one strain was inoculated with one single streak in the middle of a TSA plate and incubated for 48 h at 30°C. Then, three streaks of the second strain were perpendicularly carried out close to the previous one. Three replicated plates were prepared for each strain. Co-cultures were incubated at 30°C and checked after 24 and 48 h to evidence the strains' growth inhibition.

A nutritionally poor fruit diet composed of apple 1/3, pear 1/3 and orange 1/3 (FD, Jucker et al., 2017) was selected to evaluate the effect of bacterial administration on larval growth performance. After eclosion, larvae of BSF were fed on the FD diet for nine days until handling. Each treatment had three replicates set up in three 10.5 × 5 cm plastic containers with ∼ 60 g each of the FD fruit-based diet. Each replicate was inoculated with 10 ml of NaCl 0.9% containing 10^8 cfu/ml of i) *E. coli* DH5α pKan(DsRed); ii) *S. maltophilia* HI121; iii) *B. licheniformis* HI169; and iv) *S. maltophilia* HI121 and *B. licheniformis* HI169 (in the latter case we considered a final concentration of 10^8 cfu/ml for the two strains). Sterile saline not treated with bacteria served as negative control. One hundred and fifty 9-day-old BSF larvae were then added into each container, covered with cardboard breathable caps, and stored in the climate chamber under controlled conditions (25°C, 60–65% RH). Ten larvae were randomly selected and weighed every 2/3 days with an analytical balance (SARTORIUS CP64). Final larval weight corresponded to the mean weight of 10 larvae at the moment (day) of the appearance of the first prepupa in each replicate. Time from egg eclosion to the appearance of the first prepupa in each container was recorded. All prepupae were removed from the container at the moment of appearance and

counted. Observations continued until all larvae had entered the pupal stage or died.

Statistical Analysis

To assess the differences of insect performance among the treatments (our explanatory categorical variable; levels: Control, *E. coli* DH5α pKan(DsRed), HI121, HI169, and HI121 + HI169) we measured, as continuous response variables, larval growth, larval final weight, number of prepupae, pupal weight, and pupal length. For larval growth and the appearance of number of prepupae, we tested such differences using a generalize additive model statistic (GAM, package mgcv in 'r'; Wood, 2001), while for the larval final weight and the pupal weight and length we performed a linear mixed model where we controlled the factor batch (using the package lmerTest in 'r'; Kuznetsova et al., 2015). For the ANOVA analysis we performed a pair-wise comparison using Tukey HSD test. All statistical analyses were carried out in R (R Core Team, 2018).

RESULTS

Bacterial Isolation From BFS Larvae Gut

Bacteria were isolated from the guts of four BSF larvae reared on SD using different media specifically selected to obtain isolates with properties potentially relevant for the host nutrition. Upon appearance of colonies on the plates, the isolates were selected according to their morphology and then purified, establishing a collection of 193 pure cultures. After ITS-dereplication, we identified 77 ITS groups that were assigned to four phyla, namely Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes by 16S rRNA gene partial sequencing. Proteobacteria included 88% of all the total isolates and were assigned to the Alpha, Beta and Gamma-classes (2, 3 and 84%, respectively). Firmicutes isolates (9%) were in the class of Bacilli, whereas Actinobacteria and Bacteroidetes accounted for 2% and 1%, respectively (**Figure 1**). Within Proteobacteria the most abundant family was Morganellaceae (33%), followed by Enterobacteriaceae (29%), Moraxellaceae (8%), Xanthomonadaceae (7%), Pseudomonadaceae (6%), Alcaligenaceae (3%) Brucellaceae (2%) and Erwiniaceae (1%). Firmicutes were represented by Enterococcaceae (6%), Staphylococcaceae (2%) and Bacillaceae (1%) families. Flavobacteriaceae (1%) was the only family within the phylum of Bacteroidetes, whereas Actinobacteria were divided into Micrococcaceae (2%) and Microbacteriaceae (1%).

Within the entire collection, the most represented genera were *Providencia* (22%) and *Morganella* (6%) in the family Morganellaceae, *Klebsiella* (21%) and *Escherichia* (7%) within the family of Enterobacteriaceae, *Acinetobacter* (8%) in Moraxellaceae family, *Stenotrophomonas* (7%) in the Xanthomonadaceae family, *Pseudomonas* (6) in the Pseudomonadaceae family, and *Enterococcus* (6%) within Enterococcaceae family (**Figure 1**).

Hydrolytic Profiles and EPS Production

All the 193 isolates were screened to characterize the potential contribution of the bacterial partners to the host carbon and nitrogen uptake, as well as to investigate the bacterial ability to adhere to the gut epithelium through the production of adhesive substances, i.e., EPS (**Figure 2** and **Supplementary Table 1**).

Regarding polysaccharide degradation, we found that 15 out of a total of 193 isolates were able to degrade cellulose, 3 were amylolytic, while 47 were pectinolytic bacteria. Lipid degradation ability was present among the isolates: 21 of them were able to degrade Tween 80, 26 to utilize tributyrin and 44 were positive for the true-lipase assay on olive oil plates. Considering nitrogen diet compounds, 175 isolates, 89% of the strain collection, were able to produce ammonia from peptides, and 32 isolates could degrade proteins. The potential capacity to recycle nitrogen from insect metabolic waste compounds was identified in 63 and 31 isolates that resulted positive for the degradation of urea and uric acid, respectively. We also focused our attention on the presence of phytase activity in the bacterial collection, which could release bioavailable phosphate from diet components: we found that 119 strains were positive to the phytate-degradation screening.

The hydrolytic abilities were widespread in our collection. Some activities were specific for some taxonomical groups, i.e., pectinolytic activity for *Klebsiella* spp. and *Stenotrophomonas* spp. strains (**Supplementary Table 1**). Moreover, several strains showed multiple hydrolytic abilities: 31% of the isolates showed $\geq$ 4 activities and, among these, 13 strains showed a multi-activity profile resulting positive for 5 or 6 activities out of the 11 that we performed (**Supplementary Table 1**). Finally, we investigated the EPS production ability obtaining 59 positive strains (30% of the collection; **Figure 2**).

We pinpoint that not all the bacterial isolates obtained from cultures enriched for a specific degrading activity showed the expected enzymatic activity when tested using specific plate-based assays (**Supplementary Table 1**). This is probably due to the leaking of organic matter from the original homogenates during dilution procedure/enrichment phases or to the presence of companion strains in the enrichment cultures able to sustain the growth of the non-hydrolytic ones.

Effect of Bacterial Strains Supplementation on Larval Development

Among the 13 isolates with the higher number of metabolic activities, we selected two strains displaying synergistic and complementing abilities under the perspective to investigate the potential bacteria-mediated metabolic contribution to the holobiont. The two strains belonged to the most represented phylogenetic groups in the collection, i.e., Bacilli and Gamma-proteobacteria. *Bacillus licheniformis* HI169 showed the ability to breakdown cellulose and starch, exhibited uricolytic activity and was able to release ammonia, to dissolve tween 80 and to produce EPS. *Stenotrophomonas maltophilia* HI121, conversely, could digest casein, release ammonia, degrade organic phosphorous, breakdown pectin and had lipase activity (**Supplementary Table 1**). No inhibition was detected in direct antagonistic plate-assays between the two strains, confirming the possibility to combine them in feeding trials (treatment "*B. licheniformis* HI169 + *S. maltophilia* HI121"). The selected strains were, hence,

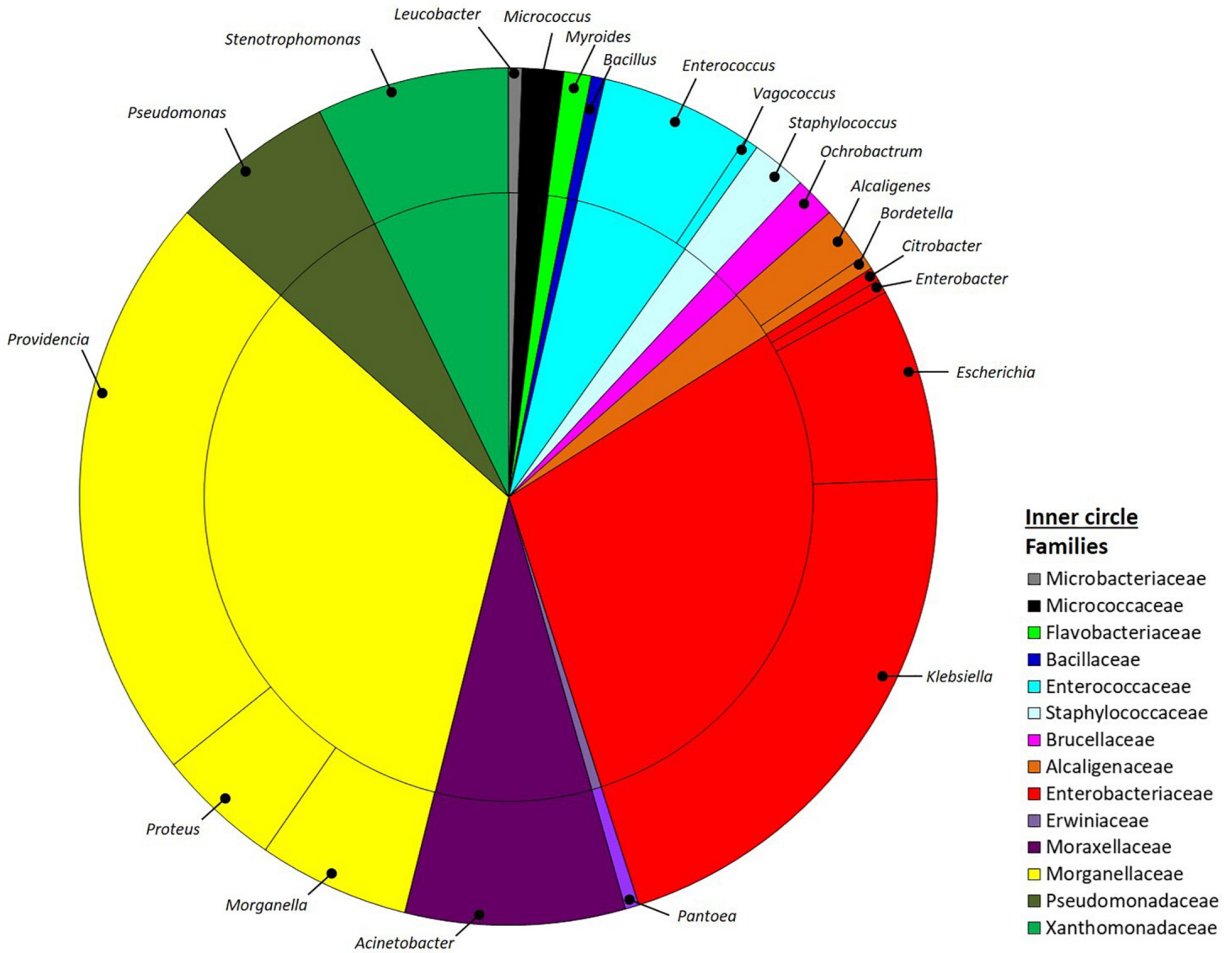

FIGURE 1 | Pie-charts representing the relative culturable bacterial diversity associated to *H. illucens* larvae. Bacterial diversity is expressed at the genus (outer circle) and family (inner circle) levels.

orally administered, alone or in combination, to BSF 9-day-old larvae reared on a nutritionally poor diet (FD) (Jucker et al., 2017): the larval growth rate and final weight, as well as the prepupal appearance, and pupal weight and length were monitored along the insect development cycle (**Figures 3**, **4**).

Bacterial supplement showed to be significant in determining the larval final weight (ANOVA, $F_{4,10} = 16$; $p < 0.001$). Particularly, pairwise analysis depicted that larvae fed with *B. licheniformis* HI169, *E. coli* DH5α pKan(DsRed) and the bacterial mix gained higher and statistically significant final weights than *S. maltophilia* HI121 addition or the control diet (**Supplementary Table 2** and **Figure 3A**). Regarding the larval growth rate, the statistical analysis unveiled a significant difference considering the treatments HI169, DH5α pKan(DsRed) and control along the time (GAM, **Supplementary Table 3**).

The pupal weight was statistically influenced by the bacterial treatment (ANOVA, $F_{4,293} = 7.5$; $p < 0.01$), but the pairwise analysis indicated that the administration of the strains HI169, DH5α pKan(DsRed) or the bacterial mix resulted comparable to the control (**Figure 4A** and **Supplementary Table 4**). Treatments were also statistically significant in determining the pupal length (ANOVA, $F_{4,295} = 9.6$; $p < 0.01$). Pupal length was maximal with the application of the bacterial mix or strain HI169, which were, however, comparable to the control (**Figure 4B** and **Supplementary Table 5**). Regarding the prepupal appearance, we recovered a significant difference considering the treatments (**Supplementary Table 6**). Statistical analysis showed that the control was significantly different from the other bacterial treatments for which we recorded a delayed prepupal appearance (GAM, **Supplementary Table 6** and **Figure 4C**).

DISCUSSION

Handling a collection of microbial strains can allow to directly test, by means of *in vitro* and *in vivo* assays, hypotheses about their importance for the insect host, an aspect that is not always possible relying solely on DNA-based techniques (Prosdocimi et al., 2015). Beyond the characterization of their function in the

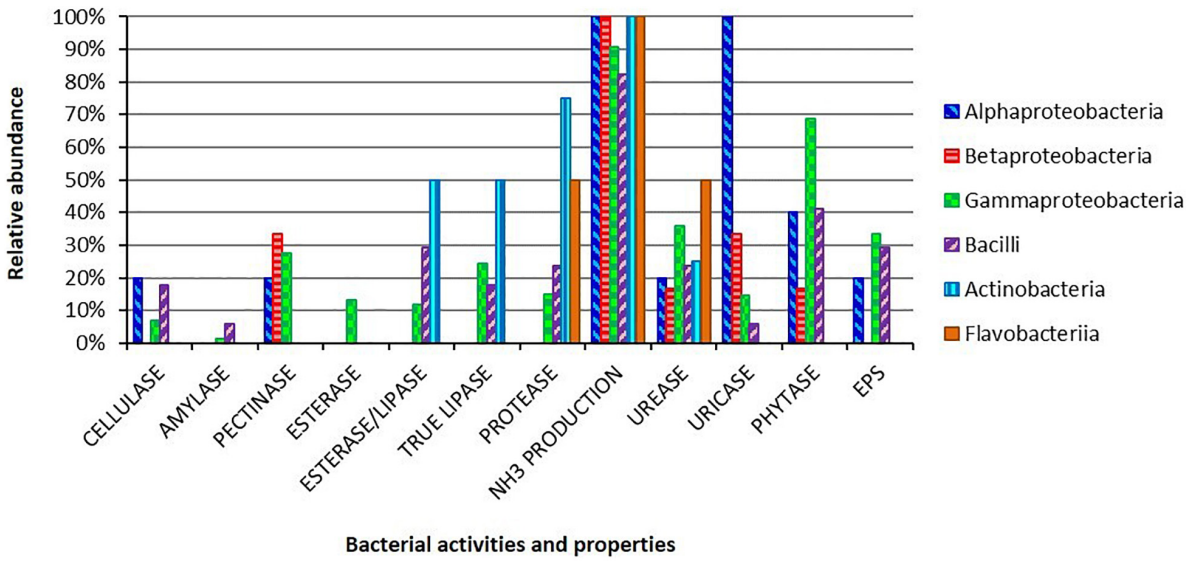

FIGURE 2 | Metabolic activities and EPS production ability for the bacterial isolates obtained from the gut of *H. illucens* larvae. Bars indicate the percentages of each bacterial class in respect to the analyzed hydrolytic activities and EPS production.

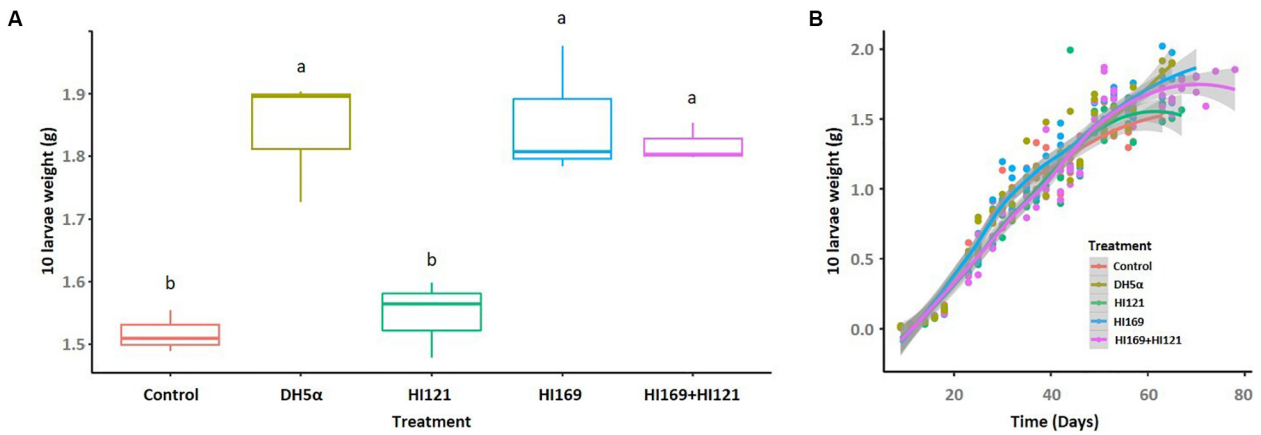

FIGURE 3 | Larval final weight and growth rate following the bacterial administration. **(A)** Final weight of 10 larvae, reported in grams, are shown for the different treatments. **(B)** Growth rate of 10 BSF larvae reared on non-sterile fruit-based diet with or without the selected bacterial strains. The horizontal axis indicates the time (days), while the vertical axis reports the weight of 10 larvae (grams). Control: non-sterile diet without any selected strains; DH5α: larvae fed with the outsider strain of *E. coli* DH5α pKan(DsRed); HI121: larvae added with *S. maltophilia* strain HI121; HI169: larvae supplemented with *B. licheniformis* strain HI169; HI169 + HI121 larvae fed with the two selected strains.

native host, culturable microorganisms can be directly exploited in different biotechnological applications (Crotti et al., 2012; Valiente Moro et al., 2013; Epis et al., 2020). For instance, they have been proposed as probiotics for honeybees to sustain insect health against specific pathogens (Crotti et al., 2013; Daisley et al., 2019) or as beneficial strains to improve the fitness of sterile male insects vs. conventional ones (Ben Ami et al., 2010; Augustinos et al., 2015). Recently, insect symbionts have been suggested as biocontrol agents against phloem-limited pathogens (Iasur-Kruh et al., 2016, 2018; Gonella et al., 2018, 2019; Lidor et al., 2018). In this work, we focused on the bacterial gut inhabitants of the BSF larvae fed on a nutritionally complete diet, on which BSF performance and fitness were previously showed to be higher than on nutritionally poor diets (Jucker et al., 2017). We assumed that the growth performance observed with the nutritionally complete diet should favor the development of a stable and well supporting gut microbiome that may include bacteria with potential probiotic value for BSF.

Our results indicated that the bacterial isolates we obtained from BSF gut encompassed six different classes for a total of 21 genera (**Figure 1**). As expected, we found an abundance of bacterial members that, as typical inhabitants of the insect digestive tract, have been also specifically retrieved in association with BSF by culture-independent methods (Jeon

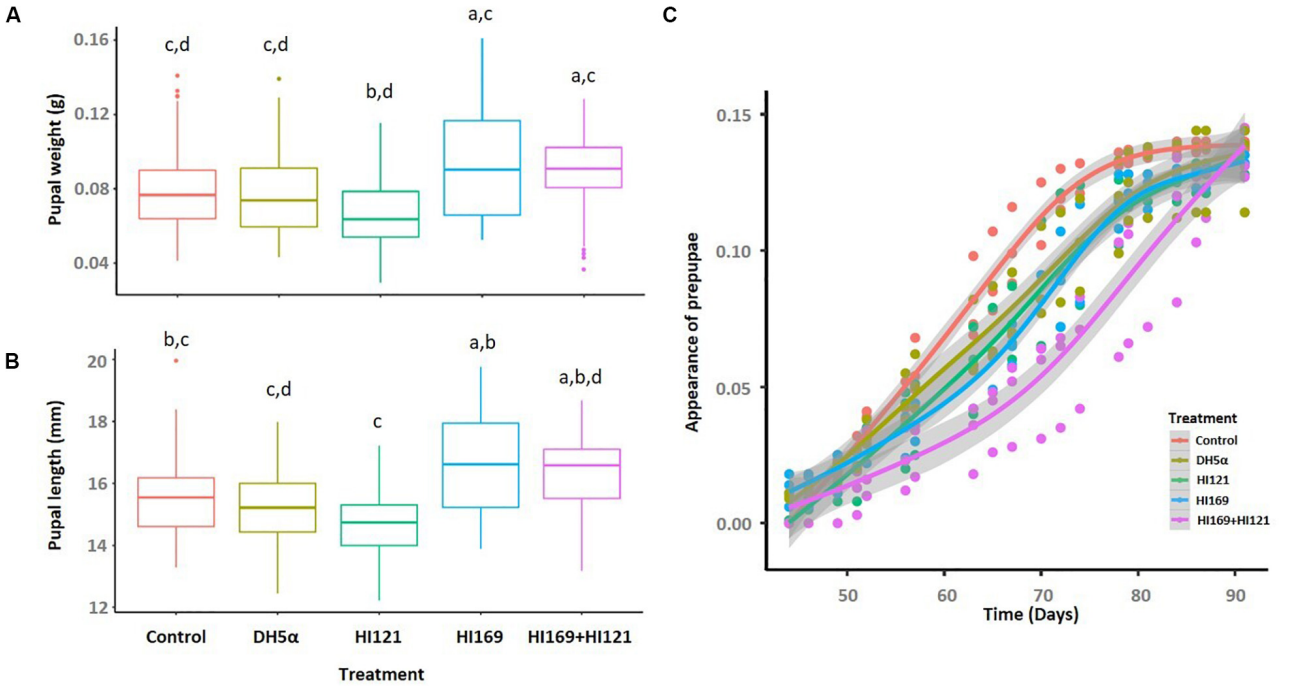

FIGURE 4 | Pupal weight, length and appearance following the bacterial administration. **(A)** Pupal weight and **(B)** length are depicted for the different treatments. **(C)** The prepupal appearance is reported as the progressive average percentages within the population of each treatment. Control: non-sterile diet without any selected strains; DH5α: larvae fed with the outsider strain of *E. coli* DH5α pKan(DsRed); HI121: larvae added with *S. maltophilia* strain HI121; HI169: larvae supplemented with *B. licheniformis* strain HI169; HI169 + HI121 larvae fed with the two selected strains.

et al., 2011). Metataxonomic analysis performed on BSF bacterial community showed that members belonging to the four phyla of Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria were consistently found in this host (Jeon et al., 2011; Zheng et al., 2013b; Bruno et al., 2019): our approach confirmed the finding of members belonging to these phyla, even if with different proportions, as reasonably expected by the cultivation-based strategy that does not claim to cover the whole bacterial diversity associated to the host, also in consideration that the isolation trials were done a small number of samples. Interestingly and in agreement with previous works (Jeon et al., 2011; Zheng et al., 2013b), we documented the presence of several strains belonging to the genus *Providencia* (**Figure 1**): Zheng et al. (2013a) have observed that, among others, gravid BSF females were attracted by members of this genus during oviposition. Conversely, we ascertained the presence of a small number of lactic acid bacteria among our isolates, likely due to the inadequacy of the media to support their growth and since media specific for this bacterial group were not used (Wynants et al., 2019).

Within the gut of larvae fed with the nutritional complete diet, the selective and enrichment media allowed the isolation of bacteria capable to produce a wide range of extracellular hydrolytic enzymes (**Figure 2**). A significant number of isolates presented multiple hydrolytic abilities, traits that potentially confer ecological advantages to the host growing on polymer-rich diets. In some cases, a specific hydrolytic activity was correlated to a specific phylogenetic group, i.e., pectinase activity in *Klebsiella* spp. and *Stenotrophomonas* spp. strains (**Supplementary Table 1**). The hydrolytic profiles obtained for BSF strains might suggest a primary role of the bacterial partners for the host nutrient supplementation (Engel and Moran, 2013; Gold et al., 2018), specifically contributing to the high levels of lipases and proteases that have been characterized in BSF gut content (Kim et al., 2011b; Bonelli et al., 2019). Taking into account its hydrolytic degradation abilities on complex substrates, BSF could be considered a potential source of enzymes with important industrial applications: recently polymer-degrading enzymes, such as cellulases and serine proteases, have been indeed characterized from *H. illucens* holobiont (Kim et al., 2011a; Lee et al., 2014). However, since strong variations of pH levels, ranging from slightly acidic conditions in the anterior part, to strong acidic values in the middle portion, and to alkaline values in the posterior region, have been reported in BSF midgut (Bonelli et al., 2019; Bruno et al., 2019) and also considering that hypoxic and anoxic conditions might occur in BSF gut (Engel and Moran, 2013; Chouaia et al., 2019), further quantitative verifications of the metabolic activities of the isolates (and of their combination) should be performed under different oxygen concentrations and pH levels, especially regarding the strain *B. licheniformis* HI169, which showed the best performances in *in vivo* tests.

Black soldier fly can be reared on a wide variety of organic substrates which can vary in their nutrient composition (De Smet

et al., 2018). For instance, the fruit-based diet used by Jucker et al. (2017), which has been also used in our work, showed a carbon-nitrogen ratio (C/N) of 22.3, suggesting to be a poor source of nitrogenous compounds for the insect. Cammack and Tomberlin (2017) evidenced that a more balanced diet in protein and carbohydrate content allows a faster larval development and a higher survival. For this reason, an "adjunctive" contribution to the nitrogen recycling and uptake provided by gut microbiota could represent a nutritional advantage for the host when grown on an unbalanced diet. Under this perspective, the hydrolytic profiles of our collection revealed that 16.1% of the isolates, mainly belonging to *Ochrobactrum*, *Providencia, Pseudomonas,* and *Stenotrophomonas* genera, showed the ability to degrade uric acid, the main nitrogenous waste compound excreted by Malpighian tubules into the insect anterior hindgut (**Supplementary Table S1**; Engel and Moran, 2013). Degradation of urea (which could derive, in turn, from uric acid utilization, Martin et al., 2018) with ammonia production was carried out by 32.6% of the strains of our collection, while the majority of the isolates (90.7%) were able to release ammonia from peptone. Protease activity, and specifically the one exerted by serine proteases, was mainly retrieved in the posterior part of the midgut, which reached a pH value of 8.3 (Bonelli et al., 2019). In our collection, 16.6% of the isolates are able to degrade proteins (i.e., milk proteins in our experiments), representing also in this case an extra enzymatic activity source for the insect. Considering a diet with an unbalanced C/N ratio, such as the fruit-based one (which could also represent an organic waste material on which BSF could be reared in a bioeconomy perspective), the gut microbiota might provide the necessary nitrogenous compounds to the host.

Regarding carbohydrate metabolism, several isolates were able to release amylolytic and cellulolytic enzymes. Degradation of carbohydrates (e.g., starch) occurs mainly in the anterior midgut and to a lesser extent in the posterior midgut (Bonelli et al., 2019): this could underline a major role exerted by insect, which led to a small number of active amylolytic and cellulolytic isolates. Conversely, further studies are necessary to characterize cellulolytic bacteria in the insect. In conclusion, as a matter of fact we observed that few strains showed to be active on complex substrates (e.g., cellulose or starch), likely sustaining other gut symbionts which could degrade simple substrates (e.g., degradation of peptone).

Finally, in our survey we found a widespread presence of phytase activity among bacterial isolates obtained from larvae fed on the standard diet, a nutritional source that can contain phytate (Magallanes-López et al., 2017; Kaplan et al., 2019). Presence of phytate in feedstuffs could reduce the availability of essential minerals, amino acids and proteins (Wodzinski and Ullah, 1996) and its degradation could release phosphate that could be available for the host or other members of the gut microbiota. Beyond its primary function as phosphorous and energy storage, phytic acid in plant tissues also plays a defensive role against phytophagous insects: Green et al. (2001) showed in fact a positive correlation between the presence of phytic acid in the diet and the mortality of three Lepidoptera species. Moreover, due to the increasing exploitation of *H. illucens* to reduce food or agricultural wastes, the degradation of phytic acid by the gut microbial community could be of interest to improve the insect growth rate and physiological status. While different studies have evaluated the positive effect derived from the supplementation of microbial phytase to the diet of broilers chickens and pigs (Dersjant-Li et al., 2013), there is still poor information about the influence of phytate on the insect growth and development, a topic that could be of pivotal importance for the emerging insect farming practices.

Under the perspective of BSF as a sustainable alternative of feed or fuel (Diener et al., 2009; Zheng et al., 2012b; Nguyen et al., 2015), different works are currently devoted to the optimization of its rearing conditions in order to obtain high amounts of insect biomass reared on low quality feed material represented by waste (Diener et al., 2009; Zheng et al., 2012b). Nevertheless, despite the well-known importance of the host-associated microbiota, few efforts have been made so far to evaluate if bacterial companions can boost the insect development and biomass gaining, also considering nutritionally unbalanced rearing conditions (Yu et al., 2011; Zheng et al., 2012a; Xiao et al., 2018; Mazza et al., 2020). In particular, addition of BSF companion bacteria such as *Bacillus* spp. strains to chicken manure resulted in a co-conversion process that shortened the manure processing and the insect developmental times, enhanced the insect biomass yield and influenced BSF nutrient accumulation (Yu et al., 2011; Xiao et al., 2018; Mazza et al., 2020). In our work we also found a positive influence on the insect performance following the administration of BSF-associated bacteria: in particular, the addition of strain *B. licheniformis* HI169 allowed an increase of the larval weight when compared to the control (**Figure 3**). Also the larval growth rate resulted higher when *B. licheniformis* HI169 was added to the diet in comparison to the control (**Figure 3**). It is noteworthy to mention that, however, following the bacterial administrations, larvae needed longer time to pupate than the control ones (**Figure 4**). Pupal weight and length were maximal with the application of the strain HI169 alone or in combination with *S. maltophilia* HI121, but they were, however, comparable to the control (**Figure 4**). Conversely, larvae and pupae fed on the diet supplemented with *S. maltophilia* HI121 did not differ from the control ones (**Figures 3**, **4**), but differed from larvae and pupae reared on the diet supplemented with *B. licheniformis* HI169 alone or in combination. We also detected a significant improvement of the larval weight following the administration of the outsider control strain *E. coli*. This could lead to hypothesize that the addition of specific bacteria may affect the nutritional quality of the insect unbalanced diet, through a supplementation of microbial proteins or cofactors, partially explaining why we observed good performances for *E. coli* DH5α pKan(DsRed) in relation to the larval weight. Otherwise, the differential promotion effect given by *B. licheniformis* HI169 could be attributed to specific activities given to the host by this strain. The hydrolytic screenings of *B. licheniformis* HI169 revealed its ability to degrade complex compounds such as

cellulose, starch and uric acid, to release esterase enzymes that degrade organic molecules and to produce EPS by which the bacterium can adhere to the surfaces, e.g., intestinal epithelium. However, a further confirmation aimed to experimentally verify that the metabolic activities can take place *in vivo* should be provided. These behaviors, especially if compared to the one exerted by *S. maltophilia* HI121, underline the need to evaluate directly the effect of the bacterial administrations on the insect performance. Finally, these data underlined the positive effect of specific bacterial administrations on the insect performance: to strengthen the outcome of the experiments here described (which were run with three replicates, following the set-up and methods already established in other works, e.g., Rehman et al., 2017; Lalander et al., 2019), it could be interesting to consider different time points to observe a consistency of our findings also in consideration that the observed performance improvement was minimal. Furthermore, it could be relevant to measure the abundance of the beneficial bacteria in the insect gut to understand their colonization ability of the host.

Selection of beneficial microorganisms with a positive impact on the host development and growth might be meaningful also in economic terms. Probiotic microorganisms are known to produce antimicrobial compounds to counteract pathogens, to stimulate the host immune system, to affect the dynamics of the gut microbial populations, to increase the digestion and absorption of nutrients and to prevent pathogens colonization (Grau et al., 2017). Application of beneficial strains in insect farming could lead to the enhancement of the host resistance, preventing the spread of pathogens in the insect rearing, and thus reducing/avoiding the cost and use of antibiotic treatments to manage disease outbreaks. Improved growth performance by the administration of beneficial strains could permit the utilization of disposal material as insect rearing substrate, leading to a valorization of the waste also from the economic point of view, reducing the cost of diet ingredients under the perspective of circular economy principles. A life cycle assessment (LCA) analysis would be thus useful to evaluate the environmental aspects and potential impacts associated with this microbe-assisted strategy in insect farming (Iribarren et al., 2012).

CONCLUSION

Gut symbionts' manipulation is a promising field of research aimed to optimize the waste disposal via insect feeding, e.g., using BSF, especially under the perspective to obtain a sustainable alternative of feed or fuel (van Huis, 2013). Under this perspective culture-dependent techniques allow to screen the culturable fraction of the host microbiota to seek for key bacteria in host nutrition and physiology.

The bacterial collection obtained from BSF larval gut was mainly constituted by members of Gamma-proteobacteria and Bacilli classes. The hydrolytic profiles of the isolates revealed their potential contribution to the host nutrition in terms of carbon, nitrogen and phosphorous recycling and uptake and allowed the selection of candidates that were further used in *in vivo* trials with the host. Our analysis supports the possibility to exploit BSF-associated strains to enhance the insect growth performance when reared on an unbalanced nutritionally poor diet (Jucker et al., 2017) that can simulate an insect rearing substrate in a bioeconomy perspective. We identified *B. licheniformis* HI169 as one of the bacteria able to amend the physiology and performance of the insect growth, highlighting the need of bacterial administration trials to provide feasible microbial solutions to improve the host growth. Furthermore, the collection of BSF-associated bacterial strains is available for future studies of insect feeding in order to evaluate the host performances when reared on different organic substrates.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, PRJEB30516.

AUTHOR CONTRIBUTIONS

EC and SS designed the study. MC, CJ, ML, and SS carried out the experiments. MC, CJ, ML, MF, SS, and EC analyzed the data. EC, SS, SB, and DD supported the research. MC and EC wrote the first draft of the manuscript. All authors contributed to the manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01965/full#supplementary-material

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Core Mycobiome and Their Ecological Relevance in the Gut of Five *Ips* Bark Beetles (Coleoptera: Curculionidae: Scolytinae)

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Bark beetles are destructive forest pests considering their remarkable contribution to forest depletion. Their association with fungi is useful against the challenges of survival on the noxious and nutritionally limited substrate, i.e., conifer tissues. Fungal symbionts help the beetles in nutrient acquisition and detoxification of toxic tree secondary metabolites. Although gut is the prime location for food digestion and detoxification, limited information is available on gut-mycobiome of bark beetles. The present study screened the gut-mycobiont from six bark beetles (five *Ips* and one non-*Ips*) from Scolytinae subfamily using high-throughput sequencing and explored their putative role in symbiosis with the host insect. Results revealed the predominance of four fungal classes- Sordariomycetes, Saccharomycetes, Eurothiomycetes, and Dothidomycetes in all bark beetles. Apart from these, Agaricomycetes, Leothiomycetes, Incertae sedis Basidiomycota, Tremellomycetes, Lecanoromycetes, and Microbotryomycetes were also documented in different beetles. Five *Ips* bark beetles share a consortium of core fungal communities in their gut tissues consisting of 47 operational taxonomic units (OTUs) belonging to 19 fungal genera. The majority of these core fungal genera belong to the phylum Ascomycota. LEfSe analysis revealed a set of species-specific fungal biomarkers in bark beetles. The present study identified the gut mycobiont assemblage in bark beetles and their putative ecological relevance. An enriched understanding of bark beetle-fungal symbiosis is not only filling the existing knowledge gap in the field but may also unleash an unforeseen potential for future bark beetle management.

Keywords: OTUs, gut-mycobiont, fungal-biomarkers, core community, Scolytinae, symbiosis, bark beetles, *Ips*

INTRODUCTION

The evolutionary success of insects is based on myriad associations with microorganisms having complementary potential that is otherwise lacking in insects and restricts them when inhabiting an ecologically challenging niche or invading new environments (Linnakoski et al., 2012; Sun et al., 2013; Douglas, 2015; García-Fraile, 2018). Hence, insects serve as an excellent model system

to study such evolutionary associations (i.e., symbiosis) concerning animal-plant ecology. Bark beetle (Coleoptera: Curculionidae: Scolytinae) and fungi association is one of such model systems. The evolution of bark beetle (BB)-fungi mutualisms is based on reciprocal interactions such as nutritional mutualisms, protective mutualisms, and dispersal mutualism (Biedermann and Vega, 2020). In BB exploiting sapwood (xylem), fungal associates often alleviate the deficiency of such plant tissue that is one of the most nutritionally-limiting and recalcitrant organic substrates on earth to thrive on (Filipiak and Weiner, 2014, 2017). The high abundance of substances like lignin, hemicellulose, cellulose (i.e., bio-polymers with rich carbon content) in the wood is not accessible to beetles until prior degradation of microbes, i.e., fungi (Kirk and Cowling, 1984).

Fungi often benefit coleopteran insects including BB by detoxifying plant allelochemicals (Dowd and Shen, 1990; Wang et al., 2013; Tsui et al., 2014; Zhao et al., 2019). It is believed that fungi and BB together involved in the exhaustion of host defense during the mass attack and subsequent tree-killing (Krokene and Solheim, 1996; Paine et al., 1997). Hammerbacher et al. (2013) showed blue-staining ascomycete *Ceratocystis polonica* helping the spruce BB, *Ips typographus* to break down the stilbene (phenolic compound) defenses of Norway spruce. The similar multipartite relationship was observed between symbiotic fungi (*Grosmannia clavigera*, *Leptographium longiclavatum*, *Ophiostoma montium*) and mountain pine beetle (*Dendroctonus ponderosae* Hopkins), where the presence of such fungal symbionts likely benefited the beetles under a wide range of environmental conditions (Ojeda Alayon et al., 2017). However, results from many other studies raise an argument against the importance of fungi for beetle fitness and killing the host trees as the pathogenic fungi are also found to be associated rather regularly with parasitic beetles that are less aggressive and do not kill the host conifers (Harrington, 1993; Wingfield, 1995). Furthermore, symbiotic fungus (i.e., *Candida nitratophila* isolated from *Ips typographus*) is also reported to oxidize pheromone compound *cis*-verbenol to *trans*-verbenol or verbenone. In return to all those favors, fungi benefit from dispersal by insect hosts (dispersal mutualism).

Bark beetles (BB) are economically important insects (Huang et al., 2020). The BB subfamily Scolytinae of weevils (Curculionidae) is composed of approximately 6000 species (Kirkendall et al., 2015). Association with fungi such as *Ophiostoma*, *Ceratocystiopsis*, *Ceratocystis,* and *Grosmannia* is very common among Scolytinae (Six, 2003; Kirisits, 2004; Zipfel et al., 2006). Some BB also associated with Basidiomycetes fungi (Whitney et al., 1987; Hsiau and Harrington, 2003). All strongly mutualistic associations are upheld generation after generation by vertically transmitting fungi through dedicated structures called mycangia (Six, 2020). Non-mycangial beetles transmit fungi via gut or body surface (Six, 2003; Stefanini, 2018). Ayres et al. (2000) showed that the N concentration of phloem tissues with *Dendroctonus frontalis* (mycangial beetle) larvae are twice than non-infested tissues indicating the importance of fungi for the growth of *D. frontalis* larvae. The same study also reveals that mycangial beetles (*D. frontalis*) construct smaller feeding galleries than non-mycangial beetle (*Ips grandicollis*), which is understandable considering the nutritional availability in host plant tissues influencing high or low consumption strategy for livelihood (Ayres et al., 2000).

Insect gut provides a typical environment for microbial inhabitation, including fungal colonization (Engel and Moran, 2013; Stefanini, 2018). Besides, herbivore insect gut is the site where plant allelochemical digestion, detoxification, and nutritional exchange occurs (Linser and Dinglasan, 2014). Gut-associated fungi can facilitate some or all of those vital processes inside the gut of its host (Dowd and Shen, 1990; Genta et al., 2006; DiGuistini et al., 2011; Wang et al., 2013; Itoh et al., 2018). Despite the functional importance of the gut inhabiting fungi in the adaptive physiology of its hosts, very few studies on BB have investigated the gut-associated fungal community exclusively. The whole-body microbiome studies, common in BB mycobiome research area, often represent a mix of biota from the digestive tract, external biota that escapes surface sterilization (mostly from mouthparts and entomogenous fungi from exoskeleton), and hemocoel and gonad rudiments (presence of fungi unknown).

Furthermore, basic information about the core fungal community in BB gut (subfamily: Scolytinae; *Ips* species) and their ecological role, including the metabolic interaction with the host is also limited. Hence, it is necessary to formulate studies on tissue-specific BB gut fungal assemblages, which are critical to a comprehensive understanding of the adaptive physiology of BB in general and their fungal symbionts inside the gut. For non-mycangial BB where gut serves as an essential site for carrying symbiotic microbes, such studies are even more crucial. Moreover, classic culture-dependent study methods are frequently biased to some specific groups of the fungal communities depending on the culture condition and laboratory processes involved downstream. Recent culture-independent, high-throughput next-generation sequencing-based screening of fungal communities provides an opportunity for exclusive identification and facilitate studying the ecological relevance of fungal species inhabiting inside BB gut that are never detected before. Furthermore, the gut fungal community assemblages may differ in same beetle species due to geographical and climatic differences between sampling sites, other biotic and abiotic factors including tree tissue, season or time of sampling, methodological strategies, and even cross-contamination during molecular processing or sample handling as described clearly in the review by Linnakoski et al. (2012). Hence, more studies are needed in different geographic locations with the same or different BB species to obtain a quick, comprehensive overview of such multipartite BB-fungal associations, which may expose surprising opportunities in bark beetle management (Popa et al., 2012).

Taken together, the current understanding of BB-fungal association and the ecological relevance of the gut tissue-specific association is incomplete, although relevant from both eco-evolutionary and applied perspectives. In Czech Republic, *Ips typographus* (IT), *I. duplicatus* (ID), and *Polygraphus poligraphus* (PP) are common spruce feeding beetles. *Ips typographus* is one of the most damaging pests in Europe, causing landscape-level mortality of spruce (Lieutier et al., 2004). *I. duplicatus* attacks

green standing trees only whereas *Polygraphus poligraphus* (PP, non-*Ips*) likes spruce trees growing under dense and shaded condition (Kolk et al., 1996). Among the pine feeding beetles in Czech Republic, *Ips acuminatus* (IAC) causes considerable damage on the top and branches of Scots pine (Davydenko et al., 2017). Being a secondary pest of pine, *Ips sexdentatus* (SX) attacks stressed and weekend tress (Kolk et al., 1996). *Ips cembrae* (IC), secondary pest of European larix population, prefers wind-blown and dying trees for colonization. However, during an outbreak, IC can colonize not only on larch but also on spruce (Kolk et al., 1996). All these beetles can attack green standing trees under drought conditions and thus possess an increasing threat to forests. Hence, in the present study, we attempt to study gut tissue-specific fungal community assemblages in six economically important bark beetles (BB) from Scolytinae subfamily [IT, ID, IC, SX, IAC, and PP] collected from forests in Czech Republic using culture-independent molecular ecological approaches based on internal transcribed spacer (ITS) sequencing. Subsequently, for the core fungal communities inside the gut irrespective of beetle species, sampling location or host plant are identified, and their potential function within the gut communities is explored. Such information fills the existing knowledge gap and provides a sound scientific basis for a future shotgun or meta-transcriptomics studies followed by downstream functional studies to unravel the fine-tuning of the metabolic exchange between gut inhabiting mycobionts and their insect host.

MATERIALS AND METHODS

Bark Beetle Collection, Dissection, and Gut DNA Extraction

Emerged adult bark beetle (Coleoptera: Curculionidae: Scolytinae) were sampled from infested trees during May and June 2018 from local forests in the Czech Republic. Precisely, *Ips typographus* (L.) (IT), *Ips cembrae* (IC), *Ips sexdentatus* (SX), *Ips duplicatus* (ID), were collected from Rouchovany (49°04′08.0″N 16°06′15.4″E, under State Forest Enterprise); *Polygraphus poligraphus* (PP) was collected from Kostelec nad Černými lesy Kostelec nad Èrnımi lesy (50°00′07.2″N 14°50′56.3″E, under Military Forest Enterprise) and *Ips acuminatus* (IAC) was collected from Libavá (49°40′18.8″N 17°31′44.1″E, under School Forest Enterprise) in the Czech Republic (**Figure 1A**). The ecology and population phase of the collected beetles were summarized in **Figure 1B**. More than 120 living and healthy beetles were collected and pooled from logs belonging to more than eight infested trees per locality to make six biological replicates. Bark beetle species identification was performed based on morphology and published classical taxonomic work (Pfeffer, 1955; Nunberg, 1981). Beetles were stored at 4°C until shock frozen under liquid nitrogen for future use. Due to the pooling of beetles during sampling, the individual colony specific variability of the beetle gut fungal diversity could not be explored. Alternatively, such an approach somewhat reduces the stochastic variations connected with the heterologous natural tissue material (i.e., bark beetles from different colonies) and enables the separation of transient OTUs using statistical methods.

Randomly selected bark beetles were surface sterilized following the standard protocol and dissected under sterile conditions in the biosafety cabinet under the microscope with a sterile entomological dissection kit. Beetle gut with apparent nematode infection was discarded. Beetle gut tissues (10 guts per replicate) were homogenized, and microbial DNA was extracted using PureLink Microbiome DNA Purification Kit from Invitrogen company. Beetle gut DNA was quantified using Qubit 2.0 Fluorometer (Thermo Scientific), and the integrity was assessed by 1% agarose gel electrophoresis. High-quality DNA from all beetle species (six biological replicates per species) was sent for high-throughput amplicon sequencing (Internal transcribed spacer, ITS) at Novogene Company, China.

Internal Transcribed Spacer (ITS) Sequencing

Amplicon sequencing was done at the Novogene company, China, following the standard company protocol. Precisely, after diluting the DNA to 1 ng/μL in sterile water, fungal ITS genes of distinct regions (ITS2) were amplified using a specific set of primers (ITS3, ITS4; amplicon size 386 bp) (White et al., 1990) with unique barcodes. PCR reactions were performed using Phusion High-Fidelity PCR Master Mix (New England Biolabs). PCR products with amplification between 400 and 450 bp were selected and mixed in equidensity ratios for gel purification. No template control in PCR reaction did not show any amplification; hence it was not used for gel purification and further library preparation. After purification of PCR products using the Qiagen Gel Extraction Kit (Qiagen, Germany), sequencing libraries were created using NEBNext Ultra DNA Library Pre-Kit from Illumina, and index codes were ligated. The library quantity and quality were analyzed using Qubit 2.0 Fluorometer (Thermo Fisher Scientific), Q-PCR and in Agilent Bioanalyser 2100 system respectively. The libraries were sequenced to generate 250 bp paired-end reads using an Illumina platform.

ITS Data Analysis

Paired-End Reads Assembly and Quality Control (QC)

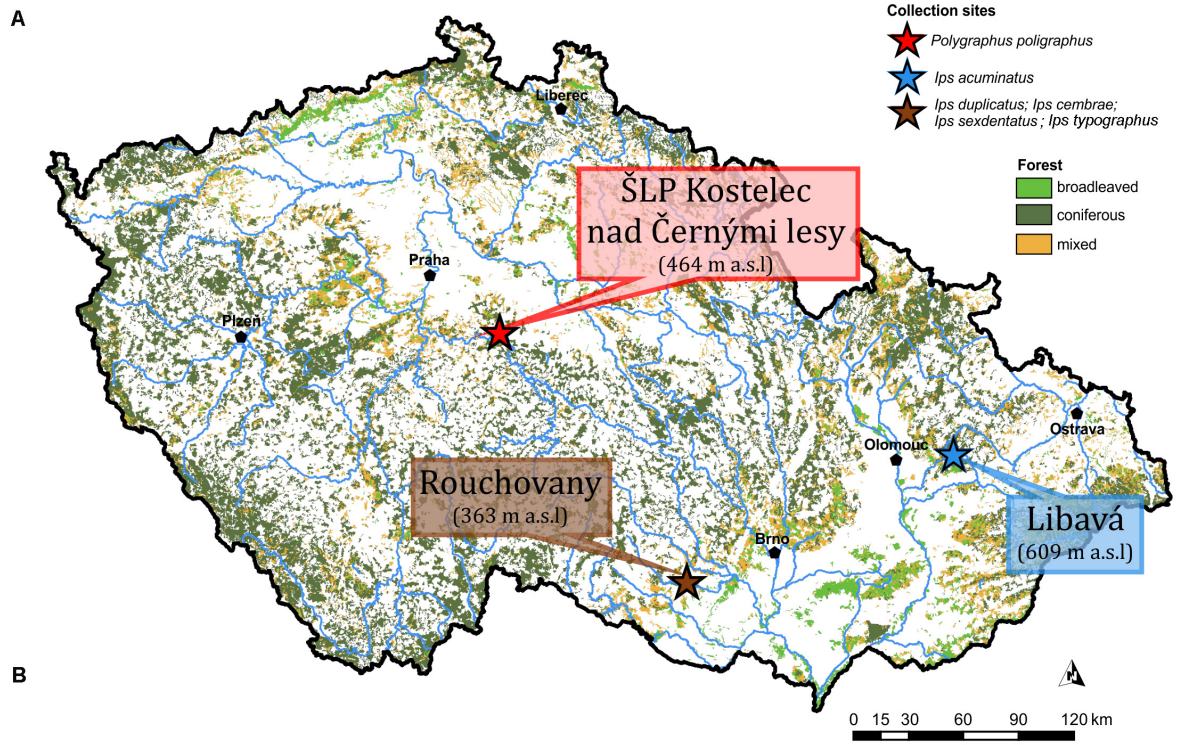
After read assignment to different samples, barcode, and primer sequence removal, paired-end reads were assembled using FLASH (V1.2.7)[1] (Magoč and Salzberg, 2011). Quality filtering was performed to obtain high-quality clean tags applying pre-set parameters (Bokulich et al., 2013) in QIIME (V1.7.0)[2] (Caporaso et al., 2010). UCHIME algorithm[3] (Edgar et al., 2011) was used to detect chimera sequences[4] taking the UNITE database (Nilsson et al., 2019) as a reference, and subsequently, the chimera sequences were removed (Haas et al., 2011), and the effective Tags were finally collected.

[1]http://ccb.jhu.edu/software/FLASH/

[2]http://qiime.org/index.html

[3]http://www.drive5.com/usearch/manual/uchime_algo.html

[4]https://drive5.com/usearch/manual/chimeras.html



Insect name	Primary host	Population type	Attack behavior	Locality
Ips typographus (IT)	Spruce	epidemic	green tree, spruces of all ages	Rouchovany
Ips duplicatus (ID)		epidemic	green tree, crown parts of spruces	Rouchovany
Polygraphus poligraphus (PP)		endemic	green tree, middle age spruces growing in dense and shaded stands	Kostelec nad Černými lesy
Ips acuminatus (IAC)	Pine	epidemic	green tree, tops and branches of Scots pine	Libavá
Ips sexdentatus (SX)		epidemic	green tree, pines of all ages	Rouchovany
Ips cembrae (IC)	Larch	epidemic	green tree, larches of all ages	Rouchovany

FIGURE 1 | (A) Sampling site. The different sampling location for bark beetle collection. *Ips duplicatus* (ID), *Ips typographus* (IT), *Ips sexdentatus* (SX), and *Ips cembrae* (IC) are collected from Rochovany, Czech Republic. *Polygraphus poligraphus* (PP) is collected from Kostelec nad Černými lesy, and *Ips acuminatus* (IAC) is collected from Libavá, Czech Republic. **(B)** Ecology, population phase and attack behavior of the collected beetles (Lieutier et al., 2004).

OTU Clustering and Annotation

Data analysis was conducted using UPARSE software (UPARSE v7.0.1001)[5] (Edgar, 2013). After assigning all sequences with ≥ 97% similarity to the same OTU, obtained OTUs were blasted against the UNITE Database for fungal species annotation. Multiple sequence alignment (MSA) was performed to explore the phylogenetic relationship of different OTUs using MUSCLE software (Version 3.8.31)[6] (Edgar, 2004). During the analysis, the singletons ($n < 2$) were discarded, and OTU abundance was normalized using the sequence number corresponding to the sample with the least sequences. The alpha and beta diversity analysis was subsequently performed based on the normalized OTU abundance data.

[5]http://drive5.com/uparse/

[6]http://www.drive5.com/muscle/

Alpha Diversity

The complexity of fungal species diversity (Alpha diversity) was estimated for each sample using standard indices such as observed-species, sequence depth (Good's coverage) (Chao et al., 1988), community richness (Chao1, ACE), diversity (Shannon, Simpson) (Magurran, 1988). These indices in all 36 tested samples were calculated in QIIME (Version 1.7.0) (Caporaso et al., 2010) and presented using R software (Version 2.15.3; R Core Team, 2013, Vienna, Austria) (R Core Team, 2013).

Beta Diversity

The differences in fungal species complexity in different beetle samples (Beta diversity) (Lozupone et al., 2007) was estimated using QIIME software (Caporaso et al., 2010) (Version 1.7.0). Weighted and unweighted UniFrac distance matrices were used to measure the dissimilarity coefficient

between pairwise samples. Non-metric multidimensional scaling analysis (NMDS) (Oksanen et al., 2010) was performed to get the principal coordinates and visualize the complex, multidimensional data. Unweighted Pair-group Method with Arithmetic Means (UPGMA) Clustering (Knight et al., 2010) was done to interpret the distance matrix using average linkage in QIIME software (Version 1.7.0) (Caporaso et al., 2010). Variation analysis of fungal community structure between different bark beetle (BB) gut was assessed by standard statistical methodologies such as Analysis of Similarity (ANOSIM) (Clarke, 1993), Multi-response permutation procedure (MRPP) analysis (Cai, 2006) and ADONIS (Anderson, 2001) using R software (Vegan package) (Oksanen et al., 2007). Furthermore, Metastats (Paulson et al., 2011) was used to observe the variation in fungal species between the different BB sample groups. The significance of observed fungal species abundance differences among groups was further evaluated by *p*-value calculated by the method of permutation test and *q*-value calculated by the method of Benjamini and Hochberg False Discovery Rate (FDR) (White et al., 2009). Lastly, LEfSe [linear discriminant analysis (LDA) Effect Size] analysis was performed to detect key fungal species with a significant intra-group variation among beetle sample groups using LEfSe software (Segata et al., 2011).

RESULTS

Sequencing Statistics

Illumina paired-end sequencing of the gut tissue of six bark beetles from the Scolytinae subfamily yielded a total of 4,564,713 reads, of which 4,400,177 reads were obtained after quality control tests (sequences with Phred Quality score < 30 were discarded). The fungal ITS2 clean-reads observed per bark beetle species were used for further downstream bioinformatic data processing (**Supplementary Excels 1, 2**).

Gut Fungal Diversity

OTU Abundance

The comprehensiveness of sampling was represented by the Good's coverage estimator (>99%), and the rarefaction curves that tend to attain a plateau indicating most of the gut fungal diversity in the bark beetles (BB) were sequenced (**Supplementary Table 1** and **Supplementary Figure 1**). The number of observed species was significantly higher in PP (176.5 ± 6.84) compared to all other beetles ($P < 0.01$) (**Supplementary Table 1**). A total of 955 fungal OTU (operational taxonomic unit) clusters at 97% similarity were observed in the six different bark beetles (**Supplementary Excel 3**). The gut mycobiome represented using GraPhlAn revealed the predominance of four different classes- Sordariomycetes, Saccharomycetes, Eurothiomycetes, and Dothidomycetes that were present in all BB (**Supplementary Figures 2,3**). Apart from these classes, Agaricomycetes, Leothiomycetes, Incertae sedis Basidiomycota, Tremellomycetes, Lecanoromycetes, Microbotryomycetes were also observed in different BB (**Supplementary Figures 2,3**). However, the GraPhlAn display of IAC was represented at the phylum level due to the presence of an increased number of different classes compare to other beetles (**Supplementary Figure 3**). Considering the spruce feeding beetles, Saccharomycetes was most abundant in ID (90.3%) followed by IT (78.9%) and lowest in PP (19%) whereas Sordariomycetes showed similar abundance in PP (11.6%) and IT (11.8%) compare to ID (5.1%). Furthermore, PP showed a high relative abundance of Eurothiomycetes (12.1%) and Agaricomycetes (4.2%) compared to other BB (**Figure 2A** and **Supplementary Excel 4**). Nonetheless, comparing the pine feeding beetles, IAC showed a similar relative abundance of Saccharomycetes (27.7%) and Sordariomycetes (21.8%) in the gut. Likewise, SX also followed a similar trend (Saccharomycetes- 11.8% and Sordariomycetes- 11.7%) (**Figure 2A** and **Supplementary Excel 4**). Additionally, the larch feeding IC documented the high abundance of Saccharomycetes (83.8%) followed by Sordariomycetes (3.4%) and Eurothiomycetes (2.8%) (**Figure 2A** and **Supplementary Excel 4**).

The evolutionary tree illustrated the relative abundance of top 100 fungal genera, and further, the top 20 fungal species identified in our study were represented in the taxonomic tree (**Supplementary Figure 4**). The relative abundance of Ascomycota was highest, followed by Basidiomycota and Zygomycota (**Supplementary Excel 4**). In particular, the presence of *Cladosporium, Penicillium, Danielozyma, Talaromyces, Fusarium Kuraishia, Blastobotrys, Yamadazyma, Cyberlindnera,* and *Nakazawaea* belonging to Ascomycota phylum were among the most abundant fungal genera present in the gut of BB (**Figure 2B** and **Supplementary Excel 4**). Other dominant genera include *Beauveria, Malassezia, Alternaria, Aspergillus, Wickerhamomyces, Ophiostoma, Ogataea, Trichoderma, Ceratocystiopsis, Peterozyma, Lecanicillium, Candida, Ceratocystis, Mortierella, Rhodotorula, Saccharomycopsis,* and *Cryptococcus* (**Figure 2C** and **Supplementary Excel 4**). Furthermore, the ternary plots representing the predominant fungal genera (top 10) among the spruce feeding BB revealed the predominance of *Yamadazyma* in ID (33.3%). In contrast, *Nakazawaea* was abundant in IT (6.9%) and *Talaromyces* in PP (9.6%). Additionally, *Cyberlindnera* was documented to be prevalent in both IT (46%) and ID (35.1%) whereas, *Blastobotrys* was dominant in ID (14.4%) and PP (12.8%) (**Supplementary Figure 5** and **Supplementary Excel 4**). Furthermore, considering the pine and larch feeding beetles (IAC, SX, and IC), IC showed the predominance of *Nakazawaea* (70%), *Blastrobotrys* (6.2%), *Yamadazyma* (2.7%) and *Penicillium* (2.4%). Nevertheless, *Cyberlindnera* (8.4%), *Kuraishia* (10.9%), and *Fusarium* (3.17%) were most abundant in IAC whereas, *Cladosporium* was highly present in SX (1.2%) (**Supplementary Figure 5** and **Supplementary Excel 4**).

Alpha Diversity

The fungal community richness and diversity present within the gut tissue of the BB was indicated by the alpha diversity indices (**Figure 3** and **Supplementary Table 1**). The Shannon and Simpson indices representing the community diversity in the gut tissue of spruce feeding BB revealed similar diversity in between *Ips typographus* (IT) (Shannon-2.67 ± 0.20 and Simpson-0.69 ± 0.05) and non-*Ips* BB, *Polygraphus poligraphus*

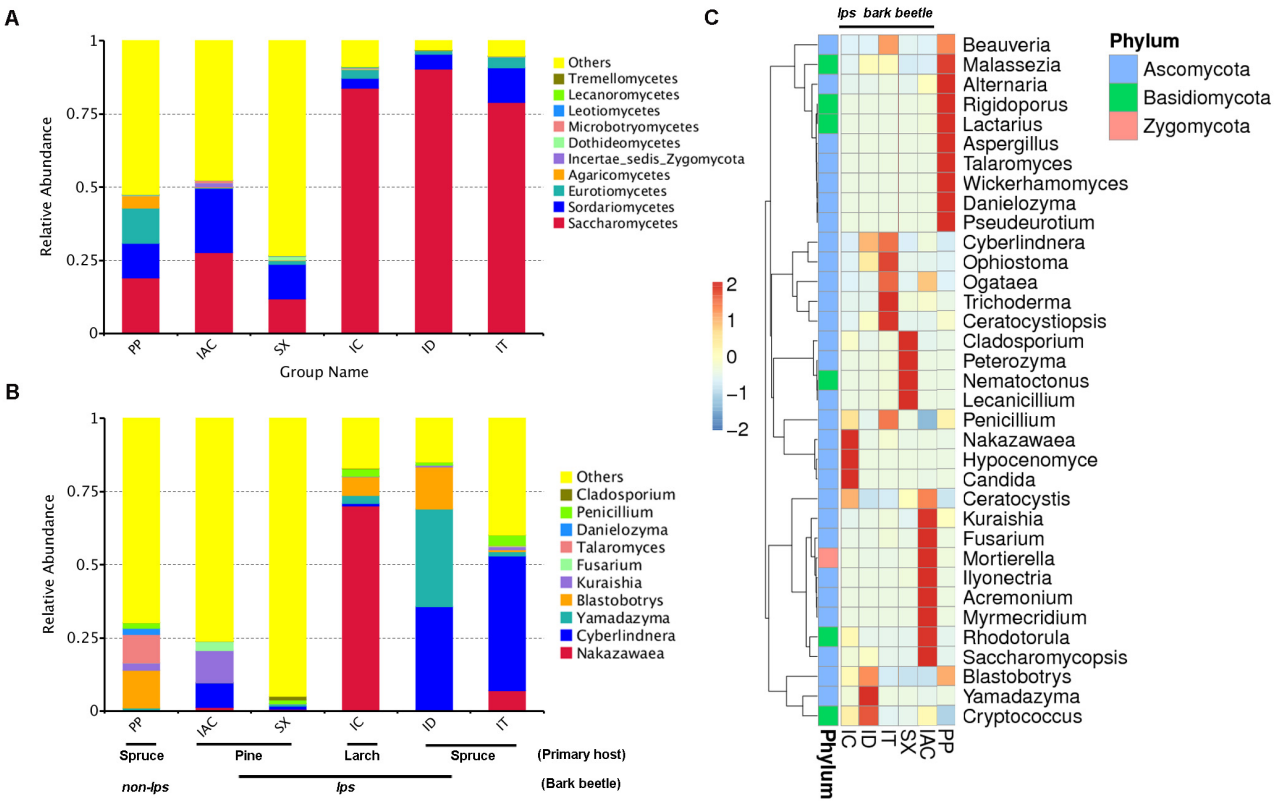


FIGURE 2 | Fungal diversity observed in the gut of different bark beetles. **(A)** The bar plot illustrating the relative abundance of the gut mycobiome at the class level (top 10). "Others" signifies the total relative abundance of the rest of the class. **(B)** The histogram depicting the relative abundance of top 10 fungal genera in the bark beetle gut. Likewise, "Others" represents the total relative abundance of rest of the fungal communities in the beetle gut. **(C)** Heatmap displaying the abundance of 35 dominant fungal genera in the gut of the bark beetles. The color gradient shows the relative OTU abundance for each bark beetle where the darker color signifies higher abundance and lighter color indicates a low abundance of the particular fungal genera.

(PP), (Shannon-2.64 ± 0.21 and Simpson- 0.67 ± 0.05) compared to *Ips duplicatus* (ID) (Shannon- 1.81 ± 0.28 and Simpson- 0.51 ± 0.08) (**Figures 3C,D**). Interestingly, the expected fungal richness (Chao1 and ACE; Wilcoxon signed-rank test) among these spruce feeding beetles documented higher richness in PP (Chao1- 210.72 ± 11.63 and ACE- 218.96 ± 13.20) compared to IT (Chao1- 128.19 ± 9.24 and ACE-128.75 ± 9.24) and ID (Chao1- 109.76 ± 6.76 and ACE- 109.76 ± 6.76) (**Figures 3A,B**). Furthermore, considering the pine feeders, *Ips acuminatus* (IAC) (Shannon- 2.84 ± 0.13 and Simpson- 0.77 ± 0.02) showed significantly higher gut fungal diversity than *Ips sexdentatus* (SX) (Shannon-1.46 ± 0.23 and Simpson- 0.42 ± 0.08) (**Figures 3C,D**). However, no significant difference in fungal richness was documented between IAC (Chao1- 136.38 ± 16.99 and ACE- 139.74 ± 17.68) and SX (Chao1- 134.34 ± 6.15 and ACE- 140.90 ± 5.45) (**Figures 3A,B**). Additionally, the gut mycobionts of *Ips* beetles showed similar community richness and diversity among them compared to PP (**Figure 3** and **Supplementary Table 1**).

Our data suggest that the BB share a consortium of core fungal communities in their gut tissues (**Figure 4**). The core mycobionts present in the spruce feeding BB (PP, IT, and ID) consists of 76 OTUs belonging to 22 fungal genera (**Figure 4A** and **Supplementary Excel 5**). Similarly, 81 OTUs assigned to 25 genera were shared between the pine beetles (SX and IAC) (**Figure 4B** and **Supplementary excel 6**). Interestingly, comparing the five *Ips* bark beetles the occurrence of 47 core fungal OTUs were observed that were assigned to 19 fungal genera constituting *Cyberlindnera, Rhodotorula, Saccharomycopsis, Yamadazyma, Blastobotrys, Talaomyces, Fusarium, Kuraishia, Penicillium, Cladosporium, Candida, Ogataea, Cryptococcus, Ceratocystis, Ophiostoma, Trichoderma, Malassezia, Cuniculitrema, and Beauveria* (**Figure 4C** and **Supplementary excel 7**). Nevertheless, the core fungal consortium compared among all of the six BB (PP, IT, ID, IC, IAC, and SX) revealed the presence of 37 common OTUs predominantly belonging to 16 fungal genera (**Supplementary Figure 6** and **Supplementary Excel 8**). Besides, the core mycobiome present in all the beetles, the occurrence of other fungal genera were also documented in this study. The existence of such fungal communities might be acquired from the environment as fungal spores or during feeding. Among these fungal communities, *Peterozyma* and *Hypocenomyce* were observed in the BB from Rouchavany (SX, IC, IT, and ID), whereas *Wickerhamomyces, Myrmecridium,* and *Lactarius* were documented in PP (Kostelec) and IAC (Libavá). Furthermore,

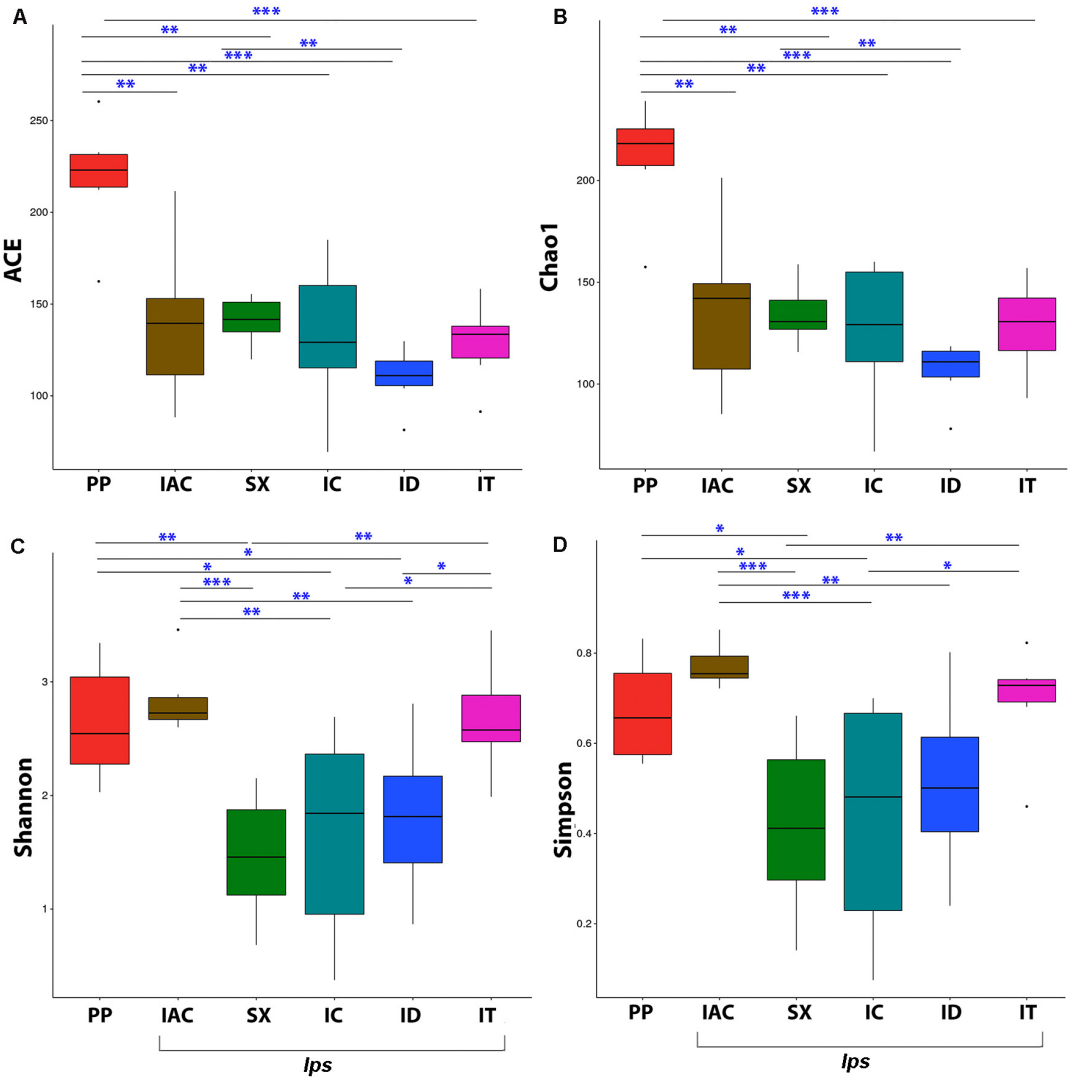


FIGURE 3 | Alpha diversity indices representing the fungal community richness and diversity among the six different bark beetles. The expected richness estimated by **(A)** ACE analysis and **(B)** Chao1 analysis indicated significant variation between *Polygraphus poligraphus* (PP) and other spruce feeding beetles while no such difference in community richness was observed between pine feeding beetles (IAC and SX). The gut fungal diversity illustrated by **(C)** Shannon index and **(D)** Simpson index documented similar diversity measures between *Polygraphus poligraphus* (PP) and *Ips typographus* (IT) compared to *Ips duplicatus* (ID) whereas *Ips acuminatus* (IAC) represented higher diversity than *Ips sexdentatus* (SX). The statistical analysis for the significant differences between groups is done by Wilcoxon signed-rank test where "*" denotes $p < 0.05$, "**" designates $p < 0.01$ and "***" resembles $p < 0.001$.

Grapium was found in all the spruce feeding BB (PP, IT, and ID), and *IIyonectria* and *Coprinellus* were observed in pine (IAC, SX) and larch feeding (IC) bark beetles, respectively (**Supplementary Excel 4**).

Beta Diversity

The beta diversity calculated based on Weighted and Unweighted UniFrac distances reflects the differences in the gut mycobiome of six different BB (**Figure 5** and **Supplementary Figure 7**). The box plot based on the Weighted UniFrac distances between the samples suggested that among the spruce feeding BB, the gut fungal communities in IT were significantly different from ID and PP ($p < 0.01$) (**Figure 5A**). However, no significant variation was observed between ID and PP. Furthermore, considering the larch feeding beetle, IC, showed a substantial difference in the gut mycobionts compare to ID ($p < 0.01$) and PP ($p < 0.001$). In contrast, no significant differences were documented between the pine feeding beetles (IAC and SX) (**Figure 5A**). However, there was considerable variation between PP and pine feeding BB (IAC, SX) ($p < 0.05$). Interestingly, the hierarchical clustering based on the Weighted Unifrac distances clustered all the *Ips* species together in one clade and placed PP (non- *Ips* species) in a different clade (**Figure 5B**) demonstrating the convergence of fungal association in *Ips* species. Furthermore, the influence of host tree feeding on the BB gut mycobiome was also observed where pine feeding (IAC and SX), spruce feeding (IT and ID), and

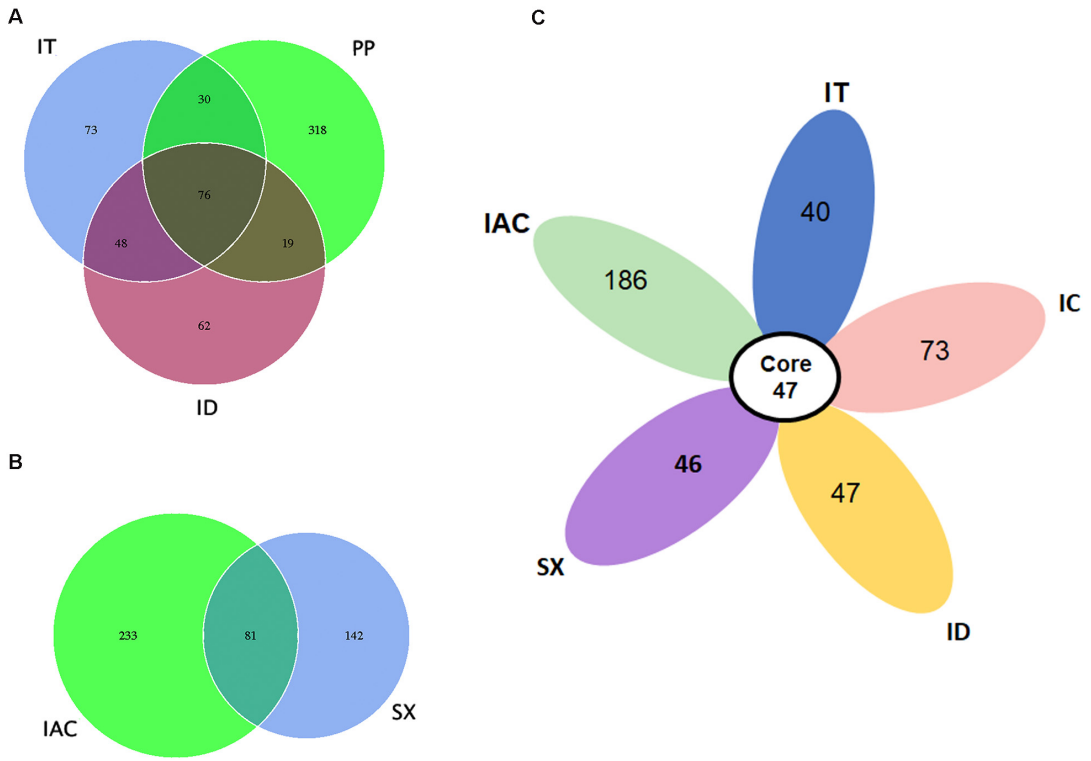


FIGURE 4 | Core gut mycobiome. **(A)** Venn diagram showing the gut fungal OTU distribution between the spruce feeding bark beetles (IT, PP, and ID) where the number of OTUs (common or unique) shared among the beetles is denoted. **(B)** Venn diagram displaying the number of common and unique OTUs between the pine feeding beetles (IAC and SX). **(C)** Flower diagram showing the presence of 47 core OTUs shared among the five different *Ips* bark beetles.

larch feeding (IC) *Ips* beetles clusters together within the same clade (**Figure 5B**).

Alternatively, Non-Metric Multidimensional Scaling (NMDS) analysis revealed the environmental influence on the beetle gut communities resulting from different sampling locations. The NMDS plot illustrated differences in the gut mycobionts of bark beetles (BB) from Rouchavany (IT, ID, IC, and SX), Kostelec (PP), and Libavá (IAC) representing three clusters (**Figure 5C**). Such observation explaining the influence of the site-specific environmental conditions on the gut communities need further experimental validation.

The fungal species-specific variation present in the gut of bark beetles represented by Metastat analysis revealed the preponderance of *Cyberlindera amylophila, Yamadazyma mexicana,* and *Ophiostoma bicolor* in the spruce feeding beetles from Rouchavany (IT and ID). In contrast, *Talaromycetes rugulosus*, *Staphylotrichum coccosporum* were significantly abundant in PP (Kostelec site) while, *Fusarium solani* was prevalent in both PP and IT. Furthermore, *Ogateae neopine, Rhodotorula hylophila,* and *Cyberlindera mississippiensis* were highly abundant in IAC (**Figure 6**). Statistical analysis (ANOSIM, MRPP, and ADONIS) illustrates significant variation between the fungal communities' present in the six BB under study (**Supplementary Tables 2,3**). The gut mycobionts differed considerably between BB with different conifer feeding and were also influenced by the environment due to different collection site. Nevertheless, our data showed that the fungal communities associated with the BB were unique among different beetle species. Although the NMDS analysis documented the clustering of the bark beetles collected from the same site nearby, there were also noteworthy differences in the gut mycobiome between these beetles (**Figure 5C** and **Supplementary Tables 2,3**).

Moreover, the presence of significantly abundant fungal communities evaluated by Linear discriminant analysis effect size (LefSe) was described as important fungal biomarkers. The LDA (Linear discriminant analysis) scores [(log10) > 4 as threshold] represented by the histogram were used for the estimation of the biomarkers (**Figure 7**). The LDA score reflected the significant abundance of fungal classes that include- Eurotiomycetes, Agaricomycetes, Saccharomycetes along with fungal families belonging to Trichocomaceae, Peniophoraceae, Ceratocystidaceae, and Pichiaceae in the spruce feeding BB (IT, ID, and PP) (**Figure 7A**). Furthermore, the prevalence of fungal species belonging to Pichiaceae, Saccharomycetales, and Sccharomycetes was identified as biomarkers for ID. In contrast, Incertae sedis Saccharomycetales were abundant in IT. Additionally, the fungal communities belonging to Trichocomaceae, Eurotiomycetes, Hypocreales, Ceratocystidaceae, Microascales, Peniophoraceae, Russulales, and Agaricomycetes were observed as dominant markers in PP (**Figure 7B**). While in the pine feeding BB (IAC and SX), the histogram of the LDA scores illustrated the significant

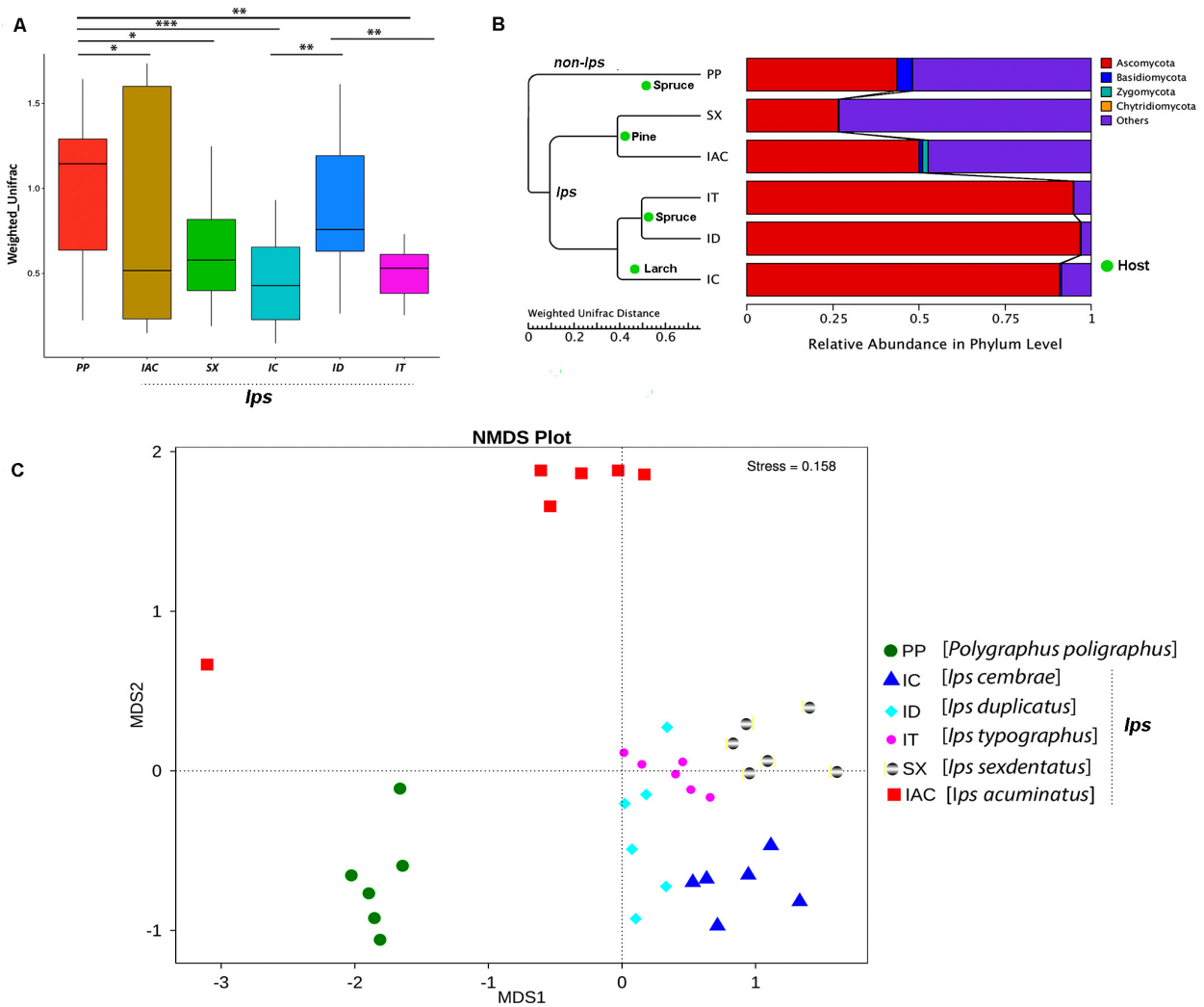


FIGURE 5 | Beta diversity analysis. **(A)** The boxplot representing the diversity between the bark beetles based on the Weighted Unifrac distances showing significant differences between the PP and other bark beetles. The statistical analysis for significance is performed by Wilcoxon signed-rank test where the significance level at $p < 0.05$ is denoted by "*," "**" designates $p < 0.01$ and "***" signifies $p < 0.001$. **(B)** The WPGMA (Weighted pair group method with arithmetic mean) tree cluster based on Weighted Unifrac distance illustrates the similarity in gut fungal communities among the *Ips* species of bark beetles (IT, ID, IC, SX, and IAC) compare to non- *Ips* bark beetle PP. Additionally, bark beetles feeding on similar conifer host are grouped. The WPGMA tree displayed with the relative abundance of the gut mycobiome at phylum level. **(C)** Non-metric multidimensional scaling analysis (NMDS) representing the variation in the gut fungal communities between the six bark beetles. The data points in the same color denote the same bark beetle species. The different bark beetles are denoted by different symbols. The bark beetles collected from Rouchovany (IT, ID, IC, SX) are clustered together and are significantly different from the bark beetles sampled from other locations, Kostelec (PP) and Libavá (IAC).

abundance of the fungal class belonging to Eurotiomycetes and Dothideomycetes and the families Trichocomaceae and Davidiellaceae in SX whereas fungal genera *Rhodotorula* and *Nakazawaea* in IAC (**Figure 7C**). Moreover, the cladogram indicated Davidiellaceae, Capnodiales, Dothideomycetes, Trichocomaceae, Eurotiales, and Eurotiomycetes as biomarkers in pine feeding beetle, SX (**Figure 7D**).

Taken together, the histogram representing all the six bark beetles (**Supplementary Figure 8**), illustrated the significant abundance of *Penicillium* and *Peterozyma* in SX while Microbotryomycetes, Sordariomycetes, Saccharomycetaceae, Ophiostomataceae, Nectriaceae, Plectosphaerellaceae, *Fusarium, Rhodotorula, Nakazawaea,* Incertae sedis Sordariomycetes in IAC. The fungal communities belonging to Eurotiomycetes, Agaricomycetes, Trichocomaceae, Trichomonascaceae, Ceratocystidaceae, Peniophoraceae were highly present in PP. Moreover, IT documented the significant abundance of Incertae sedis Saccharomycetales and *Cyberlindera,* whereas *Yamadazyma,* Microascaceae, Saccharomycetales were observed ID. The larch feeding BB (IC) showed the presence of Pichiaceae, *Candida,* and *Nakazawaea* in the histogram of LDA scores (**Supplementary Figure 8A**). Consequently, the overall cladogram representing the biomarkers from all the six bark beetles documented Incertae sedis Sordariomycetes,

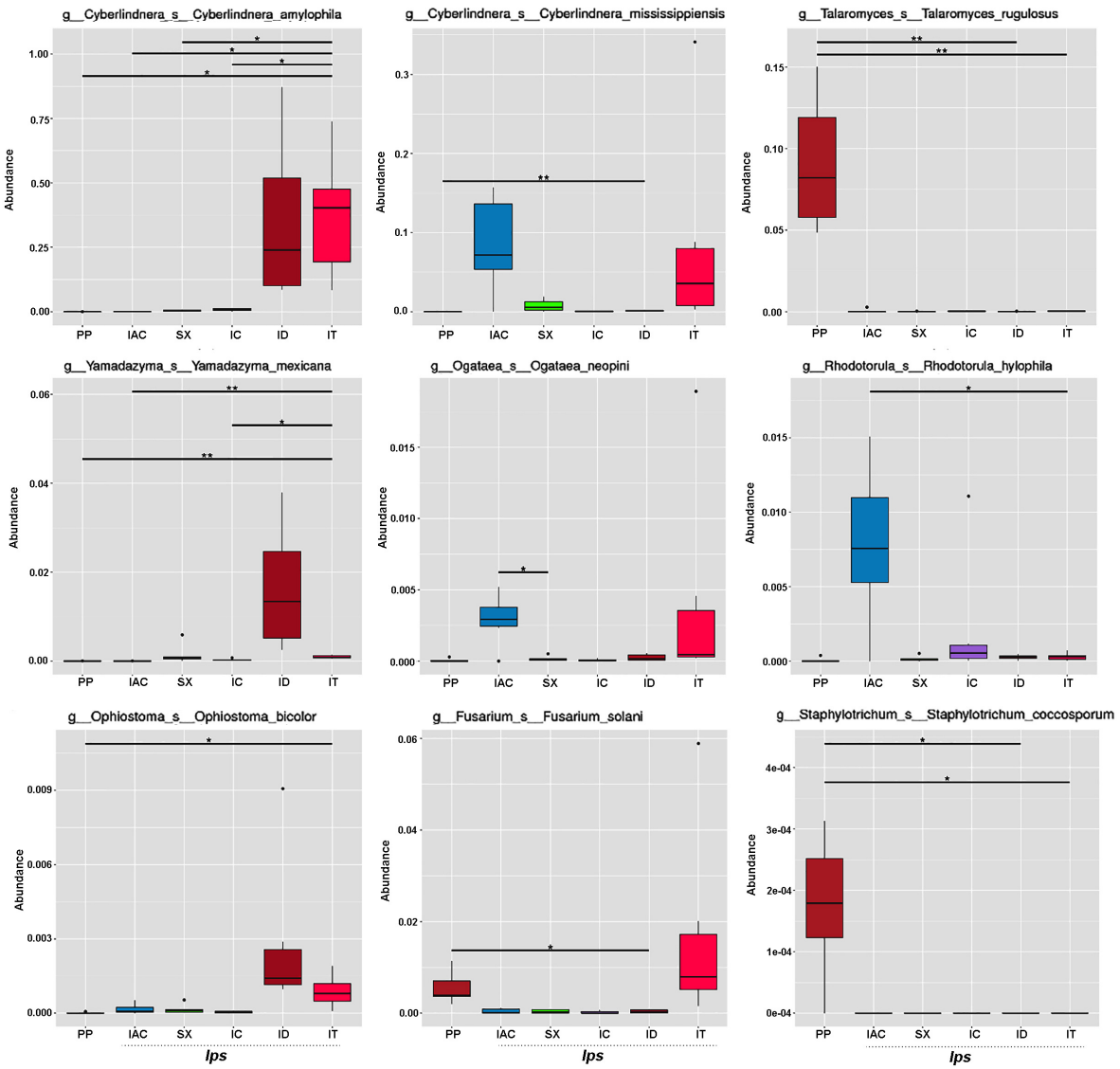


FIGURE 6 | Metastats analysis. The barplot represents the fungal species-specific variation (top 9 species) in the gut of the bark beetles. The significant differences in species abundance among beetles are evaluated by the FDR test. The horizontal line designates the two groups with significant variation. "*" represents significant difference at $q < 0.05$ while "**" denotes significance at $q < 0.01$.

Ophiostomataceae, Plectosphaerellaceae, and Nectriaceae as markers in IAC while Pichiaceae in IC. The fungal order Saccharomycetales and family Microascaceae were the biomarkers in ID, whereas Incertae sedis Saccharomycetales was the dominant marker for IT. The non- *Ips* BB, *Polygraghus poligraphus* (PP) demonstrated Trichocomaceae, Trichomonascaceae, Ceratocystidaceae, Peniophoraceae as biomarkers (**Supplementary Figure 8B**).

DISCUSSION

Fungi are often associated with bark beetles (BB) as their symbionts where fungi are either carried in specialized mycangia or on their exoskeleton (Klepzig and Six, 2004; Six, 2012). Most of the previous BB-fungal symbiosis studies are conducted non-tissue specific manner, i.e., taking the whole bark beetles comprising endo and exo-mycobiome (Kirisits, 2007; Six, 2012). Hence, we have a limited understanding of the tissue-specific assemblage and interaction between BB and its fungal associates, which is an important aspect for having a better understanding of symbiosis. Not surprisingly, BB gut serves as a rich source of mycobionts assemblage. As insect gut is the prime site for digestion and detoxification of food (Linser and Dinglasan, 2014), often gut-associated mycobionts play a crucial role in those processes and decide the fate of host-derived compounds (Itoh et al., 2018). Hence, the present study is designed to screen the gut fungal diversity in five *Ips* and one non-*Ips*

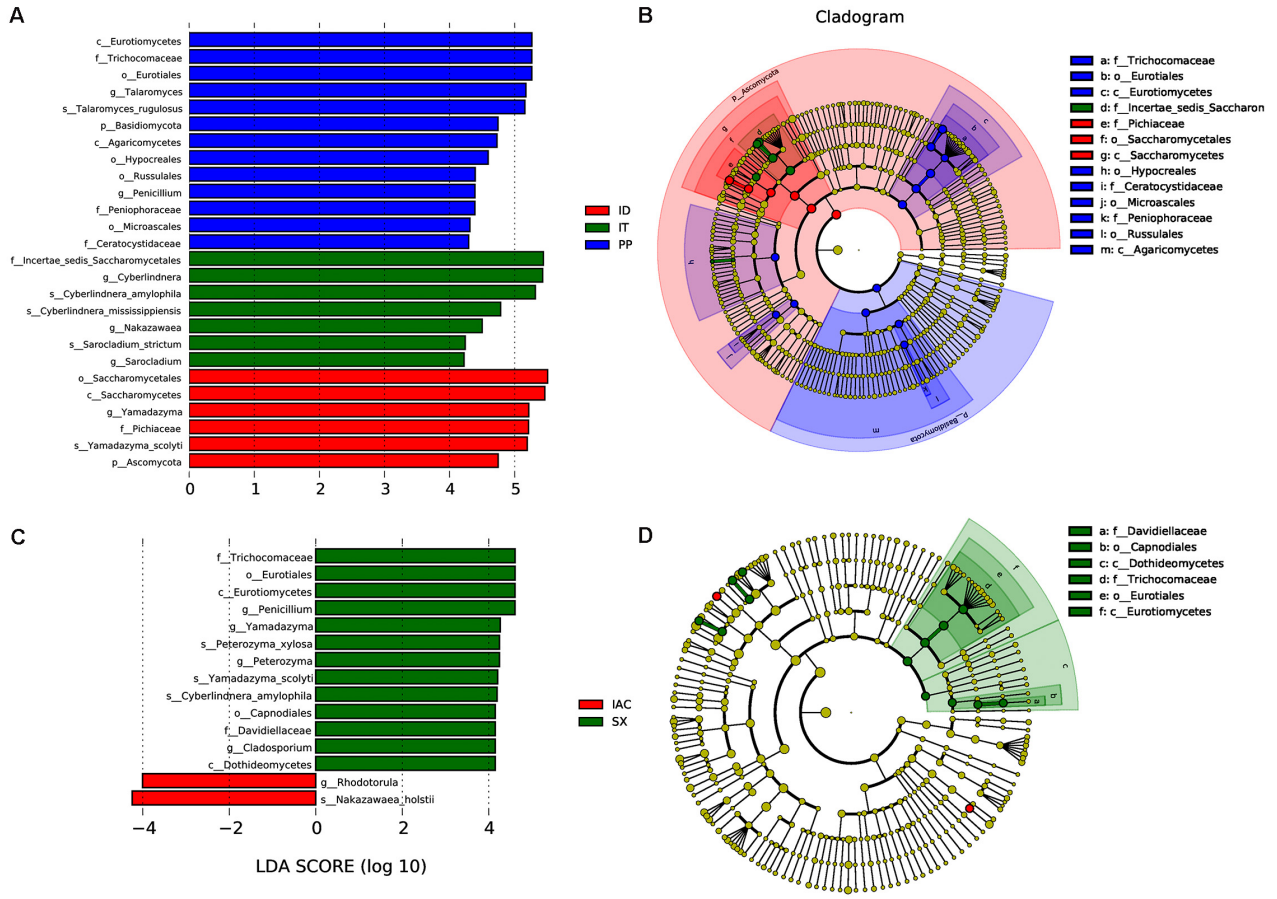


FIGURE 7 | LEfSe analysis. **(A)** The histogram of the Linear discriminant analysis (LDA) scores illustrating the differentially abundant fungal communities in the spruce feeding bark beetles (IT, ID, and PP). The LDA score at log 10 > 4 is set as threshold and the length of each bin, i.e., LDA score represents the extent to which the fungal biomarker differs among the groups. **(B)** The cladogram illustrating the presence of fungal biomarkers that are significantly different between the spruce beetles. **(C)** The LDA score plot (log 10 > 4 as the threshold) representing the significantly abundant gut fungal communities in the pine feeding beetles (IAC and SX). **(D)** The cladogram describing the fungal biomarkers in pine beetles. The circles representing from inside to outside denotes different taxonomic level (phylum to genus). Each circle resembles a distinct taxon at the respective taxonomic level. The relative abundance of a particular taxon is proportional to the size of that circle. The color of the fungal biomarkers is according to the color of corresponding bark beetle while yellowish-green dots designate the non-significant fungal species. Red and green nodes denote that these fungi contribute highly to the group. Letters above the circles denote the fungal biomarker.

bark beetles (BB) collected from three different sites (**Figure 1**) in Czech Republic. Our data reported higher fungal diversity in the gut of *Ips acuminatus* (IAC) and *Ips typographus* (IT) compared to other tested *Ips* bark beetles and *Polygraphus poligraphus* (PP) (**Supplementary Table 1**). The pine feeding BB, *Ips acuminatus* (IAC) documented higher gut fungal diversity may be due to their fungus feeding behavior at the larval stage. However, there is no apparent difference in the gut mycobiome richness compare to other pine inhabitants, *Ips sexdentatus* (SX) (**Figure 3**). Congruently, *Ips acuminatus* (IAC) considered as less aggressive yet attacked healthier coniferous trees (Wermelinger et al., 2008) compare to the other secondary pest on pine, *Ips sexdentatus* (SX) (Pineau et al., 2017). Hence, the higher mycobiome diversity in IAC could thereby contribute to coping with the toxic defensive pine compounds (Davis et al., 2018) and nutritional acquisition during development (Villari et al., 2012). On the contrary, the aggressive secondary BB (Six, 2020), *Ips typographus* (IT), exhibiting mass attack to combat the tree defense showed higher fungal diversity among the spruce feeding BB in this study. It is worth to mention here that some fungi such as *Endocondiophora polonica*, *Grosmannia penicillate*, and *Grosmannia europhioides* associated with the aggressive beetles (e.g., *Ips typographus*) often utilize the tree defensive toxic compounds as carbon source and thus reduce their concentration in addition to lessen the competition between symbiotic partners for available carbohydrate like glucose (Zhou et al., 2016; Zhao et al., 2019). Hence, the fungal association may have a dual benefit for bark beetles (BB). However, such assumptions need further experimental corroborations.

In addition to tree colonization behavior, other factors such as sampling site, microenvironment of the habitat, nutrient availability and defensive host compounds influence the gut mycobiome communities in BB. Despite all these variabilities, our data suggest the presence of core fungal communities

constituting of 19 genera that were conserved among all five *Ips* bark beetles (**Figure 4**). The majority of these core fungal genera belong to the phylum Ascomycota. Interestingly, some of the Ascomycota associates (*Grosmannia*, *Ophiostoma*, *Endocondiophora, Ceratocystis,* and *Leptographium*) in the BB (e.g., *Dendroctonus ponderosae*, *Ips typographus*) were reported to endure host tree defense mechanism and utilize these defensive secondary metabolites as carbon sources (Hammerbacher et al., 2013; Cale et al., 2016; Wadke et al., 2016; Zhou et al., 2016; Davis et al., 2018). The detoxification of defensive plant compounds and the ability to exploit these metabolites as carbon sources by the symbiotic fungi benefits the BB and boosts their fitness. Our study revealed the presence of ophiostomatoid fungi *Ophiostoma, Ceratocystis* belonging to Ophiostomataceae and Ceratocystidaceae family, as the core gut members in the BB from Scolytinae subfamily. Several studies have already documented ophiostomatoid fungi (e.g., *Grosmannia penicillate*, *Ophiostoma bicolor*, *Ophiostoma piceae*, *Ceratocystis polonica*, *Leptographium chlamydatum*) to be frequently associated with *I. typographus* (Viiri, 1997; Persson et al., 2009; Linnakoski et al., 2010). These ophiostomatoid fungi, commonly referred to as blue staining fungi, are serious tree pathogens (Wingfield et al., 1993; Kirisits, 2004), and their association with the BB has been formed over a long period (Hartig, 1878). The fungal spores of ophiostomatoid fungi are typically dispersed by BB (Masuya et al., 2009). As the BB invade the coniferous trees and the fungi get transported to the phloem and the bark where they function in nitrogen acquisition and maintains the nitrogen balance that is critical for beetle development (Ayres et al., 2000). Similarly, *D. brevicomis* beetle surviving on the outer bark largely depend on the two mutualistic fungi (*Ceratocystiopsis brevicomi and Entomocorticium* sp.) for maintaining the nitrogen and phosphorous ratios in their diet (Six and Elser, 2019). Furthermore, *Ceratocystis* sp. also contributes to the detoxification of phenolic compounds released as tree defensive response against beetle or fungal attack (Hammerbacher et al., 2013). Such association between the ophiostomatoid fungi (*Ophiostoma ulmi*) and the *Scolytus* spp. BB has been previously reported to kill numerous elm trees in Europe and North America (Hubbes, 1999; Santini and Faccoli, 2015). In contrast to *Ophiostoma* sp., the association of *Ceratocystis* sp. with the bark beetles are not very specific (Wingfield et al., 2016). The fungi *Ceratocystis* are often vectored by different insects and can infect host plants belonging to different orders (Roux et al., 2007; Lee et al., 2016). It is noteworthy that the presence of such fungal genus (*Ceratocystis*) in the core consortium could presumably pose an additional threat to forests, causing serious tree diseases such as Eucalyptus wilt (caused by *C. fimbriata s.l.*) or *Ceratocystis* wilt (caused by *C. manginecans*) (De Beer et al., 2014).

The additional presence of non-ophiostomatoid fungi belonging to the genera *Fusarium, Penicillium*, *Trichoderma, Beauveria,* and *Cladosporium* in the core communities in BB gut were also observed in this study. Similar to the ophiostomatoid fungi, *Fusarium*, an important plant pathogen, was documented to induce necrotic lesion on the phloem and disturb water conduction, ultimately killing the tree (Solheim, 1988; Yamaoka et al., 2015). Previous studies reported the association of *Fusarium* with *Ips sexdentatus* (SX) and detected their presence in SX galleries (Bezos et al., 2018). Moreover, studies showed mutualistic interaction between *Fusarium* with *Hypothenemus hampei* infesting coffee beans (Morales-Ramos et al., 2000). The occurrence of *Penicillium* and *Trichoderma* was also demonstrated in the previous study done by Krokene and Solheim (1996). It is interesting to note that *Fusarium* and *Beauveria* associated with the bark beetle core gut mycobiome in our study are entomopathogenic (Teetor-Barsch and Roberts, 1983; Zimmermann, 2007). The presence of such entomopathogenic fungus in the BB gut might occur through wounds or by sub-lethal feeding of the fungal spores by BB (Reay et al., 2005). Congruently, the presence of entomopathogenic fungus associated with bark beetles (BB) could eventually serve as a potential candidate for biocontrol strategies against forest pests.

Besides, the filamentous fungi, our results revealed the occurrence of yeasts in the BB gut microbiome. The existence of yeasts symbionts (Suh et al., 2003; Lee et al., 2006) was often attributed to nutrient acquisition, detoxification of defensive plant compounds, and production of volatile organic semiochemicals in BB (Davis et al., 2011). Several yeast genera present within the core mycobiome communities include *Kusarishia, Ogataea, Yamadazyma, Candida, Cyberlindnera,* and *Cryptococcus.* The presence of *Kusarishia* and *Ogataea* was also observed in the mountain pine beetle, *D. ponderosae* (Davis et al., 2011). Interestingly, these yeasts are capable of converting *cis*- and *trans*-verbenol to verbenone, anti-aggregation pheromone (Hunt and Borden, 1990). Similarly, *Kusarishia* and *Candida* enabled such interconversion in spruce feeding *I. typographus* (Leufvén et al., 1984). Besides, some *Candida* species have been reported to produce lipases that hydrolyze the long-chain triacylglycerols and play a significant role in metabolic processes (Suh and Blackwell, 2004). The presence of such fungal species in BB gut might serve a similar function. However, such possibilities demand further experimental corroborations.

Often symbionts interact with each other to fine-tune their co-existence within the host, i.e., *Yamadazyma* and *Candida* identified in the core consortium in the present study has been shown to influence the growth of other fungal symbionts *O. montium* in *D. ponderosae* (Adams et al., 2008). The previous studies suggested that yeast isolated from *I. typographus* outcompetes the fungus *Ophiostoma* in culture. Such influence of yeast on other BB fungal symbionts could compromise the beetle performance as this blue staining fungus may act in parallel to contribute to the death of the beetle-infested trees (Furniss et al., 1990). The yeast *Cyberlindnera,* earlier reported as a common symbiont of *Dendroctonus* beetles (Rivera et al., 2009), detected in our study, can degrade starch and lipids. It can also abide by the secondary metabolites in pine and detoxify them (Briones-Roblero et al., 2017). Additionally, the presence of *Cryptococcus* observed in this study was documented previously in *Ips pini* and *Ips typographus,* having a putative role in the production of semiochemicals (Leufvén and Nehls, 1986).

The persistence of core fungal consortium in the bark beetles (BB) in the present study suggested the involvement of gut mycobiome directly or indirectly in host physiology and

performance. Nonetheless, the variation in the gut mycobiome illustrated by the β-diversity may be contributed by the factors such as different feeding habits, environmental impact due to different sampling locations and differences in BB species (**Figure 5**). The BB belonging to *Ips* and non-*Ips* species showed significant differences in their gut mycobiome. Interestingly, different conifer host feeding also shaped the gut fungal communities where *Ips* bark beetle feeding on spruce (IT and ID) were clustered in one clade similar to pine feeders, IAC and SX. It is worth to mention here that, in the present study, autochthonous mycobiota of the digestive tract of BB cannot be distinguished from the mycobiome that is associated with the diet of beetles. Furthermore, the NMDS analysis revealed that BB gut fungal diversity varied significantly based on their sampling locations and was clustered in three distinct groups. The non- *Ips* species PP collected from Kostelec clustered distantly similar to *Ips acuminatus* (IAC) collected from Libavá. The fungus feeding behavior and the influence of varying sampling location possibly placed apart IAC in a separate cluster than other *Ips* species after NMDS analysis. In contrast, separate clustering of PP may be due to the combined contribution of species and sampling location difference. Although bark beetles (BB) collected from Rouchovany were grouped, there was a distinct separation between different *Ips* species collected from the same site. This observation reflects the feeding behavior of BB on the different coniferous hosts and conserved species-specific mycobiont association within them. However, such conclusions need to be investigated further.

It is interesting to note that each of the BB in the present study represented a set of fungal biomarkers that play a crucial role in their physiology and the performance during and after tree infestation. For instance, the biomarker belonging to the fungal family Ceratocystidaceae was able to detoxify the defensive tree compounds released on a beetle or fungal attack (Hammerbacher et al., 2013). Moreover, the genera *Ceratocytosis* belonging to the family Ceratocystidaceae identified as the core member of the gut mycobiome have a broad tree host range, i.e., this fungus was pathogenic to a wide array of trees (Juzwik et al., 2008; Tsopelas et al., 2017) thereby delivers the BB with the opportunity to infest and survive on different tree hosts. Similarly, the fungal species belonging to Ophiostomataceae family was also considered as a potential biomarker contributing to nutrient acquisition through detoxification of secondary metabolites of host trees and thereby supporting in beetle development (Ayres et al., 2000; Cale et al., 2016; Wadke et al., 2016; Zhou et al., 2016). Other yeast biomarkers such as Pichiaceae and Saccharomycetaceae observed in the BB have been reported to impact the growth of other fungal symbionts in the bark beetles (Adams et al., 2008). Such yeast species were also considered to have a putative role in the biosynthesis of anti-aggregation pheromone in beetles (Hunt and Borden, 1990).

CONCLUSION

Taken together, the present study identified and explored the core mycobiome community in five *Ips* bark beetle (BB) species of economic importance. Present findings increase the current understanding of fungal composition and α-diversity in BB (five *Ips* and one non-*Ips*) gut. β-diversity analysis further pinpoints toward the importance of sampling location, host-feeding and species differences as critical contributors for shaping up the BB gut-mycobiont. Further screening the mycobiome within beetle food (phloem), decaying wood and wood from BB galleries may provide higher insight into beetle-fungus interaction. Nevertheless, the present study could be of considerable value and interest in forest entomology, providing insights into the gut-specific core fungal communities and offering a foundation for future follow ups on the functional role that core mycobiome community could have in the gut of *Ips* bark beetles.

DATA AVAILABILITY STATEMENT

The datasets generated during this study are available under NCBI Bio-project PRJNA632703 (Bio-sample accessions SAMN14917923–SAMN14917958, SRA accession PRJNA632703; https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA632703).

AUTHOR CONTRIBUTIONS

AC, FS, and AR planned the research. AC and AR conducted the experiments, data analysis, and biological interpretation of results. RM, MA, and JS collected the beetles. MA and JS dissected the beetles. AC, RM, FS, and AR prepared the manuscript. All authors approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.568853/full#supplementary-material

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Artificial Larval Diet Mediates the Microbiome of Queensland Fruit Fly

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Larval diets used for artificial rearing can have a significant effect on insect biology. The Queensland fruit fly (aka "Qfly"), *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), is one of the greatest challenges for fruit growers in Australia. The sterile insect technique (SIT) is being developed to manage outbreaks in regions that remain free of Qfly and to reduce populations in regions where this species is endemic. Factory scale rearing is essential for SIT; however, artificial larval diets are known to affect the microbiome of Qfly, which may then affect fly performance. In this study, high-throughput Illumina sequencing was used to assess the Qfly microbiome in colonies reared, for five generations from nature, on two common artificial diets (carrot and gel). At generation five (G5), the microbiome was assessed in larvae, pupae, adult males and adult females and standard fly quality control parameters were assessed together with additional performance measures of mating propensity and survival under nutritional stress. At the genus level, bacterial communities were significantly different between the colonies reared on the two larval diets. However, communities converged at Phyla to family taxonomic levels. Bacterial genera of *Morganella, Citrobacter, Providencia,* and *Burkholderia* were highly abundant in all developmental stages of Qfly reared on the gel diet, when compared to the carrot diet. Despite abundance of these genera, a greater percentage of egg hatching, heavier pupal weight and a higher percentage of fliers were found in the Qfly reared on the gel diet. Mating propensity and survival under nutritional stress was similar for adult Qfly that had been reared on the two larval diets. Overall, our findings demonstrate that the artificial larval diet strongly influences the microbiome and quality control measures of Qfly, with likely downstream effects on performance of flies released in SIT programs.

Keywords: Tephritidae, gut bacteria, Illumina sequencing, development, domestication, mating, stress tolerance, artificial diet

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INTRODUCTION

Insects brought into the laboratory from nature and reared over multiple generations are confronted by a new environment that is very different from nature, and are exposed to significant selection pressures that lead to laboratory adaptation ("domestication") (Chambers, 1977; Hoffmann et al., 2001). In tephritid fruit flies, adaptation to artificial rearing conditions has

been reported to have significant influence on genetic diversity and numerous life history traits,including development, stress tolerance and reproductive behavior. Mass reared fruit flies tend to mature at a younger age than wild type flies and may have reduced sexual competitiveness or compatibility with wild populations and reduced environmental tolerance (Gilchrist et al., 2012; Zygouridis et al., 2014; Schutze et al., 2015; Pérez et al., 2018). These changes, resulting from domestication, are anticipated to have important implications for the success of the sterile insect technique (SIT), an environmentally benign pest management technique in which millions of sterile insects are released to induce reproductive failure in females of pest populations (Knipling, 1955; Hendrichs et al., 1995; Vreysen et al., 2006).

Tephritid fruit flies are amongst the world's most economically damaging insect pests (Norrbom et al., 1999). SIT has proven an effective means to manage some of the most economically damaging fruit flies including Mediterranean fruit fly (Medfly) *Ceratitis capitata* (Wiedemann) (Reyes et al., 2007), melon fly *Zeugodacus cucurbitae* (Coquillett) (Kakinohana, 1994; Yosiaki et al., 2003), Oriental fruit fly *Bactrocera dorsalis* (Hendel) (Orankanok et al., 2007), and Mexican fruit fly *Anastrepha ludens* (Loew) (Orozco-Dávila et al., 2015). In Australia, SIT has been implemented to eradicate outbreaks of Queensland fruit fly (Qfly) *Bactrocera tryoni* (Froggatt) in regions where this species has not yet established, and to suppress established populations (Dominiak et al., 2003; Fanson et al., 2014). With increasing restrictions on the use of insecticides for fruit fly control due to concerns about environmental and human health (Dominiak and Ekman, 2013), there is a growing need for the development of viable alternatives. There has been substantial interest and investment in development of SIT as a sustainable and environmentally benign solution.

Insects, including tephritid flies, commonly host a large diversity of microbes that can influence insect health (Jurkevitch, 2011). Microbial communities are often highly abundant in insect digestive systems (Dillon and Dillon, 2004), especially bacteria (Broderick et al., 2004; Robinson et al., 2010). In many cases, symbiotic bacteria have been found to provide nutrition that contributes to insect host fitness (Baumann, 2005; Akami et al., 2019). Microbes can provide amino acids (Nogge, 1981) and essential vitamins (Douglas, 1998), as well as nitrogen and carbon compounds (Benemann, 1973; Dillon and Dillon, 2004) to insect hosts. Some gut microbiota may have the ability to alternate between mutualism/commensalism and parasitism in response to changes in host diet (De Vries et al., 2004). Confirming the importance of the insect microbiome, elimination of resident bacteria can sharply reduce fly fitness (Ben-Yosef et al., 2008; Dinh et al., 2019).

Some microbiota may be acquired through insect diet, and diet is hence a major exogenous factor that can directly influence the composition of the insect gut microbial community and its metabolic capabilities (Chandler et al., 2011; Broderick and Lemaitre, 2012; Mason et al., 2014; Yun et al., 2014; Majumder et al., 2019). Additionally, variation in the diet nutritional composition (protein, carbohydrate and lipids) can influence both the gut microbiome biodiversity and community structure (Broderick et al., 2004; Ravenscraft et al., 2019; Woruba et al., 2019). Increasing our knowledge of these relationships may identify ways to enhance the quality of artificial diets, with the goal of improving performance in laboratory or mass-reared insects. To date, only a handful of studies have analyzed the microbiome of tephritid fruit flies reared on artificial diet (Behar et al., 2008b; Andongma et al., 2015; Morrow et al., 2015; Yong et al., 2017a; Deutscher et al., 2018; Malacrinò et al., 2018; Ventura et al., 2018; Woruba et al., 2019, Koskinioti et al., 2020). To our knowledge, however, there are no studies directly comparing the effect of different artificial larval diets on the gut bacterial community of tephritid fruit flies across the developmental stages of larvae, pupae and adult.

Larvae of the highly polyphagous Qfly develop in diverse host fruits (Hancock et al., 2000; May and Drew, 2003). In Qfly, the bacterial microbiome is largely transmitted vertically from the mother to the offspring when eggs are laid (Deutscher et al., 2018; Majumder et al., 2019). Descriptions are available characterizing the bacteria associated with wild and domesticated Qfly larvae (Deutscher et al., 2018; Majumder et al., 2019), pupae (Fitt and O'Brien, 1985) and adult flies (Thaochan et al., 2010; Morrow et al., 2015; Woruba et al., 2019). However, the effects of larval diet on changes in the Qfly microbiome through the early stages of domestication are not known. Different types of traditional solid diet, which include a biological bulking agent such as wheat meal, dehydrated carrot or lucerne chaff, have been used for rearing Qfly (Finney, 1956; Jessup, 1999; Dominiak et al., 2002, 2008). Usually, carrot-based diets have been used in moderate scale rearing and lucerne chaff-based diets used in factory-scale rearing (Jessup, 1999; Fanson et al., 2014). However factory-scale rearing and some laboratories now use a gel larval diet (Moadeli et al., 2017, 2018a,b,c, 2020). Mainali et al. (2019) found that Qfly reared on the gel diet produce better quality flies compared to solid diets containing carrot or lucerne chaff. Previous studies have assessed the bacterial populations of Qfly larvae and Qfly adults reared on a carrot diet and lucerne chaff diet (Morrow et al., 2015; Deutscher et al., 2018; Woruba et al., 2019). However, there are no studies investigating the microbial communities at all developmental stages of the Qfly reared on a gel diet compared to other larval diets, or direct comparisons through the early stages of domestication.

In the present study, high-throughput Illumina sequencing is used to investigate bacterial diversity and abundance in the microbiome of laboratory-reared Qfly at different life stages. Two Qfly colonies were established from the same wild material and were maintained through five generations of laboratory rearing separately on carrot and gel larval diets. After five generations, the microbiome was assessed, along with key quality control and performance parameters. We found that bacterial communities were significantly different between flies reared on the two larval diets, although communities converged at family taxonomic levels. Gel diets resulted in better egg hatch, heavier pupal weight and a higher percentage of fliers. Mating propensity and survival under nutritional stress was similar for the two diets. This study greatly improves our understanding of

how artificial diets affect the microbiome of laboratory reared flies, and has significant implications for factory-scale rearing in SIT programs.

MATERIALS AND METHODS

Colony Origins

Colony was established in 2017 from infested fruits (Majumder et al., 2020). Briefly, infested Pomegranate *Punica granatum*, Green Apple *Malus pumila* and Quince *Cydonia oblonga* were collected from different geographic locations in the Australian states of New South Wales (NSW) and Victoria (VIC) (**Table 1**). The infested fruits were collected from under trees, and most were over-ripe. After collection, all fruits were stored in plastic bins (60L, 447 mm× 236 mm× 663 mm, Award, Bunnings Warehouse, Greenacre, NSW, Australia) containing a 1 cm deep layer of fine vermiculite (Grade 1, Sage Horticultural, Hallam, VIC, Australia) in a controlled environment laboratory (25 ± 0.20°C, 65 ± 3% RH and 11 h: 1 h: 11 h: 1 h light: dusk: dark: dawn photoperiod). Larvae emerged from the fruit and pupated in the vermiculite. Pupae were sieved from the vermiculite, combined, and placed in a cage to emerge (Megaview BugDorm 44545, 47.5 cm× 47.5 cm× 47.5 cm, MegaView Science Co., Ltd., Taichung, Taiwan). Approximately 600 adult Qfly were obtained and from this pool 300 flies were transferred to each of two mesh cages to establish the studied colonies (Megaview BugDorm 44545, 47.5 cm× 47.5 cm× 47.5 cm, MegaView Science Co., Ltd., Taichung, Taiwan). Adult flies were provided hydrolyzed yeast (MP Biomedicals, Cat. No. 02103304) and commercial sucrose (CSR® White Sugar, Maribyrnong, VIC, Australia) separately, and water through a moist sponge.

Colony Maintenance

We reared the two Qfly colonies on different artificial larval diets; carrot and gel (Moadeli et al., 2017; Mainali et al., 2019) (see **Supplementary Tables S1, S2** for details, **Supplementary Figure S1**). The carrot diet was prepared by mixing all ingredients using a food mixer (KitchenAid, Model No: 5KSM150PSAER, United States) for 15 min (5 min slow and 10 min fast cycles) and kept at room temperature for 12–24 h before use. The gel diet was prepared as in Moadeli et al. (2017), by mixing all the dry ingredients using a blender (Kenwood, Multipro FPM810 series, China) for 5 min. Water was mixed with agar and the solution boiled. The boiled agar and the dry mixture were then mixed together. We transferred 150 g of carrot diet and 150 mL of gel diet into larvae rearing containers (17.5 cm long, 12 cm wide and 4 cm deep) (Castaway food packaging, Arndell Park, NSW, Australia).

At each generation, eggs were collected using an oviposition device comprising a 300 mL semi-transparent white soft plastic bottle (low density polyethylene). The oviposition device had numerous ∼1 mm holes through which females could oviposit, and contained 20 mL of water to maintain humidity and a few drops of natural apple juice to attract the female flies and encourage egg laying (Collins et al., 2008). Eggs were collected from 14–16 day old mature flies between 9 am and 3 pm on a single day. The oviposition device was rinsed with distilled water to wash out the eggs. The eggs were then collected using a 50 mL falcon tube and 250 μL of eggs in suspension which was transferred to the larval diet using a 1000 μL pipette (ca. 3500 eggs, ca. 23 eggs per gram of diet) (Moadeli et al., 2017). The larval rearing containers were then covered with plastic lids until the larvae reached their third instar and exited the diet to pupate. The rearing trays were then placed in a container with a 1 cm layer of fine vermiculite on the bottom. The larvae exited the rearing container and landed in the vermiculite where they pupated. Pupae were collected after sifting them from the vermiculite. In order to establish the two colonies, approximately 2000 pupae from each diet were placed in a mesh cage (Megaview BugDorm 44545, 47.5 cm× 47.5 cm× 47.5 cm, MegaView Science Co., Ltd., Taichung, Taiwan) for emergence and the rearing protocol was followed for producing subsequent generations until G5. From the colony (G5) of each type of diet, third instar larvae (N = 12), 8 days old pupae (N = 12) and 15 days old sexually mature male (N = 12) and female adult flies (N = 12) were collected for sequencing.

Sample Preparation

For sample processing, Qfly larvae, pupae and adult flies (male and female separately) from the G5 colonies were surface sterilized using 0.5% Tween 80 (Sigma-Aldrich, St. Louis, MO, United States, Cat. No. 9005656), 0.5% Bleach (Sodium hypochlorite) (Sigma-Aldrich, St. Louis, MO, United States, Cat. No. 7681529) and 80% Ethanol (Sigma-Aldrich, St. Louis, MO, United States, Cat. No. 65175) for 30 s, and rinsed 3 times in 1M sterile phosphate-buffered saline (1x PBS) again for 30 s. The PBS from the 2nd and 3rd washes were kept and 100 μL spread-plated on to five types of microbial growth medium (de Man, Rogosa and Sharpe Agar, Tryptone Soya Agar, MacConkey Agar, Potato Dextrose Agar and Yeast-dextrose Agar medium) (Sigma-Aldrich, St. Louis, MO, United States) to check the performance of the sterilization method. All plates were

TABLE 1 | Fruit types and origin for wild *Bactrocera tryoni* larvae collection.

Geographic location of collection	Fruit source and number of fruits collected	Collection date
Coomealla, NSW GPS: Lat. 34° 5′50.97″, Long. 142° 3′7.21″	Pomegranate 37 pieces	5/05/17
St. Germain's, Between Tatura and Echuca in Victoria GPS: Lat. 36°10′48.86″, Long. 145° 8′50.74″	Green Apple 41 pieces	05/05/17
Downer road between Tatura and Toolamba in Victoria GPS: Lat. 26°38′34.92″, Long. 152°56′22.99″	Quince 52 pieces	05/05/17

A total of six replicate larvae, and fruit flesh samples were collected from each fruit origin.

TABLE 2 | Taxonomic identification of the of the 14 most abundant bacterial genus in the Qfly from carrot and gel diet.

Phylum	Class	Order	Family	Genus	% Carrot diet	% Gel diet
Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Arthrobacter*	0.00	3.14
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*	12.02	19.55
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	2.35	0.57
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*	10.36	2.06
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Swaminathania/Asaia*	36.40	0.00
Proteobacteria	Gammaproteobacteria	Orbales	Orbaceae	*Orbus*	1.73	0.00
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unassigned_Enterobacteriaceae	7.93	4.00
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Citrobacter*	1.91	4.99
Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Vagococcus*	4.40	1.89
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Kluyvera*	5.77	11.19
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Morganella*	0.00	24.98
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*	2.75	3.44
Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	11.79	14.32
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Providencia*	0.00	6.70

Percentage of the carrot and gel diet represents the percentage of the average relative abundance of each genus present in the Qfly sample from the two different diets.

incubated at 32 and 35°C for 24 to 48 hr (Majumder et al., 2019, 2020). Immediately after the sterilization process, the guts of adult flies were dissected using a stereomicroscope (Leica MZ6 stereo-microscope, Leica®, Wetzlar, Germany). Using sterile pestles, larvae, pupae, and dissected guts from the adults were homogenized separately in a solution of Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, United Kingdom, Lot # 1656503) and 20% Glycerol (Sigma Aldrich, St. Louis, MO, United States, Lot # SHBG2711V) and each sample was stored in a separate cryovial tube (Simport Scientific, Saint-Mathieu-de-Beloeil, QC, Canada). All the samples are preserved at −80°C. All procedures were completed in a sterile environment (Biological air clean bench, safe 2020 1.2, Thermo Scientific, Dreieich, Germany).

Microbiome Profiling

DNeasy Power Lyzer Power Soil Kit-100 (Qiagen, Hilden, Germany, Cat. no. 12855-100) was used for the DNA extraction following the manufacturer's protocol. DNA extracts were then quantified in the Invitrogen™ Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, Eugene, OR, United States). PCR amplification and sequencing were performed by the Australian Genome Research Facility, University of Adelaide, Plant Genomics Centre, Hartley Grove, URRBRAE, SA 5064, AU. For bacterial identification, the V1-V3 16S rRNA region was amplified using primers 27F (5′AGAGTTTGATCMTGGCTCAG-3′) and 519R (3′ GWATTACCGCGGCKGCTG-5′) (Lane, 1991) as used previously in Majumder et al. (2019, 2020). Reactions contained 1X AmpliTaq Gold 360 mastermix (Life Technologies, Eugene, OR, United States), 0.20 μM of each forward and reverse primer and 25 μL DNA. PCR cycling conditions consisted of denaturation at 95°C for 7 min, 35 cycles of 94°C for 45 s, 50°C for 60 s and 72°C for 60 s, and a final extension of 72°C for 7 min. A second PCR was used to adhere sequencing adaptors and indexes to the amplicons. Primestar max DNA Polymerase was used to generate a second PCR amplicon (Takara Bio Inc., Shiga, Japan; Cat. No. #R045Q). The resulting amplicons were measured using a fluorimeter (Thermo Fisher Scientific, North Ryde, NSW, Australia) and normalized (Fouts et al., 2012). The normalized samples were pooled and quantified by qPCR prior to sequencing (Kapa qPCR Library Quantification kit, Roche, Basel, Switzerland). The resulting amplicon library was then sequenced on the Illumina MiSeq platform (San Diego, CA, United States) with 2 × 300 base pairs paired-end chemistry (Caporaso et al., 2010).

Sequence Data Processing

The Greenfield Hybrid Amplicon Pipeline (GHAP) was used to process bacterial 16s rRNA amplicon sequences (Greenfield, 2017; Sutcliffe et al., 2018). The GHAP is a publically available amplicon clustering and classification pipeline[1] (Greenfield, 2017) built around tools from USEARCH (Edgar, 2010) and the Ribosomal Database Project (RDP) (Maidak et al., 1996), combined with locally written tools for demultiplexing, trimming and generating OTU (Operational Taxonomic Unit) table. This hybrid pipeline produces a table of taxonomically assigned OTUs and their associated reads counts across all samples. First, the amplicon reads were demultiplexed and trimmed, and the read pairs were then merged (using *fastq_mergepairs*) and de-replicated (using *fastx_uniques*). The merged reads were then trimmed again and clustered at 97% similarity (using *cluster_otus*) to generate OTUs. Representative sequences from each OTU were then classified both by finding their closest match in a set of reference 16S sequences (using *usearch_global*), and by using the RDP Naïve Bayesian Classifier. The pipeline mapped the merged reads back onto the classified OTU sequences to get accurate read counts for each OTU/sample pairing and generated an OTU table complete with taxonomic classifications and species assignments. The OTU table was then summarized over all taxonomic levels, combining the counts for identified taxa across all OTUs. The pipeline finally

[1]https://doi.org/10.4225/08/59f98560eba25

classified all the merged reads using the RDP Classifier, regardless of whether they were assigned to an OTU. This last step was done to provide confidence in the clustering and OTU formation steps by providing an independent view of the community structure.

All OTUs that were assigned to "Mitochondria" at the Order level were removed from the dataset before downstream processing. The above biome table was rarefied to 10,000 reads per sample, repeating this 50 times and averaging the counts to obtain a representative rarefaction to maintain equal sequence depth among all samples. This was achieved using an in-house python script. Those samples with <10,000 reads were excluded. The data were then normalized as the percentage of relative abundance, and are henceforth referred to as the OTU table (**Supplementary File S1**). All the figures of bacterial relative abundance at different developmental stages and between generations in colonies reared on different diets were plotted in Prism 8 [version 8.0.1 (145), GraphPad software, Inc] as used previously in Majumder et al. (2019, 2020). The Illumina sequence data were deposited in NCBI database under Bioproject PRJNA647614.

Quality Control Measures

Quality control assessment was based on standard procedures (Collins et al., 2008; FAO/IAEA/USDA, 2014; Moadeli et al., 2017; Adnan et al., 2018; Mainali et al., 2019).

Egg Hatching

A 100 μL pipette was used to collect the eggs which were counted under a stereomicroscope (Leica MZ6, Wetzlar, Germany) on moistened black filter paper using a soft paintbrush to move the eggs. Five sets of 50 eggs from G5 of each Qfly colony were transferred onto a 1 cm × 3.5 cm strip of moistened black filter paper. Twenty-five milliliters of gel diet and 25 g of carrot diet were poured separately into 90 mm Petri dishes (see **Supplementary Figure S1**) with five replicates for each diet. The filter paper with the eggs was laid on top of the larval diet. Petri dishes were covered and kept in the controlled environment room. Four days later, the number of the unhatched eggs was counted. The percentage of egg hatch was calculated as {[N of egg hatched/(N unhatched + N hatched)] × 100}. The data from egg hatching rate were used to calculate pupal recovery rate.

Pupal Recovery

Twenty-five milliliters of gel diet and 25 g of carrot diet were poured separately into 90 mm Petri dishes with five replicates for each diet. Eggs were collected and handled as described above. We counted 50 eggs and immediately transferred them to the diet surface. All Petri dishes were placed in the laboratory environment with lids on. The lids were removed when larvae were ready to pupate. Plates were then placed into separate plastic containers (12 L) containing a 1 cm deep layer of fine vermiculite (Grade 1, Sage Horticultural, Hallam, VIC, Australia) and with a mesh lid. The pupae were collected every 2 days. Pupal recovery was calculated as the total number of pupae divided by the number of hatched eggs multiplied by 100.

Pupal and Adult Weight

A microbalance (Sartorius ME5, Germany) was used to assess weight of pupae and adults. Thirty pupae were collected from each colony and individually weighed on the ninth day from the day of first pupation and mean weight was calculated. To assess adult fly weight, approximately 300 pupae from each colony were placed in a mesh cage (Megaview BugDorm 44545, 47.5 cm× 47.5 cm× 47.5 cm, MegaView Science Co., Ltd., Taichung, Taiwan) for emergence. No food or water were supplied in the cages. Few flies emerged on the first day and these were discarded. On the second day of emergence, the pupae were transferred to new cages and flies that emerged over the following 2 h were collected and sexed. Thirty adult male and 30 adult females were collected from each colony, placed in a −20°C freezer and individually weighed to calculate the mean weight.

Flight Ability

Adult fly emergence, percentage of fliers, the rate of fliers and the proportion of male flies was assessed using standard flight ability assays (Moadeli et al., 2017). Two days before fly emergence, 100 pupae (8 days old, five replicates from each diet) were placed separately in 55 mm Petri dishes without lids. The dishes containing the pupae were placed in the center of 90 mm Petri dish lids with black filter paper on the base. A black 100 mm tall acrylic flight ability tube (89 mm external diameter, 84 mm inner diameter) was placed over the 90 mm Petri dish lid. Fine talcum powder on the tube interior prevented the emerged flies from walking out. The whole setup with pupae was placed in a mesh cage (dimensions 32.5 cm× 32.5 cm× 32.5 cm size, Megaview BugDorm- 43030F, MegaView Science Co., Ltd., Taichung, Taiwan) in the laboratory beneath 20-watt fluorescent lights (ca. 1250 lx at the top and ca. 900 lx at the base of the flight ability tubes). A flyback tube was placed 6 cm away from the flight ability tube. During fly emergence, we removed the flies that flew out from the tube every day in the morning and afternoon to minimize flyback. All collected flies were stored in a −20°C freezer for later assessment. We collected and counted the flies until all emergence was complete (4–5 days) and categorized them as; (1) not emerged (inside unopened pupal case); (2) partially emerged (a portion of adult body remaining stuck in the puparium); (3) deformed (fully shed the pupal case but with deformed or damaged wings or appendages); (4) non-fliers (morphologically normal flies in the flight tube minus the number of flies in the flyback tube); (5) fly-back (the number of flies inside the fly-back tube plus the same number of normal flies inside the flight tube); and (6) fliers (the number of flies that were collected from outside the tubes plus fly-back). Assessed metrics included:

- *Percentage of adult emergence:* [N pupae – (N not emerged + N partially emerged)/N Pupae] × 100.
- *Percentage of fliers:* [N pupae – (N not emerged + N partially emerged + N deformed + N non-fliers)/N pupae] × 100.
- *Rate of fliers:* percentage of fliers/percentage of emergence × 100.

Mating Performance

For mating trials, approximately 400 pupae from each colony were placed in a mesh cage (Megaview BugDorm 44545, 47.5 cm× 47.5 cm× 47.5 cm, MegaView Science Co., Ltd., Taichung, Taiwan) for emergence. After emergence, cages were supplied with water-soaked cotton wool in a 70 mL sample container. Food was provided separately as dry granular sucrose (CSR® White Sugar, Maribyrnong, VIC, Australia) and yeast hydrolyzate (MP Biomedicals, Irvine, CA, United States, Cat. No. 02103304) (3:1) on two 90 mm Petri dishes *ad libitum*. To obtain mature flies (12–16 day old) to pair with experimental flies, ca. 400 pupae from a separate laboratory colony (reared on gel larval diet for >25 generations) were placed in separate mesh cages (Megaview BugDorm 44545, 47.5 cm× 47.5 cm× 47.5 cm, MegaView Science Co., Ltd., Taichung, Taiwan) for adult emergence. Similar to flies from the experimental colonies, these flies were supplied with water-soaked cotton wool in a 70 mL sample container, dry granular sucrose and yeast hydrolyzate as food on a 90 mm Petri dish *ad libitum*. All cages of flies were sorted according to sex within 3 days after emerging by collecting and transferring individual flies in glass tubes to clear plastic 12 L cages that had a mesh-covered ca. 80 cm^2 window for ventilation. Approximately 160 flies were sorted into each 12 L cage. No calling, courting, or mating was observed in cages prior to separating the sexes.

Mating trials were conducted when flies were sexually mature (12–16 day old). Qfly mate at dusk (Tychsen and Fletcher, 1971). Four hours before the onset of dusk, 40 males and 40 females from each experimental group were placed individually in clear plastic 1.25 L containers with a mesh-covered window (ca. 28 cm^2) for ventilation. Each fly was individually paired with a sexually mature (12–16 day old) fly of the opposite sex. Virgin flies of this age fed a diet of sugar and yeast hydrolyzate show a high level of sexual receptivity (Vijaysegaran et al., 2002; Pérez-Staples et al., 2007). Periodic observations were carried out after pairs were set up, and continuous observations began 90 min prior to the onset of dusk. The time of onset of copulation for each mating pair was recorded to assess mating latency (time from the start of dusk until the onset of mating, in minutes) and observations continued until the last pair had separated to assess mating duration for each mating pair.

Survival Under Nutritional Stress

Pupae were placed in separate mesh cages (Megaview BugDorm 44545, 47.5 cm× 47.5 cm× 47.5 cm, MegaView Science Co., Ltd., Taichung, Taiwan) for adult emergence. Few flies usually emerge on the first day of emergence, and these were discarded. On the second day of emergence, pupal trays were transferred to new cages and flies that emerged in the following 2 h were used for the study. Forty females and 40 males were placed in individual, 5 mL round bottom plastic vials (Lab Australia Pty Ltd., NSW, Australia). Flies were given no access to food or water after being placed in the vials until death. The number of dead flies was recorded by visually inspecting the vials every 3 h. Flies were considered dead when they were incapable of holding onto the inner surface of the plastic vial, and when no movement of their legs or mouthparts was observed after the vials were gently flicked with a finger. Dead flies were removed at each assessment.

Statistical Analysis

Microbiome Analysis

The OTU table was imported into Primer-E v7 for analysis as described in Clarke and Ainsworth (1993); Sutcliffe et al. (2017), Majumder et al. (2019, 2020). In brief, all statistical testing was performed on fixed factors associated with developmental stage and sex (larvae, pupae, adult male and female) from which 12 replicates were collected. The DIVERSE function was used to generate univariate biodiversity metrics, species richness, Pielou's evenness and Shannon's and Simpson's biodiversity indices. Statistical differences between these metrics were assessed in JMP Statistical Software Version 10.0.0 (SAS Institute, Cary, NC, United States) using one-way analysis of variance (ANOVA) and Tukey-Kramer *post hoc* analysis. The Operational Taxonomic Unit (OTU) table was first log transformed using Primer-E v7 to observe the taxonomic compositional changes for the bacterial communities. A Bray-Curtis similarity matrix was derived from this transformed data and a permutation analysis of variance (PERMANOVA) pairwise comparison was conducted to compare all community samples. A p-value of <0.05 was considered statistically significant. Further, ordination plots of these communities were visualized using principal coordinates analysis (PcoA) in Primer-E.

We performed ANOVA and *post hoc* Tukey-Kramer tests to determine whether significant differences occurred in the relative abundance of bacterial communities at all developmental stages of the Qfly, from carrot and gel diet reared colonies. We used Benjamini–Hochberg to correct for multiple testing false-discovery rate (FDR) and an alpha threshold of 0.05 on FDR corrected ANOVA p-values was used to determine significance.

Quality Control, Mating and Survival Under Nutritional Stress

All analyses were performed using JMP statistical software (Version 10.0.0, SAS Institute, Cary, NC, United States). Qfly quality control measures were analyzed with ANOVA and pair-wise Student's t-tests. Prior to the analysis, distribution patterns were observed for all quality control data. Figures of quality control measures were plotted using Prism 8 software (1995–2018 GraphPad software, Inc., United States).

Mating probability (binary outcome) was assessed using nominal logistic regression with significance tested using likelihood ratio tests (G-test). Main effects included in the model were diet (nominal) and sex (nominal). Model parameter estimates were inspected to identify effects. Mating latency, mating duration and survival under nutritional stress (continuous outcomes) were analyzed for each treatment using least squares regression (2-way ANOVA) including diet (nominal) and sex (nominal). Initially, all interactions were included, and a backward model selection process was performed by removing non-significant interactions.

RESULTS

16s rRNA Sequence Reads and OTUs

We sequenced the bacterial microbiome of 96 Qfly samples from G5 reared on the carrot and gel diet. This included larvae (N = 12), pupae (N = 12), adult males (N = 12) and adult females (N = 12) from each diet. Among them, 79 were retained after quality control and rarefaction at 10,000 reads per sample (9 and 8 samples were removed from carrot and gel diet, respectively). After rarefaction and quality control, a total of 287 bacterial OTUs were detected across the 79 samples (**Supplementary File S1**).

Gut Bacterial Diversity

Bacterial alpha diversity metrics, species richness and Shannon biodiversity indices, were calculated for each Qfly developmental stage from the two larval diets (**Figures 1A,B**). Biodiversity metrics were insensitive to larval diet, with no significant differences at any developmental stage (**Figures 1A,B**). In contrast, beta diversity showed significant differences between diets at all developmental stages (PERMANOVA; $p < 0.05$) (**Supplementary File S1**). Principal coordinates analysis (PCoA) of Bray-Curtis similarity matrix was plotted to visualize variation in bacterial communities (**Figure 2**). Based on the PCoA ordination plot, we inferred that each developmental stage

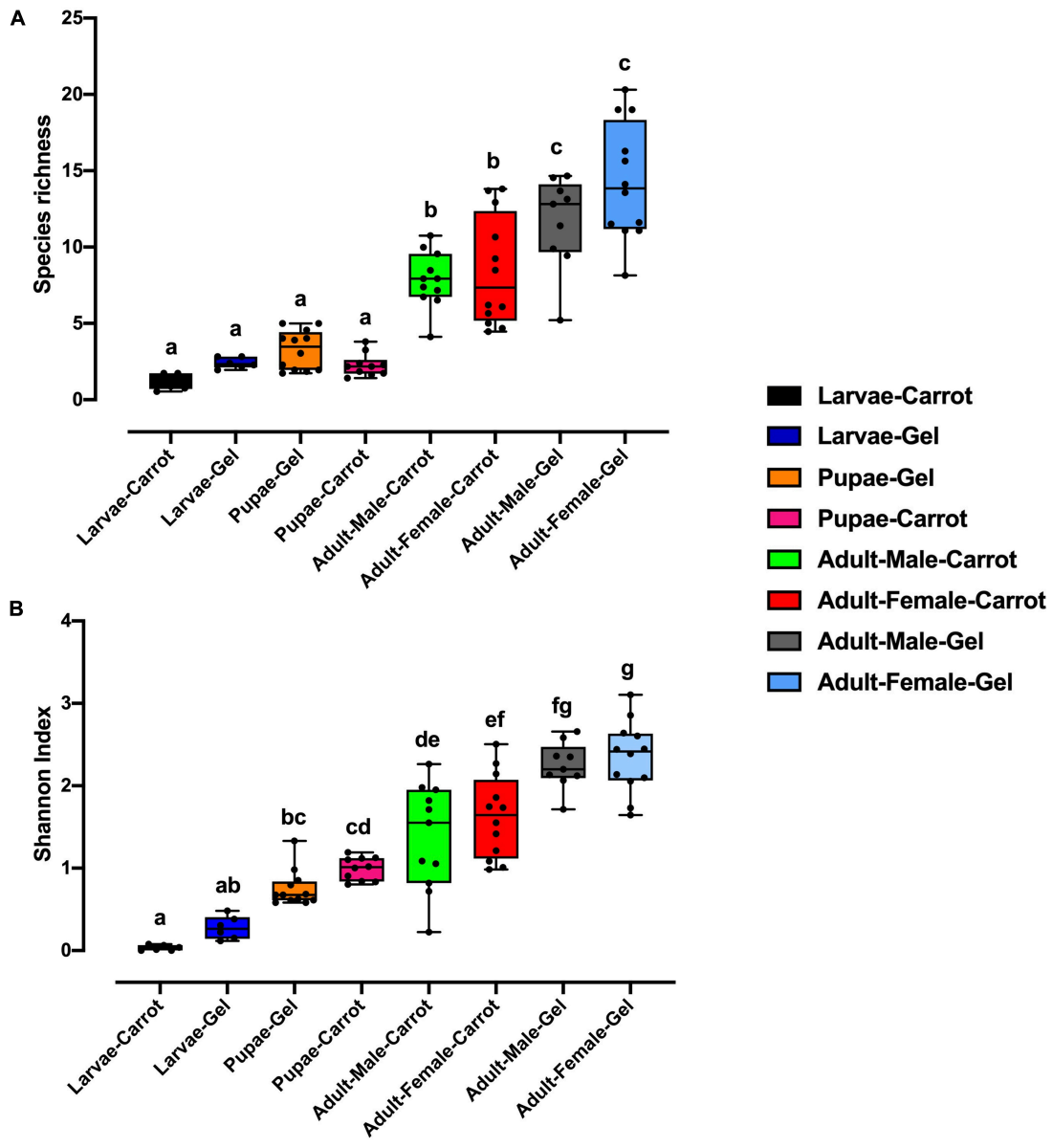

FIGURE 1 | Alpha diversity of the bacterial microbiome of the *B. tryoni* developmental stages at G5 reared on two different artificial diets, **(A)** Species richness **(B)** Shannon index. Different letters indicate significant Tukey's *post hoc* comparisons ($P < 0.05$).

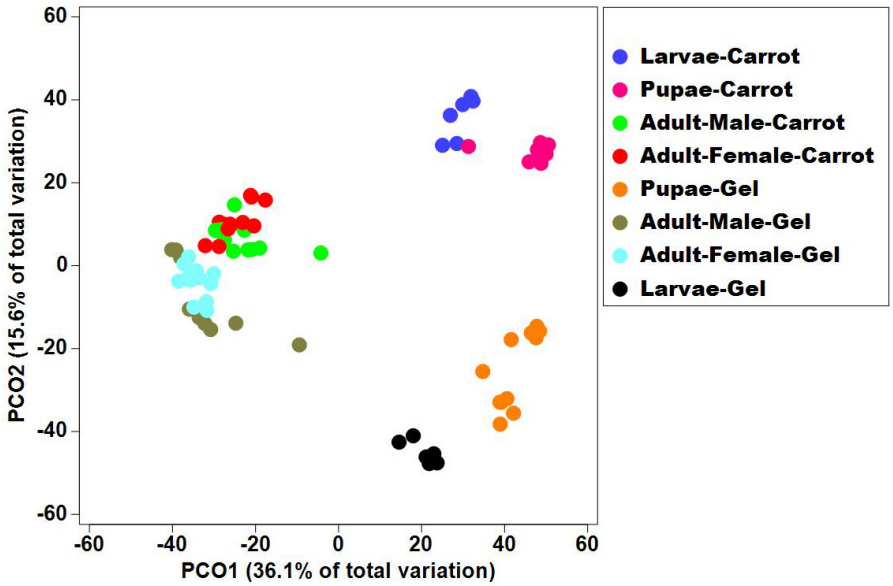

FIGURE 2 | Principal co-ordinate analysis of the bacterial communities in the Qfly developmental stages at G5 reared on carrot diet and gel diet.

of the Qfly, both from carrot and gel diet, had a distinct microbiota population. In the PCoA scatter plot, PCO1 captured 36.1% of the total variance in the dataset and corresponded with the separation of larval and pupae samples from those of the adult (**Figure 2**). The second axis, PCO2 captured a further 15.6% of variance in the data, and corresponded with the separation of communities associated with Qfly reared on different diets (**Figure 2**).

Bacterial Communities of Qfly From Carrot and Gel Based Artificial Diets

The bacterial taxa detected in the larval, pupal and adult (both male and female) stages of Qfly reared on the carrot and gel diet represented a total of 5 phyla, 12 classes, 56 families, and 115 genera (**Supplementary File S1**). At the phylum level, we detected very little difference between the two diets, or across developmental stages. For example, Proteobacteria represented ∼ 90% of all microbial communities. One exception to this was the decrease of Actinobacteria in larvae, with an average relative abundance of 3.8% for those reared on the carrot diet, compared to 0.05% on the gel diet.

We observed strong taxonomic trends at the family and genus level when comparing the two diets across developmental stages. In the larval stage, the relative abundance of Enterobacteriaceae was ∼ 99% for those reared on the gel diet. In contrast, the relative abundance of this family was <0.1% for those reared on the carrot diet (**Supplementary File S1**). Instead, the microbiomes of the carrot diet fed larvae were dominated by Acetobacteraceae, which represented almost 100% of the population, but was almost undetectable in the gel fed larvae (0.01%). These trends were mirrored at the genus level, with *Morganella* and *Providencia* (Enterobacteriaceae) being significantly more abundant in the larvae reared on the gel diet compared with those feed the carrot diet (**Figures 3**, **4** and **Table 2**). *Swaminathania/Asaia* (Acetobacteraceae) accounted for ∼ 99.9% of the larval microbiome for colonies fed on the carrot diet, but was only a minor component (<1%) of microbiomes from gel fed larvae (**Figure 4**).

Morganella, Providencia, and *Swaminathania/Asaia* were also detected in the pupae and adults, and showed similar trends to those in the larvae. Specifically, *Swaminathania/Asaia* had a significantly higher relative abundance in pupae and adults reared on the carrot diet (42%) compared with those reared on gel diet (<0.01%; FDR corrected $p < 0.0001$). *Morganella* and *Providencia* were more abundant in the colony reared on the gel diet. These differences in the bacterial genera from Enterobacteriaceae were statistically significant for larvae and adults (FDR corrected $p < 0.0001$), but not for pupae.

Another notable trend within the pupal stage (**Figure 3**) was the higher relative abundance of the Micrococcaceae in the pupae from the gel diet colony (14%), compared with the carrot diet colony (<1%). As with the Acetobacteraceae, this trend was driven by a single genus, *Arthrobacter* (**Figure 4**). This genus was more abundant in the pupal developmental stage compared with the larvae, but it was also significantly more abundant in the larvae fed on a gel diet (0.5% vs. 0%, FDR corrected $p < 0.0001$, **Figure 4**). In addition, although *Burkholderia* was highly abundant in the pupal stage, significant greater abundance was detected both in adult males and females reared on the carrot diet compared to the gel diet (FDR corrected $p < 0.0001$, **Figure 4**).

In the adult stage, a number of diet-associated differences were detected that were unique to this developmental stage and/or the sex of adults (**Figure 3**). For example, in both male and female adults, *Orbus* was significantly more abundant in colonies reared

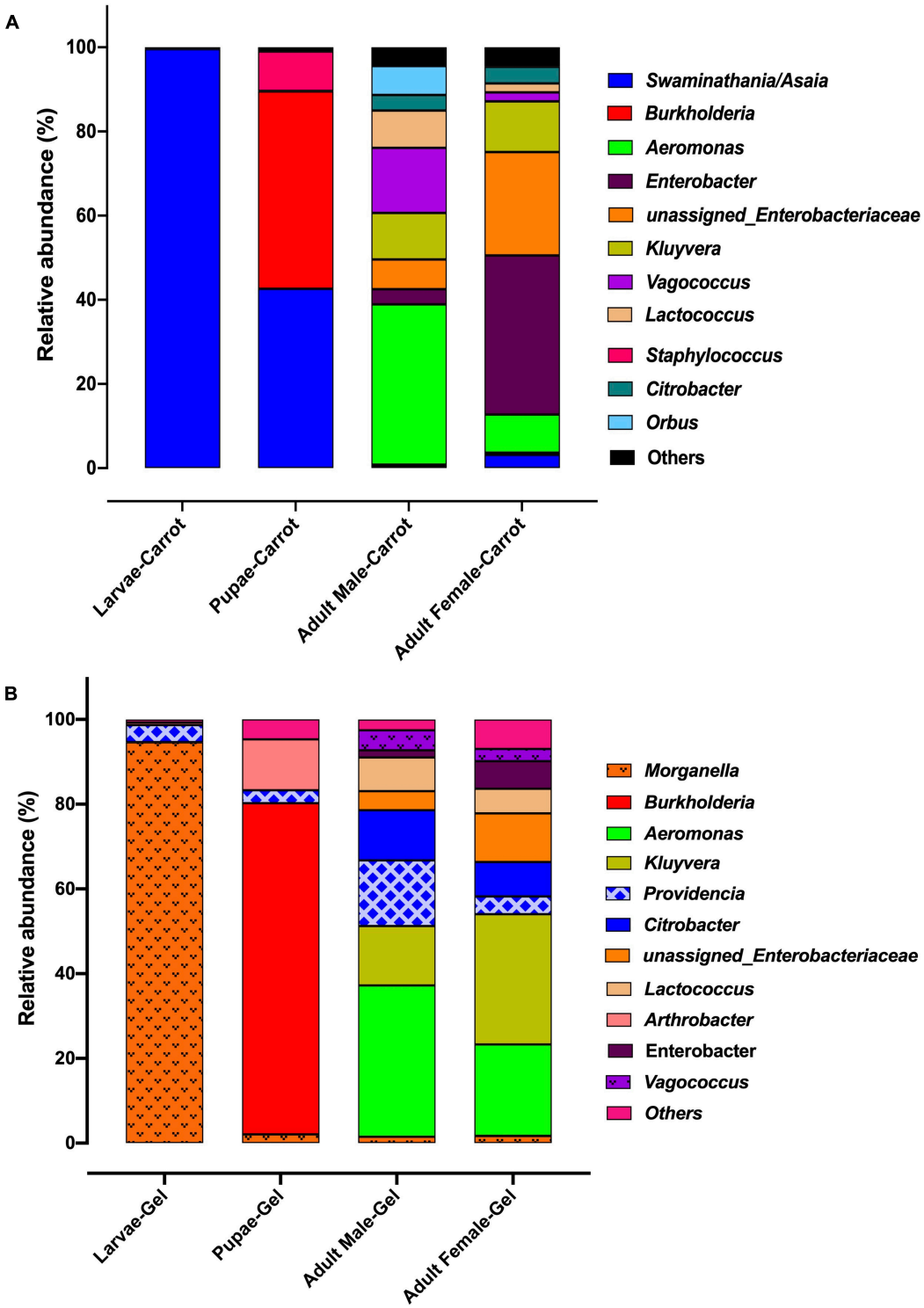

FIGURE 3 | The relative abundance of the bacterial microbiota in Qfly at different developmental stages from generation 5 reared on **(A)** Carrot based larval diet, **(B)** Gel based larval diet.

on the carrot diet (FDR corrected $p < 0.0001$, **Figure 4**). However, it is notable that this genus was much more abundant in males than in females. Several additional diet associated differences were only evident in one adult sex. For example, *Kluyvera*, *Aeromonas*, and *Erwinia* were all significantly more abundant in adult females from the gel diet colony, compared with those from

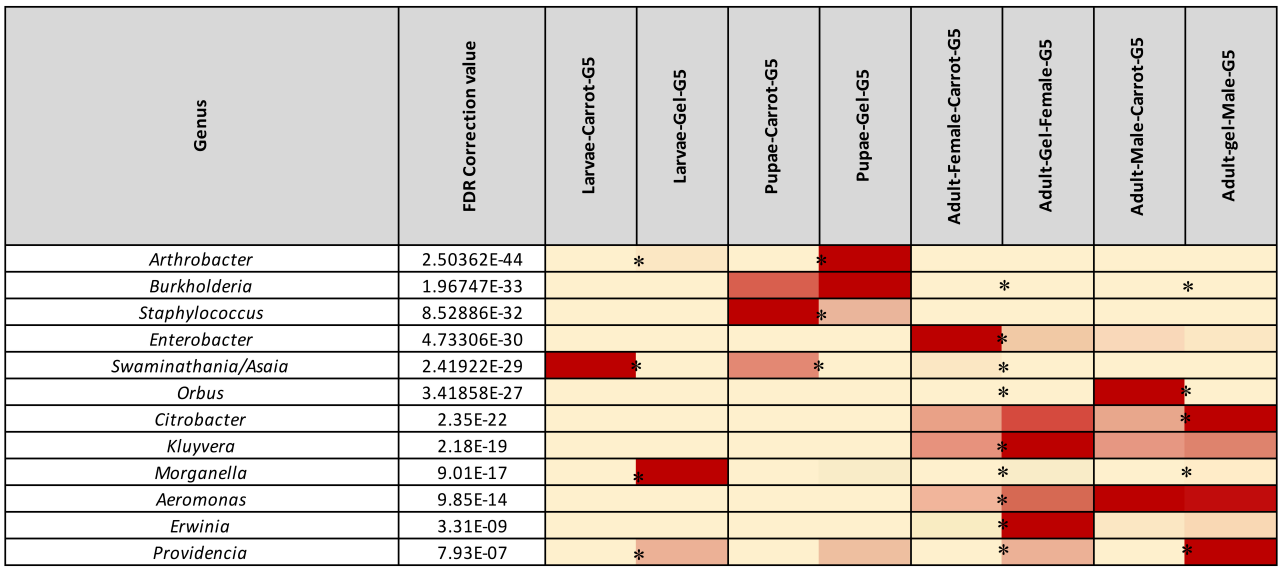

FIGURE 4 | Heatmap representing the average relative abundance of the 12 most abundant (>1%) bacterial genera of Qfly from the carrot and gel-based diet groups on 16S rRNA gene amplicon data. Relative abundances for each genus are represented by a color on a spectrum from yellow to deep red. Lowest values are highlighted yellow, highest are represented as a deep red. Asterisks (∗) indicate relative abundances of each developmental stage is significantly different between the carrot and gel diet.

the carrot diet colony, but no differences were found in males. Conversely, *Citrobacter* was significantly more abundant in adult males of the gel diet colony than the carrot diet colony, but no differences were evident in females (**Figure 4**).

Quality Control Measures

Percentage of Egg Hatch and Pupal Recovery

The percentage of egg hatching was not significantly different ($F_{1,8}$ = 2.82, p = 0.132) between the carrot diet (66.80% ± 1.86 SE) and gel diet (71.20% ± 1.86 SE). No significant difference ($F_{1,8}$ = 1.73, p = 0.225) was observed in the pupal recovery rate of the colony reared on carrot diet (58% ± 3.02 SE) and the colony reared on gel diet (63.60% ± 3.02 SE).

Weight

Pupae were significantly heavier ($F_{1,58}$ = 62.16, p < 0.001) in the colony reared on from the gel diet (14.39 mg ± 0.23 SE) than in the colony reared on carrot diet (11.72 mg ± 0.39 SE). No significant difference ($F_{1,58}$ = 0.07, p = 0.792) was found in the adult body weight of males reared on the carrot (7.91 mg ± 0.16 SE) and the gel diet (7.85 mg ± 0.16 SE). In contrast, adult body weight was significantly greater ($F_{1,58}$ = 13.89, p < 0.001) in females reared on carrot diet (9.09 mg ± 0.14 SE) compared to the gel diet (8.36 mg ± 0.14 SE).

Flight Ability

There was no significant difference ($F_{1,8}$ = 2.86, p = 0.129) in the percentage of adult emergence in Qfly reared on carrot diet (82 ± 1.67 SE) and gel diet (86 ± 1.67 SE). In contrast, the percentage of fliers was significantly higher ($F_{1,8}$ = 5.389, p = 0.0488) for Qfly reared on gel diet (80.80 ± 1.95 SE) than on carrot diet (74.40 ± 1.95 SE).

Mating Propensity

Mating propensity did not vary significantly between carrot and gel diet reared flies (G_1 = 0.02, p = 0.87). Also, there was no significant difference between the two diets in mating latency ($F_{1,51}$ = 0.66, p = 0.42) or mating duration ($F_{1,51}$ = 2.2, p = 0.14). Sex of the flies did not influence mating propensity (G_1 = 0.02, p = 0.87), mating latency ($F_{1,51}$ = 2.97, p = 0.09) or mating duration ($F_{1,51}$ = 1.63, p = 0.20).

Survival Under Nutritional Stress

Survival of the flies that were subjected to nutritional stress did not differ significantly between flies reared on the carrot and the gel diet ($F_{1,156}$ = 0.05, p = 0.82). In addition, survival under stress did not vary with the sex of flies ($F_{1,156}$ = 0.25, p = 0.62).

DISCUSSION

The present study provides a comprehensive analysis of how two common artificial diets affect the microbiome of Qfly through the early stages of domestication across all developmental stages, along with key quality control and performance parameters. In a previous study, Majumder et al. (2020) used high-throughput Illumina sequencing to identify and characterize the microbial communities present in the different developmental stages of wild type Qfly (G0) at the point of entry into laboratory rearing. In the present study, we are able to address meaningful questions regarding how microbial communities change after five generations of rearing on artificial larval diets. Artificial larval diet strongly modulated the microbial community structure (beta diversity) across all developmental

stages, but did not affect total biodiversity (as assessed by alpha diversity metrics: species richness and Shannon's diversity index). Previously, the bacterial phyla Proteobacteria and Firmicutes have commonly been reported in Qfly larvae and adults in domesticated colonies reared on a carrot diet (Deutscher et al., 2018; Majumder et al., 2019; Woruba et al., 2019), and in other fruit flies including *Bactrocera neohumeralis*, *Bactrocera jarvisi*, *Bactrocera cacuminata, Bactrocera oleae, Zeugodacus tau, A. ludens, Anastrepha obliqua, Anastrepha serpentina*, *Anastrepha striata,* and *C. capitata* (Morrow et al., 2015; Malacrinò et al., 2018; Ventura et al., 2018; Koskinioti et al., 2020; Noman et al., 2020). Our results are consistent with these previous findings. However, it is noteworthy that variations in diet and developmental stage had a limited impact on microbial community composition at the phylum level. Analyses at the family and/or genus level are far more informative of such variations in Qfly populations.

The fruit fly microbiome changes markedly across developmental stages (Andongma et al., 2015; Yong et al., 2017a,b; Noman et al., 2020). This is consistent with the magnitude of metabolic and physiological change that occurs during metamorphosis. Despite the substantial changes in the overall microbiome across developmental stages, some bacterial families and genera showed consistent trends at all developmental stages when comparing artificial diets. Specifically, the gel diet was associated with an increased relative abundance of the Enterobacteriaceae genera *Morganella* and *Providencia*, while these taxa were almost non-existent in colonies reared on the carrot diet (**Figure 3**). Conversely, the carrot diet resulted in an increased abundance in the Acetobacteraceae genus *Swaminathania/Asaia*, which was found only at very low abundance in the colonies reared on the gel diet. Previous studies of wild Qfly larvae (Deutscher et al., 2018; Majumder et al., 2019, 2020) and adults (Woruba et al., 2019; Majumder et al., 2020) suggested that Enterobacteriaceae and Acetobacteraceae are naturally abundant in the Qfly microbiome. In contrast, the response of some taxa to diet was limited to a specific developmental stage. This included the bacterial genera *Orbus* and *Enterobacter*, which were highly abundant in adult males and females, respectively, from the carrot diet colony. Conversely, *Kluyvera, Aeromonas,* and *Erwinia* were highly abundant in adult males from the gel diet colony. Similarly, Woruba et al. (2019) found that field-collected male and female Qfly had significant differences in bacterial diversity and in bacterial composition. In contrast, we observed that the bacterial community of larvae reared on a carrot and gel diet were clustered at a substantial distance in PCoA ordination plot (**Figure 2**). A possible explanation is that the main ingredient of the diet was plant based in both nature and the carrot diet reared laboratory colonies.

Diet is a key factor influencing the gut microbiome of fruit flies including *Bactrocera, Ceratitis, and Anastrepha* (Malacrinò et al., 2018; Ventura et al., 2018; Andongma et al., 2019; Majumder et al., 2019). Diet effects on the microbiome are also commonplace in other insects, for example: cotton bollworm (*Helicoverpa armigera*) (Xiang et al., 2006), the ground dwelling beetle (*Coleoptera*) (Kudo et al., 2019), gypsy moth (*Lymantria dispar*) (Broderick et al., 2004) and *Drosophila* (Colman et al., 2012). In our study, the starting material, rearing environments, adult diet and the generations were the same for the colonies reared on the two tested larval diets. This single difference of larval diet resulted in substantial variation in the bacterial composition across all developmental stages. In both the carrot diet and the gel diet, the antimicrobial agents of sodium benzoate and citric acid were common (Mainali et al., 2019), but the antifungal Nipagin (methylparaben) was only used in gel diet (Moadeli et al., 2017). Moreover, the yeast concentration used in the gel diet was almost double that of the carrot diet (see **Supplementary Tables S1, S2**). Yeast and yeast like fungi are key sources of amino acids for developing larvae (Martin, 1987; Nardon and Grenier, 1989; Vega and Blackwell, 2005; Moadeli et al., 2018c). Previous studies found that Enterobacteriaceae support metabolic activities in *C. capitata* and *B. oleae* larvae and support nitrogen fixation and pectinolysis (Behar et al., 2008b; Ben-Yosef et al., 2015). It might be that larvae reared on the gel diet consumed more yeast and that the high abundance of Enterobacteriaceae was needed for protein hydrolysis (Pavlidi et al., 2017). Additionally, the gel diet had added cane sugar, while in the carrot diet, sugar was only as naturally occurring sucrose and glucose. This difference in sugar content of larval diets could underlie the presence of *Swaminathania/Asaia*. Acetobacteraceae has been reported to support break down and digestion of complex glucose structure and lipid content of the larval diet in *Drosophila* (Huang and Douglas, 2015). Before the domestication process began, the wild type larvae fed on host fruits. *Swaminathania/Asaia* would have likely been transferred with the larvae used to establish colonies and then become abundant in the larval microbiome of the colony reared on the carrot diet. Deutscher et al. (2018) and Majumder et al. (2019, 2020) made similar observations. We suggest that bacteria from the Acetobacteraceae may be needed for the digestion of complex carbohydrate and are not needed with gel-based artificial diet, which contains only cane sugar, hence their absence. Bacterial taxonomic composition in the carrot diet reared colony was similar to that of wild flies. In contrast, bacterial taxonomic composition was highly altered across all developmental stages of the Qfly reared on the gel based larval diet. It could also be that the presence of nipagin in the gel larval diet, in addition to sodium benzoate and citric acid, which is present in both larval diets, significantly alters the bacterial community.

The bacterial genus *Providencia* was abundant in all developmental stages of the Qfly reared on the gel diet but was absent in the carrot diet reared Qfly. *Providencia* is a gram-negative opportunistic, non-spore forming genus that is often pathogenic (O'Hara et al., 2000), and has also been observed and isolated from many other fruit fly species including *A. ludens* (Kuzina et al., 2001; Ventura et al., 2018), *A. obliqua, A. serpentina*, *A. striata* (Ventura et al., 2018), *B. oleae* (Kounatidis et al., 2009; Koskinioti et al., 2020), and *Z. tau* (Noman et al., 2020). Also, *Providencia* has been identified in domesticated *C. capitata* (Guerfali et al., 2018) and reported to cause infection. However, to date, there is no evidence of any pathogenic effect

of *Providencia* in Qfly. Also, *Morganella* was highly abundant in the larval stages (∼99%) and less than 2% in other life stages of the Qfly fed on the gel diet, but was absent in the carrot diet fed Qfly (**Figure 3**). *Morganella* was first identified by Fulton (1943) and included in the family Enterobacteriaceae by Brenner et al. (1978). Salas et al. (2017) observed *M. morganii* as a lethal pathogenic bacterium in domesticated *A. ludens* larvae. Additionally, this pathogenic bacterial species has been detected in wild Qfly adults (Woruba et al., 2019) and in the larval stage of *Z. tau* (Noman et al., 2020). We found the same bacterial genera present in the larval microbiome from the Qflies reared on the gel diet. Although the gel diet contained more antimicrobial agents, *Morganella, Enterobacter, Citrobacter, Providencia* and *Burkholderia* were all detected at higher abundances in the Qfly fed the gel diet when compared to the carrot diet (**Figure 3**). *Morganella, Citrobacter,* and *Providencia* have been identified previously in wild Qfly adult (Woruba et al., 2019). We propose that, through continuous rearing on the same larval diet, these bacteria may develop a symbiotic relationship with the host and even contribute positively to the quality of Qfly across all developmental stages during laboratory rearing. We hypothesize that these bacterial genera might be controlled in part by the host genetics along with nutritional components present in the gel based larval diet (Noman et al., 2020). Other bacteria, found across all developmental stages of the Qfly fed on the gel diet, might follow the same trend. Additionally, given that these bacteria are found in the wild Qfly (Woruba et al., 2019), we hypothesize that these sometimes pathogenic bacteria genera may often be abundant as benign gut residents.

The relationship between the insect and its symbionts may be beneficial or harmful to the host health and fitness, and this depends on the overall microbiome composition (Kaufman et al., 2000; Marchini et al., 2002; Feldhaar, 2011; Hammer et al., 2017). Symbiotic and endosymbiotic bacteria can be important sources of nutrients to their host insects (Behar et al., 2005; Behar et al., 2008a). Further, the nutritional composition of the larval diet including yeasts and sugar, fatty acids and minerals has a strong influence on fruit fly development (Krainacker et al., 1987; Vargas et al., 1994; Aluja et al., 2001; Plácido-Silva et al., 2006; Moadeli et al., 2018a,b,c, 2020). In fruit flies, larval, pupal and adult body weight are commonly used measures of quality (Sharp et al., 1983; Churchill-Stanland et al., 1986; Fanson et al., 2014). We found that pupal weight was higher in Qfly from the gel diet. Pupal weight has been considered a key quality parameter in mass rearing of the Qfly (Dominiak et al., 2010). Greater pupal weight is generally expected to correspond to larger, healthier, adults (Mohamed et al., 2016). Our study is consistent with Mainali et al. (2019) who reported that the gel diet produced flies with better flight ability performance than the carrot diet. Moadeli et al. (2018c) reported that brewer's yeast present in the gel diet is better for larval development than torula yeast that is used in carrot diet.

In tephritid flies, numerous studies have demonstrated that gut bacteria are associated with digestion, detoxification, immune response, metabolism, sexual behavior, reproduction, and survival (Behar et al., 2005; Hosokawa et al., 2007; Ben Ami et al., 2010; Augustinos et al., 2015; Ben-Yosef et al., 2015; Akami et al., 2019). Various strains of Enterobacteriaceae have been added to artificial larval diets to improve pupal weight and mating performance, and to decrease developmental time, in *B. oleae* and *C. capitata* (Sacchetti et al., 2008; Ben Ami et al., 2010; Gavriel et al., 2011; Hamden et al., 2013; Augustinos et al., 2015). Use of *Enterobacter* sp. AA26 and *Bacillus* sp. 139 in artificial larval diet increased pupal weight, increased adult survival under stress and improved adult production of *B. oleae* (Koskinioti et al., 2020). Furthermore, in *B. oleae* supplemented with *Providencia* sp. AA31 in artificial larval diet increased pupal and adult production, enhanced male survival under stress conditions and delayed immature development (Koskinioti et al., 2020). We found the same bacterial family of Enterobacteriaceae in the Qfly microbiome in both artificial diet groups at all developmental stages, but the Q-fly reared on the two different larval diets contained different bacterial genera. It might be that the presence of the different bacterial genera of the same family in Qfly fed the carrot or gel diet reflected the ability of these taxa to accommodate the diet-dependent changes in the gut environment during domestication from the wild. This accommodation may result in functional redundancy of some microbiome components, whereby some bacteria are replaced by others (pathogenic/non-pathogenic) with similar functions (e.g., improve metabolic activity or physiological development) (Moya and Ferrer, 2016).

The present study explored differences in the microbiome of Qfly reared, from wild origins, on two common artificial larval diets. Overall, we found that larval diet strongly effects the microbial community structure of the Qfly across developmental stages as well as key quality control parameters. This knowledge can guide development of larval diet formulation and manipulation of bacteria to improve quality of mass-reared Qfly for SIT.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

RM designed the experiments, collected the data, and prepared the main draft of the manuscript. RM, BS, SA, and BM analyzed the data. TC and PT supervised the project. All authors provided inputs into the writing of the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.576156/full#supplementary-material

FIGURE S1 | Artificial larval diet **(A)** Gel based diet **(B)** Carrot based diet.

TABLE S1 | Gel based larval diet recipe.

TABLE S2 | Carrot based larval diet recipe.

FILE S1 | OTU table and Mapping file.

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MINI REVIEW
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Symbiont-Mediated Insecticide Detoxification as an Emerging Problem in Insect Pests

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Pesticide use is prevalent with applications from the backyard gardener to large-scale agriculture and combatting pests in homes and industrial settings. Alongside the need to control unwanted pests comes the selective pressure generated by sustained pesticide use has become a concern leading to environmental contamination, pest resistance, and, thus, reduced pesticide efficacy. Despite efforts to improve the environmental impact and reduce off-target effects, chemical pesticides are relied on and control failures are costly. Though pesticide resistance mechanisms vary, one pattern that has recently emerged is symbiont-mediated detoxification within insect pests. The localization within the insect host, the identity of the symbiotic partner, and the stability of the associations across different systems vary. The diversity of insects and ecological settings linked to this phenomenon are broad. In this mini-review, we summarize the recent trend of insecticide detoxification modulated by symbiotic associations between bacteria and insects, as well as highlight the implications for pesticide development, pest management strategies, and pesticide bioremediation.

Keywords: symbiosis, microbiota, insecticide detoxification, symbiont-mediated, host-microbe interactions

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INTRODUCTION

Environmental consciousness has increased awareness of pesticide use patterns, environmental stability, and off-target effects. This has resulted in consumer demands and policy implementation geared toward environmental sustainability including a push for green chemistries, biological control, and bioremediation (Tilman et al., 2002; Damalas and Koutroubas, 2018; Thomas et al., 2019). Appreciation for microbial transformation of toxins, pollutants, and pesticides has only increased with these campaigns pushing for the adoption of ecological pest management strategies (Bozkurt, 2017; Abrol and Shankar, 2019). Bacteria have been found to detoxify multiple insecticides classes including pyrethroids, neonicotinoids, and organophosphates (Sethunathan and Yoshida, 1973; Fisher et al., 1978; Serdar and Gibson, 1985; Chaudhry and Huang, 1988; Bhat et al., 1994; Nadeau et al., 1994; Hayatsu et al., 2000; Kamal et al., 2008; Boricha and Fulekar, 2009; Zhang et al., 2011;

Kong et al., 2013; Nayarisseri et al., 2015; Pankaj et al., 2016; Shetti and Kaliwal, 2016; Fernández-López et al., 2017; Gangola et al., 2018; Kumar, 2018; Aswathi et al., 2019; Meng et al., 2019).

Though bacterial pesticide degradation research has been steady over the past 50 years, understanding the association of these bacteria with insects has been limited until recently. While insecticide biotransformation by endogenous means is well documented in insects (Panini et al., 2016; Bass and Jones, 2018), bacterial symbionts as mechanism for *in insecta* insecticide detoxification was not documented until the mid-twentieth century. In 1967, organophosphate detoxification by a bacterial symbiont of the apple maggot was reported (Boush and Matsumura, 1967). This study demonstrated degradation of six different insecticide active ingredients by an insect-associated bacterium, but the significance of this finding to the insect host and whether it conferred pesticide tolerance remained unknown.

This idea, bacterial degradation of insecticides connected with an insect host, was revitalized decades later when *Burkholderia* colonizing bean bug midguts were demonstrated to confer resistance to the organophosphate pesticide fenitrothion to their insect hosts (Kikuchi et al., 2012). This study reignited interest in symbiont-mediated pesticide detoxification and introduced facultative associations with environmentally acquired bacteria as a mechanism for pesticide tolerance.

In this mini-review, we focus on documenting instances of symbiont-mediated insecticide detoxification within host insects and discussing the implications of this phenomenon. The repeated discovery of both facultative and obligate pesticide detoxifying symbioses across insect taxa emphasizes the need for more research in this area. Importantly, we largely focus on insect gut symbionts that encounter insecticides through host ingestion, but it is important to note that this distinction may be unnecessary given data regarding symbiont mitigation of topically applied pesticides (Kikuchi et al., 2012). Additionally, insect-associated bacteria capable of detoxifying pesticides will be important to consider in the design of future, novel active ingredients to ensure their long-term efficacy. Finally, as we continue to seek creative ways to mitigate the environmental impacts of pesticide use, perhaps we look no further than the bacteria in the very pests and fields targeted for an opportunity for bioremediation.

INSECT SYMBIONTS EXTEND AND EXPAND HOST PHYSIOLOGICAL CAPABILITIES

Bacterial symbionts have been found to perform many complex metabolic processes within their host insects (Su et al., 2013). In insects, bacterial symbionts are known to confer protection against antagonists (Oliver et al., 2003; Kaltenpoth et al., 2005; Parker et al., 2013), digest or supplement suboptimal diets (Gunduz and Douglas, 2009; Sabree et al., 2009; Peterson et al., 2015), and play critical roles in development (Dedeine et al., 2001; Kafil et al., 2013; Lee et al., 2017). In addition to these examples, symbionts have been implied or inferred to play roles in many other processes.

The relationships between insect hosts, their symbionts, and pesticides have been alluded to in the literature. Termites treated with a neonicotinoid insecticide are more susceptible to pathogens (Sen et al., 2015). In honey bees, the composition and abundance of gut bacteria have been linked to pesticide exposure (Kakumanu et al., 2016; Motta et al., 2018). However, these examples aim to understand the detrimental impacts of pesticides on insect microbiota rather than implicating those bacteria in the degradation of pesticides. Along those lines, herbivore-associated bacteria have long been credited with neutralizing the toxic metabolites of plants (reviewed in van den Bosch and Welte, 2017; Giron et al., 2017; Itoh et al., 2018) and linked to modulation of plant defense systems in favor of their insect hosts (Chung et al., 2013). So, while perhaps unsurprising, there is a growing number of studies that directly link insect symbionts to the detoxification of insecticides and/or confer tolerance to insecticides to their hosts.

Discovery of a Novel Role for Symbionts in Insect Hosts

Rhagoletis pomonella, the apple maggot, larvae develop in the fruit of apple trees causing significant damage. During the mid-twentieth century, organophosphate pesticide use in agriculture was prevalent (Croft, 1982). Though reports in the late 1970s and early 1980s suggested no widespread organophosphate resistance in apple pests (Croft, 1979; Croft, 1982), one study investigated potential detoxification mechanisms of organophosphate pesticide in *R. pomonella* (Boush and Matsumura, 1967). *R. pomonella* is associated with the bacterium *Pseudomonas melophthora* (Allen et al., 1934). *P. melophthora* has been found both in the gut of the apple maggot and in the surrounding soft rot of the fruit where apple maggots have taken up residence. At the time, this association between the symbiont and *R. pomonella* was attributed to the role of *P. melophthora* as a plant pathogen (Boush and Matsumura, 1967). However, after culturing and identifying the bacterial symbiont, the authors provided evidence that *P. melophthora* could degrade organophosphates and speculated bacterial esterases as the degradation mechanism (Boush and Matsumura, 1967).

While perhaps not as appreciated then, this study was the first to suggest a novel role in pesticide detoxification for symbionts within insects (Boush and Matsumura, 1967). Though the direct impact these symbionts would have on host insect resistance remained unclear. Likewise, whether the action of these bacterial esterases were selected specifically for organophosphate degrading activity or if they were simply generic detoxification enzymes recruited for this activity is not known. Following this study, no additional direct evidence of symbiont-mediated pesticide interactions within-insects would emerge for 45 years. Interestingly, the mechanism elucidated by Boush and Matsumura in the 1960s, bacterial esterase biotransformation of chemical insecticide, has been found several subsequent studies identifying bacterial mechanisms of insecticide degradation (Boush and Matsumura, 1967; Fisher et al., 1978; Kamal et al., 2008; Gangola et al., 2018; **Table 1**).

TABLE 1 | Summary of insect species, their associated bacteria, pesticides degraded by the symbionts, and the proposed detoxification mechanism utilized.

Insect host	Symbiont	Pesticide(s)	Detox mechanism(s)	References
Rhagoletis pomonella	*Pseudomonas melophthora*	Dichlorovos Diazinon Parathion Diisopropyl phosphorofluoridate Dieldrin Carbaryl	Esterase (hypothesized)	Boush and Matsumura, 1967
Lasioderma serricorne	*Symbiotaphrina kochii*	Parathion	Aryl-ester hydrolase Glucosidase Phosphatase Glutathione transferase	Shen and Dowd, 1991
Culex pipiens	*Wolbachia*	Organophosphates*	Not specified	Berticat et al., 2002
Riptortus pedestris & Cavelerius saccharivous	*Burkholderia* sp.	Fenitrothion Diazinon EPN Isoxathion	Not Specified	Kikuchi et al., 2012
Spodoptera frugiperda	Gut microbiota*	Deltamethrin λ-Cyhalothrin Chlorpyrifos Spinosad Lufenuron	Monooxygenase Esterase Hydrolase Transferase	de Almeida et al., 2017
Anopheles stephensi	*Pseudomonas* *Aeromonas* *Exiguobacterium* *Microbacterium*	Temephos	α-Esterase Glutathione S-transferase Acetylcholine esterase	Soltani et al., 2017
Bactrocera dorsalis	*Citrobacter* sp.	Trichlorphon	Phosphatase	Cheng et al., 2017
Plutella xylostella	*Enterococcus* sp.	Chlorpyrifos	Not specified	Xie et al., 2018
Blatella germanica	Gut microbiota*	Indoxacarb	Not specified	Pietri et al., 2018
Nilaparvata lugens	*Arsenphonus*	Imidacloprid Buprofezin	Not specified	Pang et al., 2018
Callosobruchus maculates	Gut microbiota*	Dichlorvos	Not specified	Akami et al., 2019

**Indicates details of specific taxa or pesticides are not known or not explicitly stated.*

Bean Bugs and *Burkholderia* Reignite the Study of Symbiont-Mediated Pesticide Detoxification

The burgeoning field of insect-symbiont associations at the turn of the century was facilitated with the advancement of culture-independent technologies. As mentioned above, the appreciation for bacteria-mediated physiological capabilities in association with and in complementarity to insect hosts exploded in the last 20 years. Perhaps it was only a matter of time before the observation of Boush and Matsumura was revisited. In 2012, a population of bean bugs, *Riptortus pedestris*, were suddenly resistant to fenitrothion within a single field season. Subsequent investigation revealed a correlation between particular *Burkholderia* symbionts and resistant *R. pedestris* individuals (Kikuchi et al., 2012). *Burkholderia* symbionts are known associates of true bugs (Kikuchi et al., 2007, 2011). These bacteria are harbored in specialized regions of the midget and are link to proper growth and development of the insect (Kikuchi et al., 2007, 2011). Perhaps most importantly, host insects could acquire these *Burkholderia* symbionts directly from the soil and by harboring them in the crypts of their digestive tract, tolerate both fenitrothion ingestion and topical application. Fenitrothion treatment enriched soil for degradation activity and another species, *Cavelerius saccharivous,* collected from fields regularly sprayed with fenitrothion are also associated with degrading strains of *Burkholderia.* From this, authors asserted that environmental pressure plays a large role in the acquisition of pesticide degrading *Burkholderia* symbionts by true bugs, like *R. pedestris* and *C. saccharivorous* (Kikuchi et al., 2012).

Patterns of Symbiont-Mediated Pesticide Detoxification Emerge Across Insect Taxa

Use of chemical pesticides has long been associated with the development of resistance, but that resistance to a chemical insecticide could sweep through a population within a single generation, as was observed in bean bugs, is alarming (Kikuchi et al., 2012). This was striking, because a facultative, environmentally-acquired symbiont being link to such a fast-moving phenotype was unprecedented. These *Burkholderia* symbionts were also found to degrade other organophosphate pesticides suggesting a possible broader impact on insecticide resistance within host insects (Kikuchi et al., 2012). Though previous papers had shown interplay between insect microbiomes and natural enemies, like *Bacillus thuringiensis,* this mechanism for pesticide tolerance had not been observed since Boush and Matsumura (Boush and Matsumura, 1967; Oliver et al., 2003; Broderick et al., 2006; Peterson and Scharf, 2016).

Subsequent studies and their resulting insights highlight the complexity and extent of insect-microbe collaboration regarding pesticide tolerance and biotransformation. These findings, their caveats and insights, and some perspectives on their impact on biotechnology were reviewed recently (Pietri and Liang, 2018). Briefly, *Lasioderma serricorne, Culex pipiens, Anopheles stephensi*, *Bactrocera dorsalis, Plutella xylostella, Spodoptera frugiperda, Nilaparvata lugens, Blatella germanica,* and *Callosobruchus maculatus* have all been found in association with bacteria mediate or modulate increased pesticide tolerance (Shen and Dowd, 1991; Berticat et al., 2002; Soltani et al., 2017; Cheng et al., 2017; de Almeida et al., 2017; Xie et al., 2018; Pang et al., 2018; Pietri et al., 2018; Akami et al., 2019; **Table 1**). Using reductive antibiotic treatment approaches and *in vitro*, culturing assays many of these studies directly link the presence and/or metabolic capabilities of symbiotic microbes to pesticide detoxification abilities (Shen and Dowd, 1991; de Almeida et al., 2017; Cheng et al., 2017; Soltani et al., 2017; Pietri et al., 2018; Xie et al., 2018; Akami et al., 2019; **Table 1**). And, similar to the example in *R. pedestris*, specific *Arsenophonus* strains have being linked to pesticide susceptibility in a leafhopper species (Pang et al., 2018). These examples come from diverse insect groups with distinct life histories and ecological interactions. This reemphasizes that this emerging phenomenon is not restricted to any particular taxon or niche. This discovery renewed interest in the interactions of insect symbionts with chemical pesticide and provided context for a new perspective on insecticide resistance and pesticide development.

IMPLICATIONS FOR INSECTICIDE DEVELOPMENT, PEST CONTROL, AND BIOREMEDIATION

The diverse examples of pesticide degradation via insect symbionts (**Table 1**) have many applications, particularly for the futures of pest control and bioremediation. Pest control is a dynamic arms race: on one side farmers, pesticide developers, agribusiness, and scientists all work to maintain crop protection and encourage production (Damalas and Koutroubas, 2018) and on the other side insects and other pests follow their biological drive to live and reproduce. With the intense selective pressures on pest species with heavy insecticide use, we see the potential for control failures, cross-resistance, and off-target impacts. Using the knowledge of symbiont-mediated detoxification, there is potential for the development of symbiont targeted pesticides which exploit the interplay of symbiont-host interactions. This concept has foundations in our understanding of insect gut microbiota as it relates to pro-insecticide metabolism (Daisley et al., 2018) and presents another opportunity to synergize our understanding of insect-bacteria associations with our need to control pest populations. Particularly, leveraging known mechanisms of bacterial colonization and adherence within hosts could be important for the development of active ingredients rather than deploying antibiotics into the environment. Development of such pesticides would be relevant across a variety of environments as well both urban and agricultural due to the variety of hosts associated with such bacteria (Broderick et al., 2006; van den Bosch and Welte, 2017; Pietri and Liang, 2018). In addition to exploiting knowledge of these insect-microbe symbioses as control targets, when disassociated, these bacteria may be useful for the restoration of environments contaminated with out-of-use active ingredients.

Potential Strategies for Using Insect-Associated Bacteria for Bioremediation

Using bioremediation developed for oil and other environmental contaminants as a guide, we can begin to explore how insect-associated bacteria may be deployed for environmental clean-up. The addition of either one particular bacterium or a bacterial consortium to soil or waterways reduces the half-life of these contaminants (Vasudevan and Rajaram, 2001; Singh et al., 2004; Gentili et al., 2006; Shivaramaiah and Kennedy, 2006). Importantly, the source of these bacteria are often indigenous to contaminated environments. The practice of bioaugmentation, that is increasing the density and abundance of bacteria capable of bioremediating environmental contaminants, has been successful with oil-degrading species like *Alcanivorax burkumensis* (Hassanshahian et al., 2014; Kadri et al., 2018). Using these practices as a guide, insect-associated, insecticide-degrading bacteria are advantageous because they are linked with the use of the active ingredient. Partnered with genetic engineering and large-scale production, deployment of bacterial symbionts from insects may be well-suited for bioaugmentation efforts in areas where defunct active ingredients linger (Pieper and Reineke, 2000). This would allow for remediation of areas such as agricultural fields or restored areas with a history of pesticide utilization. This may be particularly useful for the environmental cleanup of products where further use has been banned or ecological impacts persist (like off-target effects or bioaccumulation).

Any *in situ* bioaugmentation or inoculation to control environmental contaminants should be coupled with a monitoring program (Naik and Dubey, 2013). To monitor for the presence of pesticide degrading bacteria, diagnostic PCR must be developed and regular testing of soil, water, and potential host insects could be implemented (Kohno et al., 2002; Kikuchi et al., 2012). To mitigate the concerns of horizontal gene transfer, these methods of bioremediation would be utilized under circumstances where a pesticide has been discontinued. Additionally, if utilizing bioengineered or optimized bacteria there may be potential to include an exclusion system to prevent subsequent conjugation events and limit lateral gene transfer to other bacteria in the environment (Avello et al., 2019).

Limitations and Areas of Need for the Deployment of Insect-Associated Bacteria for Bioremediation

While the potential impact these symbiotic microbes have is great, it is important to recognize the current limitations of this concept. Chiefly, the research in this area is ongoing

and extremely limited. Though we have discussed evidence of symbiont-conferred pesticide tolerance and/or direct biotransformation of pesticides by insect-associated bacteria, the amenability of these microbes to this type of manipulation and the specific pathways utilized for chemical conversion remain unknown. The potential for bioremediation is likely reliant on the cultivability of the symbionts of interest. This is not a reality for many insect-associated bacteria and, as such, the dependence on culturable, tractable organisms for bioremediation is a caveat that reduces its present feasibility and may be prohibitive.

Many of the organisms associated with pesticide tolerance have yet to be identified. Instead, reduced pesticide tolerance of a host is often the result of antibiotic treatment causing dysbiosis (**Table 1**). This strategy may overestimate the role individual symbionts play in conferring pesticide tolerance. Additionally, the culture-independent approaches utilized in many studies are several steps behind practical utility in bioremediation development pipelines, as described above.

However, by dedicating time and energy to identifying, characterizing, culturing, and potentially engineering these insect symbionts, they could provide a means of pesticide clean-up in saturated agricultural fields, watershed, and soils. With further development, inoculation or bioaugmentation of detoxifying bacteria could serve to reduce pesticide residuals in the environment thereby improving ecosystem health while lessening the deleterious effects of lingering pesticides. Insect-associated, pesticide-degrading bacteria have the potential for a larger impact on environmental stewardship in multiple spheres of research including, but not limited to, pest control and bioremediation.

CLOSING REMARKS

The impact strides in the field of insect-associated microbiota have made related to pesticide degradation should inform the methods, monitoring, and development of ecological pest management strategies. Given the diversity of insects and symbionts implicated, this mechanism of chemical detoxification by bacteria is pervasive. The arms race between us and pests must be informed by these discoveries and pest control strategies must expand to consider the multifaceted nature of these ecological interactions. Additionally, symbionts also could be the key for bioremediation of pesticides particularly when they are isolated from those same environments. Environmental clean-up efforts could potentially use these identified organisms to help guide their research, development and deployment plans for remediating natural settings. In the end, the key to stewardship of chemical pesticide is and always has been environmental awareness. This includes monitoring the responses sustained use triggers in the environment not only in insect pest species, but their microbial partners.

AUTHOR CONTRIBUTIONS

AB and BP developed, wrote, and revised the ideas and content presented in this manuscript. Both authors approved the publishing of this manuscript and take responsibility for all of its contents.

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Gut Bacteriome Analysis of *Anastrepha fraterculus* sp. 1 During the Early Steps of Laboratory Colonization

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Microbial communities associated to insect species are involved in essential biological functions such as host nutrition, reproduction and survivability. Main factors have been described as modulators of gut bacterial community, such as diet, habit, developmental stage and taxonomy of the host. The present work focuses on the complex changes that gut microbial communities go through when wild insects are introduced to artificial rearing conditions. Specifically, we analyzed the effect of the laboratory colonization on the richness and diversity of the gut bacteriome hosted by the fruit fly pest *Anastrepha fraterculus* sp. 1. Bacterial profiles were studied by amplicon sequencing of the 16S *rRNA* V3–V4 hypervariable region in gut samples of males and females, in teneral (1-day-old, unfed) and post-teneral (15-day-old, fed) flies. A total of 3,147,665 sequence reads were obtained and 32 bacterial operational taxonomic units (OTUs) were identified. Proteobacteria was the most abundant phylum (93.3% of the total reads) and, *Wolbachia* and *Enterobacter* were the most represented taxa at the genus level (29.9% and 27.7%, respectively, of the total read counts). Wild and laboratory flies showed highly significant differences in the relative abundances of bacteria. The analysis of the core bacteriome showed the presence of five OTUs in all samples grouped by origin, while nine and five OTUs were exclusively detected in laboratory and wild flies, respectively. Irrespective of fly origin or sex, a dominant presence of *Wolbachia* was observed in teneral flies, whereas *Enterobacter* was highly abundant in post-teneral individuals. We evidenced significant differences in bacterial richness and diversity among generations under laboratory colonization (F0, F1, F3 and F6) and compared to laboratory and wild flies, displaying also differential patterns between teneral and post-teneral flies. Laboratory and wild *A. fraterculus* sp. 1 harbor different gut bacterial communities. Laboratory colonization has an important effect on the microbiota, most likely associated to the combined effects of insect physiology and environmental conditions (e.g., diet and colony management).

Keywords: NGS, taxonomic identification, core bacteriome, bacterial diversity, bacterial richness, fruit fly, SIT

INTRODUCTION

Insects can be considered as multiorganismal entities. Their microbiota accounts for up to 10% of the insect's biomass and is involved in essential physiological roles modulating host fitness (Zilber-Rosenberg and Rosenberg, 2008; Feldhaar, 2011; Douglas, 2015; Morris, 2018; Carthey et al., 2019; Brown et al., 2020). Insect gut microbiome comprises obligate and facultative symbionts, opportunistic parasites, and mutualistic microbes (Bourtzis and Miller, 2003; Pontes and Dale, 2006), a complex structure that is mainly affected by host environment and taxonomy (Colman et al., 2012; Engel and Moran, 2013; Augustinos et al., 2019). Gut bacteria are particularly involved in metabolic pathways that enable the host to utilize nutrient-poor or unbalanced diets, providing essential amino acids (EAAs), vitamins, lipids and co-factors (Dillon and Dillon, 2004; Wu et al., 2006; Ankrah et al., 2017). Bacteria also participate in enzymatic functions, such as, hydrolysate of xylan, lipids, and esters, or fermentation of complex polysaccharides (Engel and Moran, 2013). In addition, gut bacteria are able to perform degradation of natural toxins such us phenolic compounds and complex terpenoids involved in plant defense (Hammer and Bowers, 2015; Berasategui et al., 2016; Jing et al., 2020) or chemical toxins facilitating host insecticide resistance (Vontas et al., 2011).

Tephritidae (Diptera) is a rather large family (ca. 5000 species, ref.) that includes about 70 species of fruit flies of economic importance. These species are considered pests because larvae develop inside a wide range of fruit species including many commercial ones (White and Elson-Harris, 1992). In most tephritid fruit flies examined so far, Enterobacteriaceae has been recognized as the dominant taxonomic group, also including a range of culturable and unculturable bacteria associated to the different life styles of the host species (Behar et al., 2008; Jurkevitch, 2011; Müller, 2013; Augustinos et al., 2015; Ventura et al., 2018). A greater richness of digestive symbiotic species is associated to polyphagous flies including microbes belonging to Proteobacteria and Firmicutes phyla (Morrow et al., 2015).

Previous studies on tephritids strongly suggested that symbionts associated to the digestive tract positively affect the host fitness. The majority of the studies have focused on the effect of adult and larval diet, host taxonomy and developmental stage (Augustinos et al., 2019; Noman et al., 2020). These studies showed that gut bacteria are able to improve parameters related with survivability and reproduction, such as male sexual performance, flight ability, female fecundity, foraging behavior and longevity under starvation (Ben-Yosef et al., 2008a,b; Ben-Ami et al., 2010; Gavriel et al., 2011; Augustinos et al., 2015; Kyritsis et al., 2017, 2019; Akami et al., 2019; reviewed by Noman et al., 2020). On the other hand, certain gut bacteria have been found to be pathogenic for the host (Behar et al., 2008).

Anastrepha fraterculus Wiedemann (Diptera: Tephritidae), known as the South American fruit fly, has a wide geographic distribution ranging from northern Mexico to central Argentina (Steck, 1999). This fruit fly pest is recognized as a complex of cryptic species composed by at least eight morphotypes (Selivon et al., 2005; Hernández-Ortiz et al., 2012, 2015; Devescovi et al., 2014). Particularly in Argentina, only one member of this complex is present, the *A. fraterculus* sp. 1 or Brazilian 1 morphotype (Selivon et al., 2005; Goday et al., 2006; Hernández-Ortiz et al., 2012). This morphotype is considered a serious threat to fruit production and trade in Argentina and several South American countries. Currently, only chemical control strategies or insect traps are implemented to manage the pest in integrated management programs, which prompted the development of specific and environmentally friendly methods such as the sterile insect technique (SIT). The SIT involves the mass rearing, sterilization and release of sterile insects to compete and mate with wild individuals resulting in non-viable offspring and a gradual reduction of the pest population (Metcalf and Luckmann, 1994; Hendrichs and Robinson, 2009). Significant progress has been made to set the basis of knowledge needed to implement the SIT against *A. fraterculus*. In particular, mass rearing protocols (Salles, 1999; Jaldo et al., 2001; Sobrinho et al., 2006; Vera et al., 2007; Hernández et al., 2010; Walder et al., 2014), irradiation procedures (Allinghi et al., 2007a,b) and behavioral and physiological studies to improve the competitiveness of sterile males (Segura et al., 2007, 2009, 2013; Abraham et al., 2011, 2013; Liendo et al., 2013; Vera et al., 2013; Bachmann et al., 2015, 2017, 2019) have been addressed. Nonetheless, previous studies seldom considered *A. fraterculus* as a multiorganismal entity and only Juárez et al. (2019) focused on the importance of gut bacteria in *A. fraterculus* sp. 1 physiology and behavior.

The implementation of a successful SIT strategy requires that insects adapt to mass rearing conditions without losing valuable traits that allow them to survive and mate in natural conditions (Ochieng-Odero, 1994; Cayol, 2000; Meats et al., 2009). During the introduction of a wild population to artificial rearing conditions (laboratory colonization), variations in the environment and diet could affect biological parameters of flies and the presence and abundance of symbionts that are key determinants of their sexual competitiveness and survival (Bartlett, 1984; Cayol, 2000). In natural habitats, the development of immature stages of fruit fly species takes place first inside the fruit and then, after pupation, buried into the ground. In laboratory conditions, the eggs are deposited by females in artificial oviposition units (OUs) then transferred to artificial larval diet and pupation substrate (Walder et al., 2014). Likewise, under laboratory rearing, adult flies are provided artificial food (including a protein supply and water offered *ad libitum*), which extensively differs from the available resources of the wild environment. Such artificial conditions (among others) could affect gut bacterial diversity and the vertical transmission of symbionts from parents to offspring, typically from mothers through the eggs when females are forced to lay eggs in artificial oviposition devices (Lauzon et al., 2009; Jaenike, 2012). Likewise, artificial rearing media (both at larval and adult stages) can potentially affect the diversity of bacteria in the gut, favoring the presence of specific taxonomic groups that are not common in nature.

In the present work, we hypothesized that the composition of gut bacterial taxa hosted by *A. fraterculus* sp. 1 is modified during the laboratory colonization and vary among diverse origins, maintaining a shared central core of bacteria. Our approach included the analysis of bacterial diversity and abundance

regarding sex, feeding status and origin, studied in adult insects from a wild population, flies through the first six generations during the laboratory colonization process, and flies reared under laboratory conditions.

MATERIALS AND METHODS

Laboratory Insects

Adult insects were obtained from the *A. fraterculus* colony kept at the Institute of Genetics "Ewald A. Favret" (IGEAF). This colony (hereafter LAB flies) was established in 2007 and maintained for ca. 60 generations. No wild material has been introduced to refresh its genetic background. This fruit fly strain derived from a colony kept at Estación Experimental Agroindustrial Obispo Colombres (Tucumán, Argentina) established in 1997 with wild pupae recovered from infested guavas (*Psidium guajava*, Myrtaceae) collected in Tafi Viejo (Tucumán, Argentina). Flies used in the present study were collected at the pupal stage. After emergence, adult insects were handled in the same way as described above and kept under the same experimental conditions.

Wild Insects Collected From Traps

Adult individuals of *A. fraterculus* sp. 1 were collected using McPhail traps lured with torula yeast. Traps were hanged from an ubajay tree (*Hexachlamys edulis*, Myrtaceae), located within INTA (Instituto Nacional de Tecnología Agropecuaria) experimental field (34°36′24.7″S 58°40′07.6″W), during the fruiting season (December) and were daily checked. Flies were collected with an aspirator and then taken to the laboratory, sorted by sex and their digestive tract immediately dissected (see below). The samples were named as WU (wild ubajay) (**Supplementary Table S1**).

Wild Insects Collected From Fruits

Feijoa (*Acca sellowiana*, syn. *Feijoa sellowiana*, Myrtaceae) fruits infested with *A. fraterculus* sp. 1 larvae were sampled in Hurlingham, Buenos Aires, Argentina (34°36′40.2″S 58°40′20.9″W). Fruits were transported to the laboratory and placed on perforated plastic trays which fit in larger trays with sand as substrate for pupation. Twice a week, pupae were recovered and transferred to 3-liter glass containers and kept under controlled environmental conditions (temperature [temp]: 25 ± 1°C; relative humidity [RH]: 70 ± 10%) until adult emergence.

Emerged adults were identified at the species level according to their morphology following Hernández-Ortiz et al. (2010) and Norrbom et al. (2012). Those flies that belong to *A. fraterculus* were placed in standard cages under controlled conditions (temp: 25°C ± 1°C; RH: 75 ± 5%; photoperiod: 14:10 [light:dark]) with water but no food. Fifteen adult individuals of each sex (males and females) were randomly sampled from the cage in two different feeding status: unfed individuals collected the first day after emergence (from now on called "teneral" [T] flies) and, 15-day-old (sexually mature and fed) individuals (from now on called "post-teneral" [PT] flies) (**Figure 1**). Post teneral flies were provided with water and artificial adult diet (a mix of hydrolyzed yeast:hydrolyzed corn:sugar in a 1:2:4 ratio, and vitamins [Dayamineral, Abbott]).

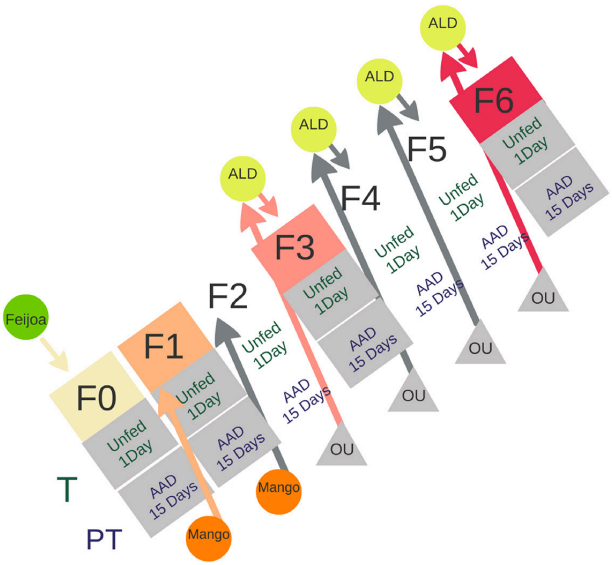

FIGURE 1 | Schematic description of the protocol followed to establish an *A. fraterculus* sp. 1 laboratory colony using wild flies recovered from feijoa infested fruits (green circle). F0 to F6 indicate the generations of flies reared under laboratory conditions. T (teneral) and PT (post-teneral) individuals, represent feeding states of adult insects. Mango and artificial oviposition units (OUs) were used to collect eggs for the next generation. ALD, artificial larval diet; AAD, artificial adult diet.

Wild flies that emerged in the laboratory were considered the first generation of artificial rearing (F0). This population was used to reproduce insects and obtain the following generations (F1 to F6) (**Figure 1**). In order to offer an adequate oviposition substrate, females from F0 to F2 generations were provided ripe mango fruits (*Mangifera indica*, Anacardiaceae). Mangoes were washed with neutral detergent and tap water and then immersed in a 0.06% w/v sodium benzoate solution before exposure to females. Exposure lasted 24 h. Fruit were then placed in sealed boxes with a layer of vermiculite as pupation substrate. Pupae were recovered two times per week and used to establish the next generation. Further generations (F3 to F6) were obtained using artificial OUs according to Parreño et al. (2014) (**Supplementary Table S1**). OUs consisted of cylindrical plastic vials (2 cm in height, 2.5 cm in diameter) filled with water colored with red food dye (Fleibor, Tablada, Buenos Aires, Argentina) and covered with Parafilm M (Pechiney Plastic Packaging, Chicago, IL, United States). After 24–48 h, eggs were recovered from the OUs and transferred to containers (4 cm × 2.5 cm × 3 cm) filled with artificial larval diet (Percent composition [% w/v] of the following supplies: Torula type B (Bioserve Inc.) 6.0; sugar 6.0; wheat germ 6.0; cholesterol 0.05; agar–agar 3.0; methyl paraben 0.1; Be Na 0.1; water 70 to 80; and HCl 36% 0.5 ml to maintain the pH between 3.7 and 4.0 [Salles, 1999]). These containers were placed over a thin layer of vermiculite as pupation substrate. Pupae were recovered two times per week and used to establish the next generation.

Digestive Tract Dissection and DNA Isolation

Adult flies were washed twice in 70% ethanol and sterilized distilled water. The gut and the subesophageal bulb were identified and dissected with sterile dissecting forceps in PBS 1X under a stereoscopic microscope (Olympus SZ30, 40× zoom) according to Caetano et al. (2006). We performed three replicates for each evaluated condition with five guts per sample (see more details in **Figure 1** and **Supplementary Table S1**). We considered three main factors: gender; origin (F0, LAB and WU) and feeding status (1-day-old and unfed individuals, and 15-day-old and fed flies); except in the case of WU for which feeding status was unknown.

Total DNA extractions of gut samples were performed following Juárez et al. (2019) based on the procedure described by Baruffi et al. (1995) with the following modifications related to sample size: (i) all volumes were reduced to half; (ii) final elution was reduced to 10 μL of TE buffer (Tris base 10 mM; EDTA 1 mM, pH 8.1). Briefly, five guts were ground and incubated (65°C) in a lysis buffer (NaCl 100 mM, Sucrose 200 mM, Tris-HCl pH 9.1 100 mM, EDTA 50 mM, SDS 0.5%) with proteinase K (final concentration of 100 μg/ml) (USB). The incubation was stopped by adding potassium acetate 8M and centrifuged. The recovered supernatant was treated with RNase (1 mg/ml) (USB). DNA was precipitated with 100% ethanol and centrifuged, the pellet was washed with 100% ethanol and 70% ethanol, and finally dried and resuspended in TE buffer.

Library Preparation and Illumina MiSeq Sequencing

The quantity and quality of the extracted DNAs were measured using NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, United States). A quantity of 50 ng of DNA per sample was used as template to generate amplicons corresponding to the V3–V4 hypervariable region of the bacterial 16S *rRNA* gene. A first round of PCR amplification was performed using KAPA HiFi HotStart PCR Kit (Kapa Biosystems) and MiSeq primers 341F and 805R (Klindworth et al., 2013). Negative controls were included in DNA extractions and PCRs were performed under the same conditions as the rest of the samples but without any genetic material. No amplicons were obtained from these negative controls. PCR products obtained were separated in a 1.2% w/v agarose gel electrophoresis to verify the size. The amplification products were visualized in Bio-Rad's Gel Doc^{TM} XR + system. Positive PCR fragments were then purified from primers and primer dimers using a 20% PEG, 2.5 M NaCl solution, centrifuged at 14.000 × *g* for 20 min and the precipitate was washed twice with 125 μl of a 70% v/v ethanol solution and centrifuged at 14.000 × *g* for 10 min as previously described (Ntougias et al., 2016). The dried precipitates were suspended in 15 μl of sterile deionized water and the concentration was measured with a Quawell Q5000 micro-volume UV-Vis spectrophotometer, diluted up to 10 ng/μl and used as template in a second round of PCR. In this step, indexed adapters were added to the ends of the 16S rDNA amplicons, as well as the Illumina adaptors. The combinatorial use of index primers resulted in unique samples that were pooled and sequenced on one Illumina MiSeq run. The resulting amplicons were cleaned-up by AMPure XP beads (Agencourt, United Kingdom) and diluted to 2.66 ng/μl. Finally, they were pooled equimolarly and mixed into indexed library following the 16S-metagenomic library preparation guide 15044223-b (Illumina Inc., 2013). Massive amplicon sequencing was performed using an Illumina MiSeq sequencing platform by Macrogen.

Data Analysis

The pre-processing of reads was carried out using USEARCH v10. Paired Fastq files were assembled by using algorithms implemented in USEARCH v10 using -fastq_mergepairs command with -fastq_maxdiffs, -fastq_pctid, -fastq_minmergelen, and -fastq_maxmergelen options set at default values. All reads were trimmed and filtered by quality using -fastq_filter, with the -fastq_maxee option set at 1.0, and -fastx_uniques commands. Unique sequences were identified, and all samples were clustered at increasing similarities of 97% using UPARSE-OTU algorithm (Edgar, 2013). Using this algorithm, chimera filtering and operational taxonomic units (OTUs) clustering were carried out simultaneously. For the clustering, a minimum abundance (value = 2) was used discarding singletons. For the OTU Table trimming, we defined 0.001 as the minimum frequency for an OTU. The OTU frequency was calculated as follow: (number of count reads for an OTU/total number of count reads)*100. For the OTU Table trimming, we defined 0.001 as the minimum frequency for an OTU. The taxonomy assignment was performed against a reference database (SILVA release 119; Quast et al., 2013). UNCROSS2 algorithm was run to detect and filter crosstalk (Edgar, 2018).

Diversity estimates including observed OTUs and Good's coverage were calculated using final count data. Richness (Chao1), diversity (Simpson and Shannon), dominance (Berger–Parker) and evenness (Pielou) indices of alpha diversity, which reflect the diversity of individual samples were calculated based on "vegan" R package (Oksanen et al., 2019) and were plotted using the "ggplot2" R Package (Wickham, 2016).

Phylogenetic diversity (Faith index) was estimated using the Picante package in R (Kembel et al., 2010). Alpha diversity indices were compared by pairwise Kruskal–Wallis test in R (R Core Team, 2020). Core bacterial OTUs shared by LAB, WU, F0 and F1–F6 were identified by comparing OTUs from the different origins following Andongma et al. (2019).

Beta diversity was analyzed to evaluate the similarity of bacterial communities from different locations using Generalized UniFrac distance (Chen et al., 2012) and visualized via Non-metric Multidimensional scaling (NMDS) plot using the RHEA pipeline in R (Lagkouvardos et al., 2017). A permutational multivariate analysis of variance using distance matrices was calculated using "adonis" function from "vegan" R package to determine significance differences between the separated groups. Statistically significant differences between samples were identified with permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001), *p*-values of PERMANOVA test indicating the significance of group separations and the dissimilarity scale of the grid, $d = 0.1$ means that the distance

between two grid lines represent approximately 10% dissimilarity between the samples. The Bonferroni–Hochberg method was used to correct for multiple PERMANOVA testing. The Non-parametric Wilcoxon rank sum test (Kruskal, 1957) was used to perform pair-wise comparisons of mean relative abundance of bacteria between gut samples.

RESULTS

Overall Data Analysis

After an ultimate and strict trimming process, a total of 3,147,665 high quality reads were achieved from a total of 66 *A. fraterculus* sp. 1 gut samples. A set of 32 bacterial OTUs were identified, clustered at 97% sequence similarity (**Table 1** and **Supplementary Table S1**). Members of three bacterial phyla were identified: Patescibacteria, Proteobacteria and Firmicutes. Proteobacteria was the most abundant taxonomic group (93.3% of reads), followed by Firmicutes (6.5%) and Patescibacteria (0.2%). Within Proteobacteria, two taxonomic classes dominated: Alpha- and Gammaproteobacteria. At the genus level, *Wolbachia* and *Enterobacter* were the most abundant taxa (29.9% and 27.7% of the obtained reads, respectively), followed by *Providencia* (8.3%), *Aeromonas* (7.1%), *Citrobacter* (5.9%) and *Burkholderia–Caballeronia–Paraburkholderia* (4.4%) (**Table 1**). Good's coverage index was 98%, suggesting that the majority of bacterial phylotypes in the insect digestive tract were included in this study. In addition, three OTUs (15, 124 and 19) were placed in three distinct phylogenetic positions, showing lower than 97% similarity to known described species of the genera *Raoultella* sp., *Klebsiella* sp. and the phylum of *Saccharibacteria*, respectively.

TABLE 1 | Representation and classification of OTUs identified in *A. fraterculus* sp. 1 gut bacteriome. The OTUs highlighted compose the *A. fraterculus* sp. 1 gut bacterial core proposed in the present work.

OTU ID	Read counts	% read counts	Phylum	Class	Order	Family	Genus
OTU 1	942423	29.9	Proteobacteria	Alphaproteobacteria	Rickettsiales	Anaplasmataceae	*Wolbachia*
OTU 2	860684	27.3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*
OTU 3	141265	4.5	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*
OTU 4	259733	8.3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Providencia*
OTU 5	67142	2.1	Firmicutes	Bacilli	Lactobacillales	Enterobacteriaceae	*Enterococcus*
OTU 6	72793	2.3	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Burkholderia–Caballeronia–Paraburkholderia*
OTU 7	32959	1.1	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*
OTU 8	35181	1.1	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Caulobacter*
OTU 9	64879	2.1	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Burkholderia–Caballeronia–Paraburkholderia*
OTU 10	81701	2.6	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*
OTU 11	41643	1.3	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
OTU 12	27726	0.9	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*
OTU 13	29721	0.9	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	*Bradyrhizobium*
OTU 14	20969	0.7	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*
OTU 15	65801	2.1	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Raoultella*
OTU 16	10523	0.3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Serratia*
OTU 17	12157	0.4	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Mesorhizobium*
OTU 18	5227	0.2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*
OTU 19	6261	0.2	Saccharibacteria	Unknown	Unknown	Unknown	Unknown
OTU 20	19166	0.6	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*
OTU 21	5358	0.2	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Mesorhizobium*
OTU 22	4207	0.1	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Commensalibacter*
OTU 24	8207	0.3	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*
OTU 26	8951	0.3	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Neisseriaceae	*Neisseria*
OTU 31	4088	0.1	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*
OTU 35	6508	0.2	Firmicutes	Bacilli	Lactobacillales	Enterobacteriaceae	*Enterococcus*
OTU 44	3323	0.1	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Neisseriaceae	*Neisseria*
OTU 52	5783	0.2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*
OTU 65	184274	5.9	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Citrobacter*
OTU 124	107352	3.4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Klebsiella*
OTU 136	4119	0.1	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Rheinheimera*
OTU 168	7541	0.2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*

For OTU19 taxonomic assignment was not feasible below the phylum level.

Core Bacteriome Analysis

A total of five OTUs (*Rheinheimera*, *Enterobacter* [OTU2], *Acinetobacter* [OTU31], *Enterococcus* and *Providencia*) made up the core bacterial community (36.1% of read counts). We defined the core bacteriome of *A. fraterculus* sp. 1 as constituted by taxonomic units present in all the samples grouped by origin (LAB, WU, including samples involved in the laboratory colonization assay F0 and F1–F6) (**Figure 2A** and **Table 1**). The relative abundance of taxonomic groups from the core bacteriome varied in the examined samples ranging from 9.5% reads (40% samples) in average for LAB, 7.9% (50% of the samples) in average for WU, 6.1% (50% of the samples in average) in average for F0 and 6.7% (33.3% of the samples) in average for F1–F6 (**Figure 2A** and **Supplementary Tables S2A,B**). In addition, when LAB and WU were compared, six OTUs (*Rheinheimera*, *Klebsiella* [OTU124], *Enterobacter* [OTU2], *Acinetobacter* [OTU31], *Enterococcus* and *Providencia*) were shared. Furthermore, nine OTUs (*Wolbachia*, *Bradyrhizobium*, *Sphingomonas*, two OTUs of *Mesorhizobium*, *Acinetobacter* [OTU18], *Staphylococcus*, *Burkholderia–Caballeronia–Paraburkholderia* [OTU6] and *Caulobacter*) were found in LAB but not in WU samples and, five OTUs (*Lactobacillus*, *Lactococcus*, *Raoultella* [OTU15], *Enterobacter* [OTU168] and *Citrobacter*) were detected in WU samples but not in LAB flies (**Figure 2B**; see details in **Supplementary Tables S2A,B**). Additionally, when total values were compared, five OTUs (*Enterobacter* [OTU2], *Wolbachia, Rheinheimera, Enterobacter* [OTU168] and *Citrobacter*) were represented in at least 40% of the analyzed samples (**Figure 2C**). This overall analysis showed two out of five OTUs (*Enterobacter* [OTU2] and *Wolbachia*) with a high percentage of read counts and the other three OTUs (*Rheinheimera, Enterobacter* [OTU168] and *Citrobacter*) were represented by a low percentage of read counts (**Figure 2C** and **Table 1**).

Gut Bacterial Community and Sex

Statistical comparisons of samples grouped by sex evidenced that there are not significant differences in the gut bacteriome composition between females and males of *A. fraterculus* (UniFrac distance; PERMANOVA $p > 0.05$; **Supplementary Figure S1**). Males and females showed the same distribution of bacterial OTUs and abundance, with *Enterobacter* and *Wolbachia* observed in a high relative

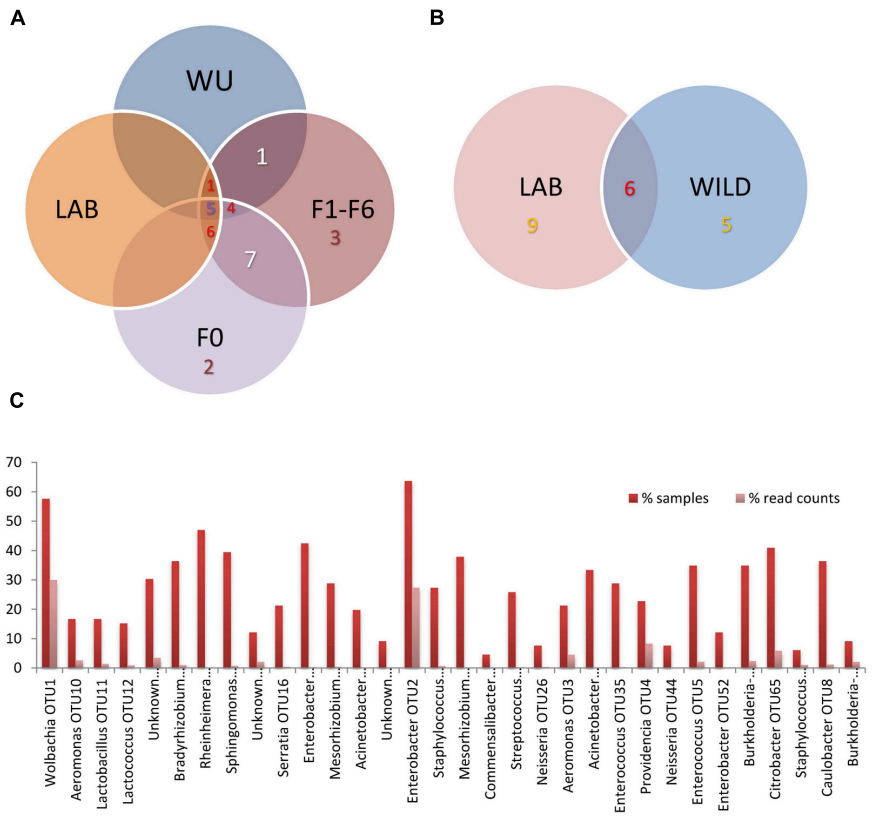

FIGURE 2 | Distribution of bacterial OTUs among *A. fraterculus* sp. 1 gut samples. **(A)** samples pooled in four groups (LAB, WU, F0, and F1–F6) according to their origin. **(B)** Samples grouped in LAB and WILD (F0 + WU). LAB, individuals from the laboratory colony; WU, wild individuals from ubajay with unknown feeding status; F0, individuals collected from infested feijoa fruits; F1–F6, individuals from generations 1, 3 and 6 reared under laboratory conditions. **(C)** OTU representation: % of samples and reads per OTU considering all analyzed samples ($N = 66$) (See more details in **Supplementary Tables S2A–D**).

abundance (**Supplementary Figure S2**), and similar values of Chao1, Shannon and Simpson indices (**Supplementary Figures S3A,B,D**). In addition, non-significant differences were observed between sexes for evenness (Pielou index), phylogenetic diversity (Faith index), and dominance (Berger–Parker index) (**Supplementary Figures S3C,E,F**). Therefore, sex was not considered for further analyses performed in this study.

Effect of the Feeding Status and Fly Origin on the Gut Bacteriome

Significant differences in gut bacterial composition were observed between individuals with different feeding status (teneral [T] and post-teneral [PT]) (UniFrac distance; PERMANOVA $p < 0.05$; **Figure 3A**). In addition, we observed a differential distribution of bacterial OTUs and abundance between T and PT samples. *Wolbachia* was detected at a high prevalence (60.0% of reads) in T samples, whereas, *Enterobacter* (42.0% of reads), *Providencia* (18.3% of reads) and *Aeromonas* (15.2% of reads) dominated the gut bacteriome of PT individuals (**Supplementary Figure S4**). Furthermore, differential values of Chao1 and Simpson indices were observed between T and PT samples, showing higher values in T flies (**Supplementary Figure S5**). Additionally, evenness (Pielou index), phylogenetic diversity (Faith index), and dominance (Berger–Parker index) evidenced the same trends of significant differences between T and PT flies (**Supplementary Figures S5C,E,F**). Conversely, Shannon index showed no significant differences between T and PT flies (**Supplementary Figure S5B**).

In relation to the fly origin and feeding status, significant differences were observed among gut samples from the laboratory (LAB_T and LAB_PT), and wild flies (WU) collected in ubajay trees with unknown feeding status (UniFrac distance; PERMANOVA $p < 0.05$; **Figure 3B**). The gut bacteriome of WU samples was dominated by *Enterobacter* OTU2 (37.86% of reads), *Raoultella* (21.75% of reads) and *Citrobacter* (19.50% of reads) (**Supplementary Table S2B**) whereas LAB_PT gut bacteria was mainly represented by *Enterobacter* OTU2 (39.02%)

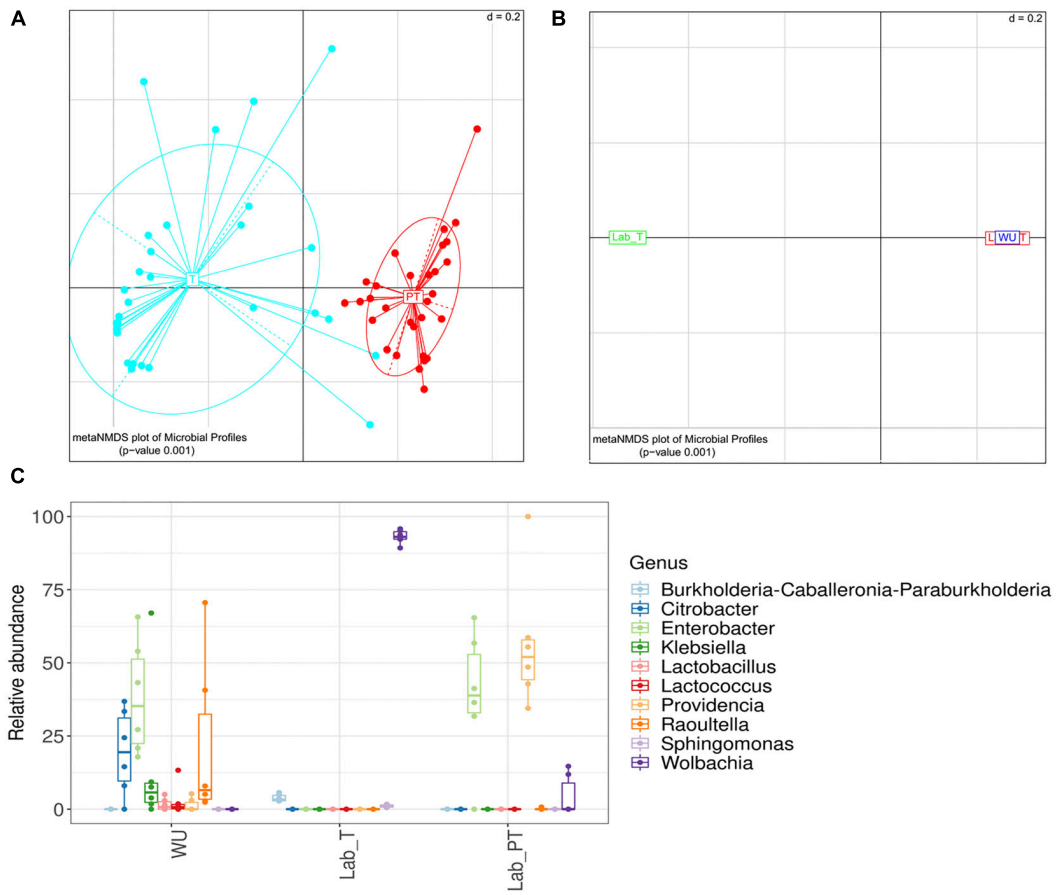

FIGURE 3 | Effect of the feeding status and fruit fly origin on the gut bacteriome. **(A,B)** Meta-Non-metric Multidimensional scaling (meta NMDS) plot representing sample groups according to: **(A)** the feeding status (T, teneral; PT, post-teneral); **(B)** the origin (LAB, laboratory individuals with different feeding status [T, teneral; PT, post-teneral]; WU, wild individuals from ubajay with unknown feeding status). Significance *p*-value from PERMANOVA analysis; $d = 0.2$. **(C)** Relative abundance of bacterial genera associated to the digestive tract of flies from two different environmental origins (Lab, laboratory samples with different feeding status (T and PT); WU, wild samples from ubajay. Error bars correspond to standard error of the mean.

and *Providencia* (56.09%) (**Supplementary Table S2C**). LAB_T gut community was dominated by *Wolbachia* (93.12% of reads) (**Figure 3C**).

Bacterial richness and diversity of teneral (T) and post-teneral (PT) flies from LAB, and WU showed significant differences in paired comparisons. Chao1 index and phylogenetic diversity (Faith index) showed non-significant differences between Lab_T and WU flies (**Figure 4A** and **Supplementary Figure S6D**). However, WU showed significantly higher values of Shannon index than LAB_T (**Figure 4B**). The same statistical differences were also observed for the Simpson index and evenness (Pielou index) (**Supplementary Figures S6A,B**). Congruently, Lab_T showed significantly higher values of dominance (Berger–Parker index) than WU (**Supplementary Figure S6C**).

In Lab_PT vs. WU comparisons, WU samples evidenced significantly higher values of Chao1, Shannon indices and phylogenetic diversity (Faith index) (**Figures 4C,D** and **Supplementary Figure S6H**). Conversely, additional analyses of diversity (Simpson index), evenness (Pielou index), and dominance (Berger–Parker index) showed non-significant differences between compared samples (**Supplementary Figures S6E–G**).

Changes in Gut Bacteriome During Laboratory Colonization

Significant differences in the gut bacterial profile were observed across generations under artificial rearing conditions (F0–F6) both in teneral (T) and post-teneral (PT) flies (UniFrac distance; PERMANOVA $p < 0.05$; **Figures 5A,B**). Considering T flies from F0–F6, we observed that bacterial profiles differed between generations, and in all cases, were different from LAB_T and WU flies (PERMANOVA; $p < 0.05$; **Table 2** and **Figure 5A**). For PT flies, F0–F6 generations differed in paired comparisons between them and among LAB_PT, and WU. Interestingly, bacterial profiles of teneral and post-teneral F6 were significantly different from teneral and post-teneral LAB samples, respectively (**Table 2** and **Figures 5A,B**).

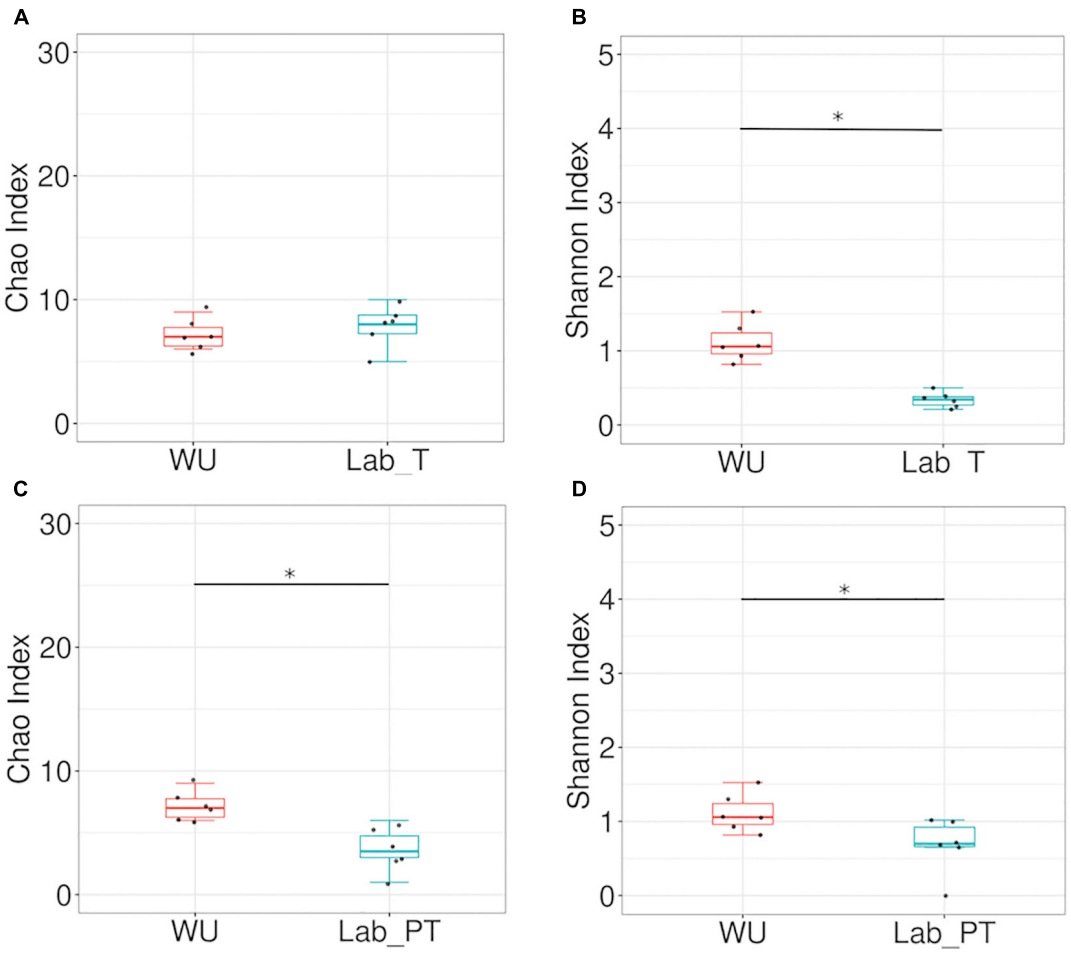

FIGURE 4 | Effect of the fruit fly origin and feeding status on the gut bacterial community. Box plots representing bacterial richness and diversity among origins. Chao index **(A,C)** and Shannon index **(B,D)** in teneral (T) and post-teneral (PT) individuals. WU, wild individuals from ubajay with unknown feeding status; Lab, individuals from the laboratory colony. Dots indicate observed values and box plots depict means and standard deviation of the data. Bars with asterisks above boxes indicate significant *p*-values (paired comparisons, Kruskal–Wallis test).

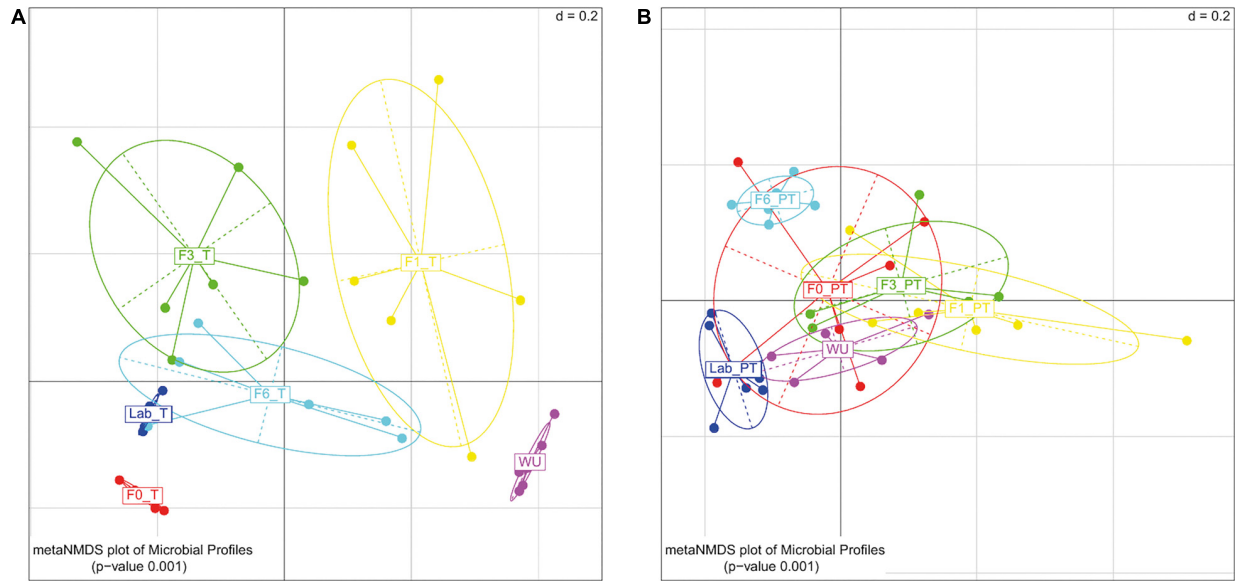

FIGURE 5 | Gut-associated bacterial communities of *A. fraterculus* sp. 1 during laboratory colonization. Meta-Non-metric Multidimensional scaling (metaNMDS) plot of bacterial profile representing samples grouped by generation under artificial rearing (F0, F1, F3, and F6) and feeding status. **(A)** Teneral (T) samples. **(B)** Post teneral (PT) samples. WU, wild individuals from ubajay with unknown feeding status; Lab, individuals from the laboratory colony. Significance *p*-value from PERMANOVA analysis (d = 0.2).

The gut bacteriome associated with T flies was dominated by *Wolbachia* sp. (>50% of the total reads, found in all samples), with the exception of F1_T, in which, members of *Burkholderia–Caballeronia–Paraburkholderia* (OTU9) were the most dominant taxon (32.2% of reads) (**Figures 6A–C** and **Supplementary Table S2C**). Gammaproteobacteria was the most representative class in PT samples (>75% of reads, found in all samples) (**Figures 6D,E**). The same taxonomic classes with a differential relative abundance were observed in F0–F6_PT flies compared to F0–F6_T flies (**Figures 6B,E**). At the genus level, *Wolbachia* was detected in a low relative abundance in PT flies (<15%, detected in 50% of the samples) (**Supplementary Tables S2C,D**). Significant differences were observed when the *Wolbachia* relative abundance was compared between T and PT flies in each generation (Wilcoxon Rank Sum Test; $p < 0.05$), except for F1_T vs F1_PT (**Supplementary Table S4**). In addition, *Wolbachia* was not detected in WU samples (**Figure 6F** and **Supplementary Tables S2A,B**).

Klebsiella sp. and *Providencia* were noticeably abundant in F0_PT (>20%). In addition, we detected *Citrobacter* highly represented in WU, which was also found in F1_T and F6_T flies and in PT samples from F0, F1, and F3.

Teneral (T) and post-teneral (PT) flies presented different patterns of Chao1, Shannon and Simpson indices across the laboratory colonization process. For T flies, F1 and F3 showed the highest Chao1, Shannon and Simpson values (**Figures 7A,B**, **Supplementary Table S3**, and **Supplementary Figure S7A**) and F3 resulted significantly different from F0 and LAB (for the three indices, **Figures 7A,B** and **Supplementary Figure S7A**). In addition, LAB_T showed significant differences in Chao1 index compared to F0, F1 and F3. LAB_T and WU showed the lowest values for this index. In the case of Shannon and Simpson indices analysis, differentiation between paired comparisons of LAB_T and F1–F6_T flies and LAB_T-WU was detected. LAB_T and F0_T flies displayed the lowest Shannon index values but non-significant differences were detected between them (**Figure 7B** and **Supplementary Figure S7A**). With regard to PT flies, significant differences were observed between LAB_PT and WU, and each paired comparisons of LAB_PT with F0, F1, and F6 PT for Chao1 index. Differential values were also observed in F0–F1, F0–F3, F1–F3, and F3–F6 comparisons for this parameter. In sum, T flies showed a tendency to a reduction of Chao1, Shannon, Simpson, Faith and Pielou indices, as generations under artificial rearing increased (F1–F6), congruently with an increase of dominance estimated by Berger–Parker index (**Figures 7A,B** and **Supplementary Figures S7B–D**). Laboratory flies showed the lowest values for Shannon, Simpson, Chao1, phylogenetic diversity (Faith) and evenness (Pielou) estimators together with the highest values recorded for Berger–Parker index. This tendency was not detected in PT flies (**Figures 7C,D** and **Supplementary Figure S7**).

DISCUSSION

In the present work, we analyzed the bacterial community associated with the digestive tract of *A. fraterculus* sp. 1 adults using 16S *rRNA* amplicon sequencing. We were able to identify 32 OTUs in this gut bacteriome. From these classified taxonomic units, 29 OTUs were described at the genus level, one OTU (OTU19) remains unclassified under phylum level, identified as unknown Saccharibacteria and two other OTUs (15 and

TABLE 2 | PERMANOVA analysis – pair-wise comparisons.

Comparison	*p*-Value	Corr. *p*-value
F0_T-F1_T	0.005	0.006
F0_T-F3_T	0.001	0.003
F0_T-F6_T	0.003	0.0045
F0_T-LAB_T	0.003	0.005
F0_T-WU_unk	0.003	0.0045
F1_T-F3_T	0.005	0.0057
F1_T-F6_T	0.009	0.010
F1_T-LAB_T	0.002	0.006
F1_T-WU_unk	0.005	0.00625
F3_T-F6_T	0.002	0.021
F3_T-LAB_T	0.004	0.005
F3_T-WU_unk	0.004	0.006
F6_T-LAB_T	0.01	0.011
F6_T-WU_unk	0.004	0.0066
LAB_T-WU_unk	0.001	0.006
F0_PT-F1_PT	0.017	0.024
F0_PT-F3_PT	0.021	0.024
F0_PT-F6_PT	0.017	0.0225
F0_PT-LAB_PT	0.004	0.008
F0_PT-WU_unk	0.019	0.023
F1_PT-F3_PT	0.053	0.053
F1_PT-F6_PT	0.005	0.0125
F1_PT-LAB_PT	0.001	0.0085
F1_PT-WU_unk	0.007	0.011
F3_PT-F6_PT	0.002	0.0064
F3_PT-LAB_PT	0.004	0.0085
F3_PT-WU_unk	0.004	0.0428
F6_PT-LAB_PT	0.002	0.01
F6_PT-WU_unk	0.003	0.0056
LAB_PT-WU_unk	0.004	0.012
F0_T-F1_T	0.005	0.006
F0_T-F3_T	0.001	0.003
F0_T-F6_T	0.003	0.0045
F0_T-LAB_T	0.003	0.005

124) were placed in two distinct taxonomic positions, showing lower similarity than 97% with *Raoultella* sp. and *Klebsiella* sp., respectively.

Overall analyses suggested that the gut bacterial profile was dominated by Proteobacteria, particularly by Alpha and Gammaproteobacteria; followed by Firmicutes and Saccharibacteria. Within the Gammaproteobacteria, the Enterobacteriaceae family was dominant in the gut of *A. fraterculus*, in agreement with previous reports in Tephritidae fruit flies (Behar et al., 2008; Jurkevitch, 2011; Müller, 2013; Morrow et al., 2015; Ventura et al., 2018; Augustinos et al., 2019; Koskinioti et al., 2019). The most abundant genera, were *Wolbachia* (Alphaproteobacteria) and *Enterobacter* (Gammaproteobacteria), followed by other members of the Gammaproteobacteria such as *Providencia*, *Aeromonas*, *Citrobacter, Burkholderia–Caballeronia–Paraburkholderia, Klebsiella* and *Raoultella.* Tephritid literature reports these genera of Proteobacteria with specific functions: *Wolbachia* with cytoplasmic incompatibility and male killing (Boller and Bush, 1974; Riegler and Stauffer, 2002; Zabalou et al., 2004, 2009; Apostolaki et al., 2011; Conte et al., 2019; Devescovi et al., 2019; Mateos et al., 2020), *Enterobacter*, *Citrobacter*, *Burkholderia*, *Klebsiella* and *Raoultella* with nitrogen metabolism (Murphy et al., 1988; Behar et al., 2005; Raza et al., 2020), and *Providencia* with pathogenic effects (Msaad Guerfali et al., 2018; Ksentini et al., 2019). In addition, *Acinetobacter* and *Rheinheimera* (Gammaproteobacteria) were detected in low abundance (0,1% of total reads) but in a high percentage of samples (33.33 and 46.97% of total samples, respectively) and wide distribution (Lab, WU, F0, F1–F6 flies). Despite *Acinetobacter* having been reported in several studies on fruit flies, its role is so far unclear (Kounatidis et al., 2009; Deutscher et al., 2019).

The gut bacteriome of *Anastrepha* genus was previously addressed using different tools. In *A. ludens*, *A. obliqua*, *A. serpentina*, and *Anastrepha striata*, *Escherichia* was one of the dominant genera (Ventura et al., 2018). Nonetheless, *Escherichia* was not identified in our samples, nor in *A. grandis*, *A. ludens*, and two morphotypes of *A. fraterculus* studied by Augustinos et al. (2019). For *A. fraterculus*, Proteobacteria dominate the gut bacterial community according to Augustinos et al. (2019) and our results. However, Müller (2013) found that the gut bacteriome of a Brazilian wild population of *A. fraterculus* was dominated by *Actinobacteria*. This author targeted a different region of the 16S *rRNA* gene, which could partially explain the differences; however, the main explanation is probably associated to environmental variation (Augustinos et al., 2019).

Despite some differences in the three studies on *A. fraterculus*, a rather clear pattern seems to emerge with Enterobacteriaceae being consistently the most abundant family associated to *A. fraterculus,* and particularly *Enterobacter* as the genus with the highest abundance. Recent studies have revealed the importance of *Enterobacter* spp. associated to Tephritidae (Kyritsis et al., 2017, 2019; Noman et al., 2020). As an example of essential contribution of this symbiont to fruit fly physiology, multiple traits have been addressed, including: nitrogen fixation and pectinolytic activity (Behar et al., 2005; Aharon et al., 2012) as well as, provision of essential and non-essential amino acids and vitamins (Azis et al., 2019) and its role on host behavior and fitness (Hamden et al., 2013; Augustinos et al., 2015; Kyritsis et al., 2017, 2019; Raza et al., 2020). These beneficial effects might explain why adding *Enterobacter* spp. to the larval diet in *C. capitata* improved pupal and adult productivity, as well as a faster development, particularly of males (Hamden et al., 2013; Augustinos et al., 2015). Further studies on the physiological role of *Enterobacter* spp. in *A. fraterculus* sp. 1 will bring valuable information to be applied to mass rearing protocols and environmentally safe control strategies.

Within Firmicutes, *Enterococcus* was the most abundant genus in the *A. fraterculus* gut bacterial community. A recent publication revealed that diet enriched with *Enterococcus* reduced the duration of the larval stage, increased pupal weight, and increased longevity in *Bactrocera dorsalis* (Khaeso et al., 2017). Finally, within the Saccharibacteria phylum, we detected an unclassified taxon that was exclusively found in F0_T flies. Similar results were observed by Koskinioti et al. (2019) who

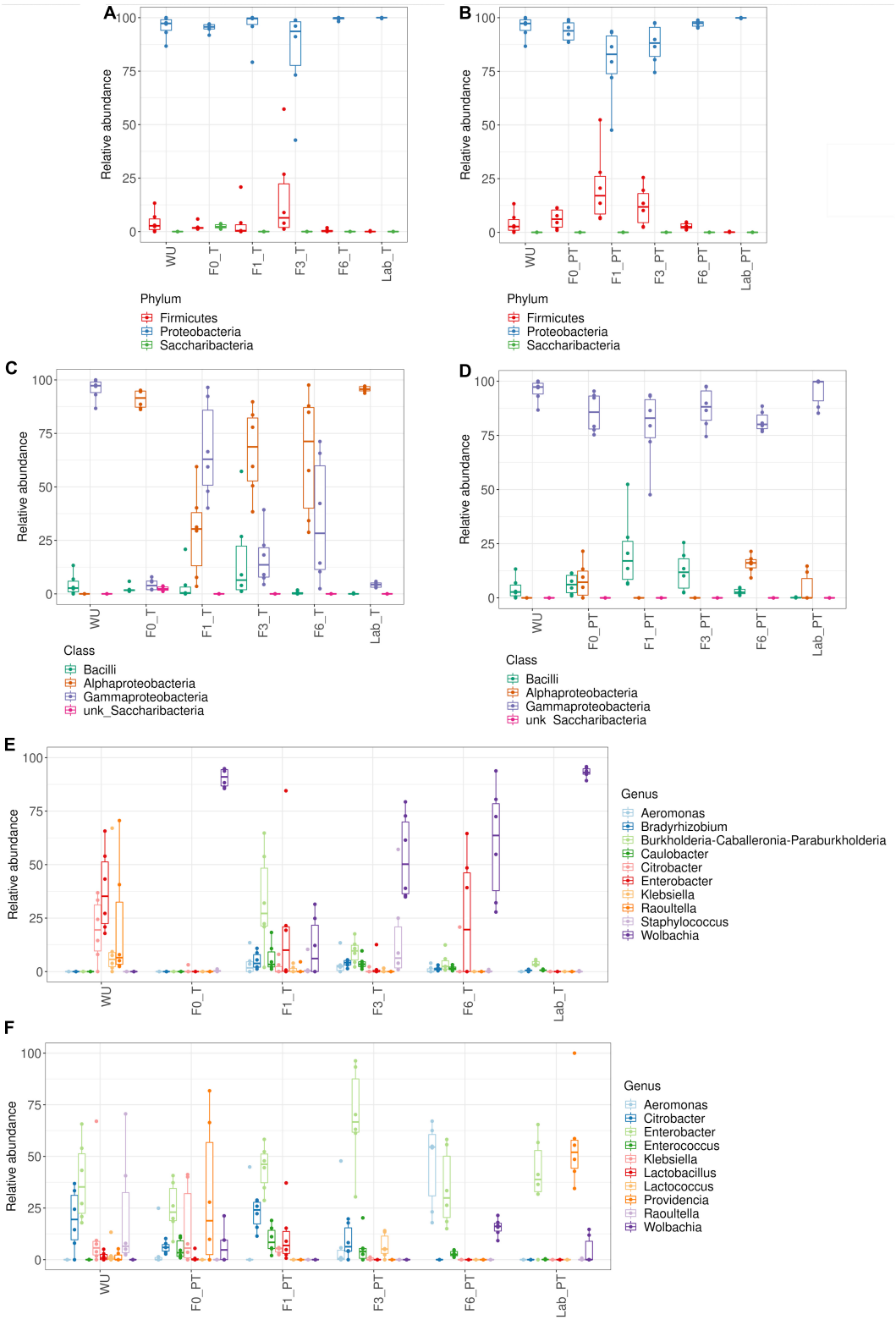

FIGURE 6 | Gut-associated bacterial communities during of *A. fraterculus* sp. 1 laboratory colonization and feeding status. Relative abundance of the ten main OTUs identified. Taxonomic identification at the Phylum, Class and Genus levels for teneral (T) (**A–C**, respectively) and post-teneral (PT) (**D–F**, respectively) flies from F0, F1, F3, F6 generations under laboratory conditions. WU, wild individuals from ubajay with unknown feeding status; Lab, individuals from the laboratory colony.

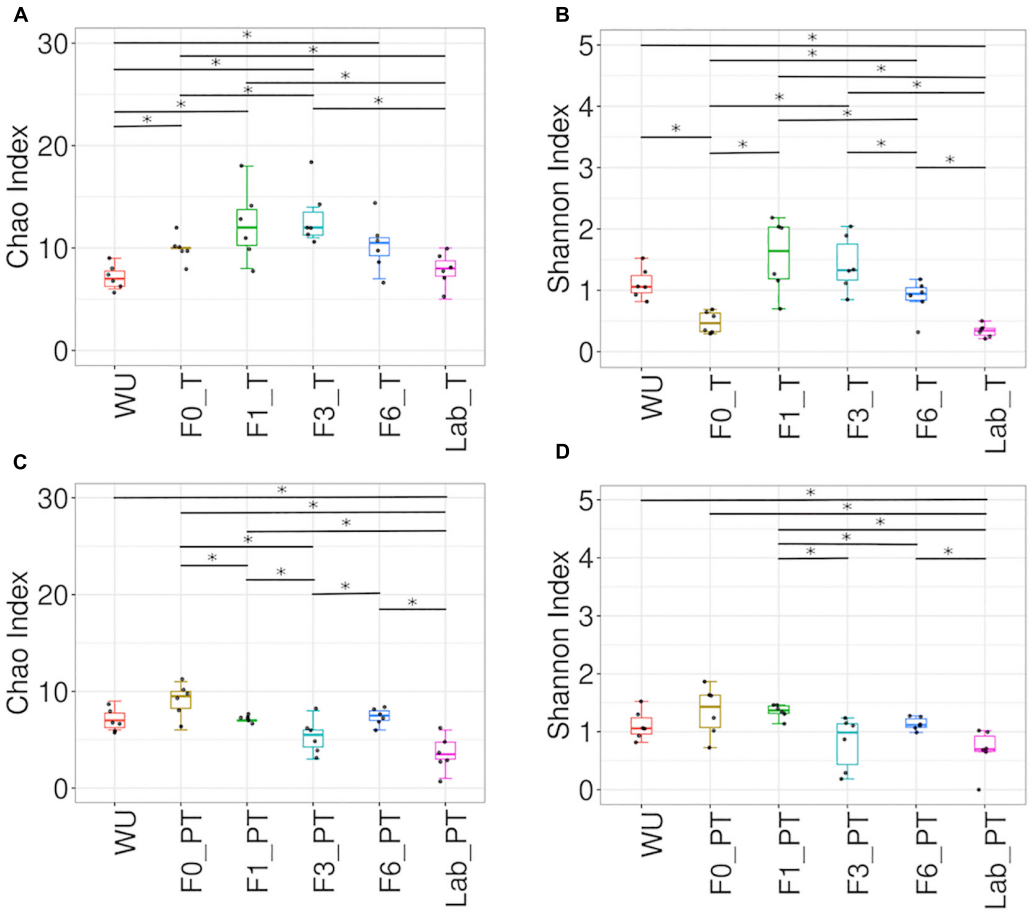

FIGURE 7 | Bacterial community of the *A. fraterculus* sp. 1 digestive tract and laboratory colonization process. Bacterial richness and diversity associated to the digestive tract of F0–F6 individuals and laboratory flies. Chao index **(A,C)** and Shannon index **(B,D)** in teneral (T) and post-teneral (PT) individuals. WU, wild individuals from ubajay with unknown feeding status; Lab, individuals from the laboratory colony. See **Figure 4** for dots and box plots description. Bars with asterisks above boxes indicate significant *p*-values (paired comparisons, Kruskal–Wallis test).

detected an unknown family of the order Saccharimonadales in the gut symbiotic communities of *Bactrocera oleae* wild samples.

Our results evidenced the presence of five common bacterial OTUs (*Enterobacter* [OTU2], *Providencia, Enterococcus, Rheinheimera* and *Acinetobacter* [OTU31]) described as the core bacteriome associated to the gut of *A. fraterculus* sp. 1 adult individuals. Digestive core bacteria and their contribution to the host biology have been addressed in other Tephritidae species including *C. capitata*, *B. minax,* and *Zeugodacus cucurbitae* (Wang et al., 2014; Yong et al., 2017; Andongma et al., 2019; reviewed by Deutscher et al., 2019). Particularly, our study showed an initial evaluation of the taxonomic composition of *A. fraterculus* sp. 1 core bacteria, with a restricted sampling considering different origins. In addition, we detected non-shared bacteria between laboratory and wild populations (WU, F0) and a population under adaptation (F1–F6), which could bring useful information to perform a further characterization and selection of bacterial isolates with potential benefits to improve mass rearing protocols and fitness of adults in the field in assistance to the SIT development against *A. fraterculus* sp. 1.

Changes in gut bacterial composition during laboratory colonization were strongly associated to the feeding status and generation. After six generations of laboratory rearing, the bacterial community is still changing and seems to maintain the differentiation from LAB flies. In our experiment, females from F0, F1, and F2 did not lay eggs in artificial OUs, and thus were offered mangoes. Artificial units were used from F3 onward. Our results are in line with previous studies conducted in other fruit fly species (Deutscher et al., 2019) and are strongly associated to the feeding behavior. Accordingly, the lack of differences in gut bacterial composition between females and males obtained in our work and in other related articles (Wang et al., 2014; Morrow et al., 2015; Augustinos et al., 2019) supported a substantial role of nutrition on the digestive microbiota composition, however, the bacterial diversity associated to different origins remained to be an important point of differentiation. These findings were also supported by studies performed in *B. oleae* (Koskinioti et al., 2019; revised by Deutscher et al., 2019).

We identified two OTUs (OTU22 assigned to *Commensalibacter* and OTU19, classified as an unknown

member of the *Saccharibacteria* phylum) exclusively present in teneral wild flies from feijoa host fruit. These OTUs were no longer detected in F1 onward. Similarly, *Citrobacter* (OTU65) and *Klebsiella* (OTU124) were found in high abundance in wild adult flies (WU) and wild flies from feijoa (F0), but then slowly decreased as laboratory colonization progressed (F1 to F6); not being detected at all in LAB fruit fly guts. In other fruit fly species, *Citrobacter* [as all Enterobacteriaceae (Octavia and Lan, 2014)] has been shown to be capable of reducing nitrate to nitrite (Borenshtein and Schauer, 2006). Furthermore, the analysis of tryptic soy broth culture filtrates of *Citrobacter freundii* isolated from *A. ludens* showed that it contained greater amounts of nitrogenated compounds (Robacker and Bartelt, 1997). Because in nature nitrogen is a rather scarce resource (Mattson, 1980) and most tephritids need protein to achieve sexual maturation, gut bacteria may play a key role by making nitrogen available to their hosts. However, under laboratory conditions flies had unlimited access to a highly rich peptide source (hydrolyzed yeast) and the presence of some of these bacterial groups may no longer be essential. This might indicate that these OTUs are relevant for the host fitness in nature but are probably not needed under laboratory conditions. Alternatively, vertical transmission of these OTUs could be compromised under artificial rearing (Sacchetti et al., 2014; Augustinos et al., 2019; Deutscher et al., 2019). Under this scenario, some gut bacteria might have disadvantageous conditions under the laboratory, and this situation could have negatively impacted on their diversity. However, more work is needed to address potential negative selection on specific bacterial groups as a consequence of drastic environmental changes, such as those suffered by wild flies when are brought to the laboratory and breed under captivity. Understanding the physiological role of these OTUs might shed light on important attributes of wild flies that are lost because of domestication.

Teneral flies of the F0 generation had the lowest values of diversity estimated through different indices among F0–F6 generations; whereas F1 and F3 showed the highest values for these parameters. This may be related with stochastic drift processes emerging after disturbs and associated to environmental factors that may have compromised the dominance of *Wolbachia* at least in F1, right after the introduction of the host to novel conditions (Staubach et al., 2013). After F3, and in F6 and LAB teneral flies, *Wolbachia* dominated the gut bacteria community. On the other hand, in post-teneral flies (15-day-old, fed individuals) the community is dominated by Enterobacter. This might indicate that the community is stabilized in post-teneral flies, and this status would be less exposed to environmental stressors. In agreement with our results, a recent study performed in *A. obliqua* (whole body) showed *Wolbachia* sp. more abundant in larvae and *Enterobacter* sp. in adults (Gallo-Franco and Toro-Perea, 2020).

Changes in the composition of digestive bacteria during the domestication process could affect the physiology and behavior of the host eventually leading to limitations in the context of the SIT, which requires a productive mass rearing but also sexually competitive. Previous studies in *C. capitata* highlighted the contribution of gut bacterial symbionts associated to the digestive tract to male sexual performance, flight ability and longevity under starvation and enhancement of the SIT (Niyazi et al., 2004; Ben-Yosef et al., 2008a; Ben-Ami et al., 2010; Gavriel et al., 2011; Augustinos et al., 2015; Kyritsis et al., 2017). Understanding the replacement of gut bacteria associated with domestication is an initial step to determine which bacterial symbionts are the most affected. This characterization would allow to design domestication protocols that either maintain key players in the gut of domesticated insects or restore them as part of the rearing process (i.e., use specific bacteria as dietary probiotics). Because the insect gut microbiome includes not only the gut bacteriome, but also virus, protozoa, fungi, yeasts that might interact not only with the host but also among them (Gurung et al., 2019), future studies should aim at disentangling these complex interactions and enrich our understanding of the physiology and behavior of this important fruit pest of South America.

CONCLUSION

Our work revealed that the origin and feeding status could shape the gut bacterial community of *A. fraterculus* adults. We observed a dynamic interaction between *A. fraterculus* and its microbiota. Upon emergence, the gut is dominated by *Wolbachia* but as flies feed and age, other genera such as *Enterobacter*, *Providencia*, and *Citrobacter* become more abundant, and the whole community more diverse, reaching a seemingly stable composition. We evidenced gradual changes during the first steps of the laboratory colonization process keeping, however, a degree of differentiation between flies under adaptation and a well-established laboratory strain. The taxonomic identification of the gut bacterial community of *A. fraterculus* sp. 1 from Argentina and the analysis of key factors modeling the structure and composition of the gut bacteriome provide valuable and novel information. Understanding the dynamic interaction between a tephritid host and digestive bacterial symbionts will enable to improve environmentally safe control strategies against fruit fly pest species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. 16S *rRNA* gene sequences reported in this study have been deposited in the NCBI under BioProject number PRJNA624812.

AUTHOR CONTRIBUTIONS

SL, LP, GT, KB, and DS conceived and designed the study. JS, LP, FM, RR, EA, and PS conducted the experiments. JS, DS, SL, and GT analyzed the results. JS, DS, SL, KB, JC, and GT drafted the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.570960/full#supplementary-material

Supplementary Figure 1 | *A. fraterculus* sp. 1 digestive bacterial community and sex. Meta-Non-metric Multidimensional scaling (metaNMDS) plot of bacterial profile representing samples grouped by sex: male in blue; female in red color. PERMANOVA analysis *p*-value (*d* = 0.2).

Supplementary Figure 2 | Relative abundance of OTUs (at genus level) of bacterial community associated to the digestive tract of flies grouped by sex.

Supplementary Figure 3 | Bacterial diversity found in *A. fraterculus* sp. 1 digestive tract of adult individuals grouped by sex. **(A)** Chao index. **(B)** Shannon index. **(C)** Faith index. **(D)** Simpson index. **(E)** Pielou index and **(F)** Berger index. See **Figure 4** for dots and box plots description.

Supplementary Figure 4 | Relative abundance of OTUs (at genus level) of bacterial community associated to the digestive tract of flies grouped by feeding status: T (teneral); PT (post-teneral).

Supplementary Figure 5 | Bacterial diversity found in *A. fraterculus* sp. 1 digestive tract of adult individuals grouped by feeding status: T (teneral); PT (post-teneral). **(A)** Chao index. **(B)** Shannon index. **(C)** Faith index. **(D)** Simpson index. **(E)** Pielou index and **(F)** Berger index. See **Figure 4** for dots and box plots description. Bars with asterisks above boxes indicate significant *p*-values (paired comparisons, Kruskal–Wallis test).

Supplementary Figure 6 | Bacterial diversity among origins - additional parameters. Simpson index **(A,E)**; Pielou index **(B,F)**; Berger-Parker index **(C,G)**; and Faith index **(D,H)** in teneral (T) and post-teneral (PT) individuals. WU, wild individuals from ubajay with unknown feeding status; Lab, individuals from the laboratory colony. Dots indicate observed values and box plots depict means and standard deviation of the data. Bars with asterisks above boxes indicate significant *p*-values (paired comparisons, Kruskal–Wallis test).

Supplementary Figure 7 | Bacterial diversity during laboratory colonization - additional parameters. Simpson index **(A,E)**; Pielou index **(B,F)**; Berger–Parker index **(C,G)**; and Faith index **(D,H)** in teneral (T) and post-teneral (PT) individuals. WU, wild individuals from ubajay with unknown feeding status; Lab, individuals from the laboratory colony. See **Figure 4** for dots and box plots description. Bars with asterisks above boxes indicate significant *p*-values (paired comparisons, Kruskal–Wallis test).

Supplementary Table 1 | Identification of gut samples and description of rearing conditions of *A. fraterculus* sp. 1 laboratory colonies and wild flies used in this work. F0–F6 indicates samples from generations under laboratory colonization. Lab, laboratory samples; WU, wild samples from ubajay; T, teneral feeding status; PT, post-teneral feeding status; M, male; F, female; Unk, unknown, without information on age, feeding status, and diet (at larval and adult stages).

Supplementary Table 2 | OTU description and representation in *A. fraterculus* sp. 1 gut bacteriome. **(A)** OTU representation – Percentage of samples. **(B)** OTU representation – Percentage of read counts. **(C)** OTU representation – Percentage of read counts considering feeding status. **(D)** OTU representation in % of samples considering feeding status. OTUs belonging to the bacterial core proposed are highlighted.

Supplementary Table 3 | Bacterial diversity and richness (Shannon and Chao indices).

Supplementary Table 4 | Relative abundance of *Wolbachia* (OTU 1) pair-wise comparisons (teneral [T] vs post-teneral [PT]) using Wilcoxon Rank Sum Test. Group compared: generation F0–F6 and laboratory flies (Lab). Mean values of each compared group were considered to the statistical analysis.

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Environmental Nutrients Alter Bacterial and Fungal Gut Microbiomes in the Common Meadow Katydid, *Orchelimum vulgare*

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Insect gut microbiomes consist of bacteria, fungi, and viruses that can act as mutualists to influence the health and fitness of their hosts. While much has been done to increase understanding of the effects of environmental factors that drive insect ecology, there is less understanding of the effects of environmental factors on these gut microbial communities. For example, the effect of environmental nutrients on most insect gut microbiomes is poorly defined. To address this knowledge gap, we investigated the relationship between environmental nutrients and the gut microbial communities in a small study of katydids (n = 13) of the orthopteran species *Orchelimum vulgare* collected from a costal prairie system. We sampled *O. vulgare* from unfertilized plots, as well as from plots fertilized with added nitrogen and phosphorus or sodium separately and in combination. We found significantly higher Shannon diversity for the gut bacterial communities in *O. vulgare* from plots fertilized with added sodium as compared to those collected from plots without added sodium. In contrast, diversity was significantly lower in the gut fungal communities of grasshoppers collected from plots with added nitrogen and phosphorus, as well as those with added sodium, in comparison to those with no added nutrients. There was also a strong positive correlation between the gut bacterial and gut fungal community diversity within each sample. Indicator group analysis for added sodium plots included several taxa with known salt-tolerant bacterial and fungal representatives. Therefore, despite the small sample number, these results highlight the potential for the gut bacterial and fungal constituents to respond differently to changes in environmental nutrient levels. Future studies with a larger sample size will help identify mechanistic determinants driving these changes. Based on our findings and the potential contribution of gut microbes to insect fitness and function, consideration of abiotic factors like soil nutrients along with characteristic gut microbial groups is necessary for better understanding and conservation of this important insect herbivore.

Keywords: insect microbiome, fungal microbiome, nutrient limitation, bacterial microbiome, katydid, grasshopper, *Orchelimum vulgare*

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INTRODUCTION

Recently, the rise in the research interest on insect microbiomes has helped reveal novel insights and understanding of insect ecology. Both resident and transient microbes exhibited effects on the health and fitness of their insect hosts (McKenney et al., 2018), such as improved nutrition (Brune, 2014; Paniagua Voirol et al., 2018), changes to host behaviors (Dillon et al., 2000), increased reproductive success (Kaltenpoth et al., 2009), and protection from environmental pathogens and pesticides (Paniagua Voirol et al., 2018). Conversely, behaviors of the insect host like feeding and social roles can be reflected in variations in their microbiome (Yun et al., 2014; Jones et al., 2018; Kakumanu et al., 2018; Obadia et al., 2018). Recently, we demonstrated that orthopterans, specifically katydids and grasshoppers, share a characteristic bacterial community dominated by Proteobacteria, Firmicutes, and Actinobacteria (Muratore et al., 2020). Because these organisms were collected from a coastal prairie under a fully factorial fertilization experiment, we had a unique opportunity to further dissect how the microbial community composition responds to nutrient modifications in the environment.

When an organism is limited by a particular nutrient, it is expected that an addition of that nutrient to the environment will result in increased biomass of that organism (van der Ploeg et al., 1999). For example, when plants are experiencing nitrogen and phosphorus limitation, the addition of nitrogen and phosphorus to the soil results in increased plant biomass. At the community level, in contrast, when an essential nutrient like nitrogen is increased, fast-growing, nitrophilic plant species often increase in abundance to the exclusion of other slower growing species (Southon et al., 2013), resulting in declines in plant community richness and diversity (Clark and Tilman, 2008; Soons et al., 2017; Midolo et al., 2019). Despite our understanding in how plant communities respond to nutrient limitation and subsequent increases, how consumer communities respond to nutrient fluctuations is less established. An early field study found that fertilization of grasses with ammonium nitrate in a Nebraska prairie resulted in increases in both the nitrogen abundance in the foliage as well as the biomass of grasshoppers (Heidorn and Joern, 1987). Similarly in a feeding trial study, grasshoppers fed with nitrogen- or phosphorus-enriched grasses from a Kansas tallgrass prairie ecosystem showed increased growth rates (Rode et al., 2017). At our field study in a Texas coastal prairie, grasshoppers also showed an increase in abundance in plots treated with nitrogen, phosphorus, and sodium, an observation indicative of nutrient co-limitation experienced by the herbivores ecosystem (Prather et al., 2018). However, a concomitant increase in the herbivore richness and diversity was also observed in this ecosystem (Prather et al., 2018). While specific mechanisms, such as nutrient status of plants and soil or insect feeding behavior, underlying these observations remain to be determined, it is clear that environmental perturbations of nutrients may indeed affect grasshopper ecology and potentially reflect the nutrient status experienced by these animals in a manner that does not fully follow the conventional wisdom with plants.

In addition to nutrients like nitrogen and phosphorus, sodium plays a key role in insect ecology. While typically considered non-essential for most plants (Maathuis, 2014), sodium is essential for animal physiology, controlling the osmolarity of body fluids (Geerling and Loewy, 2008) and regulating growth and reproduction. Sodium is also crucial for proper nervous system function (Liebeskind et al., 2011). Animals, including insects, have been known to satisfy their needs for sodium through activities beyond normal feeding behaviors (Smedley and Eisner, 1995; Morris et al., 2008). Grassland plant consumers, with limited sodium present in their food source, potentially experience sodium limitation and may exhibit sodium-seeking behaviors (Kaspari et al., 2017; Welti et al., 2019). Sodium levels in the soil can change naturally due to sodium carried by ocean winds in a coastal system, or anthropogenically by the additions of chemicals like ice-melting road salts (Franzén, 1990; Snell-Rood et al., 2014). Our study showing that grasshopper abundance and diversity were significantly higher in areas where sodium, nitrogen, and phosphorus were added strongly argues for nutrient colimitation in these animals and highlights the importance of sodium in a high-sodium grassland ecosystem (Prather et al., 2018).

Orchelimum vulgare is an insect herbivore that plays pivotal roles in the grassland ecosystems of North America (Báldi and Kisbenedek, 1997; Branson et al., 2006). *O. vulgare* is a key consumer of plant biomass as well as a key food source for predators – thus contributing to nutrient cycling and plant community composition in grasslands (Prather et al., 2017). Therefore, it has both economic and ecological significance. Though primarily feeding on plants, *O. vulgare* has been observed feeding on carcasses of insects and other small animals (Campbell et al., 2010; Zeng et al., 2016). The flexible feeding behavior might affect the composition of the gut microbiome, which can further impact the animal's fitness and functions. While our earlier work identified a "core" bacterial community in the gut of *O. vulgare* (Muratore et al., 2020), whether the microbiome composition of *O. vulgare* can be modulated by environmental nutrient conditions has not been determined.

In this study, we used a small cohort of samples (3–4 individuals per nutrient condition) to begin to explore the impact of environmental nutrient conditions on the gut microbiome of *O. vulgare* by analyzing the bacterial and fungal gut microbiome of individuals collected from large experimental treatment plots in a coastal tallgrass prairie. These treatment plots were amended with two different fertilizer treatments – nitrogen and phosphorus together (NP) and sodium alone (Na) each at two levels (ambient or added). If the gut microbiomes in *O. vulgare* experienced nutrient limitation and subsequently responded to added nutrient treatments, their compositions would be expected to change in individuals collected from added nutrient plots than those collected from ambient plots. More specifically, if the gut microbial communities responded to added nutrients similarly as their hosts, we would expect to see a significant increase in microbial diversity. Alternatively, if the gut microbial communities responded to added nutrients similarly as plant communities, we would expect to see a significant decrease in microbial diversity.

MATERIALS AND METHODS

Sample Collection

Orchelimum vulgare were collected from a coastal prairie, which is part of a large-scale fertilization experiment to study orthopteran communities at the University of Houston's Coastal Center near Houston, Texas. Many orthopteran species have been documented in this prairie, of which *O. vulgare* is one of the most common members (Prather et al., 2018). This species is an omnivore, and eats a mixed diet of plants and insect prey. The prairie topography is generally flat with a maximum of 2 cm gradient in elevation separating the experimental plots. The experimental site follows a fully factorial design manipulating nitrogen and phosphorus (N and P together at two levels, ambient and added) and sodium (Na at two different levels, ambient or added) with eight replicates in each treatment (n = 2 levels of NP × 2 different levels of Na × 8 replicates = 32 experimental plots). The prairie was divided into large plots (30 × 30 m^2) which were subsequently treated with fertilizers. Fertilizers were applied in March of 2016 and 2017 before the beginning of the growing season. We added nitrogen (in the form of urea) and phosphorus (in the form of monoammonium phosphate) and sodium at rates of 10 g/m^2 to bring the top 10 cm of soil to approximately 30% higher than ambient levels (Heidorn and Joern, 1987; Southon et al., 2013). We collected *O. vulgare* individuals from as many replicates as possible via sweep-netting during 1-day of sampling in June of 2017. Our sample size was limited by the number of individuals we caught in each plot that day, which was determined by local abundance at the time. In total we included 13 *O. vulgare* individuals at 4th instar or later in development in this study: three individuals from plots with no added nutrients (None), four individuals from plots treated only with sodium (Na), three from plots treated with only nitrogen and phosphorus (NP), and three from plots treated with nitrogen and phosphorus and sodium (NP × Na). The insect samples were shipped on ice to the University of Dayton (Dayton, OH) and stored frozen at −20°C until dissection. Dissection consisted of removal of the entire gut, including contents, from the crop to the hindgut using instruments sterilized in 95% (v/v) lab-grade ethanol between each dissection. Gut samples were stored at −20°C until DNA extraction.

DNA Extraction

A detailed description of this procedure was explained elsewhere (Muratore et al., 2020). Briefly, frozen *O. vulgare* gut samples were homogenized into smaller pieces for DNA extraction using the Qiagen DNeasy Blood and Tissue Kit (Qiagen 69504) following the manufacturer's protocol. Concentration of total DNA in each sample was measured by a nanophotometer (Implen, Denville Scientific Inc.) and then the extracted DNA sample was stored at −20°C until sequencing.

Small Subunit rRNA Gene Sequencing and Identification

As explained in a previous publication (Muratore et al., 2020), high throughput DNA sequencing was performed by Zymo Research (Irvine, CA). Before the library construction took place, extracted DNA was quantified using nanodrop and the 2100 Bioanalyzer System (Agilent). The V3–V4^{TM} region of the bacterial and Archaeal 16S rRNA gene which was amplified using the Quick-16S Primer Set V3-V4 (Zymo Research, Irvine, CA). The ITS2 region in fungal species was amplified using the ZymoBIOMICS Services ITS2 Primer Set.

The sequencing library prepared by Zymo utilized real-time PCR to prevent chimera formation and to control cycles. The PCR products were quantified with qPCR fluorescence readings. These products were pooled together based on equal molarity, and the library was cleaned up with Select-a-Size DNA Clean & Concentrator^{TM}, then subsequently quantified with TapeStation® and Qubit®. The libraries were sequenced on the Illumina HiSeq2500 platform in "Rapid Run" mode with a v3 reagent kit (600 cycles), using 100 bp paired end sequencing, with an average of 10.2 million reads per sample. Samples were collected in 4 cycle intervals until sufficient amplification had occurred, or processing was ended at 42 cycles if no amplification occurred. PCR Single nucleotide differences were distinguished among sequences and used along with the Greengenes database (gg_13_8) to establish taxonomic identification. Unique amplicon sequences were inferred, and chimeras removed using the DADA2 pipeline (Callahan et al., 2016). Taxonomy assignments were made using Uclust from Qiime (v.1.9.1) (Caporaso et al., 2010) and an internally curated research database (Zymo). Information about the number of reads per sample is listed in the **Supplementary Table S1**. The rarefaction curves of bacterial and fungal species for each sample as well as the species accumulation curves are shown in the **Supplementary Figures S1, S2**.

Data Analysis

Statistical analysis was performed in R (version 3.6.2). Diversity calculations, including Shannon and Inverse Simpson diversity as well as richness, were performed using the *vegan* package in R. To test for differences between treatments, we used ANOVA with two independent variables (NP or Na) at two levels (ambient and added) at the species level. To look at effect sizes, we calculated Cohen's d using the cohen.d function in the *effsize* package in R at the species level. Regression comparing bacterial and fungal diversity was carried out in base R. Non-metric multidimensional scaling (NMDS) was performed at the species level using the *vegan* package in R. NMDS plots were used if the model arrived at convergence. Analysis of similarity (ANOSIM) was used to measure Bray-Curtis dissimilarity, corresponding with the NMDS plots was also performed using R. Indicator values were calculated using the indval function in the *labdsv* package in R. All indicator values specified had a p value of less than 0.05. Species accumulation was calculated using the specaccum function in the *vegan* package of R. In order to highlight dominant groups, bacterial groups are classified as "other" when they are present at less than 2% of average relative abundance in at least two treatment groups and did not appear in every treatment with the exception of Entomoplasmatales families, which appeared in only three treatments but at high relative abundance. Fungal orders are classified as "other" when they are present at less than 2% of

average relative abundance in at least two treatments and did not appear in all treatments.

RESULTS

Bacterial Communities

A total of 171 bacterial and 99 fungal species-level operational taxonomic units (OTUs) were identified in the 13 samples (**Table 1**). Curiously, no archaeal species were detected in any of the samples. A mean bacterial richness of 31.6 (± 3.9) was observed across 13 samples. Bacterial phyla present in all four treatments (None, NP, NP × Na, and Na) included Proteobacteria, Firmicutes, and Actinobacteria. While Proteobacteria was the most abundant phylum in all four treatment groups (**Figure 1A**), the most notable shift in bacterial phyla was observed in the predominance of Tenericutes in the NP treatment group. There was a total of 11 bacterial families present at an average relative abundance of 2% or higher in all four treatment groups (**Figure 2A**) with the 6 most abundant families being Enterobacteriaceae, Lactobacillaceae, Listeriaceae, Methylobacteriaceae, Pseudomonadaceae, and Rhizobacteriaceae. Uncategorized family OTUs from the order Entomoplasmatales were present in four out of the 13

TABLE 1 | Gut microbiome richness and diversity for four treatments.

Treatment group	Sample	Bacterial richness	Bacterial inverse simpson index	Bacterial shannon index	Fungal richness	Fungal inverse simpson index	Fungal shannon index
None	#1	40	10.35	2.81	42	10.58	2.94
	#2	40	3.18	1.74	50	6.5	2.61
	#3	17	6.15	2.23	22	5.63	2.04
NP	#4	21	4.19	2.12	24	2.12	1.39
	#5	19	2.13	1.18	21	1.36	0.75
	#6	7	1.17	0.39	7	1.01	0.04
NP × Na	#7	30	8.32	2.68	25	3.78	1.88
	#8	37	14.59	3.03	16	9.45	2.44
	#9	65	3.58	2.44	42	4.97	2.32
Na	#10	26	11.17	2.76	11	6.71	2.12
	#11	35	4.84	2.26	19	8.23	2.41
	#12	30	6.3	2.4	26	5.14	2.04
	#13	41	12.81	2.92	25	5.28	2.23

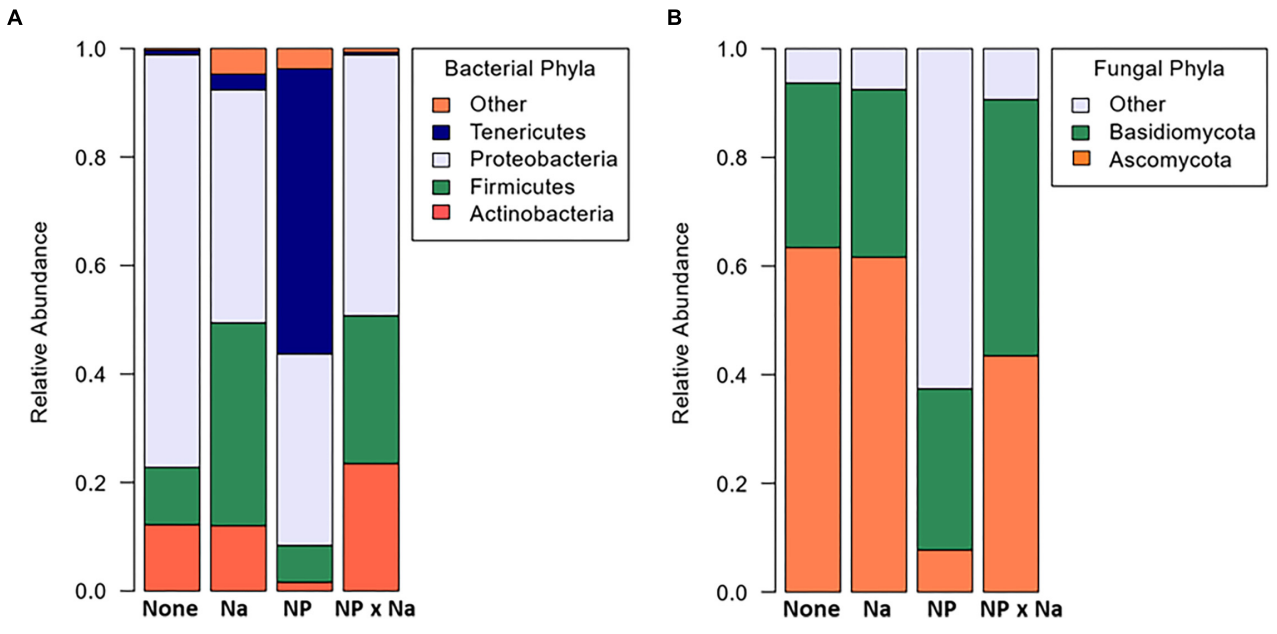

FIGURE 1 | Average relative abundance of microbial phyla in grasshopper samples from four treatments. The four treatments include: no added nutrients or "None" (n = 3), added sodium or "Na" (n = 4), added nitrogen and phosphorus or "NP" (n = 3), and nitrogen and phosphorus added with sodium or "NP × Na" (n = 3). The "Other" category is comprised of groups that do not appear in all four treatments and comprise less than 2% of reads. **(A)** Relative abundance of bacterial phyla. **(B)** Relative abundance of fungal phyla.

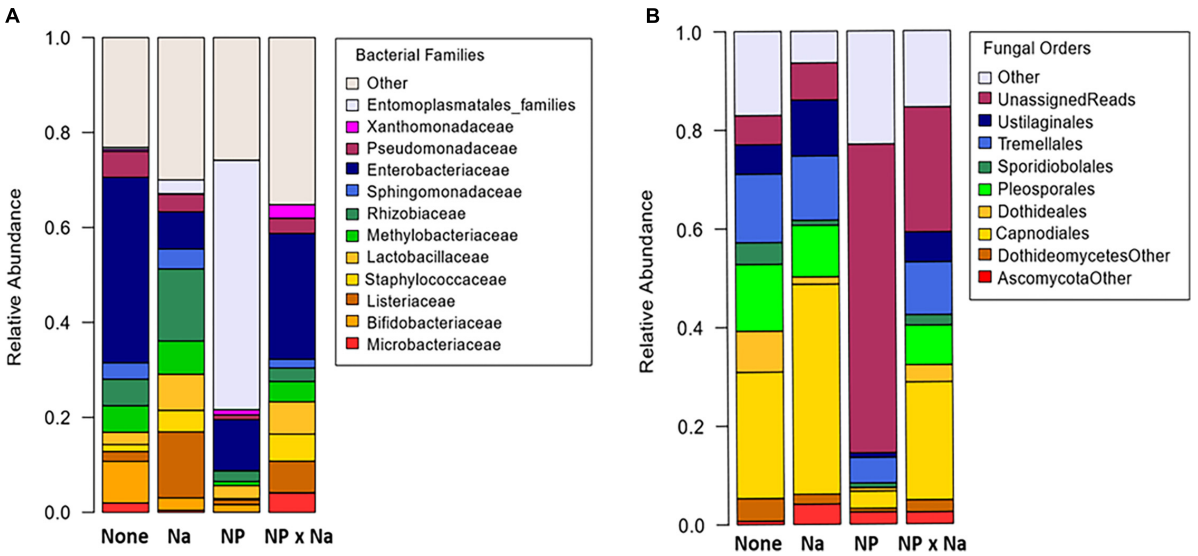

FIGURE 2 | Average relative abundance of microbial taxa in grasshopper samples from four treatments. The four treatments include: no added nutrients or "None" (n = 3), added sodium or "Na" (n = 4), added nitrogen and phosphorus or "NP" (n = 3), and nitrogen and phosphorus added with sodium or "NP × Na" (n = 3). In all The "Other" category is comprised of groups that do not appear in all four treatments and comprise less than 2% of OTUs. The only exception is the Entomoplasmatales families included which were not present in the nitrogen and phosphorus with sodium treatment, but which were present in the other three treatments. **(A)** Relative abundance of bacteria families. **(B)** Relative abundance of fungal orders.

samples, representing three of the four treatment groups. They comprise a large proportion of families in the NP treatment group but not the Na or NP × Na treatment groups. Five families, including Streptococcaceae, Propionibacteriaceae, Phyllobacteriaceae, Listeriaceae, and Corynebacteriaceae (indicator values, respectively: 0.9979, 0.9801, 0.9627, 0.8745, 0.7094), are clear indicators of bacterial communities in the Na treatment group. Only one of these, Listeriaceae, is found at high relative abundance. In contrast, only one family, Sphingomonadaceae, was an indicator of the NP treatment group (indicator value, 0.7986).

Alpha diversity of bacterial communities was assessed in terms of taxa richness, Shannon Index, and Inverse Simpson Index (**Table 1**). Shannon diversity of bacterial taxa assessed at the species level (**Figure 3A**) was significantly higher for gut samples from the NP treatment group (p = 0.014) with a large treatment effect size (Cohen's d = 1.04) compared to individual communities from plots with no added nutrients. Moreover, there was a moderate, but not significant, interaction between added Na and added NP groups (p = 0.08).

Beta diversity of bacterial communities was assessed using non-metric multidimensional scaling (NMDS) performed at the species level (**Figure 4A**). There appeared to be an overlap in characteristic community between the control group (None) and the two added Na treatment groups (Na and NP × Na). The characteristic communities of the NP treatment group shared less similarity with the characteristic communities of the other three treatments (ANOSIM, R = 0.299). Beta diversity was further characterized by assessment of shared species level OTUs between treatment groups (**Figure 5A**), revealing that 60% of total bacterial species level OTUs were unique to each treatment group. Conversely, only 5.8% of bacterial OTUs were shared amongst all four treatment groups. The relative abundance of unique bacterial OTUs were notably higher in added Na treatment groups, at 46.5% (NP × Na) and 40.7% (Na), compared to 27.6% (None) and 10.8% (NP) in no added Na groups.

Fungal Communities

A total of 99 OTUs were identified as fungal organisms with a mean fungal richness of 25.4 (± 3.3) among the 13 samples. The two most dominant fungal phyla in all four treatments were Ascomycota and Basidiomycota, but a relatively high number of fungal OTUs in the NP treatment group were unassigned (**Figure 1B**). Fungal data had a high number of specified OTUs at the order level of taxonomic classification (**Figure 2B**). Again, there was a high number of unspecified fungal OTUs for the NP treatment group even at order level. There were six predominant fungal orders that appeared in every treatment group: Capnodiales, Dothideales, Pleosporales, Sporidiobolales, Tremellales, and Ustilaginales. An unspecified species of *Cladosporium* was indicative of the Na treatment group (indicator value = 0.7806).

Alpha diversity of fungal communities was also assessed in terms of taxa richness, Shannon Index, and Inverse Simpson Index (**Table 1**). Shannon diversity of fungal species (**Figure 3B**) was significantly lower in the added Na treatment groups (Na and NP × Na, p = 0.035) and the added NP treatment groups (NP and NP × Na, p = 0.006), with a large negative treatment effect (Cohen's d = −1.12 and −1.28, respectively). Notably for fungal communities, there was a significant interaction between added Na and added NP (p = 0.004). More specifically, the interaction between added NP and added Na in the NP × Na treatment group

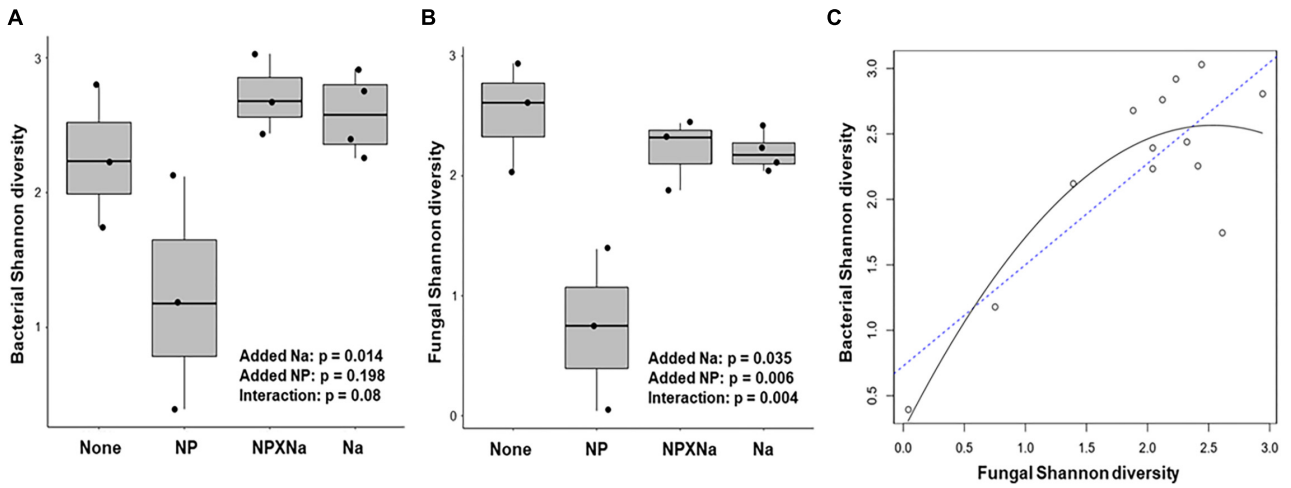

FIGURE 3 | Analysis of diversity over four treatments. **(A)** Bacterial species Shannon diversity for each treatment was calculated with the *vegan* package in R. Two-way ANOVA in base R was used to compare levels of diversity between treatments and test for significant difference. Treatment effect size, or Cohen's d, as calculated in R with the *effsize* package for Na compared to control was large (1.04). Effect size for NP was smaller (–0.32). **(B)** Fungal species Shannon diversity for each treatment was also calculated in a similar manner. Cohen's d effect size for Na as compared to control was large (–1.12), as was NP compared to control (–1.28). **(C)** Regression analysis performed in R indicates a significant positive correlation between bacterial Shannon diversity and Fungal Shannon diversity among all 13 samples (R^2 = 0.75).

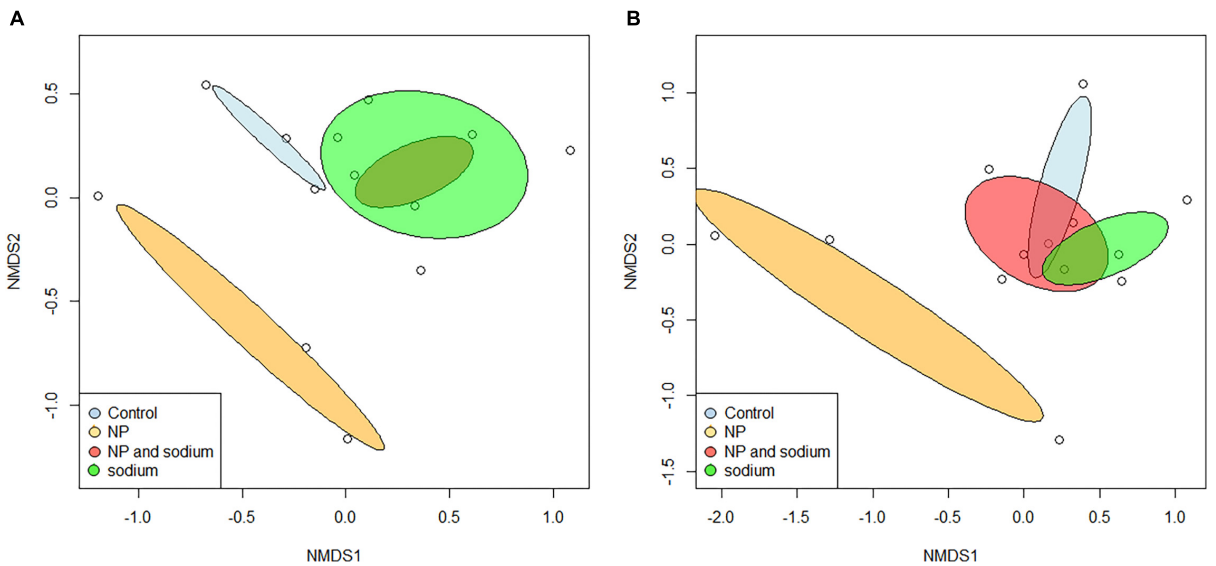

FIGURE 4 | Nonmetric multidimensional scaling of grasshopper gut community assemblages at the family level (n = 13) was plotted using the vegan package in R. Dispersion ellipses are placed for each treatment group. Bray-Curtis dissimilarity was well-preserved in two dimensions. **(A)** Bacterial characterization. Analysis of Similarity (ANOSIM) was performed with a Bray-Curtis dissimilarity measure and showed an overall significant difference in bacterial families among treatments (R = 0.299). **(B)** Fungal characterization. ANOSIM was performed with a Bray-Curtis dissimilarity measure and showed an overall significant difference in fungal families among treatments (R = 0.335).

restored fungal diversity present in the NP treatment group to the same level as the Na treatment group. Regression analysis (**Figure 3C**) of bacterial species diversity versus fungal species diversity showed a strong positive correlation between the two indices (R^2 = 0.75, p = 0.0006).

Beta diversity was also assessed using NMDS performed at the species level for fungi (**Figure 4B**). As with the bacterial communities, the characteristic communities of the NP treatment group again shared less similarity with the characteristic communities of the other three treatments (ANOSIM, R = 0.335). Beta diversity was also further characterized for fungal communities in terms of shared species with 51.5% of fungal species level OTUs unique to each treatment group (**Figure 5B**). In contrast, 18% of all fungal OTUs were

shared amongst all four treatment groups. The control group (None) contains the highest number of unique OTUs (39.4%), compared to the NP × Na, NP, and Na treatment groups (39.4, 17.1, and 23.3, respectively).

DISCUSSION

Environmental nutrients significantly altered the gut bacterial and fungal communities in this exploratory study using a small number of field-collected katydids. These patterns of changes in bacterial and fungal diversity did not mimic the changes that we saw in grasshopper densities in this experiment (Prather et al., 2018). In particular, katydid densities increased when both NP and Na were added to soils. Here, bacterial community diversity declined with added NP, but increased with added Na, while fungal community diversity declined in response to both NP and Na. Despite the limited sample number, these contrasting observations in katydid densities and katydid gut microbiome argued for further investigations to better establish the connections between environmental nutrient perturbations and the ecology of the host animals with their gut microbiomes. Here, we would like to offer some interpretations of our preliminary findings by first considering the role NP and Na on plant communities as a functional intermediate.

NP affected the plant community in ways that could have altered grasshopper feeding and density. Plant community biomass was higher with added NP (Prather et al., 2018), and NP addition changed functional composition of the plant community (Prather et al, unpublished data). In contrast, while increases in Na led to higher concentrations of soil Na, this nutrient did not affect plant biomass (Prather et al., 2018), nor did Na affect plant diversity or the relative abundance of different functional groups of plants (Prather et al., unpublished data). However, plant quality (i.e., chemistry) did change in response to Na – the relative abundance of N:Na declined with additional Na, and further declined when NP and Na were added in combination (Prather et al., 2018). This increase of Na in plants could have alleviated the grasshoppers from Na limitation, a phenomenon that has been repeatedly shown for herbivores and omnivores (Clay et al., 2017; Kaspari et al., 2017; Welti et al., 2019, 2020; Kaspari, 2020). These changes to plant chemistry could have also altered the diet of the katydids, and the katydids could have changed the relative amounts of plants and prey in their diet. In turn, this could have caused changes to the microbiome communities inside the katydids' guts. Because the bacterial and fungal communities exhibited distinctly different responses, we discuss below specifics about how these communities changed, and proposed potential investigations to further establish the mechanisms underlying the changes.

Bacterial Communities

For the gut bacterial communities, there are distinct differences between the effects of added NP and added Na. For NP, it has been observed that soil bacterial and fungal communities similarly respond directly to added NP with increased growth and shifts in community composition (Lv et al., 2017; Nottingham et al., 2018). More specifically, added NP was shown to contribute both directly and indirectly to a decrease in microbial richness and shifts in microbial communities in fertilized soils (Campbell et al., 2010; Zeng et al., 2016). The resulting shifts in community functional traits could affect nutrient cycling by the microbial communities in added NP soil (Leff et al., 2015). Therefore, for our observation where gut bacterial community samples from plots with added NP (NP and NP × Na) were less diverse than ambient controls, one possible interpretation would be that the gut bacterial communities respond to NP addition in a way similar to how the soil bacterial communities respond to NP addition. Furthermore, considering that nitrogen addition to the soil is known to decrease the diversity of plants, as well as animals in the affected area (Heidorn and Joern, 1987; Southon et al., 2013), the connectivity of the NP status in soil, plant, animal,

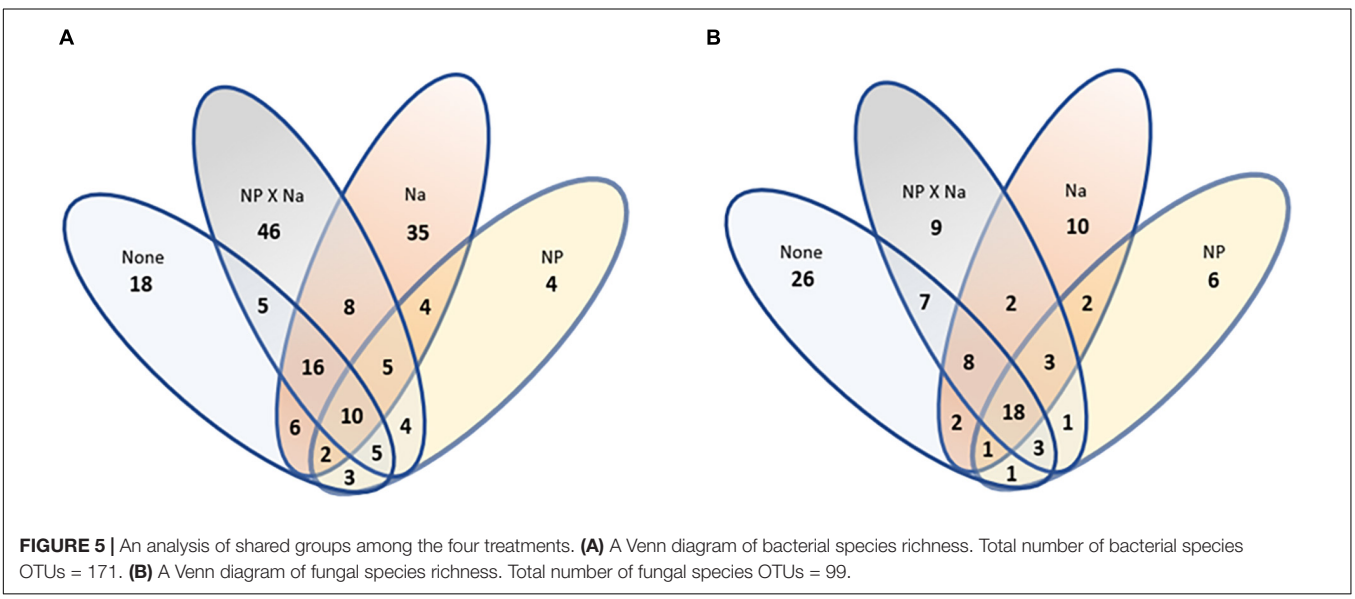

FIGURE 5 | An analysis of shared groups among the four treatments. **(A)** A Venn diagram of bacterial species richness. Total number of bacterial species OTUs = 171. **(B)** A Venn diagram of fungal species richness. Total number of fungal species OTUs = 99.

and the gut microbiome of the animals might be stronger than previously recognized and will require additional investigations to establish the relationship.

One specific piece of evidence provided by our study involves Sphingomonadaceae – the only indicator group for added NP. Sphingomonadaceae belongs in the Alphaproteobacteria class and can be found in a variety of habitats, including soil and plant phyllosphere and rhizosphere. On leaf tissues, the abundance of Sphingomonadaceae could be significantly increased by herbivory (Humphrey and Whiteman, 2020). Therefore, it is possible that if NP treatments uniquely stimulated higher herbivory, the higher levels of Sphingomonadaceae would be transferred into the gut of the herbivores. In addition, a more general piece of evidence we observed was the shifts in the bacterial community from relatively high abundance of Proteobacteria and Firmicutes to a predominance of Tenericutes in the NP cohort. Tenericutes are Gram-positive bacteria with members known to have association with plants and animals (Gupta et al., 2018). Therefore, these NP-elicited changes, including the abundance of Sphingomonadaceae or Tenericutes, may be the result of shifts in plant and soil microbiomes in combination with potential changes in insect feeding behaviors.

In contrast to NP treatments, bacterial community diversity increased in hosts collected from added Na plots. Also, the level of diversity is much higher in the NP × Na treatment samples compared to that in the NP treatment samples, suggesting that the effect of added Na is overriding the effect of added NP. If the Na treatment resulted in an increase influx of Na in the host diet, it is possible that the higher diversity is a direct consequence of added Na, perhaps as a stressor to select and enrich for a halophilic or halotolerant community. Alternatively, the higher diversity may be an indirect effect of changing insect behaviors, either sodium seeking or avoidance, that bring *O. vulgare* into contact with a higher variety of microbes as it feeds and scavenges. Salinity has been associated with changes in fitness and abundance of soybean aphids and oviposition choices in tiger beetles (Hoback et al., 2000; Eichele-Nelson et al., 2018). Moreover, in soil communities, increased salinity can lead to increases in richness and diversity of bacterial and fungal community composition (Mohamed and Martiny, 2011; Thiem et al., 2018), observations supporting a direct connection between soil and the animal gut microbiome.

Curiously, a deeper look into the Na indicator group seems to support the environmental Na playing a role in influencing the katydid gut microbiome through influencing soil as well as plant microbial communities. There are several unique taxa observed with added Na gut bacterial communities (both Na added and NP × Na added treatments). Several indicator groups for added Na communities were identified, including *Corynebacterium* – a Gram-positive bacterium in the Actinobacteria phylum. While multiple *Corynebacterium* species are notable human pathogens (Bernard, 2012), other research has identified isolates of this genus that are highly associated with sugarcane rhizosphere with increased soil salinity (Pirhadi, 2018). Phyllobacteriaceae and Streptococcaceae were also indicator groups for added Na and are bacterial families previously identified to be associated with high saline soil microbiomes (de León-Lorenzana et al., 2017; Genderjahn et al., 2018). Listeraceae and Propionibacteriaceae, additional indicator groups for added Na, are also families with species that are capable of growth in relatively high concentrations of Na (Labadie et al., 2003; Liu et al., 2005). Again, despite the limited number of samples in our study, the identification of these indicator groups presents a novel line of inquiry to identify the role of the gut microbiome in host fitness and behavior upon Na perturbations.

Our previous work has indicated that *O. vulgare*, when compared to other related grasshoppers, contains characteristic bacterial communities that may depend on diet or evolutionary lineage (Muratore et al., 2020). While almost nothing is known about the mode of transmission for bacterial communities in *O. vulgare*, it can be assumed that some of these resident bacteria come from diet, environmental contact, and feeding behaviors (Billiet et al., 2016; Krams et al., 2017). A large study investigating over 200 different insect species has indicated that gut bacterial communities among omnivorous insects are more diverse in general than those in strict herbivores or carnivores (Yun et al., 2014). This general conclusion implies that for the omnivorous *O. vulgare*, a shift in host feeding behaviors, perhaps from fewer plants to scavenging or vice versa, may correlate with a shift in the host microbiome. Beyond diet and feeding behaviors, it is also possible that many of the microbes in the *O. vulgare* gut community originate from soil and therefore shifts in gut microbial communities represent shifts in the soil microbial communities. In feeding trials, caterpillars who fed on intact dandelion plants had a microbiome that more closely resembled the soil microbiome than the phytobiome of the dandelion (Hannula et al., 2019). With previous evidence suggesting that both soil and plant microbiomes are affected by changes in environmental nutrients like NP and Na (Lv et al., 2017; Thiem et al., 2018), better understanding of the nutrient levels inside the grasshopper gut (Holmes et al., 2017; Bier et al., 2018) will provide insight into whether changes to gut microbiome are the direct result of changing environmental nutrient status.

Fungal Communities

The decrease in fungal diversity in both added NP and added Na treatment groups suggests that the gut fungal microbiome in *O. vulgare* is also susceptible to environmental NP and Na perturbations. However, whether this is a direct or indirect relationship is, again, difficult to determine. Increased soil salinity has been indicated as a cause of decreased fungal diversity, especially in estuary soils (Mohamed and Martiny, 2011; Thiem et al., 2018). Therefore, changes in the soil fungal community in response to Na addition may indirectly contribute to the community shifts observed inside *O. vulgare*.

To delve deeper into our data, the number of fungal OTUs shared by all groups (18) was lower than the total number of unique fungal OTUs (51), suggesting a small "core" fungal community in these animals. Some of these shared groups, such as the order Capnodiales, have established associations with insects like scale and aphids (Stephenson, 2012). Other shared taxa, such as genera *Cryptococcus* and *Hannaella*, have been previously associated with plants (Wen et al., 2017). The one unique group distinctive as an indicator

of added Na gut fungal communities were members of the hyphomycete genus *Cladosporium*, which is categorized as an osmotolerant mold (Araújo et al., 2020) and are found in a wide range of environmental habitats, including hypersaline waters (Bensch et al., 2012).

The *O. vulgare* gut samples in this study yielded a total of 99 species-level OTUs. However, because taxonomic resolution is still relatively low for fungal communities, many fungal OTUs were unspecified or uncategorized. This in part due to the lack of well-established databases of fungal ITS sequences, and in part due to the convolutions of fungal taxonomy (Tang et al., 2015; Gdanetz et al., 2017). Moreover, historically speaking, research into the microbiome of insects has been biased towards bacterial community characterization, rather than characterization of the whole gut microbiome. This one-domain approach to learning about insect microbiomes has inevitably led to gaps in our understanding of the contributions by other Eukaryotic members in the insect microbiome (Gurung et al., 2019). Also, the characterization of the host-fungal microbiome relationship is largely informed by the studies of insect fungal pathogens or plant fungal pathogens transmitted by insects. Characterization of the fungal microbiome of insects like *O. vulgare* helps us to begin to gain a more comprehensive awareness of these fungal communities and their potential functions.

CONCLUSION

Here we demonstrated that abiotic factors, in this case, environmental shifts in soil nutrients like NP and Na, result in changes the gut microbiome of *O. vulgare*. Although our sample number is limited, significant differences in microbial community composition between treatment and control groups were observed, supporting a continuity of microbial communities across soil, plant, animal and raising the hypothesis that nutrient limitation may also exist in the gut microbiome. We further demonstrated that the fungal microbiome of *O. vulgare* responded to environmental perturbations very differently than the co-existing bacterial microbiomes. We identified salt-tolerant genera *Corynebacterium and Cladosporium*, as well as families like Streptococcaceae, Propionibacteriaceae, Phyllobacteriaceae, and Listeriaceae as indicators of insect microbiomes exposed directly or indirectly to increases in environmental sodium. While these results may be considered preliminary in nature, they provide an important insight into insect gut microbiome structure and function upon environmental perturbations. Future studies to better establishing mechanisms contributing to characteristic insect gut microbiomes under the influence of changing ecosystem conditions, such as changing nutrient levels, will help us identify the roles of microbes in sensing and responding to disruptions that may threaten insects or their ecosystems.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Dryad Repository, https://doi.org/10.5061/dryad.t4b8gthzp.

AUTHOR CONTRIBUTIONS

All authors participated in the writing and preparation of the manuscript. Insect collection and prairie fertilization were performed under the direction of CP. Sample preparation and data analysis were performed by MM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.557980/full#supplementary-material

Supplementary Figure 1 | Rarefaction curves for bacteria **(A)** and fungal **(B)** taxa in each sample. Analysis performed in Qiime (v.1.9.1).

Supplementary Figure 2 | Species accumulation curves for bacteria **(A)** and fungal **(B)** communities calculated using specaccum function in vegan package of R (v.3.6.2).

Supplementary Table 1 | Number of reads per *Orchelimum vulgare* sample..

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High-Throughput Cultivation for the Selective Isolation of Acidobacteria From Termite Nests

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Microbial communities in the immediate environment of socialized invertebrates can help to suppress pathogens, in part by synthesizing bioactive natural products. Here we characterized the core microbiomes of three termite species (genus *Coptotermes*) and their nest material to gain more insight into the diversity of termite-associated bacteria. Sampling a healthy termite colony over time implicated a consolidated and highly stable microbiome, pointing toward the fact that beneficial bacterial phyla play a major role in termite fitness. In contrast, there was a significant shift in the composition of the core microbiome in one nest during a fungal infection, affecting the abundance of well-characterized *Streptomyces* species (phylum Actinobacteria) as well as less-studied bacterial phyla such as Acidobacteria. High-throughput cultivation in microplates was implemented to isolate and identify these less-studied bacterial phylogenetic group. Amplicon sequencing confirmed that our method maintained the bacterial diversity of the environmental samples, enabling the isolation of novel Acidobacteriaceae and expanding the list of cultivated species to include two strains that may define new species within the genera *Terracidiphilus* and *Acidobacterium*.

Keywords: termites, *Coptotermes*, core microbiome, natural products discovery, Acidobacteria, underexplored phyla, social insects, termite-associated microbes

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INTRODUCTION

Subterranean termites play a key role in the decomposition of plant biomass (Scheffrahn et al., 2015; Kuwahara et al., 2017). Their ingestion and degradation of wood can change the composition of soils and remodel entire landscapes (Bonachela et al., 2015). Furthermore, some termites cause damage valued at more than US$ 22 billion p.a. by attacking wooden structures (Chouvenc et al., 2011; Chouvenc et al., 2016). The genus *Coptotermes* contains the largest number of economically destructive termite species (Scheffrahn et al., 2015), including *C. formosanus* (Su, 2003) and *C. gestroi* (Jenkins et al., 2007; Vargo and Husseneder, 2009), both of which are native to Asia but have spread to other areas as invasive pests. Furthermore, *C. testaceus* is the dominant termite species infesting living trees in the central Amazonian rain forests (Apolinário and Martius, 2004).

Termites are eusocial insects with worker, soldier and reproductive castes. Their social lifestyle includes allogrooming and trophallaxis, which requires frequent direct contact among individuals

and facilitates the spread of microbes (He et al., 2018). Accordingly, termites have evolved behaviors to prevent infections, such as the removal of corpses from the nest (Davis et al., 2018). Furthermore, more than 150 million years of coevolution has established a beneficial relationship between termites and their surrounding microbial community (Bourguignon et al., 2015; Legendre et al., 2015). These microbes not only facilitate the digestion of wood (Long et al., 2010; Poulsen et al., 2014; Wang et al., 2016), but also suppress the growth of entomopathogenic bacteria and fungi (Traniello et al., 2002; Chouvenc et al., 2013; Mevers et al., 2017).

Social insects—especially termites—are protected by parts of the symbiotic and stable microbiota, particularly natural products (NPs) producing organisms, therefore they are discussed as a fruitful source for NPs discovery (Klassen et al., 2019). Such specialized microbial communities are essential for the lifestyle of social insects (Audisio et al., 2005; Kaltenpoth, 2009; Chouvenc et al., 2018). For example, several actinomycete mutualists associated with leaf-cutting ants produce antifungal natural products such as dentigerumycin or antimycotic polyenes (Haeder et al., 2009; Devine et al., 2017). Similar mechanisms have been reported for higher and lower termites (Stroeymeyt et al., 2014; Benndorf et al., 2018). Termite soldiers not only prevent invasion by predators (Husseneder and Simms, 2014), but they also possess a broad range of chemical defense mechanisms including the production of antibacterial agents (Sobotnik et al., 2010; Terrapon et al., 2014; He et al., 2018). Bacteria that synthesize antimicrobial compounds have been isolated from the nest material of *C. formosanus* (Mevers et al., 2017; Chouvenc et al., 2018). Bacterial genera known to form symbiotic interactions with social insects include *Streptomyces* and *Pseudonocardia*, both of which are actinomycetes known to synthesize natural products (Chouvenc et al., 2013). However, termites are also associated with *Burkholderia* species, representing the well-characterized phylum Proteobacteria (Santos et al., 2004), as well as less-studied phyla with the potential to synthesize as yet unexplored natural products (Makonde et al., 2015; Su et al., 2016). A balanced consortium of beneficial microbes in the environment is therefore necessary to maintain the health of the colony (Chouvenc et al., 2011; Rosengaus et al., 2011; Peterson and Scharf, 2016).

Here we carried out a systematic analysis of the microbial core community at different levels in the nest of three *Coptotermes* species, revealing the stability of the core microbiome and its impact on colony fitness. Selected bacterial strains were enriched and cultivated in a high-throughput microplate-based format to gain insight into the roles of less-studied bacterial phyla that are generally underrepresented in culture, such as the Acidobacteria.

MATERIALS AND METHODS

Termite Ancestry, Rearing, and Sampling

Captive colonies of *C. testaceus* (± facing fungal burden), *C. formosanus* and *C. gestroi* were reared for more than 15 years at the Federal Institute for Materials Research and Testing in Berlin (BAM) in separate metal tanks with a volume of ∼2 m^3 (**Supplementary Figure 1**). *C. gestroi* was reared at 26 ± 2°C, 87 ± 5% relative humidity (*RH*), and was fed on birch wood, which was refreshed every 3 months. *C. testaceus* and *C. formosanus* were reared at 29 ± 2°C, 75 ± 5% *RH*, and were fed on pine wood as above. All three species were identified based on their morphological characters (Scheffrahn et al., 2015).

Samples from the carton nest material of *C. testaceus* were collected at four different time points over 2 years. Sterile plastic spatulas were used to transfer 200–300 g of nest material into sterilized glass Petri dishes. At first, surface material was collected (*surface*), then wood pieces from feeding events from below the nest surface were placed aside and biofilms were sampled from the fresh eroded base of the wood pieces (*wood*) (**Supplementary Figure 2**). Autoclaved steel double spatulas were used to excavate sample material from the carton nest (*carton nest*). For all termite samples, first, filter carton paper traps were placed in the nests to separate the termite soldiers from the nest material. Using soft tweezers, the termite specimens were collected in 50 mL tubes and directly frozen (*termites*). All samples were frozen and stored at –50°C for processing and at 4°C for cultivation purposes.

Sample Processing and Environmental DNA Extraction From Nest Material and Termites

Nucleic acids were extracted using the NucleoSpin soil DNA purification kit (Macherey Nagel, Düren, Germany) to combine a gentle mechanical and chemical disruption of samples. To increase the yield, 200–500 mg of nest material or 12–28 termites as a whole were weighed into the NucleoSpin bead tubes before adding 700 µL of lysis buffer SL2. The tubes were vortexed horizontally for 15 min at 40 Hz using a Top Mix 11118 (Fisher Scientific, Schwerte, Germany) and then centrifuged at 12,000 × *g* for 2 min. Thereby, the samples were mechanically broken and lysed. Subsequent extraction steps were carried out according to the manufacturer's recommendations. Finally, the yield and purity of the received DNA from the termites and from nest material was checked using a NanoDrop ND-1000 UV/Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). In the following, we will use the term environmental DNA (eDNA) for these DNA samples and differentiate between the origin (e.g., termites and nest material).

16S rRNA Gene Amplicon Sequencing and Data Processing

PCR amplification and Illumina 300 bp paired-end read sequencing of the eluted eDNA extracts were carried out by LGC Genomics (Berlin, Germany) using an Illumina (San Diego, CA, United States) MiSeq V3 system. The variable V3-V4 region was amplified using forward primer U341F (5′-CCT AYG GGR BGC ASC AG-3′) and reverse primer U806R (5′-GGA CTA CNN GGG TAT CTA AT-3′) (Klindworth et al., 2013). Data pre-processing, including the demultiplexing of all libraries, was carried out using Illumina bcl2fastq v1.8.4. Reads were sorted by amplicon inline barcodes unique to each sample, allowing one mismatch per barcode and discarding those with missing or one-sided barcodes or conflicting barcode pairs. Reads with a final length < 100 bases were discarded during the clipping of the

sequencing adapter from all reads. During primer detection and clipping, three mismatches were allowed per primer, and pairs of primers had to be present in each sequence fragment. If primer-dimers were detected, the outer primer copies were clipped from the sequence. The sequence fragments were converted to forward-reverse primer orientations after removing the primer sequences. The forward and reverse reads were combined using BBMerge v34.48[1].

Each read was aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN revision 21008) (Pruesse et al., 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast et al., 2013). Reads < 300 aligned nucleotides and reads with >2% of ambiguities, or 2% of homopolymers, respectively, were excluded from further analysis. All reads containing a low alignment quality (50 alignment identity, 40 alignment score reported by SINA), were identified and excluded from downstream processing. After this quality control, identical reads were dereplicated, the unique reads were clustered (OTUs), on a per sample basis, and the reference read of each OTU was classified. Dereplication and clustering was done using cd-hit-est (version 3.1.2)[2] (Li and Godzik, 2006) running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.98, respectively.

Classification was achieved by running a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset release 132[3] using blastn v2.2.30+[4] with standard settings (Camacho et al., 2009).

The classification of each OTU reference read was mapped onto all reads that were assigned to the respective OTU. This yields quantative information (number of individual reads per taxonomic path), within the limitations of PCR and sequencing technique biases, as well as, multiple rRNA operons. Reads without any BLAST hits or reads with weak BLAST hits, where the function "(% sequence identity + % alignment coverage)/2" did not exceed the value of 93, remained unclassified. These reads were assigned to the meta group "No Relative" in the SILVAngs fingerprint and Krona Charts (Ondov et al., 2011)[5]. Data were corrected by excluding all reads affiliated to Archaea (5.7%), chloroplasts (0.003%), mitochondria (0.003%), Eukaryota (3.4%), or *No Relative* (0.25%) from further analysis (9.35% excluded in total). Reads affiliated to the domain Bacteria were set to 100%.

Statistical Analysis

Statistical evaluation was carried out using PAST v3.18[6] (Hammer et al., 2001) including non-metric multidimensional scaling (nMDS) (Taguchi and Oono, 2005) and one–way analysis of similarities (ANOSIM) via 9999 permutations with a statistical significance test (Clarke, 1993) at genus-level resolution for operational taxonomic units (OTUs) assigned to phylogenetic groups, representing clusters of uncultivated bacteria or genera. ANOSIM and nMDS scores were computed using the Bray-Curtis similarity index. The calculations were used to illustrate the ratio between within-group and between-group dissimilarities of microbial communities associated with nest materials, termite samples, different sampling time points, before and after a 5 μm filtration step during Nycodenz density gradient centrifugation. A ternary plot and heat map charting the relative abundance of phylogenetic groups were used to find overlapping bacterial genera among the three *Coptotermes* species and to address which nest level represents the best source of underexplored bacterial phyla. We calculated diversity indices such as Chao 1, Shannon, dominance, and evenness, considering the number of phylogenetic groups and the number of individual reads per phylogenetic group to show similarities within the non-infected nest material and differences among all samples (Harper, 1999).

Retrieval of Living Cells From Nest Material Using Nycodenz Density Centrifugation

Living cells were retrieved from the *C. testaceus* carton nest material by density gradient centrifugation using a 60% (w/v) Nycodenz solution (Axis Shield, Dundee, United Kingdom) as previously described (Hevia et al., 2015). We transferred 1.0–2.0 g of nest material aseptically into four 50-mL tubes, and added 20 mL of autoclaved Milli-Q ultrapure water to each tube. The samples were homogenized three times at 225 Hz for 5 s using an S25 KD 18 G dispersal tool connected to an Ultra-Thurrax T25 basic (both provided by IKA Werke, Staufen im Breisgau, Germany) to a fineness of 10–50 μm. We added another 10 mL of Milli-Q ultrapure water to each tube and centrifuged briefly at 450 × *g* at 4°C using an A4-81 swing-out rotor (Eppendorf, Hamburg, Germany). We then transferred 25 mL of the debris-free supernatant to a fresh 50-mL tube. The homogenate was carefully underlain with the autoclaved 60% Nycodenz solution. The tubes were then centrifuged at 3,050 × *g* for 60 min at 4°C in a swing-out rotor without acceleration and deceleration to form the desired layer of bacteria (Berry et al., 2003). The layer containing the bacteria was passed through a 5-μm cellulose acetate Minisart syringe filter (Sartorius, Göttingen, Germany) and collected in a 5-mL reaction tube.

Cultivation Media

For the cultivation of Acidobacteria from the *C. testaceus* carton nest material, we used VL55 medium (de Castro et al., 2013) (DSMZ no. 1266) supplemented with 0.05% (w/v) xylan instead of glucose (Sait et al., 2002) and used $FeCl_3 \cdot 6\ H_2O$ instead of $FeCl_2 \cdot 4\ H_2O$ for trace element solution SL-10 (Tschech and Pfennig, 1984).

Microplate-Based Cultivation

To estimate the total number of cells per well, the Nycodenz cell phase was diluted 1,000-fold in phosphate buffered saline (PBS) and analyzed by flow cytometry on a FACSCalibur (BD Bioscience, San Jose, CA, United States) following the protocol of the Bacteria Counting Kit (B7277, Thermo Fisher Scientific)

[1] http://bbmap.sourceforge.net
[2] http://www.bioinformatics.org/cd-hit
[3] http://www.arb-silva-de
[4] http://blast.ncbi.nlm.nih.gov/Blast.cgi
[5] https://github.com/marbl/Krona/wiki
[6] https://folk.uio.no/ohammer/past/

and visualized using FlowJo v10.4.2 (FlowJo, Ashland, OR, United States). Based on a combination of cell enumeration and the live/dead ratio, calculated using the LIVE/DEAD BacLight Bacterial Viability Kit (L34856, Thermo Fisher Scientific), 40 μL of cell suspension containing an average of 40 cells was distributed using a Matrix WellMate (Thermo Fisher Scientific) into 64 × 384-well microplates (Greiner Bio-One, Kremsmünster, Austria) minus the media controls on each plate (24,384 wells in total). Following incubation for up to 14 days at 28°C, 70 ± 5% *RH*, growth was verified by measuring the optical density at 600 nm (OD_{600}) in a Wallac 1420 Victor2 Microplate Reader (Perkin Elmer, Waltham, MA, United States). The threshold was set by averaging the media controls on each plate to establish the media background. All cultures with verified growth were automatically transferred into 96-deepwell microplates (Corning, New York, NY, United States) pre-filled with 1.5 mL medium using a Precision XS liquid-handling system (BioTek Instruments, Bad Friedrichshall, Germany). Then, plates were incubated at 28°C using a Duetz System holder (Adolf Kühner, Birsfelden, Switzerland), shaking at 220 rpm with 2.5 cm deflection for 7 days.

Rapid Identification of Cultures by 16S rRNA Gene Sequencing

Culture broth from the above mentioned cultivation step was divided into aliquots for OD_{600} measurements, DNA extraction, and cryo-conservation using the VIAFLO 384 system (Integra Biosciences, Zizers, Switzerland). Glycerol stocks were prepared by first pre-filling tubes with 300 μL 80% glycerol using the Matrix Wellmate and then adding 200 μL of culture broth. For DNA extraction, 200 μL of broth was transferred to microtubes (Qiagen, Hilden, Germany) containing 2.3-mm zirconia beads (Carl Roth, Karlsruhe, Germany) and the cells were disrupted by 2 × 1 min pulses at 30 Hz using a TissueLyser II (Qiagen). The tubes were centrifuged for 2 min at 4,000 × *g*, incubated at 70°C for 45 min and centrifuged again as above. The supernatant was used for 16*S* rRNA gene amplification with primer pair E8F (5′-GAG TTT GAT CCT GGC TCA G-3′) and 1492R (5′-ACG GYT ACC TTG TTA CGA CTT-3′) (Lane, 1991).

Phylogenetic Classification of Isolated Acidobacteria

Liquid cultures affiliated to Acidobacteria via 16*S* rRNA sequencing (only using the reverse primer), were prioritized to obtain pure cultures. Thus, the liquid cultures were streaked onto solid VL55 medium containing 1.5% (w/v) agar no. 1 (Oxoid Deutschland, Wesel, Germany) and in parallel onto Reasoner's 2A medium (R2A) prepared from DSMZ no. 830 by reducing the pH to 5.5 using 1.95 g/L 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich, St. Louis, MO, United States). The 16*S* rRNA gene sequence of FhG110202 was used to detect the 40 most similar sequences from the NCBI 16*S* ribosomal RNA sequences database using BLAST[7]. Multiple sequences were aligned using ClustalW with default parameters, including nearly full-length 16*S* rRNA gene sequences of FhG110206, FhG110214 and representatives of different subgroups of Acidobacteria. The phylogenetic tree was calculated using MEGA v7.0.26[8], by applying the maximum-likelihood method using the Tamura-Nei model (Kumar et al., 2016) with 1,000 bootstrap replications. We used iTOL v4.4.2[9] for graphical modifications and annotations (Letunic and Bork, 2019).

RESULTS AND DISCUSSION

Experimental Rationale and Strategy

Termite nests maintained for decades at the BAM, offer a unique opportunity to assess a community of microbes, unaffected by abiotic factors. We were inspired by the hypothesis that a stable microbial composition confers protection on a colony and is an indicator of fitness among eusocial insects (Koch and Schmid-Hempel, 2011; Lanan et al., 2016). We compared the microbiomes of three *Coptotermes* species in order to (i) identify the bacterial core microbiome, (ii) analyze the stability of the bacterial community over time, (iii) observe any shifts in the composition when the termite colony was infected by fungi, and (iv) achieve the selective cultivation of underexplored bacterial phyla like Acidobacteria. Our main goal was to screen this resource for underexplored bacterial phyla like Acidobacteria, making them more accessible and thus facilitating the future analysis of their natural products. To this end, we applied a standardized, high-throughput cultivation approach and adapted the conditions to favor the recovery of underexplored and hard-to-cultivate phyla, focusing on the Acidobacteria (Crits-Christoph et al., 2018).

Bacterial Core Microbiome of Three Domesticated *Coptotermes* spp.

To characterize the core microbiomes of three *Coptotermes* species [*C. testaceus* (Ct), *C. formosanus* (Cf) and *C. gestroi* (Cg)] and their nest material, eDNA was isolated from the termites and from different levels of their nests [surface (S), wood (W), and carton nest (C)]. Three samples were taken from each nest level and from corresponding termites. Illumina amplicon sequencing of isolated eDNA yielded 4,724,006 sequences in total, 0.25% of which could not be classified and were defined as *No Relative*. Rarefaction analysis confirmed adequate coverage for statistical calculations (**Supplementary Figure 3**). Although the rearing temperature and type of wood provided as food differed between Cg and the other species, the termite and nest materials were highly comparable among all three species, with the different nest levels and termites (**Figure 1** and **Supplementary Figure 4**).

Illumina amplicon sequencing revealed that sample *C. testaceus* surface (CtS) featured the greatest number of phylogenetic groups at the genus level (605) and sample *C. gestroi* termite (CgT) featured the least (242) (**Table 1**). To calculate the ratio between within-group and between-group dissimilarities a one-way ANOSIM was performed including all non-infected

[7]https://www.ncbi.nlm.nih.gov/

[8]https://www.megasoftware.net

[9]https://itol.embl.de/

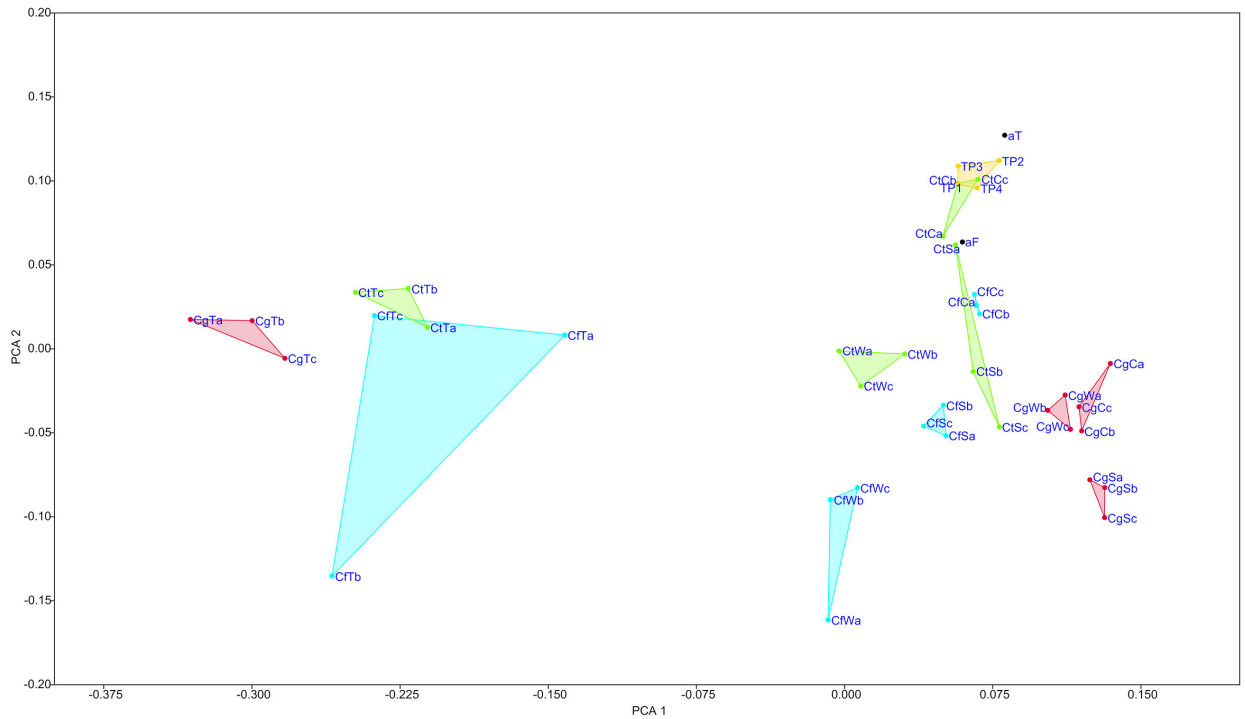

FIGURE 1 | Non-metric multidimensional scaling (nMDS) of all samples. Analysis includes data showing relative abundances of combined replicates from termite microbiomes, their nest material at different levels, cells before and after filtration during Nycodenz density centrifugation (each $n = 1$), and four different time points of *C. testaceus* carton nest material. Triplicates from different nest levels and termites are localized distinctly in the grid. All the *C. testaceus* carton nest samples, analyzed at different time points and filtration steps, form a distinct cluster indicating a similar bacterial composition. TP1, CtC replicate b; TP1–4, sampling time points; Ct, *C. testaceus*; Cg, *C. gestroi*; Cf, *C. formosanus*; C, carton nest material; W, wood material; S, surface; T, termite; aT, after turrax; aF, after 5 μm filtration during Nycodenz density centrifugation. Stress value = 0.124.

samples. No significant differences between sample means were calculated ($p > 0.2$) except the termite group ($p < 0.01$). The Shannon indices were similar among the samples of nest material (2.9–3.7) and among the termites (2.3–2.6), but the termite samples showed lower overall microbial diversity (**Table 1**). This indicates that the termite samples inhabit a narrower selection of microbes than the surrounding nest material, probably reflecting selection for exo- and endosymbionts with specific metabolic roles (Brune and Dietrich, 2015).

In all termite samples, Bacteriodetes was the most abundant phylum (52%), followed by Alphaproteobacteria (12%), Spirochetes (11%), Firmicutes (7%), and Actinobacteria (6%) (**Figure 2**). Compared to the captive termites of this study, those phyla were already observed in comparable abundances in free-living *C. gestroi* harvested in Vietnam (Do et al., 2014) and *C. testaceus (formerly C. niger)* (Dietrich et al., 2014) as well as *C. curvignathus* guts *from Malaysia* (King et al., 2014). Particularly, the genera *Alistipes*, *Desulfovibrio*, *Treponema*, *Dysgonomonas*, *Mycoplasma,* and *Burkholderia* (**Supplementary Table S1**) and order Actinomycetales, Xanthomonadales, Pseudomonades (**Figure 2**) were also described as highly abundant in other studies (Brune and Dietrich, 2015); Among the Bacteroidetes, the order Bacteroidia was most abundant (up to >98%), whereas Candidatus Azobacteroides represented the most abundant genus (>50%) (**Supplementary Table S1**). Some Bacteroidia are known as obligate intracellular symbionts that live within cellulolytic protists in the termite gut (Hongoh et al., 2008). They are essential for nitrogen fixation and the degradation of complex carbon sources, but are not particularly enriched in natural product gene clusters (Stingl et al., 2004; Sun et al., 2015).

In all nest samples, the most abundant phyla were Actinobacteria, Alphaproteobacteria, Gammaproteobacteria and Acidobacteria, which together represented more than 90% of all phylogenetic groups (**Supplementary Figure 4**). The most abundant genera/families were present in all nest samples from all three *Coptotermes* species, and did not change significantly over time. Ranked by relative abundance based on our heat map and ternary plot, the most prevalent groups were *Conexibacter*, uncultivated Xanthobacteraceae, *Acidicaldus*, *Acidibacter*, *Acidothermus*, *Sphingomonas*, uncultivated Acetobacteraceae, *Roseiarcus*, *Occallatibacter*, *Candidatus Solibacter*, *Bryobacter*, *Xiphimenatobacter*, *Nocardioides*, *Gryllotalpicola*, and *Frankia* (**Figures 2**, **3**). We therefore propose, that these taxonomic genera are part of the core microbiome.

Although the phylum Actinobacteria was the most abundant in our nest samples, the classical members associated with natural product synthesis were rare [mean < 0.5%, standard error (SE) = 0.01]. In particular, streptomycetes were ∼10-fold underrepresented (<0.15%, *SE* = 0.12) compared to

FIGURE 2 | Relative abundances of the 80 most abundant phylogenetic groups. Samples of nest material were collected from the surface, wood and carton nest (n = 3), and corresponding termites ($n \geq$ 12–23) of three species (*C. formosanus*, *C. gestroi*, and *C. testaceus*). If the assignment of phylogenetic groups to the genus level was not possible it was left blank. Replicates were combined for visualization. Abundances indicate microbial communities shared among all nest levels or associated with termites. Ct, *C. testaceus*; Cg, *C. gestroi*; Cf, *C. formosanus*; C, carton nest material; W, wood material; S, surface; T, termite.

TABLE 1 | Richness and diversity indices based on amplicon sequencing data from termite and termite nest samples.

Termite species	Level	Number of sequences[a]	Number of phylogenetic groups[b]	Simpson[c]	Shannon[d]	Evenness[e]	Chao 1[f]
C. gestroi	CgS	322,114	549	0.92	3.7	0.05	245
	CgW	318,647	549	0.93	3.6	0.06	221
	CgC	309,675	392	0.93	3.5	0.06	187
	CgT	210,185	242	0.78	2.3	0.03	128
C. testaceus	CtS	896,649	605	0.92	3.0	0.03	232
	CtW	218,641	432	0.95	3.6	0.06	146
	CtC	450,553	404	0.89	2.9	0.04	222
	CtT	306,701	260	0.68	2.3	0.03	125
C. formosanus	CfS	257,399	444	0.91	3.3	0.04	220
	CfW	231,098	392	0.91	3.2	0.05	212
	CfC	217,688	279	0.90	3.0	0.05	136
	CfT	174,613	277	0.75	2.6	0.04	150
Nycodenz	aT	139,879	241	0.85	2.3	0.06	169
	aF	245,690	438	0.90	3.0	0.05	110
Sampling time points	TP1	105,574	217	0.85	3.1	0.05	132
	TP2	35,320	204	0.90	2.4	0.05	196
	TP3	95,298	242	0.86	2.8	0.05	232
	TP4	53,558	275	0.89	2.6	0.05	152
C. testaceus nest material	eS	34,709	442	0.97	4.2	0.20	325
facing fungal burden	lS	100,015	483	0.90	3.4	0.12	238

Replicates of nest materials and termites were combined for analysis. Statistical analysis was performed using PAST v3.18.
Sample abbreviations: Ct, C. testaceus; Cg, C. gestroi; Cf, C. formosanus; C, carton nest material; W, wood material; S, surface; T, termite; TP, time point; aT, before 5 μm filtration; aF, after 5 μm filtration during Nycodenz density centrifugation; eS, early stage of fungal infection; lS, late stage of fungal infection.
[a] *Total number of sequences per sample derived from Illumina sequencing.*
[b] *Total number of different phylogenetic groups in each sample.*
[c] *Simpson index represents the diversity. If the value is 0, diversity is at zero level, whereas 1 is the highest.*
[d] *Shannon describes the diversity of the data and considers the abundance and number of phylogenetic groups.*
[e] *Evenness represents the equal distribution of phylogenetic groups per sample: 0 = equal and 1 = unequal distribution.*
[f] *Estimated species richness of each sample.*

soil habitats (Zhang et al., 2019). To exclude the possibility that spore-forming actinomycetes were under-detected due to an experimental bias in eDNA isolation, the eDNA extraction method was evaluated using an actinomycete spore suspension. This confirmed the detection of actinomycete DNA and ruled out the artifactual exclusion of DNA from spore-forming bacteria (data not shown). Furthermore, nMDS analysis revealed overlaps in the composition of the surface, wood and carton nest microbiomes, suggesting that microbes are transferred within the nest. Compared to the other samples, the carton nest showed the least diverse bacterial microbiome, indicating a specialized bacterial composition (**Figure 1** and **Table 1**).

The total proportion of Acidobacteria in all nest materials (mean = 6.4%, *SE* = 2.3) was higher than in the termites (mean = 1.5%, *SE* = 1.99). For example, the abundance of Acidobacteria in sample CtC (7.8%, *SE* = 2.2) was greater than in sample CtT (2.7%, *SE* = 2.8) (**Supplementary Figure 4**). The Acidobacteria in the nest material were also more diverse than those in the termites. The major subgroups of Acidobacteria present in CtC were subgroups 1 (35%), 3 (50%), and 4 (11%), whereas 2% of the reads could not be assigned to a specific subgroup (**Supplementary Figure 5**). Comparison of the termite and nest material samples revealed clearly distinguishable dissimilarities (**Figures 1**, **4**).

Taken together, these experiments showed that all nest materials had similar diversity indices, indicating that the material was exposed to limited external influences and allowed a diverse microbial community to flourish. The taxonomic diversity of Acidobacteria (in terms of different subdivisions) was not greatly enhanced in CtC compared to the other nest materials, however, their nest material was selected for further investigation, because the statistical analysis and the abundance of Acidobacteria (7.8%, compared to a mean of 6.0%) made it a promising source for the isolation and cultivation of strains representing this phylum.

Stability of the *C. testaceus* Carton Nest Microbiome

Abiotic factors such as aeration, temperature and humidity, which are regulated by the architecture of termite nests, play a key role in microbiome stability (Marins et al., 2016). Among the various nest compartments, the carton nest is expected to contain the most adapted microbiota because it consists of salivary secretions and feces (Enagbonma et al., 2020). Furthermore, the carton nest is frequented by termites and protected from external contact except during feeding. We used the carton nest material as an accessible resource that enables routine sampling and the generation of comparative datasets over time. Therefore, we first investigated the temporal stability of the microbial community.

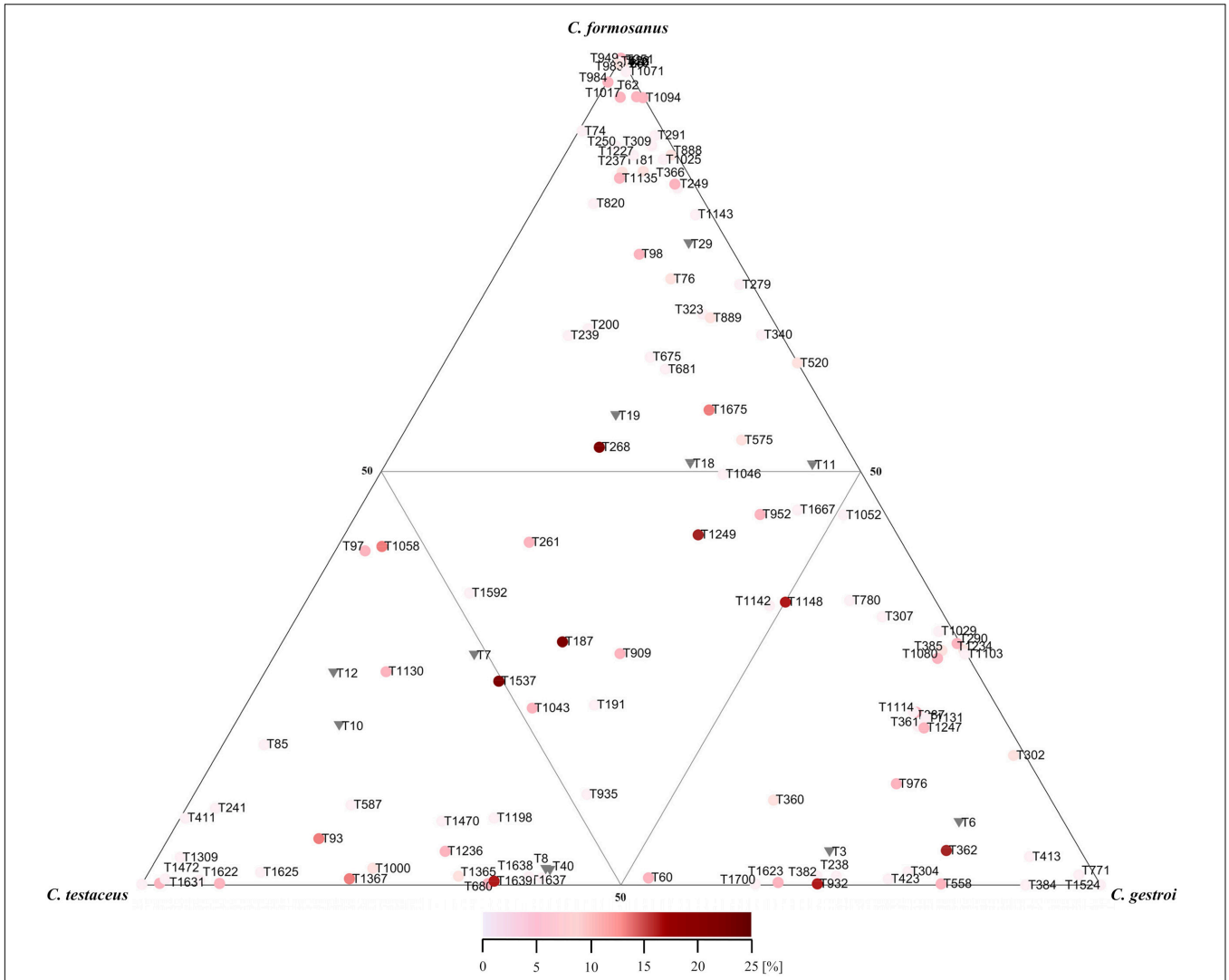

FIGURE 3 | Ternary plot of the relative abundances of shared bacterial phylogenetic groups among all nest materials of three domesticated *Coptotermes* species. Mean values of replicates ($n = 3$) from each nest level were combined. Only phylogenetic groups with a relative abundance >0.1% in at least one nest were included in the analysis. Colors indicate the relative abundance per sample of each phylogenetic group as indicated by the scale bar. Shared phylogenetic groups with the same abundance appear in the inner triangle. Phylogenetic groups belonging to the phylum Acidobacteria are marked with inverse triangles. An assignment of each phylogenetic group is provided in **Supplementary Table S1**.

The *C. testaceus* carton nest material was assessed at four time points over a period of 2 years to determine the comparability and statistical robustness of the amplicon sequencing data (**Figures 1**, **4**). Krona pie charts were constructed to compare the composition of the microbiomes over time (**Supplementary Figure 6**). The most abundant families were ranked as follows: Solirubrobacteraceae, Acetobacteraceae, Xanthobacteraceae, an unknown family of the class Gammaproteobacteria, Solibacteraceae, Acidobacteriaceae, Xipinematobacteraceae, Gemmataceae, Microbacteriaceae, Nocardioidaceae, IMCC26256 and Acidotermaceae. The stability of this microbial community was confirmed by their mean species evenness of 0.04 ($SE = 0.01$), their Shannon indices (**Table 1**), their nMDS scores (**Figure 1**), and the absence of significant changes identified by ANOSIM ($p > 0.05$, $R < 0.35$). The stability of the community is likely to be promoted by the unchanging abiotic and biotic conditions in the carton nest, including the constant temperature, humidity and light, and the consistent diet, which determines the activity of termites and the content of their secretions and excretions. The remarkable stability of the microbiome over time ensured that Acidobacteria were constantly present in the carton nest material.

Relationship Between Microbiome Stability and Termite Fitness

To determine whether the health status of termite populations has an effect on the stability of the microbiome, we studied a second *C. testaceus* nest at this time, which unintentionally suffered from a fungal infection that became progressively more

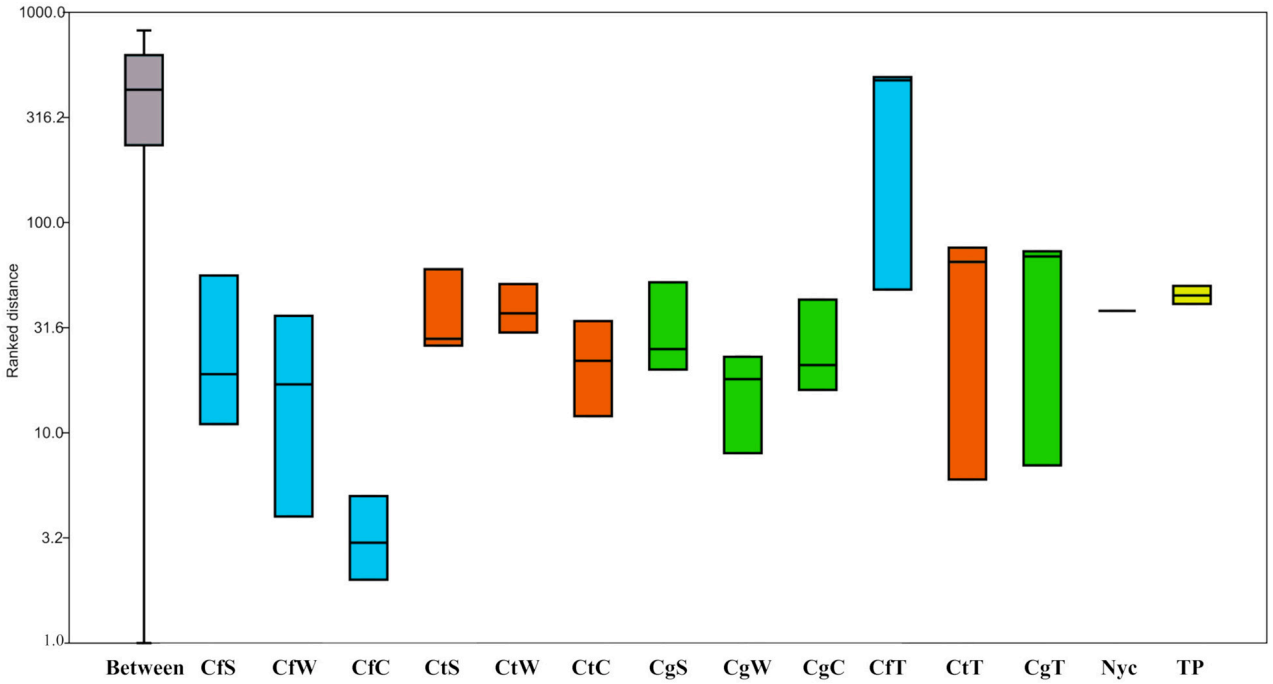

FIGURE 4 | Analysis of similarities (ANOSIM) of all nest materials taken from three captive *Coptotermes* species. One-way ANOSIM shows the ratio between within-group and between-group dissimilarities. Replicates of each sample, time point, and step (before and after filtration) were grouped together for analysis. PAST v3.18 was used for the calculations based on the Bray-Curtis similarity index. Box-plots were calculated using the interpolated quartile method. No significant differences between two or more groups exist except the termite group ($p < 0.01$). Cf, *C. formosanus* (blue); Ct, *C. testaceus* (orange); Cg, *C. gestroi* (green); C, carton nest material; W, wood material; S, surface; T, termites; Nyc, before and after 5 μm filtration; TP, four different sampling time points for the *C. testaceus* carton nest (yellow).

severe between sampling intervals (**Supplementary Figure 7**). Over time, the number of individual termites in the colony declined, ultimately to zero. No live specimens or even corpses were recovered from the nest material facing a fungal burden at the final sampling point. The microbiome of soldier termites retrieved from non-infected and infected showed little change during the early stages of infection (**Supplementary Figure 8**). In contrast, there was a remarkable shift in the composition of the nest material microbiome, with particular shifts in the abundance of Chloroflexi, Acidobacteria and the genus *Streptomyces* (**Figure 5**) compared to healthy nest material (**Supplementary Figure 4**). The abundance of *Streptomyces* spp. increased by more than 30-fold between the early and late stages of infection, which has been reported before in *C. formosanus* nests due to opportunistic mutualism between the termites and *Streptomyces* spp. with antifungal activity (Chouvenc et al., 2013, 2018). We also found that, during the early stage of infection, Acidobacteria were twice as abundant compared to uninfected nests (**Figure 5** and **Supplementary Figure 4**). Accordingly, we surveyed Acidobacteria in the different *C. testaceus* nest compartments. The carton nest showed generally broad taxonomic diversity and was enriched for Acidobacteria and other underexplored phyla, such as members of the PVC superphylum (Wagner and Horn, 2006), which accounted for more than 10% of the bacterial consortium (**Supplementary Figure 4**). Genome and metagenome analysis have shown that underexplored phyla such as Acidobacteria also carry biosynthetic gene clusters for the synthesis of specialized metabolites such as modified ribosomal peptides (Skinnider et al., 2016; Eichorst et al., 2018), polyketides, and non-ribosomal peptides (Crits-Christoph et al., 2018).

The comparative analysis of these gene clusters has shown a remarkable degree of genetic diversity compared to well-characterized gene clusters in other bacteria (Crits-Christoph et al., 2018). The genetic divergence among these clusters is often correlated with structural diversity of the corresponding secondary metabolites, providing confidence that such bacteria may lead to the discovery of new chemical entities (Medema et al., 2014). The opportunity to find gene clusters for secondary metabolism increases in bacteria with genome sizes exceeding 8 Mb (Baltz, 2017). The largest genome reported for a cultivated member of the phylum Acidobacteria is 9.9 Mb (Ward et al., 2009), whereas the mean size of the 23 published type strains of Acidobacteria[10] genomes is ∼5.2 Mb. Furthermore, horizontal gene transfer has been reported among the Acidobacteria, which indicates a potential to acquire functions with a defensive or regulatory advantage in their environment (Challacombe et al., 2011). For example, chitinolytic and cellulolytic activities are widespread among the Acidobacteria, which would be useful for the exploitation of

[10]https://www.ezbiocloud.net/search?tn=acidobacteria

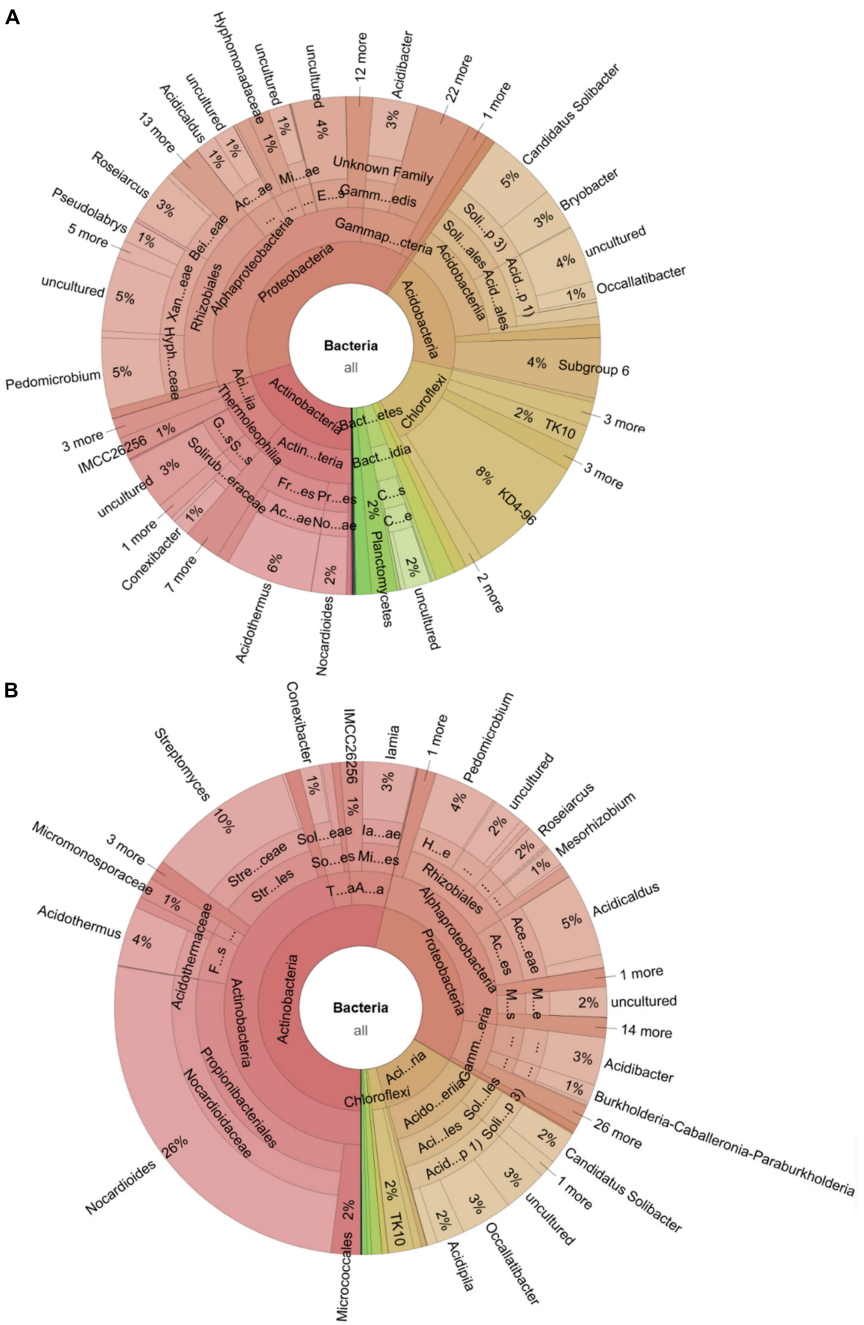

FIGURE 5 | Microbiome shift during the spreading of a fungal infection in a *C. testaceus* carton nest. The Krona chart shows the microbiome of the infected nest material during **(A)** the early and **(B)** the late stage of infection. The abundance of Acidobacteria and Chloroflexi during the early stage of infection was significantly higher compared to a healthy nest microbiome. The abundance of *Streptomyces* (early 0.03–10% late), Acidobacteria (early 19–12% late) and *Nocardioides* (early 2–26% late) was higher compared to the healthy nest material. The abundance of phylum Chloroflexi decreased from 14 to 2% from the early to the late infection stage.

nutritional niches in termite nests (Kielak et al., 2016; Belova et al., 2018). This may also benefit termites facing infections, as already reported for *Streptomyces* spp. associated with termite nest material (Carr et al., 2012; Chouvenc et al., 2018; Klassen et al., 2019). Although, Acidobacteria is the most abundant phylum in some habitats (Chan et al., 2006; Weijers et al., 2009), fewer than 60 Acidobacteria strains have been cultivated thus far (Kielak et al., 2016), whereas four classes have been proposed with validly published names[11]. Most of the isolates belong to subgroup 1, but still the

[11]https://lpsn.dsmz.de/phylum/acidobacteria

number of available strains is only ∼20 (Damste et al., 2017). A larger number of Acidobacteria strains with broader diversity must be made available in order to exploit their genetic and metabolic repertoire for natural product discovery. Given that the abundance of Acidobacteria doubled to 19% during the early stages of the fungal infection and that they remained more abundant (12%) than *Streptomyces* spp. (10%) even at the later stage (**Figure 5**), we sought to increase their general accessibility by a combination of targeted and high-throughput cultivation.

Targeted Cultivation and Phylogenetic Classification of Enriched Acidobacteria

The core bacterial community of the healthy *C. testaceus* carton nest material remained stable for 2 years. It showed broad taxonomic diversity but was enriched for Acidobacteria, and this phylum was further enriched by fungal infection. We chose the healthy *C. testaceus* carton nest material as the source for our targeted cultivation process to circumvent the fungal bias. To enable high-throughput cultivation, we used a small-scale microplate format. Thus, cells were retrieved based on Nycodenz density centrifugation used to enrich cells from complex environmental matrices, therefore, to enable subsequent cell enumeration via flow cytometry. Previous reports have indicated that using Nycodenz density centrifugation for soil samples can introduce bias (Holmsgaard et al., 2011). However, this may reflect the sample origin, given that the same method did not affect cell viability, or the distribution and proportion of the microbial community, during the analysis of fecal samples (Hevia et al., 2015). Accordingly, we first evaluated our adapted method to determine whether the retrieval of living cells from termite nest matrices influenced the composition of the bacterial community. We compared crude nest material with a homogenate and with cell layers before and after filtration (**Figure 6**). Statistical analysis using ANOSIM of the amplicon data revealed no significant shift in the bacterial community ($p > 0.1$), which was supported by bacterial composition analysis (**Figure 6**). Furthermore, nMDS analysis confirmed that the pivotal steps before and after filtration in the Nycodenz gradient centrifugation protocol are similar compared to the *C. testaceus* carton nest samples (**Figure 1**). The material was passed through a 5-μm syringe filter to separate cells from residual matrix particles. This is necessary for accurate cell counting by FACS, otherwise particles of the nest matrix can produce background noise. Following this treatment, the composition of the bacterial community (**Supplementary Figure 4**), the Shannon index (**Table 1**), and the ANOSIM and nMDS clustering profiles, indicated no remarkable changes (**Figure 1**). Indeed, the cell suspension derived from the Nycodenz and filtration steps were similar to the untreated nest material samples of *C. testaceus* (aF and aT, **Figure 1**). This confirms that the procedure we used was able to extract living cells from termite nest material with negligible experimental bias, and our target phylum was reliably extracted from the matrix (**Figure 6**). In the next step, cells were counted by staining with SYTO9 followed by analysis using flow cytometry, which indicated a total cell concentration (living and dead) of ∼8 × 10^8 cells/mL (**Supplementary Figure 9**).

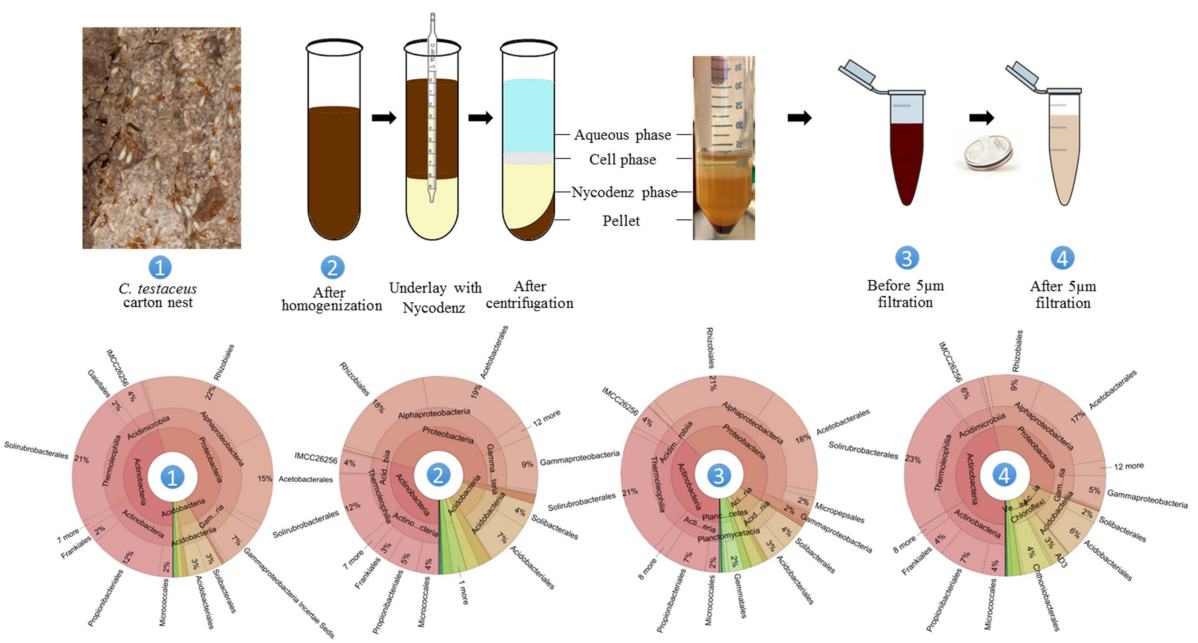

FIGURE 6 | Retrieval of living bacteria from *C. testaceus* carton nest samples. The microbiome composition was analyzed at four key steps of the Nycodenz density gradient centrifugation method. No bias in bacterial diversity was observed. Filtration using a 5-μm nylon syringe filter was necessary to remove particles for subsequent cell counting. The statistical comparison of these data with other data from this study can be found in **Figures 1**, **4**. Adapted with permission (Barra Caracciolo et al., 2005).

Based on earlier studies concerning the success of cultivating bacteria from environmental samples (Epstein, 2013; Ling et al., 2015), and taking the live/dead ratio (∼70:30) into account, we estimated that ∼40 cells should be distributed into each well of a 384-well plate containing 40 µL of medium per well. This was based on the assumption that only a small fraction would be able to grow in the synthetic VL55 medium (pH 5.5) supplemented with xylan as the only carbon source, a medium already shown to be suitable for the cultivation of Acidobacteria (Campanharo et al., 2016). This medium suppresses the growth of bacteria that cannot grow on complex carbon sources at low pH, and therefore favors the growth of the majority of Acidobacteria (Belova et al., 2018; de Chaves et al., 2019). Furthermore, Acidobacteria appear to thrive when presented with a lower concentration of trace elements than normally found in complex media (de Chaves et al., 2019). An additional advantage of the process described here is its ability to propagate the cultures quickly in order to reduce the risk of competition from faster-growing bacteria that take longer to become established due to the chosen medium, such as certain Proteobacteria (George et al., 2011; Campanharo et al., 2016).

Following the pipeline, 4,291 wells in total were re-inoculated from 384- to 96-deepwell microplates. After seven days of incubation, 4,028 wells were determined as grown via turbidimetry (93.9%). In total, 3,456 individual cultures (85.8%) were successfully sequenced via 16*S* rRNA gene sequencing (Sanger method, using the reverse primer only).

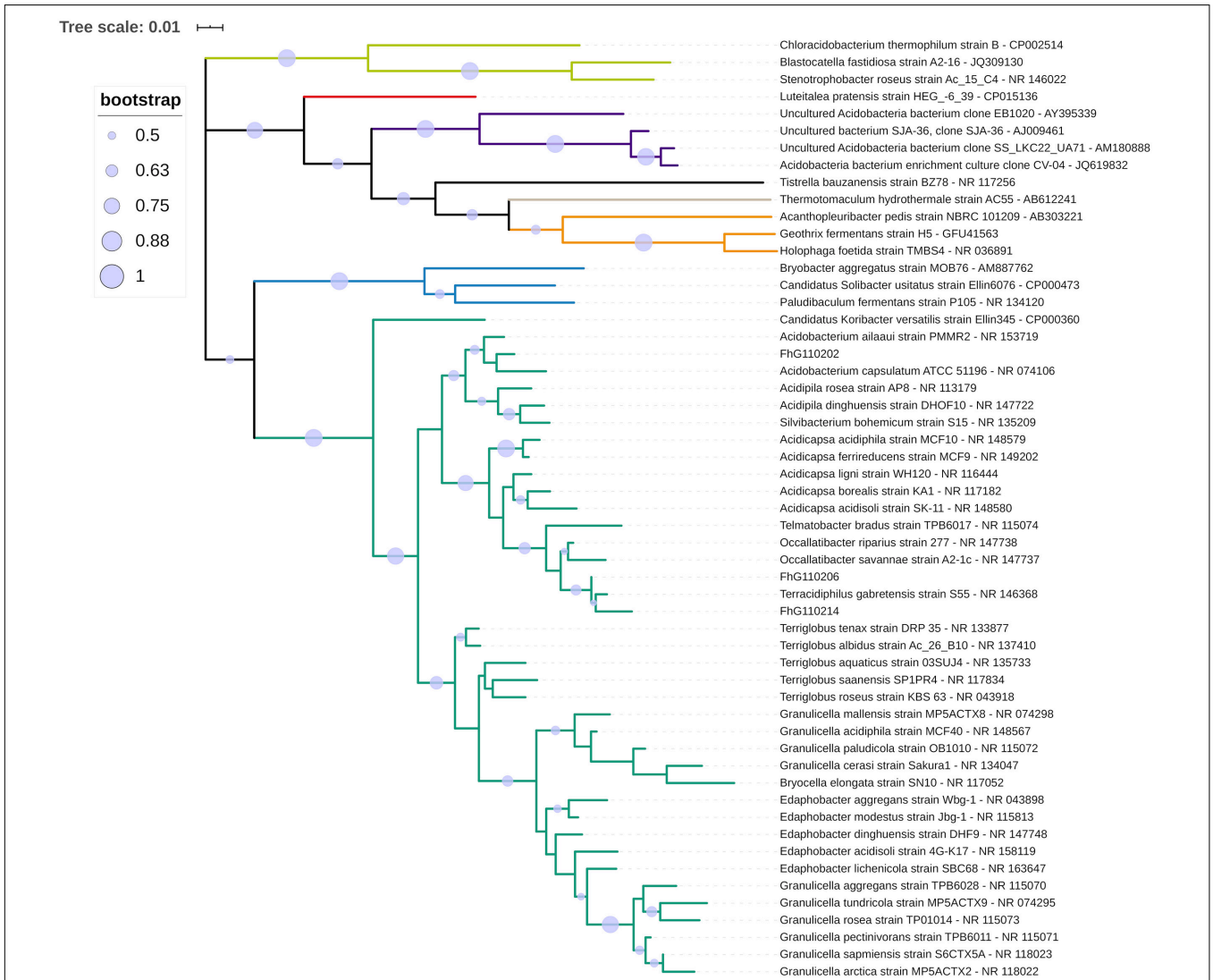

FIGURE 7 | Phylogenetic classification of the isolated strains FhG110202, FhG110206, and FhG110214 within the phylum Acidobacteria. The tree is based on a ClustalW alignment of available 16S rRNA gene sequences between positions 113 and 1,357 [based on *Escherichia coli* 16S rRNA gene numbering (Brosius et al., 1978)] from the 40 most similar sequences to the reference strain FhG110202, and also includes FhG110206, FhG110214, and representatives of Acidobacteria subgroups 3, 4, 6, 7, 8, and 10. The tree was calculated using MEGA v7.0.26 with the maximum-likelihood method and Tamura-Nei model. Circles on the tree branches indicate bootstrap values of 1,000 bootstrap replicates with a bootstrap support of more than 50%. Subgroup affiliations are indicated by colors. The new isolates are indicated by a green arrow. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Among these, 235 wells (6.8%) were affiliated with the target phylum Acidobacteria. After reduction for redundancy—based on 16*S* rRNA sequencing (Sanger method, using forward and reverse primer)—the unique wells were propagated onto agar plates. Finally, four unique strains were isolated. However, only three could be propagated furthermore and were therefore integrated into our strain collection with the IDs FhG110202, FhG110206, and FhG110214.

The phylogenetic classification of the three strains within the phylum Acidobacteria based on nearly full-length 16*S* rRNA gene sequences revealed their assignment to three different genera within subgroup 1 (**Figure 7**). FhG110202 was most closely related to the type strain of *Acidobacterium ailaaui* (97.7%), a bacterium that was isolated from a geothermally active microbial mat on Hawaii (Myers and King, 2016). FhG110214 was most closely related to *Terracidiphilus gabretensis* (97.1%), which was isolated from a boreal forest in a Czech national park (Garcia-Fraile et al., 2016). FhG110206 was most closely related to *T. gabretensis* (98.9%), *Occalibacter riparius* (98.4%), and FhG110214 (97.9%). A sequence identity threshold of 98.65% indicates species differentiation (Kim et al., 2014). The sequence identity between the 16*S* rRNA genes of FhG110214 and strain of *T. gabretensis* S55^T (97.1%), and FhG110202 and strain of *A. ailaaui* PMMR2T^T (97.7% identity), suggests that further experiments should be carried out to determine whether those strains represent new species.

Further investigations should also be carried out to determine the beneficial role of Acidobacteria for xylophagous *Coptotermes* spp. in terms of their potential to produce natural products cohering with their enrichment during the early and late stages of a fungal infection such as *Streptomycetes*. Our work therefore adds to the number of strains from the interesting phylum of Acidobacteria that are available for further analysis.

CONCLUSION

In this study, we evaluated the use of laboratory-bred termite colonies (*Coptotermes* spp.) and their nest materials as bioresources for the isolation of underexplored Acidobacteria, which are currently difficult to access or cultivate with sufficient diversity for bioprospecting. This is a key requirement because the likelihood of discovering new chemical entities is thought to be higher in underexplored phyla compared to classical phylogenetic groups that are already known to synthesize natural products. Our in-depth analysis at the genus level of microbial communities associated with three different termite species revealed the carton nest as the best source of Acidobacteria, with a 10-fold enrichment compared to the termites themselves. The microbial community showed high temporal stability in the healthy colony but underwent a profound shift during the late stage of a fungal infection favoring the proliferation of *Streptomyces* spp. and Acidobacteria. In summary, we applied a high-throughput cultivation process adapted to the metabolic repertoire of Acidobacteria. This led to the successful isolation of three novel strains of Acidobacteria, which may shed light on their biological relationship with xylophagous lower termites due to their accessibility for natural product discovery.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the following online repositories. The data can be accessed under the BioProject PRJNA657759 and the 16S rRNA sequences can be accessed under following numbers: MT895693, MT898545, and MT895791 (https://www.ncbi.nlm.nih.gov/genbank/).

AUTHOR CONTRIBUTIONS

MO, MS, and JG conceived and designed the experiments. JG initiated the isolation of Acidobacteria. MO, CZ, TC, MS, BL, and SM contributed to the cultivation and isolation experiments. MO, CZ, TS, JG, and SG analyzed the data. BL and MO implemented the liquid handling processes. MO drafted the first manuscript. RP and DM were responsible for the rearing, supply of termites, and revised the manuscript. MO, TC, JG, and RP performed sampling campaigns. TS, PH, and AV organized the manuscript writing. JG and TS supervised the research, helped to draft the manuscript, and revised it. AV acquired funding from the state of Hesse. AV and PH initiated the public-private partnership between Fraunhofer and Sanofi. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.597628/full#supplementary-material

Supplementary Table 1 | Taxonomic classification and the respective calculated relative abundance using Illumina amplicon sequencing data and additionally the SILVAngs v1.3 analysis pipeline.

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REVIEW
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Disruption of Host-Symbiont Associations for the Symbiotic Control and Management of Pentatomid Agricultural Pests—A Review

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The family Pentatomidae (Hemiptera: Heteroptera) includes several invasive stink bug species capable to attack a large number of wild and cultivated plants, causing several damages to different crops. Pentatomids rely on obligate symbiotic associations with bacteria of the family *Enterobacteriaceae*, mainly of the genus *Pantoea*. A distinctive trait of these associations is the transmission route: during oviposition, females smear egg masses with symbiont-containing secretions, which are ingested by newly hatched nymphs, allowing the symbiont to pass through their digestive tract and establish in the crypts of the posterior midgut. Preventing newborns from orally acquiring symbionts seriously affects their fitness and survival. This symbiont inheritance process can be manipulated to develop innovative pest control measures by sterilization of egg masses prior to nymph hatching. This review summarizes the recent knowledge advances concerning the gut primary symbionts of pentatomids, with a specific focus on the most troubling pest species for agriculture. Current understanding of host colonization dynamics in pentatomids is presented, as well as the phenotypic effects determined in different insect species by the alteration of vertical transmission. Details on the current knowledge on the whole bacterial communities accompanying primary symbionts are analyzed. The recent research exploiting the perturbation of symbiont acquisition by pentatomid nymphs is discussed, by considering published work on laboratory and field trials with several active substances. These translational strategies are presently regarded as promising for limiting the populations of many important pentatomid pests in a sustainable way.

Keywords: stink bug, gut symbiont, *Pantoea*, vertical transmission, egg smearing, pest control

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INTRODUCTION

Obligate symbioses with bacteria are widespread in many insect orders, and have particular evolutionary significance in species with nutritionally restricted diets. A main function of obligate symbionts is nutrient supply, upgrading the biosynthetic properties of the hosts and consequently their feeding potential (Buchner, 1965; Jing et al., 2020). A crucial phase for insect-microbe

obligate symbioses is symbiont inheritance, which is essential for the evolutionary conservation of symbiosis-related advantages (Salem et al., 2015). This process is allowed by transgenerational (vertical) transmission (i.e., the bacterial transfer from mother to offspring), which may take place in different ways according to the bacterial localization within the host body (Hurst, 2017). Intracellular symbionts can be transmitted from the mother to the offspring by entering the ovaries and the germline (i.e., transovarial transmission). One of the most widespread transovarially transmitted symbionts is the alphaproteobacterial *Wolbachia* that, by colonizing the egg cytoplasm, induces a number of manipulations of the host reproduction to the advantage of infected females and its own dissemination (Werren et al., 2008). Extracellular symbionts undergo alternative transmission strategies, including physical deposition of symbiont-containing substances close to the eggs after oviposition (e.g., egg smearing or capsule production), or environmental reacquisition of symbiotic cells by each generation (Hurst, 2017; Hosokawa and Fukatsu, 2020). Extracellular transmission routes imply that newborns are aposymbiotic after emergence, being exposed to possible failures of symbiont acquisition. The manipulation of symbiont vertical transmission in insect pests has been recently regarded as a promising method for implementing pest control strategies. Target insects for the application of such an approach must retain the following biological and behavioral traits: (i) nutritional dependence on bacterial symbiont; (ii) vertical transmission based on symbiont reacquisition by newborns; and (iii) well-delimited reacquisition sites. Insects characterized by transmission of associated symbionts through maternal secretions possess all of these features, therefore they are amenable to vertical transmission disruption.

Inherited symbiotic relationships are very common in the order Hemiptera. Associations are well-recognized in Auchenorrhyncha and Sternorrhyncha and, in the Heteroptera, they are usually found in infraorders Cimicomorpha and Pentatomomorpha (Hosokawa et al., 2006, 2019; Duron and Noël, 2016). In this review, a special focus is given to inherited symbionts of the Heteroptera, as they include many important pests in agriculture, such as the stink bugs (Pentatomidae; Knight and Gurr, 2007; Palumbo et al., 2016; Leskey and Nielsen, 2018; Sosa-Gómez et al., 2020), shield bugs (Scutelleridae; Davari and Parker, 2018), and plataspids (Plataspidae; Dhammi et al., 2016). More specifically, we describe host colonization and vertical transmission of gut symbionts of the family Pentatomidae. This family encompasses a number of agricultural pests that are responsible for huge economic losses worldwide, as they feed on a variety of fruits and seeds, seriously affecting crop yield and quality (Conti et al., 2020). Heavy attacks are recorded on almost all economically relevant crops, including commodity crops such as cotton, rice, maize, soybean, and wheat (Leskey and Nielsen, 2018; Zerbino and Panizzi, 2019; Defensor et al., 2020; Sosa-Gómez et al., 2020), fruit trees (Leskey and Nielsen, 2018; Mi et al., 2020; Powell, 2020), nuts (Bosco et al., 2018; Mehrnejad, 2020), and vegetables (Palumbo et al., 2016; Leskey and Nielsen, 2018). Furthermore, the invasive potential of many pentatomid species, such as *Halyomorpha halys* (Stål), *Nezara viridula* L., *Erthesina fullo* Thunberg, or *Bagrada hilaris* (Burmeister), makes them major agricultural threats in several areas outside their native range (Palumbo et al., 2016; Leskey and Nielsen, 2018; Conti et al., 2020; Mi et al., 2020).

Hemipteran species are associated with different bacterial taxa of Actinobacteria, *Alphaproteobacteria*, *Bacteroidetes*, *Betaproteobacteria*, *Gammaproteobacteria*, and Firmicutes (Sudakaran et al., 2017; Kashkouli et al., 2020). In Heteroptera, the ubiquitous inherited endosymbiont *Wolbachia* has been recorded in several species (Kikuchi and Fukatsu, 2003), even though it is not so widespread as in other insect taxa, and its role on the hosts biology has been poorly investigated (Kikuchi and Fukatsu, 2003; Matsuura et al., 2012; Becerra et al., 2015).

Gammaproteobacteria are one of the most represented taxa in the Heteroptera suborder hosts (Bansal et al., 2014; Karamipour et al., 2016; Kashkouli et al., 2020). These symbionts are essential for growth, development, and survival of the host and generally exhibit particular genomic features, such as A+T enriched genomes, fast-tracked molecular evolution and drastically reduced genome size to <1 Mb (Nikoh et al., 2011).

In Pentatomidae, bacteria of the family *Enterobacteriaceae* are often related to the genus *Pantoea* (Duron and Noël, 2016). These symbionts are obligate mutualists, provide their hosts with missing nutrients in their diets (Kenyon et al., 2015; Otero-Bravo et al., 2018), and inhabit crypts of the terminal portion of midgut, named region V4 (Fukatsu and Hosokawa, 2002; Prado et al., 2006; Karamipour et al., 2016). The maintenance of such primary symbionts in the host is ensured by egg smearing, i.e., deposition of maternal secretions upon egg surface, containing symbiont cells, which are orally acquired by newborns after hatching (Prado et al., 2006; Tada et al., 2011; Bansal et al., 2014; Otero-Bravo and Sabree, 2015). During the transmission process, the persistence of symbionts in the extrachorion matrix—outside the insect tissues—is supported by genomes that, even though reduced with respect to their free-living counterparts, are larger than those of intracellular primary symbionts commonly found in other Hemiptera. Their genomes retain genes encoding essential factors for autonomous life, such as those for the cell wall synthesis (Bergmann et al., 2014). These symbionts are adapted to multiple lifestyles, such as the symbiotic and the environmental lifestyles. Under an operational framework of sustainable pest control strategies, the environmental phase of the life cycle of these bacteria can be exploited to interfere with the symbiont acquisition process by the newborns that may impair their development (Taylor et al., 2017; Gonella et al., 2019; Kashkouli et al., 2019b).

SYMBIOTIC ASSOCIATIONS IN PENTATOMIDS

A growing number of studies highlighted the importance of gut symbioses in stink bugs, with emphasis on their peculiar transmission routes. According to the behavioral traits regulating the inheritance of pentatomid gut symbionts, upon hatching, first-instar nymphs remain aggregated around the egg masses and orally ingest the symbionts laid on the eggs (**Figure 2**) (Kashkouli

et al., 2019a; Oishi et al., 2019). During the host development the ingested symbionts colonize the inner cavities of the crypts in the posterior midgut of the second instar (Hosokawa et al., 2006; Oishi et al., 2019). Initial investigations of the adult digestive tract revealed that the symbiont-inhabited gut crypts are attached to the main gastric region by means of connective tissue, with no evident communication between these structures, suggesting that establishment in the crypts occur immediately after ingestion (Nikoh et al., 2011; Bansal et al., 2014). Indeed, a study of the spatiotemporal dynamics of the symbiont colonization process during the early developmental stages of the host showed the establishment of the gut bacteria in the posterior midgut already in the first instar (Oishi et al., 2019). Thickening and folding of the midgut epithelium proceed all throughout the immature development, with formation of several crypts and the spatial isolation of posterior midgut from the anterior part. In the final adult stage, bacteria are confined in the crypt cavities and isolated from the remaining gut compartments (Oishi et al., 2019). Morphological studies of the midgut of adult stink bugs highlighted a differentiation between males and females, with females displaying enlarged posterior midgut and bacteria occurring in the main tract outside of crypts, to allow egg smearing of symbiotic cells during oviposition (Hayashi et al., 2015).

Molecular and genomic methods identified through sequences of the 16S rRNA gene the primary symbiont of different stink bugs (Bansal et al., 2014), which were mostly classified within the genus *Pantoea,* closely related to plant- and insect-related strains (Prado and Almeida, 2009a) (**Figure 1**). Phylogenetic analyses of pentatomid symbionts indicated that they are polyphyletic, suggesting that several events of taxonomically-related symbiont replacements took place among different hosts during evolution. Evidences of different levels of dependency on symbiotic relationships have been provided for distinct stink bug species (Prado and Almeida, 2009a).

A considerable number of pentatomid primary symbionts and their importance for the host life cycle completion have been described, and a number of aberrant biological traits have been identified in response to a missed symbiont acquisition (**Table 1**). Some of the currently described host-symbiont systems, which have been studied in detail due to the major economic relevance of the stink bug host, are examined below.

Halyomorpha halys

The brown marmorated stink bug *H. halys* is an invasive Asiatic pentatomid recently introduced in North America and Europe. Its marked polyphagy—it attacks more than 300 species of plants—, high reproduction potential and high mobility make this insect a major pest of many crops (Rice et al., 2014; Leskey and Nielsen, 2018). The gut primary symbiont of *H. halys* is "*Candidatus* Pantoea carbekii" (*P. carbekii*) (Bansal et al., 2014), one of the few stink bug symbiotic bacteria whose genome is currently available (Kenyon et al., 2015). *P. carbekii* genome analysis, besides showing the genetic potential for nutrient provisioning to the host, indicated that this bacterium shares some genomic traits with intracellular primary symbionts of insects, such as a reduced genome size (0.7–0.9 Mb) and a low G+C content (Kenyon et al., 2015). These are both distinctive features of endosymbionts with stable host-restricted lifestyles (Moran et al., 2008). However, *P. carbekii* still encodes functional genes for essential extracellular life style traits, such as the metabolic pathways for the peptidoglycan synthesis, the generation of ATP by aerobic respiration, and other primary metabolic processes (Kenyon et al., 2015).

Prevention of symbiont acquisition through surface-sterilization of *H. halys* eggs results in nymph developmental delays in the first generation, providing direct evidence of its high dependence on *P. carbekii* (Taylor et al., 2014). Furthermore, in the second generation, only few individuals reach the adult stage, and surviving adults show longer pre-oviposition periods and produced less eggs, which in turn drastically reduces hatching rates and juvenile survivorship (Taylor et al., 2014).

Nezara viridula

The southern green stinkbug *N. viridula* is a cosmopolitan species distributed in different regions of North and South America, Africa, Asia, Australia, and Europe, and known as an important agricultural pest that damages a large number of crop plants (Tada et al., 2011). An unnamed symbiotic bacterium allied to the *Enterobacteriaceae* was reported as the gut symbiont of this stink bug (Hirose et al., 2006; Prado et al., 2006). Its involvement in *N. viridula* life cycle is debated, since different stink bug populations resulted either affected or not by symbiont removal (Prado et al., 2006; Tada et al., 2011). In facts, studies on Hawaian populations showed no clear fitness decrease on the offspring emerging from surface-sterilized egg masses, as emerged nymphs reached adulthood, finally producing viable eggs, despite they were symbiont-free (Prado et al., 2006). In contrast, similar treatment on egg masses from a Japanese population resulted in high mortality, with only few individuals reaching the adult stage (Tada et al., 2011). In this population, heat-induced symbiont suppression induced several fitness abnormalities, including retarded growth, reduced size, and altered body color (Kikuchi et al., 2016).

Plautia stali

The brown-winged green stinkbug *Plautia stali* Scott is a harmful pest of several fruit trees and crops (Oishi et al., 2019). *P. stali* was firstly reported to be associated with a single specific gammaproteobacterial symbiont, allied to *Pantoea* sp. This is an uncultivable bacterium with a small genome and it is essential for normal growth, fecundity, and survival of the insect host (Hosokawa et al., 2008; Oishi et al., 2019). Interruption of symbiont vertical transmission seriously hampers the development of nymphs hatched from sterilized eggs, due to the arise of opportunistic infections (Abe et al., 1995). Genomic analysis of the *P. stali* symbiont suggests that it is capable of producing lipopolysaccharides (LPS), important antigenic components of the Gram-negative bacteria cell wall, which are likely exploited as a defense during the environmental lifestyle, and as trigger for innate immune response of the host against pathogens (Kobayashi et al., 2011).

P. stali and its associated symbiont are currently regarded as a laboratory model for studying the insect-microbe gut symbiosis

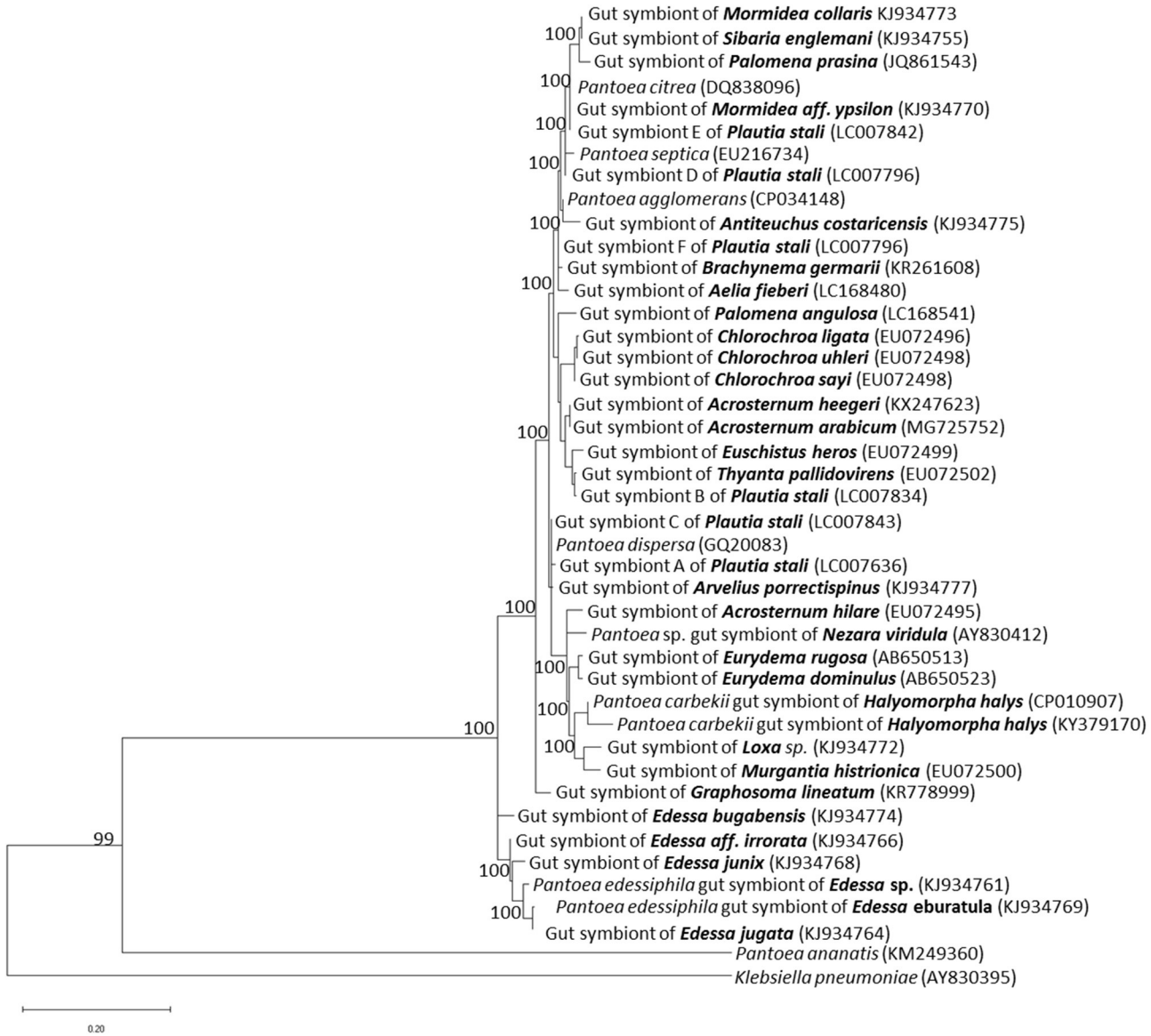

FIGURE 1 | Phylogenetic placement of gut primary symbionts of pentatomid stink bugs. The phylogenetic tree was constructed by the maximum likelihood method, using the MEGA X software. The 16S rRNA gene sequences of pentatomid gut symbionts, available in public databases, were selected according to: Hirose et al. (2006), Prado and Almeida (2009a), Kikuchi et al. (2012), Kenyon et al. (2015), Bistolas et al. (2014), Hosokawa et al. (2016), Karamipour et al. (2016), Otero-Bravo and Sabree (2018), and Kashkouli et al. (2019a). Allied *Pantoea* species are included. Names of pentatomid hosts are indicated in bold; accession numbers of each sequence included in the analysis are indicated in parenthesis. Numbers at each node represent percentages of bootstrap replications calculated from 1,000 replicate trees. The scale bar represents the sequence divergence. *K. pneumoniae* was used as an outgroup in the family *Enterobacteriaceae*.

(Oishi et al., 2019). Phylogenetic investigations on *P. stali* symbionts showed six distinct bacterial linages, differing for their genome size, cultivability and prevalence in the host populations. All the six lineages were demonstrated to be individually essential for the host survival: after the elimination of the original symbionts, cross colonization of the host by alternative lineages completely restored insect development and growth, suggesting the possibility of multiple replacements. Notably, aposymbiotic nymphs were able to acquire symbionts from the environment (for example from the food source), suggesting also a possible horizontal route for symbiont replacements in nature (Hosokawa et al., 2016; Nishide et al., 2017).

Pistachio Green Stink Bugs

Pentatomid stink bugs are abundant and serious pests of pistachio nuts in most of pistachio plantation areas. They are reported to cause heavy losses in Iran due both to direct damage by the insect and the transmission of the fungal

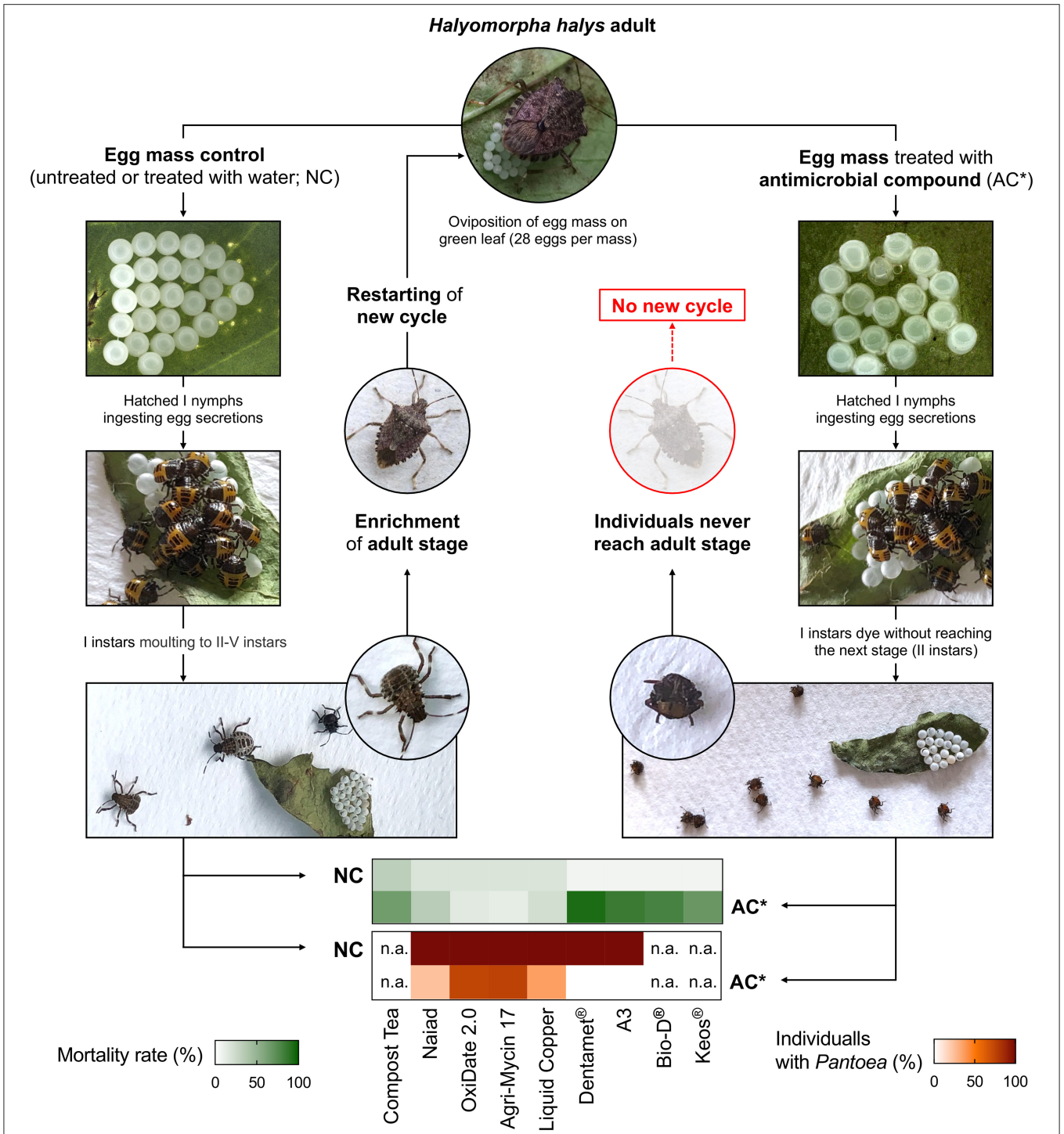

FIGURE 2 | Graphical representation of the laboratory trails testing the anti-*P. carbekii* activity of commercial products with antimicrobial activity, interrupting the life cycle of *H. halys* (Mathews and Barry, 2014; Taylor et al., 2017; Gonella et al., 2019). The effect of treating with antimicrobial compounds (AC) is compared with a negative control (NC), which shows no alteration of stink bug development. Mortality rates induced by different substances, as well as P. *carbekii* infection rates related to treatments, are depicted in the heatmaps.

TABLE 1 | List of described gut primary symbionts of pentatomid hosts.

Host insect	Primary symbiont	Symbiont suppression modality	Symptoms of symbiont suppression	Symbiont-targeted management	References
A. arabicum	Unnamed	Heat treatment; surface sterilization (ethanol + bleach)	>Nymphal mortality; <Adult fecundity	Laboratory trials	Kashkouli et al., 2019b
A. heegeri	*Pantoea* sp.	Heat treatment; surface sterilization (ethanol + bleach)	>Pre-adult development; < Adult longevity; <Adult fecundity	Laboratory trials	Kashkouli et al., 2019a,b
Acrosternum hilare	Unnamed	Surface sterilization (ethanol + bleach)	> Developmental time; > Nymphal mortality; < Adult fecundity	N. a.	Prado and Almeida, 2009a,b
Antiteuchus costaricensis	Unnamed	N. d.	N. d.	N.a.	Bistolas et al., 2014
Arvelius porrectispinus	Unnamed	N. d.	N. d.	N. a.	Bistolas et al., 2014
B. germari	*Pantoea* sp.	Heat treatment; surface sterilization (ethanol + bleach)	>Pre-adult development; <Adult longevity; < Adult fecundity	Laboratory trials	Kashkouli et al., 2019a,b
Chlorochroa ligata	Unnamed	N. d.	N. d.	N. a.	Prado and Almeida, 2009a
Chlorochroa sayi	Unnamed	N. d.	N. d.	N. a.	Prado and Almeida, 2009a
Chlorochroa uhleri	Unnamed	N. d.	N. d.	N. a.	Prado and Almeida, 2009a
Edessa bella	"*Ca.* Pantoea edessiphila"	N. d.	N. d.	N. a.	Bistolas et al., 2014; Otero-Bravo et al., 2018
Edessa bugabensis	Unnamed	N. d.	N. d.	N. a.	Bistolas et al., 2014
Edessa eburatula	"*Ca.* Pantoea edessiphila"	N. d.	N. d.	N. a.	Bistolas et al., 2014; Otero-Bravo et al., 2018
Edessa aff. irrorata	Unnamed	N. d.	N. d.	N. a.	Bistolas et al., 2014
Edessa jugata	Unnamed	N. d.	N. d.	N. a.	Bistolas et al., 2014
Edessa junix	Unnamed	N. d.	N. d.	N. a.	Bistolas et al., 2014
Edessa loxdalii	"*Ca.* Pantoea edessiphila"	N. d.	N. d.	N. a.	Otero-Bravo et al., 2018
Edessa n. sp.	"*Ca.* Pantoea edessiphila"	N. d.	N. d.	N. a.	Otero-Bravo et al., 2018
Eurydema dominulus	Unnamed	N. d.	N. d.	N. a.	Kikuchi et al., 2012
Eurydema rugosa	Unnamed	Surface sterilization (ethanol + formaldehyde)	Retarded growth; < Body weight; Abnormal body color	N. a.	Kikuchi et al., 2012
Euschistus heros	Unnamed	N. d.	N. d.	N. a.	Prado and Almeida, 2009a
Euschistus sp.	Unnamed	N. d.	N. d.	N. a.	Bistolas et al., 2014
Graphosoma lineatum	Unnamed	Surface sterilization (ethanol + bleach)	>Developmental time; <Lifespan; <Adult fecundity	N. a.	Karamipour et al., 2016
H. halys	"*Ca.* Pantoea carbekiii"	Surface sterilization (bleach); treatments with several antimicrobials and surfactants	>Nymphal mortality; >Developmental time; <Adult fecundity; <Progeny survivorship	Laboratory and field trials	Mathews and Barry, 2014; Taylor et al., 2014, 2017; Gonella et al., 2019
Loxa sp.	Unnamed	N. d.	N. d.	N. a.	Bistolas et al., 2014
Mormidea collaris	Unnamed	N. d.	N. d.	N. a.	Bistolas et al., 2014

(Continued)

TABLE 1 | Continued

Host insect	Primary symbiont	Symbiont suppression modality	Symptoms of symbiont suppression	Symbiont-targeted management	References
Mormidea aff. ypsilon	Unnamed	N. d.	N. d.	N. a.	Bistolas et al., 2014
Murgantia histrionica	Unnamed	Surface sterilization (ethanol + bleach)	>Developmental time	N. a.	Prado and Almeida, 2009a,b
N. viridula	*Pantoea* sp.	Surface sterilization (ethanol + bleach; ethanol + formaldehyde); heat treatment	=Fitness >Nymphal mortality; Retarded growth; <Size; Abnormal body color	N. a.	Prado et al., 2006; Tada et al., 2011; Kikuchi et al., 2016
P. guildinii	Unnamed	N. d.	N. d.	N. a.	Husseneder et al., 2017
P. stali	*Pantoea* spp.	Surface sterilization (ethanol + formaldehyde)	>Nymphal mortality	N. a.	Abe et al., 1995; Hosokawa et al., 2016
Sibaria englemani	Unnamed	Surface sterilization (bleach)	>Duration of II instar; <Growth rate; Aberrant gut morphology	N. a.	Bistolas et al., 2014
Thyanta pallidovirens	Unnamed	N. d.	N.d.	N. a.	Prado and Almeida, 2009a

The modalities tested for symbiont suppression and the resulting effects are indicated, as well as the current advancement of development of pest management strategies, when available. N. d., not determined; N. a., not available; =, no significant changes observed; >, significant increment; <, significant reduction; Ca., Candidatus.

pathogen, *Nematospora coryli* Peglion, a pathogen of pistachio nuts (Pourkhatoon et al., 2016). This group of stink bugs includes the species *Acrosternum arabicum* Wagner, *Acrosternum heegeri* Fieber, *Acrosternum millieri* Mulsant & Rey, *Apodiphus amygdali* Germar, *Brachynema germari* Kolenati, and *Brachynema segetum* Jakovlev (Kashkouli et al., 2019a; Mehrnejad, 2020). Most of information concerning the associations with gut primary symbionts regards *B. germari* and *Acrosternum* spp., in consideration of their primary importance as pests (Kashkouli et al., 2019a). In *B. germari*, a peculiar bacterial distribution was observed in the V4 midgut region, with the obligate symbiont sheltered in the intercellular space rather than intracellular cytoplasm (Hosokawa et al., 2006). Molecular phylogenetic analyses of 16S rRNA gene of *B. germari* and *A. heegeri* symbionts placed these bacteria in the genus *Pantoea* (Kashkouli et al., 2019a, 2020). A comprehensive study of the effects on hatched nymphs of these species after surface sterilization or heat treatment of egg masses showed high nymph mortality, slow growth, reduced fitness and reduced fecundity (Karamipour et al., 2016; Kashkouli et al., 2020).

OBLIGATE SYMBIONTS AS PART OF PENTATOMID MICROBIOMES

Compared to the relatively large body of information on the stink bug obligate symbionts, few data are available concerning the other members of the gut microbial community and their interactions with the main symbionts. Studies of the gut microbiome have been conducted on adult individuals of few pentatomid species, like *H. halys* and *N. viridula*, revealing that the bacterial community of the crypt-harboring midgut portion (V4) is largely dominated by the primary symbionts (Kenyon et al., 2015; Medina et al., 2018), but yet colonized by other bacterial phyla in many stink bug hosts. For instance, in a survey on seven pentatomid species, different Actinobacteria were detected in the terminal midgut portion, mostly in the genera *Corynebacterium*, *Dietzia*, *Citricoccus*, *Mycobacterium, Propionibacterium*, and *Streptomyces*. These bacteria are thought to be involved in the protection of the microbial community, by producing bioactive metabolites which may limit the invasion of pathogens (Zucchi et al., 2012).

In the other midgut compartments (named V1-V3), the primary symbionts are much less abundant (Medina et al., 2018), and many other bacteria have been found. For example, in the digestive tract of the red-banded stink bug *Piezodorus guildinii* (Westwood) several bacteria putatively involved in nutrient provision and digestion have been identified, such as *Klebsiella oxytoca*, *Clostridium butyricum*, and *Citrobacter farmeri*, along with the candidate primary symbiont *Pantoea dispersa* (Husseneder et al., 2017). The analysis of different *N. viridula* populations from Brazil, Hawaii, California and Japan, confirmed that the terminal midgut ventriculus was dominated by a single bacterial type of the family *Enterobacteraceae*, while in the remaining compartments of the gut other Enterobacteria and Enterococci were detected (e.g., *Klebsiella pneumoniae, Enterococcus faecalis,* and *Yokenella* sp.), possibly being involved in detoxification of the food source (Medina et al., 2018). Many stink bugs are recognized as vectors of different plant pathogens, which often colonize the digestive tract, representing an additional component of the gut microbiota (Mitchell, 2004; Esquivel et al., 2010; Esquivel and Medrano, 2014; Medrano et al., 2016).

Even though a number of reports have described a relatively complex microbial community accompanying the primary symbionts of pentatomid bugs, at present no studies tackled

the questions related to their ecological role and the possible interactions among these microorganisms and the primary symbionts to maintain the host fitness, or the mechanisms ruling microbial compartmentalization in the distinct gut portions. Future studies on the machinery of symbiotic homeostasis and microbiome dynamics will allow a better comprehension of the phenotypic effects observed in stink bugs in response to disruption of the association with the obligate symbionts.

DISRUPTION OF SYMBIONT INHERITANCE FOR PEST CONTROL APPLICATIONS

Based on scientific evidences showing the harmful effect of preventing symbiont acquisition by nymphs through surface-sterilization of stink bug eggs (Abe et al., 1995; Tada et al., 2011; Taylor et al., 2014; Kashkouli et al., 2020), the creation of symbiotic control programs was envisaged against some pentatomid pests of major economic relevance (**Table 1**). For example, the use of surface-sterilizing agents was proposed as management tactic against pistachio green stink bugs in Iran (Kashkouli et al., 2019b). However, most of the studies on symbiotic control programs were conducted on *H. halys* (**Figure 2**), which is one of the most damaging stink bug agricultural pests in North America and Europe, due to its high polyphagy and invasive potential (Leskey and Nielsen, 2018). The first substances tested to suppress the primary symbiont *P. carbekii* were compost teas, i.e., biologically-active organic matter emulsions, commonly used for pathogen management in organic agriculture (Mathews and Barry, 2014). The application of different compost teas, deriving from poultry manure and mushroom waste, resulted in high nymphal mortality in the first and second instar, especially when egg masses were treated few days after deposition (Mathews and Barry, 2014). These authors suggested that the observed reduction of insect survival could be related to antagonistic effects exerted by the complex microbiota hosted by the compost teas. However, besides a putative anti-*P. carbekii* activity, a direct insecticidal effect was reported against *H. halys* eggs, resulting in reduced egg hatching (Mathews and Barry, 2014).

A vast array of substances was tested by Taylor et al. (2017) on *H. halys* egg masses for their effects on the fitness of newly hatched nymphs. A laboratory assay was conducted with available products for plant pathogen control in the USA, including surfactants, insecticides, and different antimicrobials. The application of antimicrobial and surfactant formulates caused high nymphal mortality related to missed symbiont acquisition, providing evidence of the potential use of these products for the management of *H. halys*. However, the same substances only affected egg hatch rate in field experiments, showing no reduction of symbiont acquisition and nymphal survival (Taylor et al., 2017), and indicating the need to optimize control strategies (e.g., by identifying the most efficient number of treatments and doses) before proposing symbiont-targeted control options.

A different set of active substances, commercially available in the European agriculture as micronutrient fertilizers, were used against *H. halys* through primary symbiont elimination (Gonella et al., 2019). Egg mass treatments with a zinc, copper and citric acid biocomplex under laboratory conditions removed *P. carbekii*, as confirmed by molecular diagnosis, resulting in high mortality rates of the I instar nymphs. The suppressive effect was attributed to the anti-microbial activity of the Zn- and Cu-hydracid complexes contained in these micronutrient fertilizers, whose components are well-known antibacterial agents used for the control of plant pathogens (Gonella et al., 2019). However, these results still need to be experimentally validated under field conditions.

Notably, a significant negative relationships between the mortality rate and *P. carbekii* infection rate is highlighted by examining the results of laboratory tests conducted on *H. halys* egg masses by Taylor et al. (2017) and Gonella et al. (2019) using substances with an antimicrobial activity, suggesting commonalities in the nymph suppression processes exerted by different compounds that target the symbiont. The significant suppressive effect reported for several products, and the clear correlation observed between mortality rates and percentage of individuals carrying *P. carbekii*, further underline the importance to design symbiont-targeted control strategies against *H. halys*.

CONCLUDING REMARKS

The family Pentatomidae includes many cosmopolitan and invasive species capable of large infestations, causing intense damage to several crops (Knight and Gurr, 2007; Palumbo et al., 2016; Leskey and Nielsen, 2018; Sosa-Gómez et al., 2020). Invasions in new areas may be very difficult to contain due to the limited knowledge of the pest life cycle in different new environments joint to the absence of effective natural enemies (McLaughlin and Dearden, 2019). Additionally, their actual control mainly relies on the use of chemical insecticides, which often determine hidden costs due to the environmental impact and the effect on human health. Another major management measure against pentatomid pests is biological control, taking advantage of specialized egg parasitoids (Correaferreira and Moscardi, 1995; Felipe-Victoriano et al., 2019; Moraglio et al., 2019; Sabbatini Peverieri et al., 2019). Their activity is seriously hampered by intensive use of chemicals (Lowenstein et al., 2019), leading to the need for sustainable alternatives (Brzozowski and Mazourek, 2018). The peculiar inheritance mode of bacterial symbionts of Pentatomidae is certainly an interesting target for the disruption of the stink bugs life cycle. Some of the active substances tested to interrupt symbiont acquisition by neonate stink bugs are already commercially available as antimicrobials or fertilizers, and the exploitation of their accessory effect against *H. halys* represents an actual sustainable control option, since they are allowed in organic farming (Mathews and Barry, 2014; Gonella et al., 2019). Because these products are not insecticides, they are not expected to determine unintended effects on non-target insects, including egg parasitoids, even though no studies have been published regarding the consequences of treatments with such products on non-target species. However, it must

be pointed out that some active substances proposed for the interruption of symbiont vertical transmission displayed an ovicidal effect (e.g., compost teas), and this may indirectly hamper the activity of egg parasitoids. Moreover, in some countries, regulatory issues may arise concerning the use of micronutrient fertilizers, as their use for crop protection purposes has not been regulated yet.

Additionally, future studies could implement current knowledge on potential different mechanisms of antagonistic action, exploring different formulates that are used against microbial pathogens of many crops. For example, a promising source of anti-bacterial activity against pentatomid primary symbionts is represented by microbial biocontrol agents. Indeed, several strains are the base of commercial products that are currently used against plant pathogens (Ab Rahman et al., 2018), and studies on the effects on stink bug egg masses played by this class of substances may constitute a further step toward the implementation of effective pest control methods with low environmental impact.

Finally, attention is deserved by the observations on symbiont alteration in stink bugs exposed to increased temperatures as a result of climate change (Kikuchi et al., 2016). This is certainly a major issue to be addressed within the set-up of risk maps, an important component of Integrated Pest Management measures. Indeed, temperature increase may represent a perturbation for symbiont acquisition and development similarly to the direct bacterial elimination from the egg surface, seriously affecting prediction models of distribution range in areas of new pest introduction (Kikuchi et al., 2016), with a practical relevance for stink bug management.

AUTHOR CONTRIBUTIONS

EG, BO, and RM conceived, designed, and wrote the manuscript. BO and RM prepared the table. EG and RM prepared the figures. AA and DD critically reviewed the manuscript and contributed to its improvement. All authors approved the final version of the manuscript.

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Infection of *Ophiocordyceps sinensis* Fungus Causes Dramatic Changes in the Microbiota of Its *Thitarodes* Host

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The Chinese cordyceps is a unique and valuable parasitic complex of *Thitarodes*/*Hepialus* ghost moths and the *Ophiocordyceps sinensis* fungus for medicine and health foods from the Tibetan Plateau. During artificial cultivation of Chinese cordyceps, the induction of blastospores into hyphae is a prerequisite for mummification of the infected *Thitarodes* larvae. To explore the microbial involvement in the induction of mycelia-blastospore transition, the microbiota of the hemolymph and gut from *Thitarodes xiaojinensis* larvae with or without injected *O. sinensis* blastospores were investigated by culture-dependent and -independent methods. Twenty-five culturable bacterial species and 14 fungal species, together with 537 bacterial operational taxonomic units (OTUs) and 218 fungal OTUs, were identified from the hemolymph and gut of samples from five stages including living larvae without injected fungi (A) or with high blastospore load (B), mummifying larvae without mycelia coating (C), freshly mummifying larvae coated with mycelia (D), and completely mummified larvae with mycelia (E). Two culturable bacterial species (*Serratia plymuthica, Serratia proteamaculans*), and 47 bacterial and 15 fungal OTUs were considered as shared species. The uninfected larval hemolymph contained 13 culturable bacterial species but no fungal species, together with 164 bacterial and 73 fungal OTUs. To our knowledge, this is the first study to detect large bacterial communities from the hemolymph of healthy insect larvae. When the living larvae contained high blastospore load, the culturable bacterial community was sharply inhibited in the hemolymph but the bacterial and fungal community greatly increased in the gut. In general, high blastospore load increased bacterial diversity but sharply decreased fungal diversity in the hemolymph and gut by OTUs. The bacterial loads of four culturable species (*Chryseobacterium* sp., *Pseudomonas fragi*, *S. plymuthica, S. proteamaculans*) increased significantly and *O. sinensis* and *Pseudomonas* spp. became dominant microbes, when the infected larvae became mummified, indicating their possible involvement in the larval mummification process. The discovery of many opportunistic pathogenic bacteria in the hemolymph

of the healthy larvae, the larval microbial diversity influenced by *O. sinensis* challenge and the involvement of dominant bacteria during larval mummification process provide new insight into the infection and mummification mechanisms of *O. sinensis* in its *Thitarodes* hosts.

Keywords: Chinese cordyceps, *Ophiocordyceps sinensis* fungus, *Thitarodes*/*Hepialus* moth, microbiota, hemolymph, gut

INTRODUCTION

The Chinese cordyceps, a unique parasitic complex formed by the parasitism of *Thitarodes*/*Hepialus* spp. (Hepialidae, Lepidoptera) by the *Ophiocordyceps sinensis* fungus in the Qinghai-Tibet Plateau, is a highly valuable biological resource for medicines and health foods in China but also elsewhere in the world (Yue et al., 2013; Li et al., 2016; Baral, 2017; Pouliota et al., 2018; Qin et al., 2018). Diverse bacteria and fungi in the environment and in the *Thitarodes*/*Hepialus* hosts may influence the formation of the Chinese cordyceps.

Microbial communities of the wild Chinese cordyceps have been extensively investigated using traditional culture-dependent (Zhang et al., 2010) and culture-independent methods (Liang et al., 2008; Zhang et al., 2009, 2010; Xia et al., 2015, 2016). The microbiota of the wild Chinese cordyceps and its microhabitats are highly diverse. More than 22 species belonging to 13 genera as anamorphs have been reported in wild Chinese cordyceps specimens (Jiang and Yao, 2003). Approximately 600 isolates were obtained by culture-dependent methods from different parts of the Chinese cordyceps (including stromata, sclerotia, and external mycelial cortices) and its soil microhabitats (Zhang et al., 2010). The microbiota of three different sections (stromata, sclerotia, and mycelial cortices) from wild Chinese cordyceps specimens contained 572 fungal strains and 92 putative operational taxonomic units (OTUs) by a culture-dependent method, and 118 putative OTUs by a culture-independent method (Zhang et al., 2010). A high diversity of fungal communities, with the dominant fungal phylum Ascomycota (such as *Ophiocordyceps*, *Verticillium*, *Pseudallescheria*, *Candida,* and *Ilyonectria*), inhabiting the Chinese cordyceps and its microhabitats was revealed by using Illumina high-throughput sequencing (Xia et al., 2016). High throughput sequence analysis of 16S rRNA genes and ITS regions showed that the main bacterial groups were Proteobacteria, Acidobacteria, Bacteroidetes, Actinobacteria, and Firmicutes, while the Ascomycota, Basidiomycota, and Zygomycota were the main fungal phyla in wild Chinese cordyceps (Xia et al., 2016). Bacterial diversity grouped into 23 phyla including Proteobacteria, Actinobacteria, Acidobacteria, and Verrucomicrobia, in the soils of the native habitats of the Chinese cordyceps was investigated using Illumina sequencing data (Yang et al., 2015). Apart from *O. sinensis*, *Paecilomyces sinensis* (Chen et al., 1984), *Mortierella hepiali*, *Scytalidium hepiali* (Li, 1988), *Tolypocladium sinensis*, *Cephalosporium acremonium*, *Paecilomyces hepiali* (Dai et al., 2017), *Penicillium chrysogenum,* and *Pseudogymnoascus roseus* were also found from wild Chinese cordyceps (Jiang and Yao, 2003; Zhang et al., 2010).

Insects depend to a degree on the diverse communities of microbiota in their gut for basic functions, such as nutrition, protection from parasites and pathogens, modulation of immune responses, communication and reproduction (Engel and Moran, 2013; Kwong and Nancy, 2016; Wei et al., 2017). Zhuo et al. (2004) isolated 8 bacterial genera (*Staphylococcus*, *Bacillus*, *Klebsiella*, *Pseudomonas*, *Aeromonas*, *Plesiomonas*, *Sporosarcina,* and *Neisseria*) from the guts of wild *Thitarodes gonggaensis* larvae, with *Staphylococcus* being dominant. Liu et al. (2008) also isolated eight bacterial genera (*Enterobacter*, *Carnobacterium*, *Novosphingobium*, *Acinetobacter*, *Pseudomonas*, *Klebsiella*, *Pantoea,* and *Delftia* also from the guts of wild *T. gonggaensis* larvae with dominant *Enterobacter* bacteria, based on culture-dependent and -independent methods. Yu et al. (2008) obtained several fungal genera (*Mortierella*, *Trichosporon*, *Mucor*, *Rhinocladiella*, *Cephalosporium*, *Rhodiola,* and *Mastigobasidium*) from the guts of the same ghost moth species by RFLP analysis, and *Cryptococcus magnus*, *Geomyces* sp., and *Trichosporon porosum* by culture method. In a study of the internal microbial community in unfertilized eggs of *Thitarodes pui*, 348 bacterial genera (dominant genera included *Wolbachia*, *Spiroplasma*, *Carnobacterium*, *Sphingobium*, and *Acinetobacter*) belonging to 26 phyla, 58 classes, 84 orders, and 120 families were identified from 1,294 OTUs; 289 fungal genera, mainly including *Aureobasidium*, *Candida*, and *Cryptococcus*, were identified, and they belonged to five phyla (Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, and Zygomycota), 26 classes, 82 orders, and 165 families (Liang et al., 2019). Although different bacterial or fungal communities were isolated even from the guts of the same wild *Thitarodes* species living in different locations, the functions of these microbes in the association with the host development were totally unknown. No microbes were also reported from the hemolymph of *Thitarodes/Hepialus* spp. larvae so far.

To protect this natural resource, artificial cultivation of *O. sinensis* fruiting bodies on rice media (Cao et al., 2015) and culturing of the host caterpillar *Thitarodes* sp. on artificial media (Cao and Han, 2014; Tao et al., 2016) has been successfully established in low-altitude laboratories mimicking environmental conditions of the wild habitat. After being introduced into the hemolymph of the larvae, the spindle blastospores may produce more spindle cells by budding growth and/or grow into elongate hyphal bodies (pseudohyphae) and hyphae by apical growth under the induction of some unknown factors (Liu et al., 2019, 2020), as reported in the dimorphic fungi *Candida albicans* and *Ustilago maydis* (Ruiz-Herrera et al., 2020) and the entomopathogenic fungus *Metarhizium rileyi* (Boucias et al., 2016). However, the slow mummification of the *Thitarodes*

larvae post infection is still an unresolved problem and an obstacle for commercial production of Chinese cordyceps (Li et al., 2016; Qin et al., 2018; Han et al., 2019). The living infected larvae might harbor the spindle blastospores in the hemolymph for several months, contrary to other entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* which cause the death of their host larvae within a few days (Meng et al., 2015; Rao et al., 2019).

The ability of pathogenic fungi to switch between yeast or spindle cells (blastospores) and hyphae is well-studied in plant pathogens such as *U. maydis* (Gauthier, 2015) and the human prevalent fungal pathogen *C. albicans* (Desai, 2018). This dimorphism is correlated with pathogenicity along with the infection process (Nemecek et al., 2006; Gauthier, 2015). *O. sinensis* also exhibits spindle cell-hyphae dimorphism (Liu et al., 2019, 2020). In other fungal systems, a number of external inputs such as nutrients, temperature, pH, CO_2 and chemicals and quorum sensing molecules for the morphological transition in dimorphic fungi have been identified (Gauthier, 2015; Zhu et al., 2017). Inducers responsible for hyphal conversion from blastospores in *O. sinensis* and for the mummification of the infected larvae are unclear.

To explore the possible involvement of the microbial communities in the induction of mycelial transition from blastospores, the microbiota of the hemolymph and gut from the larvae of *T. xiaojinensis*, a highly potential ghost moth species for commercial cultivation, with or without injected *O. sinensis* blastospores were investigated by culture-dependent and culture-independent methods.

MATERIALS AND METHODS

Insects

Thitarodes xiaojinensis was reared according to the methods described by Tao et al. (2016) and Liu et al. (2019). Briefly, the insect pupae were collected from 3500–4000 m altitude mountains in Xiaojin, Sichuan Province, China, and housed in plastic containers (L = 50 cm; W = 40 cm; H = 30 cm) with moist moss kept at 9–17°C and 50–80% relative humidity. The emerged adults were housed in equal proportions of males and females in small mosquito nets (L = 104 cm; W = 50 cm; H = 50 cm) for mating and oviposition. The hatched larvae were transferred to a culture room at 9–13°C in Guangzhou, Guangdong, China, and offered the roots of *Potentilla anserina* as food to obtain 5th instar larvae (average fresh weight = 0.52 ± 0.03 g) for fungal infection. The insect species was identified as *T. xiaojinensis* by using the amplified Cytochrome b sequence with the primers CB1 (TATGTACTACCATGAGGACAAATATC) and CB2 (ATTACACCTCCTAATTTATTAGGAAT (Zou et al., 2011; Tao et al., 2016).

Fungal Isolate

The KD1223 isolate of *O. sinensis* isolated from the fruiting bodies of wild *O. sinensis* in Sichuan, China was cultured on PPDA medium (liquid PPDA medium: 200 g potato extract, 20 g glucose, 10 g peptone, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$, 20 mg vitamin B_1, and 1,000 ml distilled water; solid PPDA medium: 15% agar in liquid PPDA medium) at 13°C. The fungal isolate was identified by using the amplified sequence from the internal transcribed spacer (ITS; ITS1-5.8S-ITS2) of the nuclear ribosomal DNA as described by Cao et al. (2015). The identified *O. sinensis* isolate was preserved at −80°C in the Guangdong Institute of Applied Biological Resources, Guangzhou, China.

The fungal colonies cultured on the PPDA plates at 13°C for 60 days were transferred to 250 mL flasks containing 150 mL liquid PM medium (200 g potato extract, 20 g maltose, 10 g peptone, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$, 20 mg vitamin B_1, and 1,000 mL distilled water; Liu et al., 2019). The flasks were incubated at 13°C on a 120 rpm rotary shaker, and after 50 days the blastospores from the flasks were harvested by using three layers of sterile lens papers to remove hyphae and large particles, then the filtered solution was centrifuged at 8,000 rpm for 15 min at 10°C and the supernatant discarded. Harvested blastospores were re-suspended in sterile phosphate-buffered saline (PBS; pH 7.0) at a concentration of 3.0×10^6 blastospores per mL. The blastospore suspension was kept at 4°C for less than 3 days before use for larval inoculation.

Larval Infection

An aliquot of 4 μL suspension containing 1.2×10^4 blastospores was injected into each 5th instar larva by a microinjection system (IM-31; Narishige, Japan). 150 larvae were used for each replicate and three replicates were set for each injection. Larvae injected with PBS buffer or without any injection were set as controls. The injected larvae were reared at 4°C for 1 week and then transferred to a culture room at 13°C. After 90 days, about 10 μL hemolymph of each injected larva (6th instar) was sampled for confirming the presence of the blastospores.

Culture-Dependent Microbial Community

For investigation of the culture-dependent microbial community, after 90 days post injection, the hemolymph (100 μL from each larva; H) and guts (whole gut of each larva; G) of uninjected living larvae (A), and of injected larvae with fungal growth in four stages, including living larvae with a high blastospore load (B; 4.2×10^3 blastospores per mL in the hemolymph), and freshly mummifying larvae without mycelia coating (C) were sampled, using micro-needles (pulled glass capillaries G-1 by a micropipette PC-10 puller, Narishige, Japan). The tissues of the mummified larvae coated with mycelia (D) and completely mummified larvae with mycelia (E; **Figure 1**) were sampled after the mycelial coating and cuticle of the larvae were carefully removed with sterile scalpels. All these samples were immediately placed in sterile centrifuge tubes with sterile PBS and divided into two parts: one for the culture-dependent method and another for the culture-independent method. AH, BH, and CH represented the hemolymph samples from Group A, B, and C, respectively; AG, BG, and CG represented the gut samples from Group A, B, and C, respectively; DT and ET refer to tissue samples from Group D and E. Thus, eight types of samples were collected for the analysis. For the process of hemolymph collection, the methods described by Clark (1982) and Herren et al. (2014) were employed with minor improvements. Briefly, under sterile

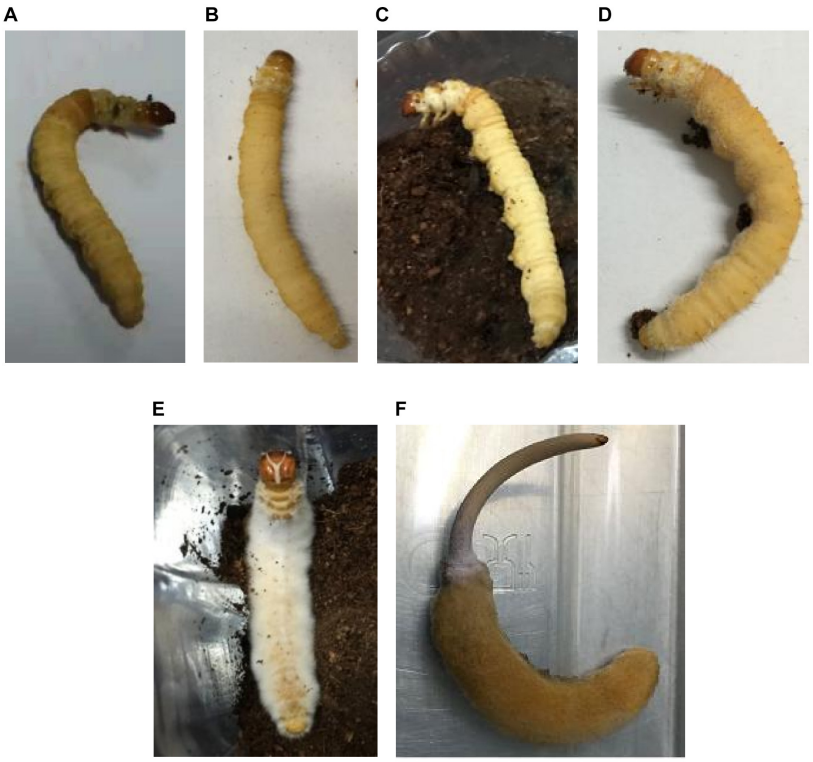

FIGURE 1 | *Thitarodes* larvae with or without *Ophiocordyceps sinensis* infection. Living larvae without injected fungi **(A)**, living larvae with high blastospore load **(B)**, mummifying larvae not coated with mycelia **(C)**, freshly mummifying larvae coated with mycelia **(D)**, completely mummified larvae with mycelia **(E)**, and Chinese cordyceps **(F)**.

conditions in a cabinet, the larvae from the laboratory culture (not from the wild) were washed three times with sterile distilled water, disinfected two times with 75% alcohol for 30 s, and rinsed three times with sterile distilled water. The larvae were dried on sterile filter paper before the hemolymph was carefully collected by sterile capillary micro-needles. For each sample type, three replicates containing three larvae per replicate were established.

The suspension was used to isolate the fungi and bacteria on the plates of LB (Lucia-Bertani), HIA (Heart infusion agar; BD, United States), G5 (PPDA supplemented with 0.5% milled fresh 6th instar *Galleria mellonella* larvae) and GSA (Gause's synthetic agar; HKM, China), by the dilution method. The pH values of all these media ranged from 6.0 to 7.0. The plates were sealed with parafilm (BEMIS, United States) and cultured in the dark in incubators with or without aeration, at 13 and 23°C, respectively. Twelve plates were set up for each treatment.

After the bacteria and fungi appeared on the plates, they were individually collected to inoculate new plates until 30 days. Microbe isolates were first grouped according to their colony and morphological characteristics on LB or PDA plates. The resulting colonies were cultured in liquid LB (for bacteria) or PDA (for fungi) and kept at -80°C with 15% glycerol.

Genomic DNA of each isolate within each group was extracted with a simple and rapid method for fungi (Zhang et al., 2010) and a routine method for bacteria (Cao et al., 2015), and the V4 region of bacterial 16S rDNA and the ITS2 region of the fungal ITS gene were specifically amplified using PCR. Primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), and ITS1 (5′–TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used specifically for bacterial and fungal fragments, respectively. The polymerase chain reaction (PCR) mixture contained 5 μL of 10 × Pfu buffer, 4 μL of dNTP mixture (2.5 mM), 1 μL of each primer (10 μM), 2 μL of deionized formamide, 1 μL of MgCl2 (25 mM), 1 μL of genomic DNA, and 0.5 μL of Pfu DNA polymerase in a total volume of 50 μL. PCR amplification was performed in a TGradient thermocycler (Applied Biosystems). For 16S rDNA and ITS, the mixture was heated for 30 s at 98°C, then subjected to 35 cycles of 10 s at 98°C, 10 s at 55°C, and 30 s at 72°C, and a final 10-min elongation step at 72°C. After confirmation of the PCR products by agarose gel electrophoresis, the fragments were cleaned using a MinElute PCR Purification Kit (Qiagen, Germany). The purified PCR products then were sequenced by Sangon Biological Engineering (Shanghai) Co., Ltd. The resulting sequences were compared with the data set in NCBI GenBank.

The sequences of one representative isolate or clone for each bacterial and fungal species were submitted to GenBank[1] and assigned accession numbers (MT626045-MT626058,

[1]http://www.ncbi.nlm.nih.gov/genbank/

MT631975-MT631999) for the sequences of cultivated isolates and clones, respectively.

Culture-Independent Community

Amplicon sequencing was employed to investigate the bacterial and fungal microbiota of the hemolymph and gut from the eight sample types, according to Liang et al. (2019). Briefly, total DNA of the hemolymph and gut samples after being ground in liquid nitrogen was extracted and purified by the MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, United States) following the instructions of the manufacturer. The purified DNA was quantified by the NanoDrop ND-3300 spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE, United States). The V4 region of bacterial 16S rDNA with primers 515F/806R and the ITS2 region of the fungal ITS gene with primers ITS3/ITS4 were specifically amplified using PCR. The primers contained a 12-bp barcode sequence at the 5′-end to distinguish the samples. All the PCR reagents were purchased from TaKaRa, Dalian, China. The PCR reaction mixture (50 μL) contained Ex Taq DNA polymerase (0.5 units), 1 × Ex Taq loading buffer (10 μL), dNTPs (8 μL), 2 μL of each primer (10 mM), and DNA template (10–100 ng). PCR was performed by the ABI Gene Amp® 9700 PCR System (Applied Biosystems, Waltham, MA, United States) with the parameters for bacterial-specific fragments: 95°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. For fungal-specific fragments, the PCR procedures included 95°C for 5 min; 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 45 s; and final elongation at 72°C for 10 min. The amplifications were conducted in triplicate for each sample and the products were pooled to minimize the PCR bias. After evaluation by 2% agarose gel electrophoresis, PCR products were mixed in equidensity ratios according to the GeneTools Analysis Software (Version4.03.05.0, SynGene). The mixed products were purified with the EZNA Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, United States).

Sequencing libraries with index codes were constructed by NEBNext Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, MA, United States) according to the instructions of the manufacturer. After assessment on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, MA, United States) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Waldbron, Germany), the libraries were sequenced on an Illumina Novo6000 PE250 platform, and 250 bp paired-end reads were generated (Berry Genomics Co., Ltd., Beijing, China). To obtain high-quality clean reads, paired-end raw reads were filtered according to the Trimmomatic (V0.33) quality control process (Bolger et al., 2014). Paired-end clean reads were merged using FLASH (v1.2.11) according to the relationship of the overlap between the paired-end reads (Magoè and Salzberg, 2011). Raw Tags were merged when at least 10 of the reads overlapped the read generated from the opposite end of the same DNA fragment, with the maximum allowable error ratio of the overlap region of 0.1. Sequences were assigned to each sample based on their unique barcodes and primers by Mothur software (V1.35.1; Schloss et al., 2009). Clean Tags were generated after barcodes and primers were removed.

Usearch software (V10; Edgar, 2010) was used to perform sequences analysis, and sequences with ≥ 97% similarity were assigned to the same OTUs (Tindall et al., 2010). The most frequently occurring sequence was extracted as the representative sequence for each OTU and was screened for further annotation. The obtained 16S and ITS amplicon data were submitted to sequence read archive (SRA) and assigned accession numbers PRJNA631104 and PRJNA631325, respectively.

For each representative sequence, the taxonomic classification (setting the confidence threshold default to ≥ 0.5) was carried out by SILVA (v. 119[2]) for bacteria and UNITE (v. 7.0[3]) for fungi. The OTU and its tags were removed when annotated as chloroplasts or mitochondria (16S amplicons) and not to the kingdom level, then the OTU taxonomy synthesis information tables for the final analysis were generated.

Based on the OTU table, the unique and the shared OTUs among the eight sample types in UpSetR plot were illustrated with UpSetR package in R software (Conway et al., 2017). The annotation ratio on each classification level was calculated to obtain the sequence composition of each sample at each classification level. Based on the relative abundance of species at each classification, the histogram was drawn with the ggplot2 package in R software.

For alpha-diversity and beta-diversity analyses, 294753 (A), 302110 (B), 275154 (C), 144858 (D) and 136746 (E) 16S rRNA tags per sample, and 219394 (A), 254271 (B), 271636 (C), 126816 (D) and 136673 (E) ITS tags per sample in OTU tables were used. QIIME (V1.9.1; Caporaso et al., 2010) was used to calculate bacterial and fungal alpha-diversity indices, including Chao1 and Simpson, to analyze the complexity of species diversity for a sample. Chao1 was selected to identify community richness. Simpson was used to identify community diversity. Beta-diversity analysis was used to evaluate differences of samples in species complexity. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis distance matrixes was used for multivariate analysis of all samples, which was performed by Vegan package of R software. Based on the OTU table, two non-parametric analyses including analysis of similarity (ANOSIM) and non-parametric multivariate analysis of variance (Adonis) using distance matrices were performed by R software, to display the extent of differences among samples and to test whether the differences were significant ($p < 0.05$). A linear discriminant analysis (LDA) effect size (LEfSe) algorithm was employed to identify the taxa in different abundances (biomarkers; Segata et al., 2011) among samples. The effect size threshold of the LDA score was set to 2.

RESULTS

Culture-Dependent Communities

Overall, 25 bacterial species belonging to 16 genera and 14 fungal species belonging to 13 genera were identified from the hemolymph, gut and tissues of 8 types of samples from 5

[2]http://www.arb-silva.de

[3]http://unite.ut.ee/index.php

infection stages (**Figure 1**). Two bacterial species (*S. plymuthica* and *S. proteamaculans*) were shared and *Pseudomonas* spp. were dominant in the larval hemolymph and gut in all samples (**Table 1**). Interestingly, hemolymph from the healthy larvae also contained as many bacterial species as did the guts.

As to the larval hemolymph, no fungi except the injected *O. sinensis* were detected from the agar plates. The detected bacterial isolates decreased sharply from 14 species in the living larvae without blastospores (AH) to 5 species in the living larvae containing a high load of blastospores (BH). *Arthrobacter* sp., *Carnobacterium maltaromaticum*, *Enterobacterales*, *Micro bacterium* sp., *Nocardiaceae*, *Pseudomonas fragi*, *Pseudomonas migulae*, *Pseudomonas* sp., *Rahnella aquatilis* and *Rhodococcus* sp., which were present in stage A, disappeared from the hemolymph in stage B (**Table 1**). It appeared that the bacterial community in the hemolymph was inhibited when the larvae were full of blastospores. Surprisingly, it was difficult to isolate *O. sinensis* by the tested media from the larvae even in the stage when they were filled with blastospores.

More colonies of *C. maltaromaticum* were detected from the gut of healthy larvae, and growing blastospores apparently stimulated the occurrence of fungal and bacterial species in the gut of the living larvae filled with blastospores (BG; **Table 1**). Three more bacterial species and 6 more fungal species including fungal species not detected from the healthy larvae were isolated from guts. Compared with AG, six bacterial species (*Kaistia terrae*, *Mycobacteroides salmoniphilum*, *Pseudomonas brenneri*, *Pseudomonas* sp., *Streptomyces* sp., and *Tsukamurella strandjordii*) and six fungal species (*Cladosporium halotolerans*, *Mucor racemosus*, *Penicillium commune*, *Pseudogymnoascus* sp., *Stachybotrys chartarum*, and *Toxicocladosporium irritans* were detected only from BG.

Interestingly, when the infected larvae (C, D and E) became mummified, four bacterial species including *Chryseobacterium* sp., *Pseudomonas fragi*, *S. plymuthica*, and *S. proteamaculans* strongly increased, whereas the fungal community in the gut sharply decreased (**Table 1**).

Flavobacterium frigidimaris was unique for freshly or completely mummified larvae. *Pseudomonas mohnii* and *Rhizobium* sp. were detected only in AG, and *Sphingobacterium kitahiroshimense* only in CH (**Table 1**). No fungal species were shared in all samples. *Arthrinium* sp., *Mucor hiemalis*, *O. sinensis*, *Paecilomyces hepiali,* and *Parengyodontium album* were unique for mummified larvae.

The percentages of the microbe species isolated from different media, temperatures and aeration conditions are presented in **Supplementary Table 1**. Generally, more bacterial and fungal species were isolated from the plates on G5 medium, at 23°C and under normoxia.

Culture-Independent Communities

Microbial Diversity

A total of 1,204,000 high-quality bacterial (16S rRNA) and 1,201,711 high-quality fungal (ITS) clean reads were obtained from 24 samples representing 8 types of samples (AH, AG, BH, BG, CH, CG, DT, and ET) with three replicates for each group and over 99% of the reads met the demand of Q30 (**Supplementary Table 2**), indicating high quality of sequencing. A total of 230,841 amplicon sequences from the V4 region of the bacterial 16S rRNA gene and 711,904 sequences from fungal ITS were obtained (**Supplementary Table 3**). The 16S and ITS sequences were clustered into 537 and 218 OTUs, respectively. For bacteria, 222 genera (dominant genera include *Pseudomonas*, *Stenotrophomonas*, *Rhodococcus*) belonging to 20 phyla (dominant phyla included Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes), 42 classes, 89 orders, and 146 families were identified from 537 OTUs. For fungi, 54 genera (mainly *Ophiocordyceps* and *Verticillium*) belonging to 5 phyla (Ascomycota, Basidiomycota, Mortierellomycota, Mucoromycota, and Chytridiomycota), 17 classes, 43 orders, and 56 families were identified from 218 OTUs (**Supplementary Table 3**).

From all samples 47 shared bacterial OTUs were detected, with one class, 7 families, 24 genera and 15 species including *Bartonella apis*, *Clostridium* sensu stricto 1, *Helicobacter ganmani*, *Mycoplasma pulmonis*, *Rhodococcus degradans*, and *Rhodococcus hoagie* (**Supplementary Table 4** and **Supplementary Figure 1**). The unique bacterial OTUs were present in all the samples, but their abundances were very low. 15 shared fungal OTUs were found, including Pseudeurotiaceae and Mortierellaceae at family level; *Chaetomium*, *Penicillium,* and *Pezicula* at genus level; and *Fusarium oxysporum*, *Mortierella humilis*, *O. sinensis,* and *Verticillium leptobactrum* at species level. Only three unique fungal OTUs (Agaricomycetes, *Oidiodendron,* and *Candida*) from BG, two OTUs (*Cenococcum* and *Ophiostoma*) from CG, and one OTU (Sordariomycetes) from ET were identified. These bacterial and fungal OTUs in all samples were considered as shared species in the larvae, whether in the hemolymph or gut, and with or without *O. sinensis*. Beside the shared OTUs, AG and BG samples shared the most bacterial and fungal OTUs among all samples (**Supplementary Figure 1**), indicating the microbial OTU structure similarity between AG and BG.

In the alpha-diversity analysis (**Figure 2** and **Supplementary Table 5**), compared with stages AH and AG, slightly higher bacterial diversities but markedly lower fungal diversity (as indicated by the Simpson index) were detected in BH and BG; bacterial and fungal diversity decreased when the larvae became mummified. Bacterial abundance (as indicated by the Chao1 index) increased and fungal abundance was similar in the larvae injected with blastospores. These results are consistent with rank-abundance curves (**Supplementary Figure 2**). Rarefaction curves for all samples indicated that the sequence depth was reliable for both bacterial and fungal identification (**Supplementary Figure 2**). In general, high blastospore load increased bacterial diversity but sharply decreased fungal diversity in the larval hemolymph and gut. When the larvae became mummified, the bacterial and fungal diversity decreased.

Non-metric multidimensional scaling was applied to evaluate the beta-diversity changes in the eight sample types, based on the Bray–Curtis distance matrix. For all samples, the bacterial beta-diversities could be divided into four groups. The bacterial communities of ET were significantly different from those of

TABLE 1 | Bacterial and fungal species identified in the larval hemolymph, gut and tissue by culture-dependent method.

	Microbe Species	A		B		C		D	E
		AH	AG	BH	BG	CH	CG	DT	ET
Bacteria	*Arthrobacter* sp.	+	+		+				
	Carnobacterium maltaromaticum	+	++++		+				
	Chryseobacterium sp.		+	++	+	+	++	+++	++
	Enterobacterales	+				+	+		+
	Flavobacterium frigidimaris					+	+	+	++
	Kaistia terrae				+				
	Microbacterium sp.	+	+		+				+
	Micrococcaceae		+		+				
	Mycobacteroides salmoniphilum				+				
	Nocardiaceae	+	+		+				
	Pseudomonas brenneri				+	+		+	
	Pseudomonas fragi	+	+		++	++	++++	++++	++++
	Pseudomonas migulae	+	+		+	+		++	+
	Pseudomonas mohnii		+						
	Pseudomonas poae					+	++		
	Pseudomonas sp.	+			+	+			+
	Rahnella aquatilis	+	+		+				
	Rhizobium sp.		+						
	Rhodococcus sp.	+	+		+				
	Serratia plymuthica	+	+	+	+	+	++++	++	+++
	Serratia proteamaculans	+	+	+	++	++++	++	++++	++++
	Sphingobacterium kitahiroshimense					+			
	Stenotrophomonas rhizophila	+		+					
	Streptomyces sp.				+				
	Tsukamurella strandjordii	+		+	+	+	+	+	
Fungi	*Apiotrichum porosum*		+		+				
	Arthrinium sp.						+		
	Cladosporium halotolerans				+				
	Mucor hiemalis								+
	Mucor racemosus				+				
	Ophiocordyceps sinensis					++			
	Paecilomyces hepiali							+	
	Parengyodontium album							+	
	Penicillium commune				+				
	Phialemonium inflatum		+		+				
	Pseudogymnoascus sp.				+			+	
	Stachybotrys chartarum				+				
	Sterigmatomyces halophilus		+						
	Toxicocladosporium irritans				+				
	Bacterial species	**14**	**14**	**5**	**18**	**12**	**8**	**8**	**9**
	Fungal species	**0**	**3**	**0**	**8**	**1**	**1**	**3**	**1**

AH and AG, hemolymph and gut of un-injected living larvae; BH and BG, hemolymph and gut of living larvae with a high load of blastospores; CH and CG, freshly mummifying larvae without mycelia coating; DT, tissues of the mummified larvae coated with mycelia; ET, tissues of the completely mummified larvae with mycelia. +, ≤ 100 colonies detected in the plates; ++, > 100 ≤ 1,000 colonies detected in the plates; +++, > 1,000 ≤ 3000 colonies detected in the plates; ++++, > 3000 colonies detected in the plates.

BH (ANOSIM: R = 1, p = 0.05) and BG (R = 1, p = 0.05). No significant differences in bacterial communities were found between CH and CG (R = 0.59, p = 0.12), or DT and ET (R = -0.19, p = 0.8; **Figure 3** and **Supplementary Table 6**). The fungal beta-diversities could be separated into six groups. The fungal communities of the larvae without *O. sinensis* infection (AH and AG) were significantly different from those of the larvae with *O. sinensis* infection (BH, BG, CH, CG, DT, and ET). There were significant differences of beta-diversities between the samples AG and CG (ANOSIM: R = 1, p = 0.05), AH and BH (R = 1, p = 0.06), and AH and CH (R = 1, p = 0.05). No significant differences were found between CH and CG (R = -0.111, p = 0.63), AH and AG

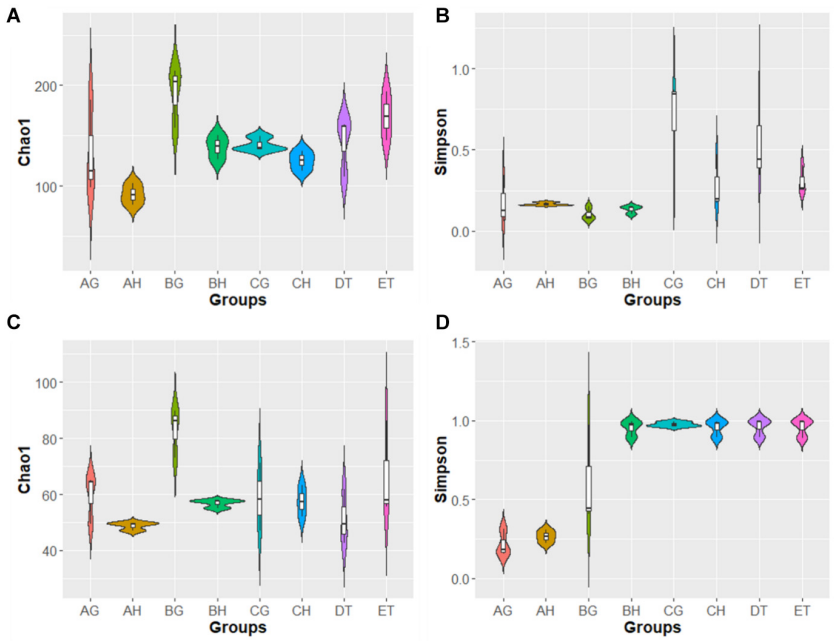

FIGURE 2 | Violin plot of Chao1 and Simpson index of bacterial **(A,B)** and fungal **(C,D)** operational taxonomic units (OTUs) among eight samples. AH, BH, and CH were represented as hemolymph samples respectively from sample A (uninjected living larvae), B (living larvae with high blastospore load) and C (freshly mummifying larvae without a mycelial coating); AG, BG, and CG were represented as gut samples respectively also from samples A–C; DT and ET indicated tissue samples from sample D (mummified larvae with mycelial coating) and E (completely mummified larvae with mycelia).

(R = 0.059, p = 0.12) or DT and ET (R = 0.111, p = 0.43). These results indicate again that *O. sinensis* infection reduced fungal diversity (**Figure 3** and **Supplementary Table 6**).

Clustering analysis was also done to evaluate the beta-diversity changes in the 8 sample types, based on the Bray–Curtis distance matrixes. The bacterial diversities could also be separated into two groups: One group consisted of the samples of BG, CH and CG, DT and ET, which contained Enterobacteriaceae and Pseudomonadaceae, and were affected by the injection and growth of the blastospores; and another group consisted of the samples without *O. sinensis* challenge, or from BH and CH, which contained top OTUs belonging to *Empedobacter* sp., *Delftia* sp., *Sphingobacterium* sp. SOZ2-4111, *R. degradans* and *R. hoagie*. The fungal diversities could be separated into two groups: One group contained the larvae with *O. sinensis* infection, whereas the other grouped the larvae without *O. sinensis* infection. In the former group, over 99% fungal richness belonged to the *O. sinensis* fungus. In the latter group, *V. leptobactrum* became the dominant fungal species (**Figure 4**).

Based on the significant differences among the samples by using LDA, 156 bacterial OTUs were selected as biomarkers, including 51 from AG, 21 from AH, 47 from BG, 23 from BH, 2 from CG, 3 from CH, 2 from DT and 7 from ET, indicating the high bacterial diversities from the living healthy larvae (AH and AG). 29 of 156 bacterial OTUs were identified to be species level, such as *Stenotrophomonas* sp. (LDA score: 5.12, group: AH), *R. hoagii* (4.84, AH) and *R. degradans* (4.67, AH; **Supplementary Table 7**). 59 fungal OTUs were selected as biomarkers, including 22 from AG, 18 from AH, 8 from BG, 2 from BH, 2 from CG, and 6 from DT, indicating the high fungal diversities from AG and AH (**Supplementary Table 7**). 10 of 59 fungal OTUs were identified to be species level, including *V. leptobactrum* (LDA score: 5.082, group: AH), *Mortierella*_sp. (4.328, BG) and *Dothideomycetes*_sp. (4.210, DT).

Bacterial and Fungal Structures

A total of 8, 12, 7, 13, 9, and 5 dominant bacterial OTUs (relative abundances > 1%) were identified from AH, AG, BH, BG, CH, and CG, respectively (**Supplementary Table 8**). One dominant bacterial species, *Stenotrophomonas* sp. I64-LFP1A9B2 was shared among six types of samples. However, the relative abundance of *Stenotrophomonas* sp. I64-LFP1A9B2 was significantly suppressed in the CG group (1.54%), being at least ten times lower than that in the other five types of samples (at least 15.59%). Two dominant bacterial species, *R. hoagii* (> 6.49%) and *R. degradans* (> 5.15%) were shared among non-mummified samples (AG, AH, BG, and BH) and the hemolymph of early mummified larvae (CH). In addition to the shared species above, the guts of non-mummified larvae (AG and BG) shared three more dominant species, including *Bartonella apis* (> 2.84%), *Lactobacillus* sp. Aahmto12 (> 1.22%) and *Gilliamella apicola* (> 1.25%). Three and nine dominant species were found in DT and ET, respectively. The above shared dominant bacterial species were significantly suppressed in the mummified stages (DT and ET), while *Sphingobacterium* sp. SOZ2-4111 (5.05%) was significantly increased in ET. Moreover, 4 dominant bacterial OTUs significantly increased in the mummified stages (CH, CG, DT, and ET), including

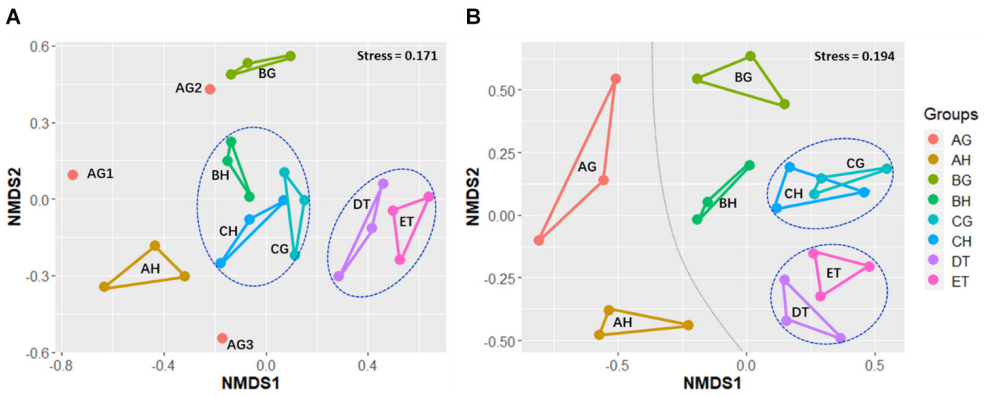

FIGURE 3 | Non-metric multidimensional scaling (NMDS) analysis of beta-diversity based on the Bray–Curtis distance matrix for bacterial **(A)** and fungal **(B)** communities of all samples, with the stress of 0.171 and 0.194, respectively. AH and AG, hemolymph and gut of uninjected living larvae; BH and BG, hemolymph and gut of living larvae with high blastospore load; CH and CG, freshly mummifying larvae without mycelia coating; DT, tissues of the mummified larvae coated with mycelia; ET, tissues of the completely mummified larvae with mycelia.

Enterobacteriaceae (family level) and three *Pseudomonas* sp. (genus level). Top 10 bacterial OTUs from all samples were listed based on the relative abundance (%; **Figure 5** and **Supplementary Table 8**), including 13 at class level; 24 at family level; *Bacillus*, *Bacteroides*, *Bartonella*, *Bifidobacterium*, *Brevundimonas*, *Carnobacterium*, *Chryseobacterium*, *Delftia*, *Empedobacter*, *Enterococcus*, *Escherichia–Shigella*, *Flavobacterium*, *Gilliamella*, *Lactobacillus*, *Parabacteroides*, *Pedobacter*, *Pseudochrobactrum*, *Pseudomonas*, *Rathayibacter*, *Rhizobium*, *Rhodococcus*, *Snodgrassella*, *Sphingobacterium*, *Stenotrophomonas,* and *Streptococcus* at genus level, with *Lactobacillus* being shared in all samples. *Pseudomonas* became the dominant bacterial genus (**Figure 5** and **Supplementary Tables 8**, **9**). The dominant bacterial OTUs at species level included *Stenotrophomonas* sp. I 64-LFP1A9B2 from all samples; *R. hoagii* and *R. degradans* from AH, AG, BH, BG, and CH; *Bartonella apis* from AG, BH, and BG; *Gilliamella apicola* from AG and BG; and *Lactobacillus* sp. Aahmto12 from AG and BG; *Bifidobacterium* sp. H6bp22N and *Apibacter* sp. wkB309 from BG (**Figure 5** and **Supplementary Tables 8**, **9**).

A total of 9, 18, and 2 dominant fungal OTUs were identified from the AH, AG and BG samples, respectively. Two dominant fungal species were shared among these samples, including *O. sinensis* (9.96, 43.41, and 91.69%) and *V. leptobactrum* (24.89, 25.03, and 3.49%). *O. sinensis* was the only dominant OTU among the BH, CG, CH, DT, and ET samples, covering more than 91% relative abundance. *O. sinensis* was abundant in all samples (9.96–99.75%), including the gut (9.96%) and hemolymph (43.41%) samples without *O. sinensis* infection (AG and AH), implying the potential inherent symbiosis between the larvae of *T. xiaojinensis* and the fungus *O. sinensis*. Meanwhile, *O. sinensis* was mainly present in the hemolymph rather than in the gut under uninfected conditions. *V. leptobactrum* was another dominant fungal species in the gut (24.89%) and hemolymph (25.03%) under normal conditions, and decreased sharply after *O. sinensis* infection (**Figure 5** and **Supplementary Table 8**). Top 10 fungal OTUs from all samples were ranked based on their relative abundance (%; **Figure 5**), including 16 at class level; 20 at family level; *Apiotrichum*, *Aspergillus*, *Candida*, *Cenococcum*, *Chaetomium*, *Clavulina*, *Fusarium*, *Inocybe*, *Lyomyces*, *Mortierella*, *Mucor*, *Myrothecium*, *Ophiocordyceps*, *Penicillium*, *Pezicula*, *Phaeotremella*, *Rhodosporidiobolus*, *Russula*, *Trichoderma*, *Tricholoma,* and *Verticillium* at genus level, with *Ophiocordyceps*, *Penicillium*, and *Verticillium* being common in all samples. High fungal diversities were detected from the hemolymph (AH) and guts (AG) of the larvae without *O. sinensis* infection, where *V. leptobactrum*, *O. sinensis*, *F. oxysporum,* and *M. verrucaria* were identified at species level, with *V. leptobactrum* and *O. sinensis* being the dominant species in AH and AG. Interestingly, *O. sinensis* sequences were detected from uninjected larvae, although they were only 1/435 of those detected from BH. For the analysis of fungal community structures, the richness of *O. sinensis* in the gut of the injected larvae (BG) was much higher than that in the hemolymph (BH; **Supplementary Table 5**). When the larvae became mummified, *O. sinensis* was the dominant fungal species (> 99.5% relative abundance) in both the larval hemolymph and gut (CH and CG), as well as in the larval tissues (DT and ET), whereas other fungi were seriously inhibited (**Supplementary Tables 8**, **9**).

DISCUSSION

During artificial cultivation of the Chinese cordyceps, the *T. xiaojinensis* host larvae were fed on the roots of *P. anserina* collected from the wild, which allowed the larval microbiota to establish. Using culture-dependent as well as culture-independent methods, the microbiota in the hemolymph and gut of *T. xiaojinensis* larvae was compared among uninjected living larvae, injected living larvae containing blastospores, and freshly mummifying larvae without mycelia coating. In addition, the tissues of larvae from mummified larvae coated with mycelia and completely mummified larvae with mycelia were also analyzed. The discovery of many

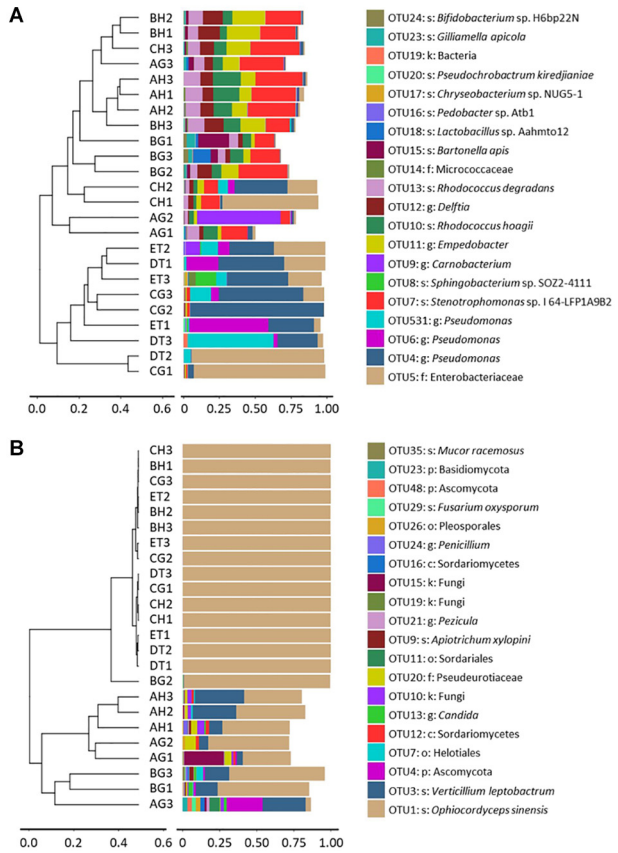

FIGURE 4 | Clustering analysis of beta-diversity based on the Bray–Curtis distance matrix for bacterial **(A)** and fungal **(B)** communities of all samples by the UPGMA method. AH and AG, hemolymph and gut of un-injected living larvae; BH and BG, hemolymph and gut of living larvae with high blastospore load; CH and CG, freshly mummifying larvae without mycelia coating; DT, tissues of the mummified larvae coated with mycelia; ET, tissues of the completely mummified larvae with mycelia.

opportunistic pathogenic bacteria in the hemolymph of the living larvae without *O. sinensis* infection, the high larval microbial diversities influenced by *O. sinensis* infection and the involvement of dominant bacteria during the mummification process of infected larvae provide new insight into the infection and mummification mechanism of *O. sinensis* in its *Thitarodes* hosts.

Microbiota in Insect Hemolymph and Gut Without *O. sinensis* Infection

Hemolymph is recognized as a key mediator of nutritional and immunological homeostasis in insects and is generally considered to be microbe-free, or nearly so, in healthy insects (Lemaitre and Hoffmann, 2007). Now, overwhelming evidence indicates that various non-pathogenic microorganisms can stably or transiently inhabit hemolymph in a diversity of insects (Blow and Douglas, 2019). The most reported hemolymph microorganisms are bacteria of the genus *Spiroplasma* (Phylum Tenericutes, Family Mollicutes) widely associated with insects in the Hymenoptera, Diptera, Lepidoptera, Hemiptera and Coleoptera orders (Clark, 1982), with reported densities of ca. 10^8 mL^{-1} hemolymph in *Drosophila melanogaster* (Herren et al., 2014). A second group of bacteria found abundantly in the insect hemolymph are members of the Enterobacteriaceae (γ-proteobacteria) in aphids, specifically *Serratia symbiotica* and the sister taxa *Hamiltonella defensa* and *Regiella insecticola* (Henry et al., 2013; Zytynska et al., 2016). Surprisingly, in the present study, from the hemolymph of healthy, laboratory-cultured *T. xiaojinensis* larvae (i.e., without *O. sinensis* infection), 14 bacterial species in 12 genera including entomopathogenic *Serratia* spp. and *Pseudomonas* spp. were isolated, using a culture-dependent method (**Table 1**). Further, 164 bacterial OTUs including the dominant species (relative abundance > 1%) *Stenotrophomonas* sp. I 64-LFP1A9B2 (32.46%), *R. hoagii* (17.07%) and *R. degradans* (11.39%), and 73 fungal OTUs including the dominant species *M. humilis* (1.07%), *O. sinensis* (43.41%) and *V. leptobactrum* (25.03%) were identified by a culture-independent method. These findings suggest that these bacteria and fungi, which are totally different from those reported from other insects (for example, *D. melanogaster* and aphids; Henry et al., 2013; Herren et al., 2014; Zytynska et al., 2016), may utilize host nutritional resources, and persist in the hemolymph by a combination of evasion and tolerance of insect immune effectors. To the best of our knowledge, this is the first study to isolate such a high number of pathogenic bacterial species from the hemolymph of healthy insect larvae. How these microorganisms can survive in the larval hemolymph of *T. xiaojinensis* and succeed in evading the insect's immune system are questions for further study.

Without *O. sinensis* injection, the gut of healthy laboratory-reared larvae contained 14 bacterial species in 11 genera and 3 fungal species (*Apiotrichum porosum*, *Phialemonium inflatum,* and *Sterigmatomyces halophilus*) in 3 genera based on the culture-dependent method (**Table 1**); and 259 bacterial OTUs including dominant species *Stenotrophomonas* sp. I 64-LFP1A9B2 (25.34%), *R. hoagie* (6.49%), *R. degradans* (6.35%), *Bartonella apis* (2.84%), *Lactobacillus* sp. Aahmto12 (1.22%) and *Gilliamella apicola* (1.25%), and 108 fungal OTUs including *F. oxysporum* (2.23%), *Myrothecium verrucaria* (1.37%), *O. sinensis* (9.96%), and *V. leptobactrum* (24.89%), based on the culture-independent method. Compared with eight bacterial genera (*Staphylococcus*, *Bacillus*, *Klebsiella*, *Pseudomonas*, *Aeromonas*, *Plesiomonas*, *Sporosarcina,* and *Neisseria*) isolated by Zhuo et al. (2004), and eight bacterial genera (*Enterobacter, Carnobacterium*, *Novosphingobium*, *Acinetobacter*, *Pseudomonas*, *Klebsiella, Pantoea, and Delftia*) by Liu et al. (2008) from the guts of *T. gonggaensis* larvae collected in the wild, more bacterial genera (14 vs. 8) were found in the present study by a culture-dependent method. Only *Pseudomonas* and *Carnobacterium* were common in wild *T. gonggaensis* larvae and laboratory-reared *T. xiaojinensis* larvae, which may be due to differences in the microbiota of wild and laboratory insect populations, different *Thitarodes* species and microbe detection methods.

Few *O. sinensis* sequences (1/435 of those detected from BH) were detected by a culture-independent method in both the hemolymph and gut of healthy larvae, i.e., without fungal

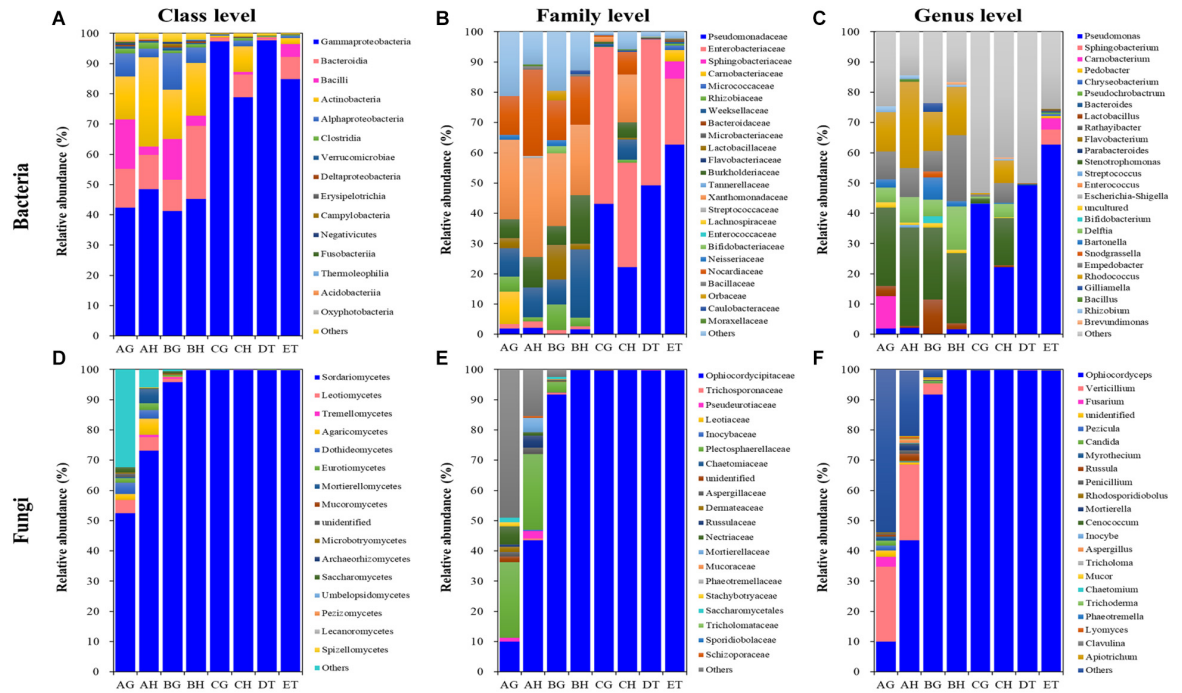

FIGURE 5 | Relative abundances of the top 10 bacterial **(A–C)** and fungal **(D–F)** classes **(A,D)**, families **(B,E)** and genera **(C,F)** in eight types of samples (AG, AH, BG, BH, CG, CH, DT, and ET). "Others" includes classes, families or genera beyond the top 10. AH and AG, hemolymph and guts of un-injected living larvae; BH and BG, hemolymph and guts of living larvae with high blastospore load; CH and CG, freshly mummifying larvae without mycelia coating; DT, tissues of the mummified larvae coated with mycelia; ET, tissues of the completely mummified larvae with mycelia.

infection. The roots of *P. anserina* may be associated with the *O. sinensis* fungus as a plant endophyte (Zhong et al., 2014; Lei et al., 2015; Wang et al., 2020). Whether few fungal spores were introduced into the larvae from the larval food (i.e., roots of *P. anserina* collected in the wild) needs further study.

Microbiota in Insect Hemolymph and Gut With *O. sinensis* Infection

The microbiota of the insect gut plays crucial roles in modulating the interactions between the insect host and intestinal pathogens. The infection of the Chinese white wax scale insect *Ericerus pela* by *Cladosporium langeronii* and *C. sphaerospermum* fungi had little effect on the fungal community but strongly influenced the bacterial community (Sun et al., 2008). The pathogenic fungus *Beauveria bassiana* interacts with the gut microbiota of mosquitoes to accelerate host mortality, and fungal infection causes dysbiosis of mosquito gut microbiota with a significant increase in gut bacterial load and a significant decrease in bacterial diversity (Wei et al., 2017). Contrary to Wei et al. (2017), here growth of *O. sinensis* blastospores in the hemolymph of live larvae greatly inhibited the culturable bacterial community in the hemolymph but stimulated the culturable bacterial and fungal community in the gut; when the living larvae contained a high load of blastospores, nine culturable bacterial species disappeared from the hemolymph, but three more bacterial species and three-times more fungal species were isolated from the gut, compared with the larvae not containing blastospores.

The bacterial abundance of *Carnobacterium* and *Lactobacillus* species in the gut was much higher than that in the hemolymph (**Figure 6A**). *O. sinensis* was the most abundant fungal species in *T. xiaojinensis*, both in the hemolymph and the gut, and in both mummified stages of the larvae. Except for *Ophiocordyceps* species, most of the fungal species were significantly suppressed in the living larvae containing high numbers of blastospores. In uninfected larvae, *Verticillium* species was evenly distributed in the gut and hemolymph with a high relative abundance, while *Myrothecium*, *Fusarium*, *Pezicula,* and *Candida* were mainly distributed in the gut, and *Ophiocordyceps*, *Aspergillus*, *Penicillium*, *Russula,* and *Mortierella* were dominant in the hemolymph (**Figure 6B**). Based on the OTU diversity of unculturable bacteria and fungi, in general, high blastospore load increased bacterial diversity but sharply decreased fungal diversity in the larval hemolymph and gut. When the larvae became mummified, the bacterial and fungal diversity declined.

Microbiota Comparison of Culture-Dependent and -Independent Methods

The culture-independent method detected more OTUs than the culture-dependent method in this study. Eight (*Carnobacterium*, *Chryseobacterium*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Sphingobacterium* and *Stenotrophomonas*) of 16 culturable bacterial genera and 4 (*Apiotrichum*, *Mucor*,

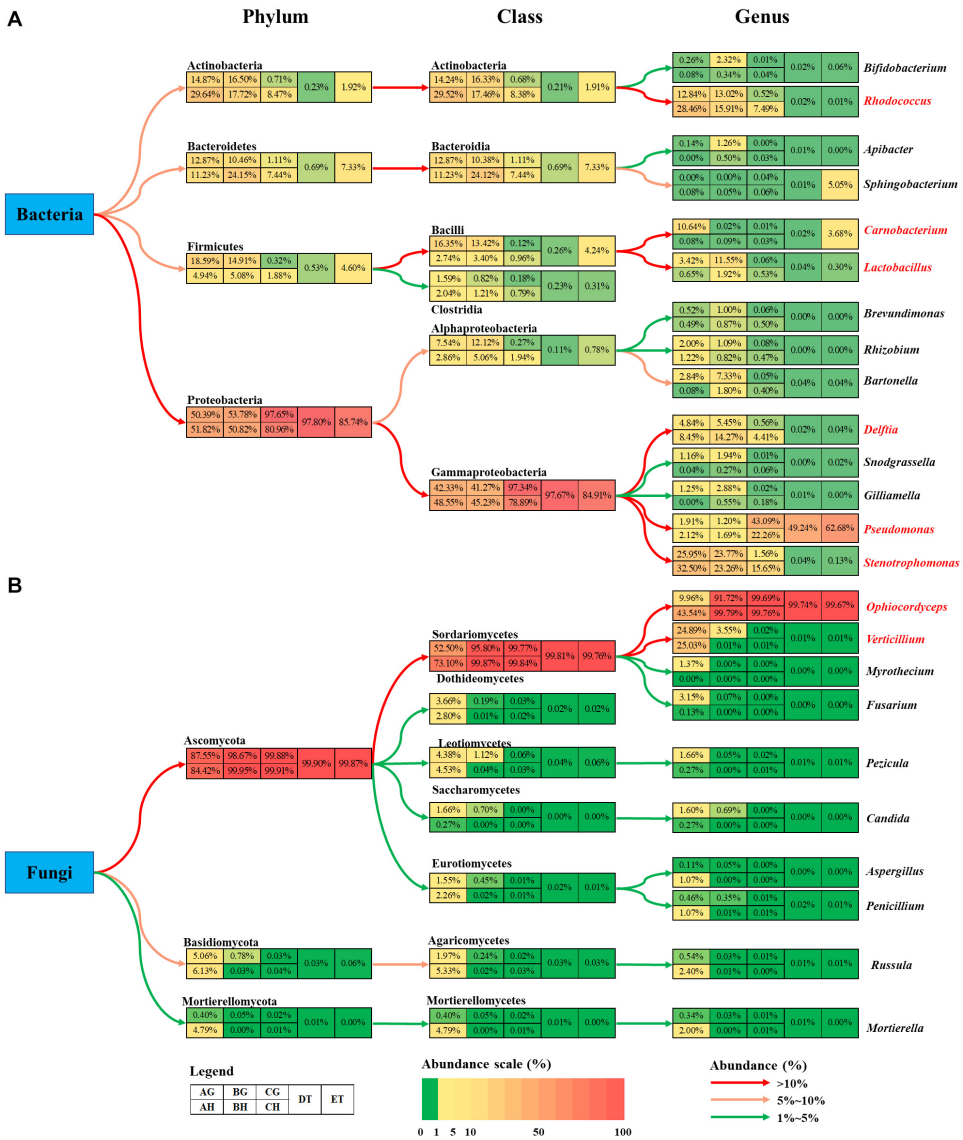

FIGURE 6 | Summary of the changes in bacteria **(A)** and fungi **(B)** community among eight types of samples. The colored boxes represent corresponding samples according to the legend. Relative abundance is indicated by color gradients; the color from green through yellow to red represents the value from low to high. Colored arrows show the abundance changes from higher taxonomic level to lower level. AH and AG, hemolymph and gut of un-injected living larvae; BH and BG, hemolymph and gut of living larvae with a high blastospore load; CH and CG, freshly mummifying larvae without mycelia coating; DT, tissues of the mummified larvae coated with mycelia; ET, tissues of the completely mummified larvae with mycelia.

Ophiocordyceps and *Penicillium*) of 13 cultural fungal genera were included in the top 10 OTU genera for all samples. *Ophiocordyceps* and *Pseudomonas* were dominant genera in both detection methods, indicating the overlapping detection of key microbes by both methods. In contrast, Zhang et al. (2010) reported that very few fungal OTUs from the naturally occurring Chinese cordyceps were shared by culture-dependent and -independent methods.

Based on OTUs, the dominant bacterial species *Stenotrophomonas* sp. I 64-LFP1A9B2, *R. hoagii* and *R. degradans* and fungal species *O. sinensis* and *V. leptobactrum* were shared in the hemolymph and gut of uninjected larvae. However, with the exception of *O. sinensis,* these microbe species were not isolated from the media plates, even when using different media (LB, HIA, G5, and GSA) in the incubators with or without aeration at 13 and 23°C, respectively. *V. leptobactrum* is a rare rock-inhabiting fungal species from serpentinic rocks playing an important role in the bioweathering (Daghino et al., 2009). A specific medium (Daghino et al., 2009) was used to isolate this fungus but this attempt was not successful. Due to its dominant occurrence in the hemolymph and gut of uninjected larvae and its shared presence in all samples, *V. leptobactrum* may be a representative fungus in *Thitarodes* moths. The microbial diversity trend appeared not to be exactly similar for

the two detection methods, which may have been influenced by microbial culturability.

Mummification-Related Bacteria and Fungi

So far, a variety of microbial communities have been identified from naturally occurring Chinese cordyceps and the gut of wild *Thitarodes/Hepialus* larvae, as well as from their microhabitats, by using traditional culture-dependent and culture-independent methods, including PCR-based molecular markers, sequencing of ribosomal DNA and high-throughput sequencing (Zhuo et al., 2004; Liu et al., 2008; Liang et al., 2008, 2019; Yu et al., 2008; Zhang et al., 2009, 2010; Xia et al., 2015, 2016). All these communities may provide the foundation for further discovery of new valuable microorganism resources for the medicine industry. However, no information is available on the direct effects of the microbiota on the regulation of artificial production of Chinese cordyceps.

Cultivation of Chinese cordyceps at commercial scale has been successfully established (Li et al., 2016, 2019; Han et al., 2019; Liu et al., 2020). However, several factors such as the degeneration of the fungus, high mortality of host larvae by pathogens, low and slow infection and mummification rate constrained the efficient production of Chinese cordyceps (Zhou et al., 2013; Lu et al., 2015; Qin et al., 2018). Low and slow mummification rate was the most important uncontrolled factor during the cultivation process (Liu et al., 2019). The microbiota in the hemolymph and gut may be involved in this process. For example, the opportunistic pathogenic bacterium *Serratia marcescens* overgrows in the midgut of *Anopheles stephensi* and translocates to the hemocoel, which promotes *B. bassiana* killing the mosquitoes (Wei et al., 2017). In the present study, four culturable bacterial species (*Chryseobacterium* sp., *P. fragi*, *S. plymuthica*, *S. proteamaculans*) were common in freshly or completely mummified larvae and actively overgrew. *F. frigidimaris* and *P. poae* were detected only in freshly and/or completely mummified larvae and *Arthrinium* sp. was the only culturable fungal species from freshly mummified larvae, indicating their high loads at this stage of infection. *O. sinensis* and *Pseudomonas* spp. became dominant microbe species, when the infected larvae became mummified. *Pseudomonas* spp. are regarded as psychrotolerant and broad-host-range entomopathogenic bacteria exhibiting insecticidal activity toward certain agricultural pests (Chen et al., 2014; Mei et al., 2016). It appears that these bacteria and fungi might be involved in the blastospore-mycelium transition during larval mummification process. Whether blastospores induce the larval immune system to inhibit the bacterial and fungal growth in the hemolymph and how the blastospore-mycelium transition destroys the larval immune system allowing bacterial outbreak warrants further study. Furthermore, as *Chryseobacterium* sp., *P. fragi*, *S. plymuthica,* and *S. proteamaculans* may be opportunistic pathogens for human beings (Kertesz and Thai, 2018), the possible presence of these bacteria in the fresh Chinese cordyceps should be carefully considered.

Interestingly, *O. sinensis* was detected only in the hemolymph of freshly mummified larvae. This phenomenon was also observed in the routine practice in the laboratory (unpublished data). It appears that not all blastospores from the hemolymph can survive well *in vitro* when they are spread onto the media plates. Possibly, the blastospores exhibit different phases in different environments to adapt growth *in vivo* and *in vitro*. *Paecilomyces hepiali* and *Isaria farinosa* were considered to be important entomopathogenic fungi for *Thitarodes* larvae during the artificial cultivation of Chinese cordyceps (Han et al., 2019). However, *I. farinosa* was not abundant in the culture plates. Perhaps *I. farinosa* is an opportunistic fungus for the moth larvae. It should be pointed out that some minor bacterial or fungal species might not have been detected from the plates under the present experimental conditions, although five media, low and temperate temperatures, and normoxia and anoxia conditions were used for the detection of microbiota in this study.

CONCLUSION

The composition of the hemolymph and gut microbiota from laboratory-reared *T. xiaojinensis* larvae with or without injected *O. sinensis* blastospores is unique. The uninfected larval hemolymph contained as many culturable bacterial species as did the larval gut. *Stenotrophomonas* sp. I 64-LFP1A9B2, *R. hoagii*, *R. degradans*, *O. sinensis,* and *V. leptobactrum* OTU species were dominant and shared in the hemolymph and gut of uninjected larvae. The growth of blastospores sharply inhibited the culturable bacterial community in the hemolymph but greatly enhanced the bacterial and fungal community in gut of live larvae. However, high blastospore load increased bacterial diversity but sharply decreased fungal diversity in the larval hemolymph and gut based on OTUs. Four culturable bacterial species (*Chryseobacterium* sp., *P. fragi*, *S. plymuthica*, *S. proteamaculans*) overgrew and *O. sinensis* and *Pseudomonas* spp. became dominant microorganisms, when the infected larvae became mummified, indicating their possible involvement in the larval mummification process.

DATA AVAILABILITY STATEMENT

The datasets generated in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

No special permits were required for sampling of organisms in this study. All samples were collected by researchers with introduction letters from the Guangdong Institute of Applied Biological Resources and with the help of local herdsmen.

AUTHOR CONTRIBUTIONS

R-CH and PD designed and coordinated the research. HW and LC collected the samples. HW conducted the research.

Z-CR and HW analyzed the data. R-CH, PD, HW, and Z-CR wrote the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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The Succession of the Gut Microbiota in Insects: A Dynamic Alteration of the Gut Microbiota During the Whole Life Cycle of Honey Bees (*Apis cerana*)

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The Asian honey bee *Apis cerana* is a valuable biological resource insect that plays an important role in the ecological environment and agricultural economy. The composition of the gut microbiota has a great influence on the health and development of the host. However, studies on the insect gut microbiota are rarely reported, especially studies on the dynamic succession of the insect gut microbiota. Therefore, this study used high-throughput sequencing technology to sequence the gut microbiota of *A. cerana* at different developmental stages (0 days post emergence (0 dpe), 1 dpe, 3 dpe, 7 dpe, 12 dpe, 19 dpe, 25 dpe, 30 dpe, and 35 dpe). The results of this study indicated that the diversity of the gut microbiota varied significantly at different developmental stages (ACE, $P = 0.045$; Chao1, $P = 0.031$; Shannon, $P = 0.0019$; Simpson, $P = 0.041$). In addition, at the phylum and genus taxonomic levels, the dominant constituents in the gut microbiota changed significantly at different developmental stages. Our results also suggest that environmental exposure in the early stages of development has the greatest impact on the gut microbiota. The results of this study reveal the general rule of gut microbiota succession in the *A. cerana* life cycle. This study not only deepens our understanding of the colonization pattern of the gut microbiota in workers but also provides more comprehensive information for exploring the colonization of the gut microbiota in insects and other animals.

Keywords: *Apis cerana*, core microbiota, colonization, relative abundance, environmental exposure

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INTRODUCTION

The gut microbiota has attracted extensive attention due to its close relationship with the host. The gut microbiota not only promotes digestion and absorption of food by the host (Cummings, 1984) but also plays an important role in host development (Sommer and Bäckhed, 2013; Lin et al., 2019), immunity (Pickard et al., 2017), aging (O'Toole and Jeffery, 2015), and resistance to pathogen invasion (Pamer, 2016). The realization of gut microbiota function depends on the composition and structure of the gut microbiota. The composition and structure of the gut microbiota change dynamically during the whole life cycle of the host. Interestingly, previous studies have shown that improving the structure of the host gut microbiota can increase the lifespan of the host (Smith et al., 2017; Han et al., 2018). For example, by transferring young African turquoise killifish (*Nothobranchius furzeri*) gut microbes into older hosts, the lifespan of the hosts was increased, and

the rate of decline in exercise ability was delayed (Smith et al., 2017). In addition, the rapid change in the abundance of subdominant bacteria in the gut is a hallmark of human aging. Interestingly, health-related bacteria were enriched in a long-lived population (Biagi et al., 2016).

There are few reports on the colonization and succession of the host gut microbiota in the natural state, and most research has focused on vertebrates, such as foals (Costa et al., 2016), goats (Lei et al., 2018), chickens (Xi et al., 2019), and southern catfish (Zhang et al., 2018). These results indicate that the structure of the gut microbiota is constantly changing with the development of the host, and the structure of the gut microbiota is significantly correlated with host age. These studies not only revealed the succession of a series of gut microbiota species but also provided an important reference value for studying the colonization of other gut microbiota species.

Insects (Arthropoda: Insecta) are the most ubiquitous and diverse animals on the planet. The relationship between the composition of insect gut microbes and the host has gradually been revealed, including gut microbes with effects on host immunity (Wei et al., 2017), metabolism (Zheng et al., 2017), environmental exposure (Wintermantel et al., 2018), and pest control (Xie et al., 2019). In addition, the succession of insect gut microbes has also been studied. For example, Duguma et al. (2015) found that the *Culex* mosquito gut microbiota had different structures in different stages of development. Similar studies have been conducted in other insects, such as the burying beetle (*Nicrophorus vespilloides*) (Wang and Rozen, 2017), the cockroach (*Blattella germanica*) (Purificación et al., 2014), the queen bee (*Apis mellifera*) (Tarpy et al., 2015) and *Drosophila melanogaster* (Han et al., 2017). The results of these studies all revealed that the structure of the gut microbiota showed a significant correlation at different stages of development in different insects. However, little is known about the natural succession of the insect gut microbiota in the natural state, such as that in honey bees.

Honey bees are important pollinators and convey great economic benefits to crop pollination worldwide (Southwick and Southwick, 1992; Kevan, 1999; Klein et al., 2007; Kleijn et al., 2015). In addition, the relationship between the bee gut microbiota and health has received considerable attention. When honey bees are exposed to pesticides, their gut microbiota is disturbed, and their mortality increases (Motta et al., 2018). Moreover, the overuse of antibiotics also leads to structural changes in the gut microbiota of honey bees, making them more susceptible to infection by pathogens and leading to greater challenges to bee survival (Raymann and Moran, 2017). In addition, when *A. cerana* were infected with *Nosema ceranae*, the steady state of the gut microbiota was disturbed, leading to an increase in the mortality rate of the bees (Huang et al., 2018). These studies all suggest that the gut microbiota plays an important role in bee health and disease. However, the gut microbes of *A. cerana* are poorly studied, and little is known about the succession rules of the gut microbiota of honey bees. This lack of knowledge limits our understanding of the gut microbiota throughout the life cycle of *A. cerana* and hinders our ability to protect resource insects.

The goal of this study was to elucidate the dynamic changes in the gut microbiota throughout the life cycle of *A. cerana* from the perspective of the composition and structure of the gut microbiota. In this study, high-throughput sequencing technology was used to sequence the gut microbiota of workers 0 days post emergence (dpe), 1 dpe, 3 dpe, 7 dpe, 12 dpe, 19 dpe, 25 dpe, 30 dpe, and 35 dpe. The purpose of this study was to elucidate the colonization rules of gut microbes in *A. cerana*, which inform important theories for improving host health through gut microbes. In addition, this study provides reference information for the study of gut microbe colonization in other animals.

MATERIALS AND METHODS

Worker Sampling

Worker samples were collected in Kunming, Yunnan Province in July 2018. To obtain bees of different ages, we conducted sample collection in accordance with the method described in previous publications (Powell et al., 2014; Guo et al., 2015). First, we identified a healthy hive, determined the age of the workers and selected a frame in which new workers were appearing. A frame of late-stage pupae (eyes were pigmented, but pupae lacked movement) was selected from the hive and moved to a sterile incubator (34°C and 90% relative humidity, mimicking hive conditions), and the pupae were allowed to eclose naturally. We first collected worker samples 0 dpe (0 days post emergence) and placed them in 1.5 mL centrifuge tubes (0 dpe individuals had no contact with the environment). Next, 300 newly emerged worker individuals from the incubator were marked with red Testors enamel paint and then returned to the original hive to allow for natural growth. Finally, according to the life cycle of the bees, samples were randomly collected 1 dpe, 3 dpe, 7 dpe, 12 dpe, 19 dpe, 25 dpe, 30 dpe and 35 dpe. Three worker samples were collected 0 dpe, 1 dpe, 3 dpe and 7 dpe, and six worker samples were collected 12 dpe, 19 dpe, 25 dpe, 30 dpe and 35 dpe. All worker samples were immediately placed in an ultralow temperature freezer (EU1DW/BD-55W321EU1, China) after collection.

In the process of sample collection, we to reduce the contamination of the samples, including the use of sterile centrifuge tubes. All experiments were conducted on a sterile ultraclean platform, and all the equipment was treated with high-temperature sterilization.

DNA Extraction and PCR Amplification

First, workers were removed from the ultralow temperature freezer and placed onto an ultraclean working table. Then, sterile tweezers were used to remove the entire gut of the workers, and the gut was then placed into a 1.5 mL sterile centrifuge tube. Then, 60 μL of Krebs Ringer buffer was added to the centrifuge tube, and the sample was ground. A soil DNA kit (Omega Biotek, Norcross, GA, United States) was used to extract the gut bacterial DNA of the workers according to the manufacturer's protocol. Finally, 60 μL of elution buffer was added to obtain the DNA sample, and the resulting DNA

sample was stored in a freezer at −20°C. The final DNA concentration and purity were determined with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, United States), and the DNA quality was determined with 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with the primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by a thermocycler PCR system (GeneAmp 9700, ABI, United States). PCR was conducted using the following program: 3 min of denaturation at 95°C; 27 cycles of 30 s at 95°C, 30 s annealing at 55°C, and 45 s elongation at 72°C; and a final extension at 72°C for 10 min. Each PCR was performed in triplicate in 20 μL of reaction mixtures containing 4 μL of 5 × FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel, further purified using an AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, United States) and quantified using QuantiFluor^{TM}-ST (Promega, United States) according to the manufacturer's protocol.

Illumina MiSeq Sequencing and Processing of Sequencing Data

Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, United States) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

MiSeq sequencing results were reported as paired-end sequence data. First, according to the overlap relation between PE reads, pairs of reads were merged into a single sequence. At the same time, the quality of the reads and the effect of merging were used as filters. Barcode and primer sequences at both ends of the sequence were used to distinguish the samples and obtain the effective sequence. In addition, the sequence direction was corrected to optimize the data.

Raw fastq files were quality filtered by Trimmomatic and merged by FLASH with the following criteria: (i) The reads were truncated at any site with an average quality score <20 over a 50 bp sliding window; (ii) sequences with overlaps longer than 10 bp were merged according to their overlap, with no more than 2 bp mismatched; and (iii) the sequences of each sample were separated according to barcodes (exactly matching) and primers (allowing 2 nucleotide mismatches), and reads containing ambiguous bases were removed. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1)[1] with a novel "greedy" algorithm that performs chimera filtering and OTU clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP Classifier algorithm[2] against the Silva (SSU123) 16S rRNA database at a confidence threshold of 70%.

[1]http://drive5.com/uparse/

[2]http://rdp.cme.msu.edu/

Statistical Analyses and Comparison of Microbial Communities

One-way analysis of variance (ANOVA) was used to analyze the diversity parameters (ACE, Chao1, Shannon and Simpson) of the gut microbiota of workers at different dpe (the time points after the emergence of workers, 0 dpe, 1 dpe, 3 dpe, 7 dpe, 12 dpe, 19 dpe, 25 dpe, 30 dpe and 35 dpe). In addition, Kruskal-Wallis H test was performed to analyze the bacteria with the highest abundance (phylum and genus taxa) in the gut of workers at different dpe. According to the gut microbiota abundance of workers of different ages, Kruskal-Wallis H test was performed to conduct hypothesis tests for different groups. In addition, the significance level of differences in species abundance was evaluated, and the species with significant differences at different ages were obtained (from analysis using the stats package in R and the SciPy package in Python). The non-metric multidimensional scaling (NMDS) distance algorithm based on Bray-Curtis distance was used to calculate the differences between the assessment of microbial communities (QIIME was used to calculate the beta diversity distance matrix, and the R language vegan software package was used for NMDS analysis and mapping).

RESULTS

Summary of the Sequencing Data

Deep sequencing of 42 gut samples from workers yielded 1,988,217 sequences with a total length of 886,569,845 bp and an average length of 445.91 bp. A total of 1670 OTUs were obtained at a 97% similarity level. Cluster analysis was conducted at the phylum and genus levels, and 30 phyla and 512 genera were obtained. Good's coverage index showed that the estimated values of all samples were over 99%, indicating that all samples reached an appropriate sequencing depth (**Table 1**). In addition, the raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (accession numbers SRR9715685-SRR9715699, SRR9715700-SRR9715726).

Changes in Gut Microbiota Diversity in Workers of Different Ages

The diversity of bacterial communities is reflected through the Shannon and Simpson indices, and the richness of the community is reflected by the Chao1 and ACE indices. The results showed that the diversity of the gut microbiota was influenced by the age of the host; moreover, the composition and structure of the gut microbiota of workers of different ages were significantly different (**Table 1**). Namely, the *P*-values of the bacterial diversity index differences were as follows: ACE, $P = 0.045$; Chao1, $P = 0.031$; Shannon, $P = 0.0019$; and Simpson, $P = 0.041$.

Composition of the Gut Microbiota in Workers of Different Ages

At the phylum level of classification, the gut microbiota of workers consisted mainly of the following bacteria:

TABLE 1 | Richness and diversity indices relative to each gut sample.

Sample name	Coverage	Alpha diversity			
		Ace[ab,bc]	Chao[ab,bc,]	Shannon[ab,bc]	Simpson[ab]
0 dpe1[a]	0.9997	211.70	208.06	2.25	0.31
0 dpe2	0.9996	228.82	243.00	2.71	0.20
0 dpe3	0.9997	277.04	277.58	2.72	0.23
1 dpe1[b]	0.9987	951.04	959.05	4.85	0.04
1 dpe2	0.9993	743.14	755.33	4.87	0.04
1 dpe3	0.9996	605.84	613.33	3.99	0.13
3 dpe1	0.9984	487.49	484.50	1.87	0.36
3 dpe2	0.9981	841.55	847.51	3.47	0.18
3 dpe3	0.9996	419.22	420.71	2.16	0.46
7 dpe1[c]	0.9983	390.13	386.23	2.08	0.19
7 dpe2	0.9985	485.01	484.51	2.32	0.17
7 dpe3	0.9990	465.36	478.69	3.10	0.11
12 dpe1	0.9975	427.56	407.17	1.98	0.23
12 dpe2	0.9989	224.41	196.08	1.93	0.18
12 dpe3	0.9977	434.01	405.45	2.00	0.21
12 dpe4	0.9971	524.17	525.26	1.82	0.26
12 dpe5	0.9973	599.82	496.44	2.13	0.20
12 dpe6	0.9981	408.75	398.33	2.36	0.17
19 dpe1[d]	0.9966	533.32	534.85	2.00	0.24
19 dpe2	0.9985	360.22	363.63	2.13	0.25
19 dpe3	0.9977	617.02	611.49	2.99	0.10
19 dpe4	0.9983	369.31	365.61	2.01	0.26
19 dpe5	0.9979	401.82	311.86	1.72	0.23
19 dpe6	0.9983	338.69	337.50	1.48	0.37
25 dpe1	0.9989	189.24	181.04	1.26	0.42
25 dpe2	0.9989	233.72	177.45	1.77	0.25
25 dpe3	0.9983	259.59	255.16	2.17	0.20
25 dpe4	0.9976	494.64	358.35	1.77	0.27
25 dpe5	0.9959	607.74	618.13	2.51	0.15
25 dpe6	0.9975	443.40	402.00	2.03	0.20
30 dpe1[e]	0.9969	914.48	922.20	3.79	0.07
30 dpe2	0.9997	572.88	579.14	5.26	0.02
30 dpe3	0.9984	240.18	202.67	1.58	0.30
30 dpe4	0.9970	523.16	497.87	1.94	0.26
30 dpe5	0.9969	492.45	502.20	2.09	0.20
30 dpe6	0.9987	388.42	382.16	1.98	0.22
35 dpe1	0.9984	304.89	307.56	1.69	0.30
35 dpe2	0.9990	169.79	159.04	1.76	0.24
35 dpe3	0.9987	199.77	175.03	1.87	0.19
35 dpe4	0.9975	484.45	389.12	1.79	0.23
35 dpe5	0.9962	855.79	878.00	3.76	0.06
35 dpe6	0.9977	387.62	354.16	2.03	0.24
P value		0.045	0.031	0.0019	0.041

[ab]*Shows that the diversity index was significantly different between 0 dpe and 1 dpe.*
[bc]*Shows that the diversity index was significantly different between 1 dpe and 7 dpe.*

Proteobacteria, Cyanobacteria, Planctomycetes, Spirochaetae, Bacteroidetes, Actinobacteria, Verrucomicrobia, Acidobacteria, Firmicutes, and Fibrobacteres. The results of this study also showed that there were significant differences in the relative abundance of some of the top 10 phyla in the gut of workers of different ages (**Table 2**). Proteobacteria had the highest relative abundance 0 dpe (71.97%) and the lowest abundance 1 dpe (15.50%) (P = 0.013). Bacteroidetes are an important and consistent components of the worker gut, and their relative abundance was lowest 0 dpe (2.45%) and highest 30 dpe (28.07%) (P = 0.021). Actinobacteria had the highest relative abundance 0 dpe (11.06%) and the lowest 1 dpe (0.72%). In addition,

TABLE 2 | Top 10 abundant phyla.

Phylum	Different post time (%)									*P* value
	0 dpe[a]	1 dpe[b]	3 dpe	7 dpe[c]	12 dpe	19 dpe[d]	25 dpe	30 dpe[e]	35 dpe	
Proteobacteria[ab]	71.97	15.50	26.73	22.21	29.58	37.75	48.01	40.61	46.77	0.013
Cyanobacteria	1.44	2.33	2.13	0.91	0.85	0.66	0.11	1.42	0.56	0.015
Planctomycetes	0.03	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.172
Spirochaetae[bc]	0.30	4.62	1.48	0.60	0.15	0.27	0.14	1.59	0.50	0.010
Bacteroidetes[ab,bc]	2.45	23.58	8.91	12.02	18.51	17.75	24.43	28.07	23.09	0.021
Actinobacteria[ab]	11.06	0.72	4.11	12.07	1.68	3.66	0.77	1.99	1.83	0.067
Verrucomicrobia[bc]	0.03	0.94	0.32	0.06	0.04	0.03	0.03	0.29	0.08	0.032
Acidobacteria	0.31	0.04	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.001
Firmicutes[ab]	5.26	47.40	54.94	51.34	48.98	39.59	26.30	23.73	26.35	0.008
Fibrobacteres	0.00	0.10	0.05	0.01	0.00	0.01	0.01	0.06	0.01	0.013
Others	7.10	4.66	1.27	0.75	0.20	0.28	0.20	2.18	0.81	

[ab]*Indicates that the relative abundance 0 dpe and 1 dpe at two developmental stages is significantly different (Student's t test).*
[bc]*Indicates that the relative abundance 1 dpe and 7 dpe at two developmental stages is significantly different (Student's t test).*

the relative abundance of Firmicutes was lowest 0 dpe and highest 3 dpe (54.94%) (P = 0.008). Moreover, the following bacteria were significantly different in the gut of workers of different ages: the relative abundance of cyanobacteria was highest at 1 dpe and lowest at 25 dpe (P = 0.015), the relative abundance of Spirochaetae was highest 1 dpe and lowest at 25 dpe (P = 0.0095), the relative abundance of Fusobacteria was lowest 0 dpe and highest 1 dpe (P = 0.011), the relative abundance of Verrucomicrobia was lowest 0 dpe and highest 1 dpe (P = 0.032), the relative abundance of Acidobacteria was highest 0 dpe and lowest at 25 dpe (P = 0.00061), and the relative abundance of Chloroflexi was highest 0 dpe and lowest 19 dpe (P = 0.0092) (**Supplementary Figure 1**).

The results of this study indicated that there were 35 genera with a relative abundance higher than 1% in the gut of *A. cerana* workers (**Figure 1A**). Among them, the genera with the highest relative abundances were *Lactobacillus*, *Gilliamella*, *Apibacter*, *Acinetobacter*, *Snodgrassella*, *Bifidobacterium*, *Peptostreptococcaceae*, *Escherichia-Shigella*, *Bacteroides*, and *Sphingomonas*. In addition, the gut microbiota 0 dpe and 1 dpe had similarities in structure, and the genera with the highest relative abundances were *Acinetobacter*, *Sphingomonas* and *Lactobacillus* (**Figure 1B**). After 3 dpe, the structure of the gut microbiota was more uniform (**Figure 1B**).

An interesting result of this study was that there were significant differences in the relative abundance at the genus level of the gut microbiota (top 14) of workers of different ages (**Table 3**). The relative abundance of *Lactobacillus* was only 0.44% 0 dpe and 48.09% 12 dpe. The relative abundances of *Gilliamella* and *Apibacter* were the lowest 0 dpe (both lower than 0.01%), and the relative abundance of *Gilliamella* had increased significantly by 3 dpe and then became stable. The relative abundance of *Apibacter* reached 17.62% 12 dpe and then stabilized. The relative abundances of *Acinetobacter* and *Sphingomonas* were the highest 0 dpe (49.91 and 10.21%, respectively). Interestingly, the relative abundance of both genera decreased significantly after 0 dpe, and although *Acinetobacter* and *Sphingomonas* still occupied a certain niche, their relative abundances in the gut of the workers were not high and tended to be stable. *Snodgrassella* and *Bifidobacterium* are important bacteria in the gut of workers. Their relative abundances were the lowest 0 dpe; the highest relative abundance of *Snodgrassella* was observed 25 dpe (14.28%), and the highest relative abundance of *Bifidobacterium* was observed 7 dpe (11.98%). The relative abundance of bacteria of the genera *Peptostreptococcaceae*, *Escherichia-Shigella*, *Bacteroides*, *Tatumella*, *Cyanobacteria*, *Fusobacterium* and *Gammaproteobacteria* in the gut of workers was higher than 1%, and the relative abundance was significantly different at different ages of *A. cerana* (**Figure 2** and **Table 3**). The abundance of *Peptostreptococcaceae* was lowest 0 dpe and highest 1 dpe (P = 0.006). The abundance of *Escherichia-Shigella* was highest 1 dpe and lowest 12 dpe. The abundance of *Bacteroides* was lowest 0 dpe and highest 1 dpe (P = 0.016). The abundance of *Tatumella* was lowest 7 dpe and highest 25 dpe. The changes in the abundances of *Cyanobacteria*, *Fusobacterium* and *Gammaproteobacteria* were significantly different; P-values were as follows: P = 0.019, P = 0.005, P = 0.012, respectively.

Interestingly, we performed statistical analysis on the intestinal flora of bees at different developmental stages, and the results showed that at the phylum and genus levels, there were significant changes in the relative abundance of 19 phyla and 236 genera, respectively. Details of the relative abundances are shown in **Supplementary Table 1**.

Beta Diversity of Gut Bacteria

The gut microbiota was analyzed based on the weighted UniFrac distance of a principal coordinates analysis (PCoA) and Bray-Curtis distance of the non-metric multidimensional scaling (NMDS) method for workers at different dpe. PCoA and NMDS analyses revealed that the compositions of the gut microbiota of workers at different dpe were different (**Figure 3**). In addition, the gut microbiota of workers 0 dpe was significantly separated from that of workers at other dpe, indicating that the composition of the gut microbiota of workers 0 dpe was significantly different from that at other dpe. Next, the gut microbiota of workers 1 dpe and 3 dpe was separated from that at other dpe, indicating

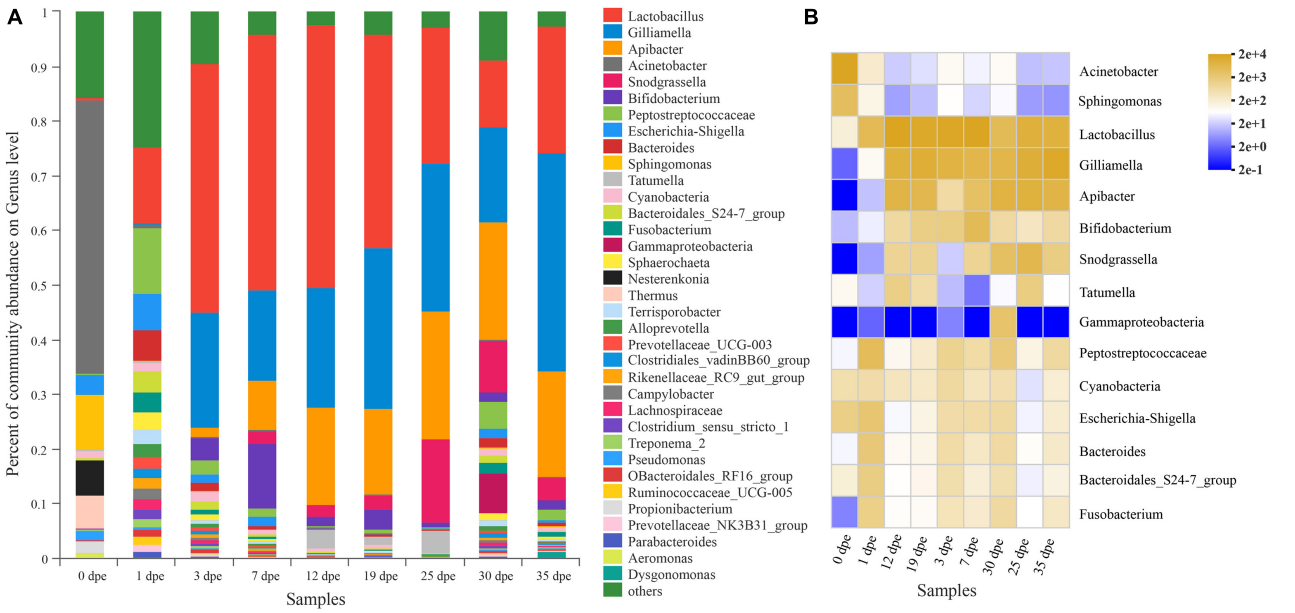

FIGURE 1 | (A) The abscissa is the sample name, and the ordinate is the proportion of species in the sample. The column of different colors represents different species, and the length of the column represents the proportion of the species. **(B)** The abscissa is the name of the sample, and the ordinate is the name of the species. The abundance of different species in the sample is shown by the color gradient of the color block. The right side of the figure is the value represented by the color gradient.

TABLE 3 | Top 13 abundant genera.

Genera	Different post time (%)									*P* value
	0 dpe[a]	1 dpe[b]	3 dpe	7 dpe[c]	12 dpe	19 dpe[d]	25 dpe	30 dpe[e]	35 dpe	
Lactobacillus	0.44	14.01	45.67	46.19	48.09	37.67	25.34	12.47	22.88	0.003
Gilliamella[bc]	0.01	0.22	20.88	16.91	21.95	30.39	26.86	16.88	40.20	0.011
Apibacter[bc]	0.00	0.04	1.64	8.91	17.62	16.39	23.73	20.71	19.63	0.006
Acinetobacter[ab]	49.91	0.58	0.21	0.12	0.04	0.07	0.04	0.21	0.04	0.005
Snodgrassella	0.00	0.02	0.05	2.28	2.23	2.68	14.28	9.48	4.11	0.005
Bifidobacterium	0.04	0.11	3.90	11.98	1.66	3.62	0.75	1.75	1.79	0.026
Peptostreptococcaceae[ab]	0.12	11.87	2.70	1.61	0.22	0.57	0.37	4.54	1.86	0.006
Escherichia-Shigella[bc,cd]	3.59	6.71	1.45	1.53	0.12	0.31	0.13	1.79	0.51	0.001
Bacteroides[ab,bc]	0.12	5.54	1.54	0.73	0.20	0.28	0.20	1.75	0.64	0.016
Sphingomonas[ab,bc]	10.24	0.31	0.17	0.06	0.02	0.04	0.02	0.15	0.01	0.002
Tatumella	0.24	0.06	0.04	0.01	3.45	1.50	4.25	0.13	0.15	0.007
Cyanobacteria	1.23	1.50	1.83	0.77	0.82	0.63	0.09	1.13	0.48	0.019
Fusobacterium[ab,bc]	0.01	3.60	0.94	0.56	0.16	0.20	0.17	1.81	0.72	0.005
Others	33.63	51.40	17.67	7.89	3.27	5.42	3.67	17.77	6.64	

[ab] *Indicates that the relative abundance 0 dpe and 1 dpe at two developmental stages is significantly different (Student's t test).*
[bc] *Indicates that the relative abundance 1 dpe and 7 dpe at two developmental stages is significantly different (Student's t test).*
[cd] *Indicates that the relative abundance 7 dpe and 19 dpe at two developmental stages is significantly different (Student's t test).*

that the composition of the gut microbiota of workers 1 dpe and 3 dpe was different from that at other dpe. Although the distances between the 7 dpe, 12 dpe, 19 dpe, 25 dpe, 30 dpe and 35 dpe groups were small, the samples of different dpe groups were clustered in their respective groups. Overall, samples from each group were concentrated in clusters of workers of different ages. In the PCoA, PC1 accounted for 38.75% of the total variance, and PC2 accounted for 22.72% (**Figure 3A**). In the NMDS, stress = 0.104, which indicated that the grouping and sampling were reliable (**Figure 3B**).

DISCUSSION

A. cerana and *Apis mellifera*, as the two largest commercial species of bees in China, not only produce an abundance of bee

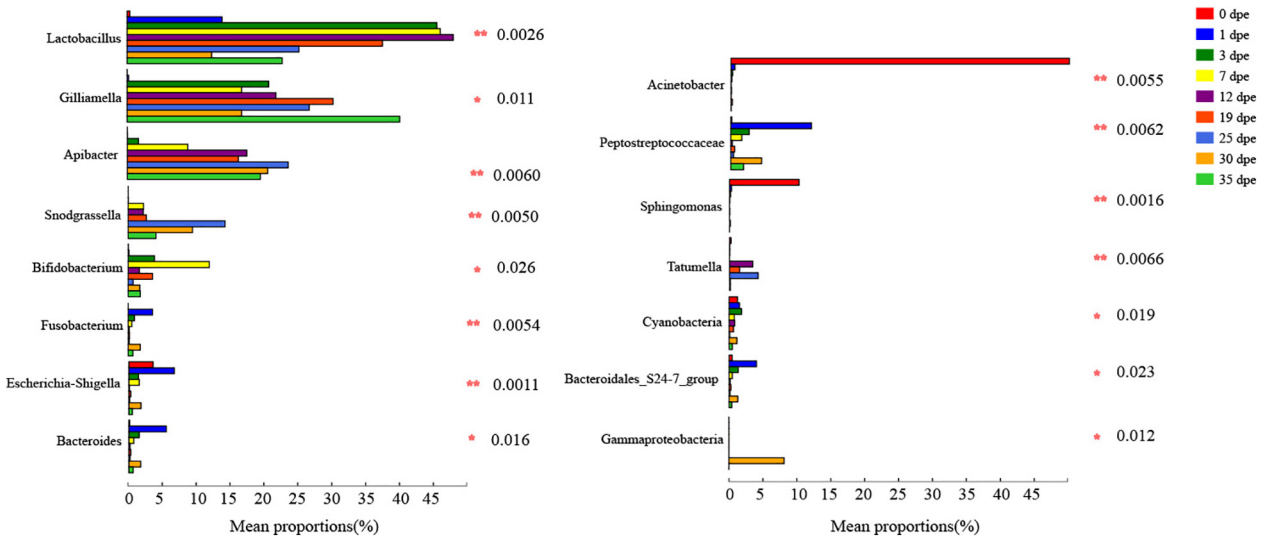

FIGURE 2 | The vertical axis represents the species names at genus classification level, the corresponding column length represents the average relative abundance of the species in various groups, and different colors represent different groups. On the far right is the value of *P*, *0.01 < *P* ≤ 0.05, **0.001 < *P* ≤ 0.01, ***$P \leq 0.001$.

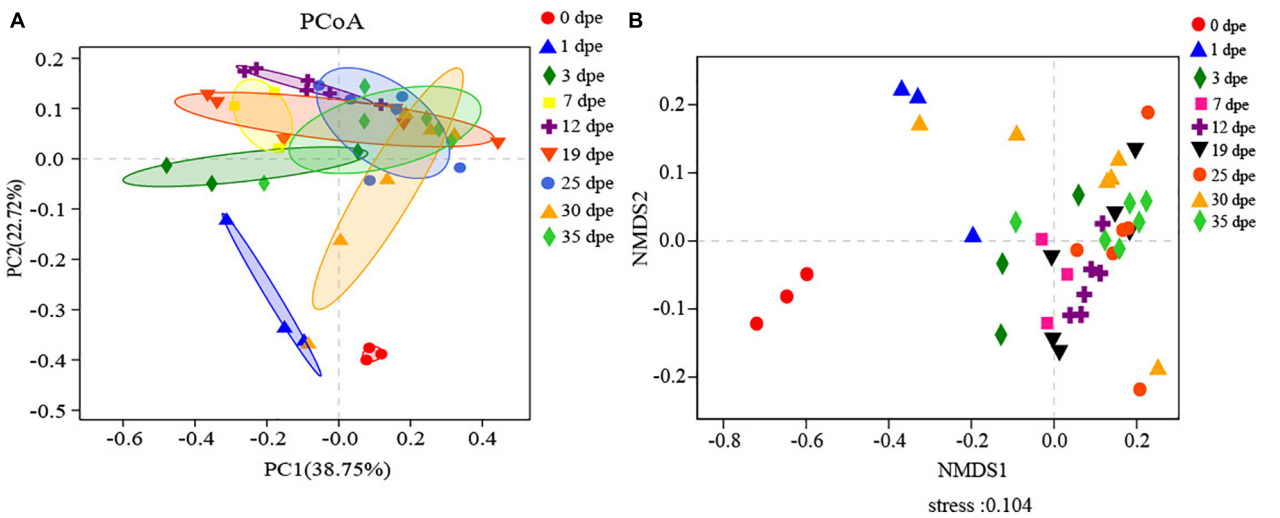

FIGURE 3 | (A,B) The horizontal and vertical coordinates represent the two selected principal coordinate components, and the percentage represents the contribution value of the principal coordinate component to the sample composition difference. The scales of the horizontal and vertical axes are relative distances and have no practical significance. Points of different colors or shapes represent samples of different groups. The closer the two sample points are, the more similar the species composition of the two samples will be.

products with huge economic value but also provide pollination services for crops. In addition, these bees exhibit similar life cycles and behaviors, including division of labor. Previous studies have shown that the gut of *Apis mellifera* consists mainly of nine types of bacteria, *Lactobacillus Firm-4*, *Lactobacillus Firm-5*, *Snodgrassella alvi*, *Gilliamella apicola*, *Bifidobacterium*, *Frischella perrara*, *Bartonella apis*, *Parasaccharibacter apium* and Alpha 2.1 (Jeyaprakash et al., 2003; Babendreier et al., 2007; Bottacini et al., 2012; Kwong and Moran, 2013; Philipp et al., 2013). In addition, many published studies have shown that gut microbes in *A. mellifera* undergo dynamic changes at different developmental stages, and there were significant correlations between microbes and host development, aging and social behavior (Dong et al., 2020). However, studies on the gut microbes of *A. cerana* at different stages of host development are rare. This fact is not conducive to the recognition and protection of this important bee species. Therefore, this study used high-throughput sequencing technology to explore the gut microbes of *A. cerana* at different stages of development.

The gut microbiota plays an important role in the health and disease of the host (Raymann and Moran, 2018), and the study of the gut microbiota is of great significance for

the protection of bees, important resources, and can further reveal the dynamic succession of the insect gut microbiota. The results showed that the diversity of the gut microbiota changed significantly throughout the life cycle of bees. Interestingly, the diversity of the gut microbiota of *A. cerana* was highest 1 dpe. Previous studies reported that the gut microbiota of honey bees was acquired mainly through social contact (Powell et al., 2014). Stephens et al. (2016) studied the gut microbiota of zebrafish at different developmental stages and found that environmental exposure in early development had the greatest impact on the gut microbiota (Stephens et al., 2016). After pupation of *A. cerana* 0 dpe, the bees were quickly in contact with the hive environment and the older bees, which may be an important reason for the significant increase in gut microbiota diversity 1 dpe. Therefore, environmental exposure has a huge impact on the diversity of the gut microbiota during the development of the host. This phenomenon is true for insects and fish. However, further research is needed to determine whether this theory applies to other animal groups. In addition, the same is true for infants who have a low gut microbiota diversity, which becomes more abundant as the infants grow (Azad et al., 2015). It can be inferred from these results that the transformation of gut microbiota diversity of *A. cerana* is similar to that of humans; it may be a common rule that gut microbiota diversity changes with host development, but further experimental exploration is needed.

In this study, it was found that 0 dpe samples lacked the core microbiota in the gut, which was consistent with previous reports (Yun et al., 2018). The results of this study further support the reliability of previous results. The gut microbiota of *A. cerana* 0 dpe is dominated by Proteobacteria, which are among the major gut microbiota constituents of other insects (Yun et al., 2014). These results indicate that the dominant role of Proteobacteria in the insect gut microbiota may be a distinctive feature of insect gut microbiota composition. Notably, this hypothesis needs to be confirmed by analysis of the gut microbiota of more insect groups. In addition, the composition of the gut microbiota of *A. cerana* and *Anoplophora glabripennis* at the phylum level is similar, with both being dominated by Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria (Schloss et al., 2006). This relatedness indicates that the gut microbial compositions of *A. cerana* and *A. glabripennis* are highly similar, and we preliminarily speculate that this structure may be the unique composition of the gut microbiota of insects.

The relative abundance of the dominant bacteria of the *A. cerana* gut microbiota at the genus level varied significantly at different developmental stages. At 0 dpe, the main components were *Acinetobacter* and *Sphingomonas*. *Acinetobacter* is an important part of the gut microbiota of many insects and has been found in the gut microbiota of *Pardosa laura*, *Pardosa astrigera*, *Nurscia albofasciata*, *Omphisa fuscidentalis* and other insects (Liu et al., 2016; Hu et al., 2019). *Acinetobacter* assists the host in digesting food and converting nitrogen (Briones-Roblero et al., 2016) and is an important insect gut probiotic. However, after 1 dpe, *Acinetobacter* was quickly replaced by other dominant bacteria, which may be due to the gut microbiota adapting to a more complex environment, as it does 0 dpe, *A. cerana* had not yet come into contact with the environment. *Apibacter* is highly abundant in the gut of *A. cerana* and bumblebees; however, its abundance in the gut of *A. mellifera* is low (Smagghe et al., 2016; Kwong et al., 2018), and *Apibacter* metabolizes mainly monosaccharides and dicarboxylic acids (Kwong et al., 2018). *Apibacter* abundance increased significantly by 7 dpe, indicating that the host began to ingest large amounts of monosaccharides or was on the verge of metabolizing and absorbing monosaccharides. *Lactobacillus*, *Gilliamella*, *Snodgrassella* and *Bifidobacterium* compose the core microbiota of bees and occupy an important niche (Babendreier et al., 2007; Bottacini et al., 2012; Kwong and Moran, 2013). The relative abundances of *Lactobacillus*, *Gilliamella*, *Snodgrassella* and *Bifidobacterium* in the gut were low 0 dpe, and they then rapidly colonized the gut 1-7 dpe. Previous studies have shown that *Lactobacillus* and *Bifidobacteria* can promote the absorption of nutrients and activate the host immune system (Alberoni et al., 2018). Therefore, an increase in the relative abundance of both microbes may indicate rapid development of workers and changes in diet. In addition, *Bifidobacterium* can stimulate the production of hormones by the host, which can affect the development of bees and accelerate the development of workers (Kešnerová et al., 2017). Therefore, the rapid increase in *Bifidobacterium* abundance in the gut of workers may coincide with the peak of *A. cerana* development. *Gilliamella* and *Bifidobacterium* are involved in the degradation of complex polysaccharides (Zheng et al., 2016; Kwong et al., 2018); however, pollen, bee bread and honey contain a variety of complex polysaccharides, and colonization by these two microbes promotes the catabolism of these compounds, indirectly promoting the development of workers. Interestingly, while workers, such as nurse bees, mainly feed larvae and old bees (Seeley, 1982; Crailsheim, 1991, 1992), the feeding material depends mainly on the metabolism of pollen and polysaccharides by these microbes. Therefore, these microbes in the gut of workers may also contribute to changes in the host's social behavior.

An interesting phenomenon in this study is that the succession of the gut microbiota of *A. cerana* occurs via the constant colonization of core microbiota at different developmental stages and the replacement of non-core microbiota 0 dpe. The colonization of the core microbiota is a dynamic process, but once established the composition of the microbiota is relatively stable.

Notably, although certain negative controls were needed to prevent erroneous results, we lacked these controls, and the absence of these controls may have affected the results of this study to some extent. A negative control was not included for the materials (tubes, swaps, etc.) used in this study; therefore, there is a potential risk of introducing a contaminating sequence. This study mainly focused on the changes in the main dominant microbiota in the gut of workers at different developmental stages. However, a negative control is certainly important for the study of gut microbiota, especially for improved data interpretation (Hornung et al., 2019). Therefore, in future gut microbial-related studies, multiple controls should be adopted to

reduce the influence of environmental factors on the conclusions. In addition, to ensure the reliability of the experimental results, it is necessary to add some positive controls such as (1) a positive control for DNA extraction, to ensure that the DNA of the contained organisms can be sufficiently extracted with the method used, and (2) a positive control for sequencing (a pre-extracted DNA mix), to ensure that the sequencing itself did not introduce any errors (Hornung et al., 2019).

In summary, exploration of the colonization characteristics of the gut microbiota in insects is an essential step for further understanding microbiota formation in the animal gut. Here, the composition and abundance of the gut microbiota were determined and quantified comprehensively in workers, and the colonization pattern of the gut microbiota and various genera was further revealed in the comparison across different time points within 35 days after worker pupation. In particular, the colonization characteristics of the gut microbiota of workers were compared with those of other species of animals (mainly vertebrates) based on the overall tendency of microbiota colonization and genera (or species), which revealed several common and characteristic colonization rules between insects and other animals. This study not only deepens our understanding of the colonization pattern of the gut microbiota in workers but also provides useful information for exploring colonization of the gut microbiota in insects and other animals more comprehensively.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRR9715685-SRR9715699 and SRR9715700-SRR9715726).

AUTHOR CONTRIBUTIONS

Z-XD wrote the article on data analysis. H-YL, Y-FC, and Q-HT took samples. JG designed the experiment. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Different Cultivation Environments Affect the Yield, Bacterial Community and Metabolites of *Cordyceps cicadae*

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Cordyceps cicadae is an entomogenous fungus with important uses in traditional Chinese medicine. However, its wild resources have not met consumers' demand due to excessive harvesting practices. Artificial cultivation is therefore an important alternative, but research on cultivating *C. cicadae* in natural habitats has not been reported. In this study, we aimed to explore the viability of cultivating *C. cicadae* in a natural habitat, in the soil of *Pinus massoniana* forest. We assessed and compared the yield, metabolite contents and bacterial community composition of *C. cicadae* grown in the *Antheraea pernyi* pupae at different growth stages, and under different cultivation conditions, in the soil of a natural habitat and in sterile glass bottles. Our results showed that cultivating *C. cicadae* in a natural habitat is feasible, with up to 95% of pupae producing *C. cicadae* fruiting bodies. The content of nitrogen compounds (amino acids) in *C. cicadae* cultivated in a natural habitat was significantly higher than in glass bottles, while the yield and carbon compound (mannitol and polysaccharide) and nucleoside (cordycepin and adenosine) contents were lower. Different bacterial genera were enriched in *C. cicadae* at different growth stages and cultivation environments, and these bacterial genera were closely related to metabolites contents during growth. This study demonstrated the viability of a novel cultivation method of *C. cicadae*, which could be used as an alternative to wild stocks of this fungus. These findings provided new insights into the growth mechanism of *C. cicadae* and its interaction with soil microorganisms.

Keywords: *Cordyceps cicadae*, cultivation in natural habitat, artificial cultivation, metabolite, bacterial community composition, PICRUSt

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INTRODUCTION

Cordyceps cicadae is an entomogenous fungus belonging to the family Claviciptaceae that has been used as a traditional Chinese medicine for over 1600 years (Liu, 2012). It has several host cicadas, such as *Cicada flammata* Distant, *Platypleura kaempferi* Fabricius, *Cryptotympana pustulata* Fabricius, *Platylomia pieli* Kato, and *Oncotympana maculatieollis* Motsch (Zeng et al., 2014, 2017),

and can form fruiting bodies on the surface of such cicada nymphs after they have been parasitized and killed by *Isaria cicadae*, the anamorphic form of this fungus (Cheng et al., 2006). *C. cicadae* mainly grows in the soil of bamboo, broad-leaved and evergreen broad-leaved forests, but also grows in mixed coniferous and broad-leaved forests. It is geographically widely distributed in provinces south of the Qinling-Huaihe River in China, including Guizhou, Yunnan, Sichuan, and Zhejiang, as well as in Japan, South Korea, Australia, Brazil, and other countries (Liu, 2012; Chen Z.A. et al., 2014; Zeng et al., 2015).

In China, *C. cicadae* is used to treat chronic kidney diseases, palpitations and children with seizures, and is also used as a food and tonic (Hsu et al., 2015; Li et al., 2019). Recent studies have found that *C. cicadae* contains metabolites with important pharmacological functions. Examples of these include the carbon compound mannitol, which is a diuretic, exhibits anti-free-radical activity, and improves cerebral microcirculation and blood flow (Lin et al., 2016; Zhang et al., 2019); polysaccharides, which are also essential carbon compounds, have antioxidant properties and play a role in treating diabetes and improving immune regulation (Lu et al., 2016; Zhang et al., 2018; Wang et al., 2019); adenosine, a nucleoside with anti-inflammatory, neuroprotective and anti-convulsive properties, which has been shown to improve cell viability and prevent and treat neurodegenerative diseases (Latini and Pedata, 2001; Nakav et al., 2008; Olatunji et al., 2016b); and cordycepin, another nucleoside with anti-cancer, immune regulation, and antioxidant functions (Olatunji et al., 2016a; Zhang et al., 2019). Owing to its use as a medicine and tonic, wild *C. cicadae* has been harvested at unsustainable levels, resulting in a significant population reduction in its natural habitats (Liu et al., 2008). The demand for *C. cicadae* cannot be met using these practices, and artificial cultivation is necessary to be an alternative to wild *C. cicadae* and ensure an adequate supply (Hsu et al., 2015; He W.W. et al., 2019; Sun et al., 2019; Nxumalo et al., 2020).

Artificial cultivation techniques for *C. cicadae* include liquid fermentation (Cheng et al., 2006, 2012) and cultivation on cereal medium (Feng, 2002; Zhang et al., 2012; Ke and Lee, 2016) or pupae (Hu et al., 2009; He Y.Q. et al., 2019). Hyphae can only be produced by liquid fermentation, and fruiting bodies only by cereal medium. Cultivation on pupae has become more popular in recent years, because *C. cicadae* with insects and fruiting bodies can be harvested by this way (He W.W. et al., 2019). However, the cultivation of *C. cicadae* on pupae has mainly been conducted in sterile environments, such as sterilized glass bottles, sand, or soil (Hu et al., 2009; Liu et al., 2018); little research has been conducted on cultivating *C. cicadae* in natural habitats, despite a preference among consumers for more "natural" production methods (Huang, 2008).

Wild *C. cicadae* grows in complex natural habitat that are rich in vegetation including *Camellia japonica*, *Camellia oleifera*, *Pyrus pashia,* and *Myrica nana* (Liu, 2012; Zeng et al., 2015). Researches have shown that the habitat soil, sclerotia, and external mycelial cortices of *C. cicadae* enrich abundant microbial communities and share some of the same bacterial genus, and some ectomycorrhizal fungi have also been detected in sclerotia (Zeng et al., 2019; Mou et al., 2021). These results suggested that wild *C. cicadae* populations are ecologically linked to the surrounding plants and soil through microorganisms, which may impact *C. cicadae* growth and production of metabolites. Previous research found that the composition of microbial communities in host varies between different growth environments (Mueller et al., 2020), and changes in microbial community composition in *Cordyceps* spp. can affect growth and metabolite production of host (Zhang et al., 2010; Qu et al., 2019a,b). Cultivating *C. cicadae* in natural habitats, rather than under sterile conditions, may therefore be a helpful experimental approach to exploring soil microbial interactions and their potential impact on growth and metabolite production in *C. cicadae*. In addition, we previously demonstrated that the relative abundance of fungi other than *Isaria* in the sclerotia of *C. cicadae* was less than 1%, while the bacterial community was more abundant and stable (Zeng et al., 2019; Mou et al., 2021). Given their relative abundance compared with fungi, exploring the composition of bacterial communities in the sclerotia of *C. cicadae* is a specific priority in this study.

This study therefore aimed to explore the feasibility of cultivating *C. cicadae* in natural habitats, the bacterial community composition of *C. cicadae* in different cultivation environments, and the relationship between bacterial communities in sclerotia of *C. cicadae* and metabolite production in this medicinally important fungal species. To achieve this, we compared the yield, bacterial community composition and metabolites content of *C. cicadae* cultivated in aseptic glass bottles versus in the soil of a natural *Pinus massoniana* forest habitat.

MATERIALS AND METHODS

Cultivation of *Cordyceps cicadae* in Two Cultivation Environments

The *Isaria cicadae* strain GZUIFR_DJS1 (preserved in Institute of Fungal Resources of Guizhou University) was activated and purified on potato dextrose agar (PDA, 200 g potato extract, 20 g glucose, 20 g agar, 1,000 mL distilled water) solid growth medium. Purified colonies were inoculated in the center of the PDA medium and cultured upside down at 25°C for 5–7 days, until they produced spores. Spore suspensions [concentration (C) = 5×10^7 spores/mL] were prepared with sterile ultrapure water containing 0.05% Tween 80 viscous liquid.

Pupae of *Antheraea pernyi* Guérin-Méneville, sourced from the Yuxi City Artificial Sericulture Base (Liaoning, China), were selected as host insects (cultivation medium) for *C. cicadae*. Pupae were soaked and scrubbed with 75% alcohol for 10 s for body surface sterilization; pupae were rinsed three times with sterile water to remove any residual alcohol, and then dried with sterile tissue. An inoculation of 0.02 mL spore suspension was made into each pupa at the intersection of the wing and the third somite (Zhang et al., 2016). Inoculated pupae were cultivated in sterile glass bottles at 25°C, each bottle contained a pupa and added a sterile moist cotton ball for maintaining humidity. After a week, the pupae were completely infected by *I. cicadae* and had become mummified. Each pupa was given one of two possible treatments for 2 weeks (**Figure 1**):

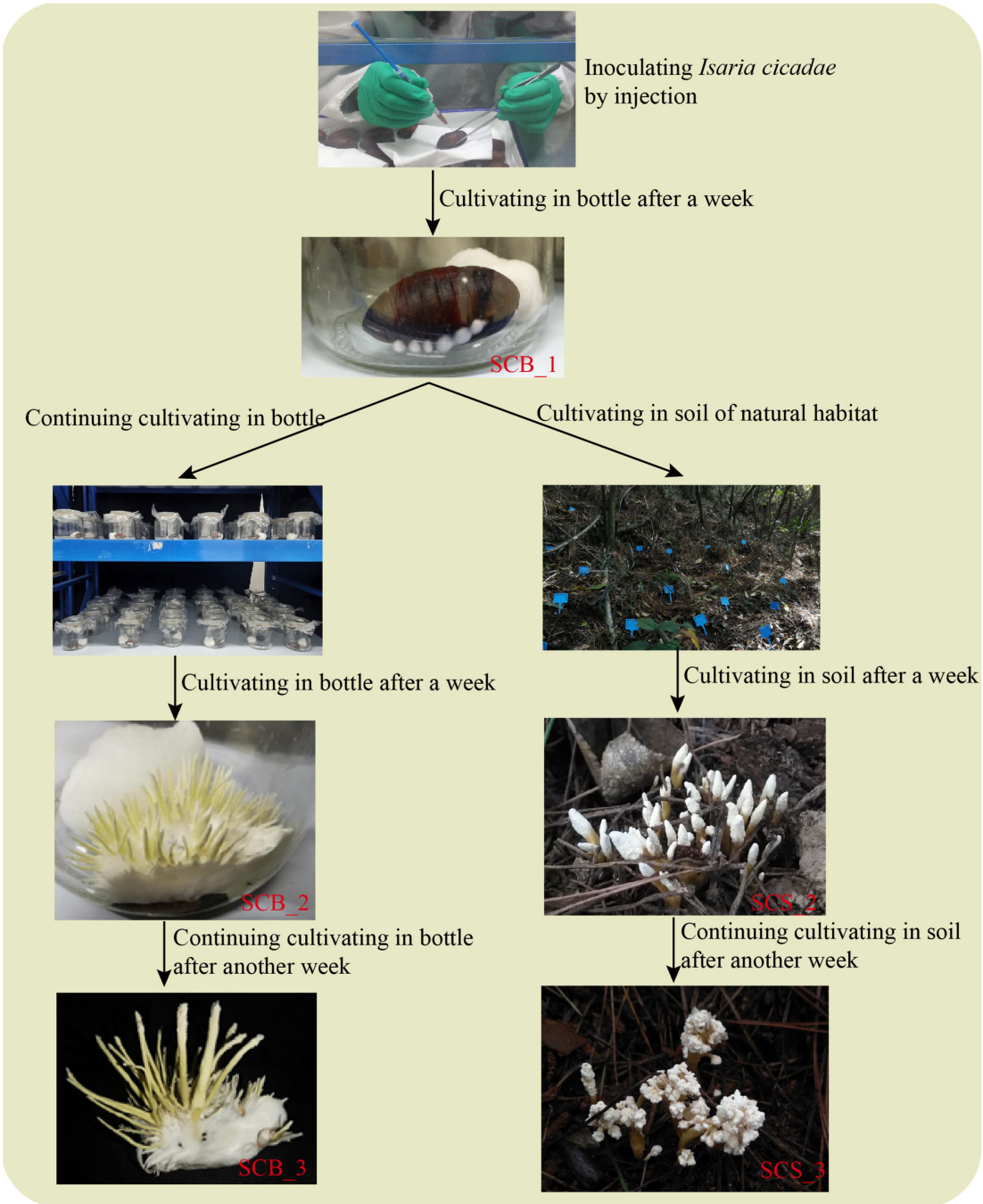

FIGURE 1 | A comparison of *Cordyceps cicadae* cultivation in sterile glass bottles and in the soil of natural habitats.

either their cultivation in glass bottles was continued (hereafter referred to as CB), or they were cultivated in the soil of natural habitat under a *Pinus massoniana* forest (CS). The experimental cultivation site for natural habitat used in this study was located in the West Campus of Guizhou University (Guizhou, China) and measured approximately 100 m^2; a detailed description of the habitat is in **Supplementary Table 1**. Mummified pupae were cultivated in natural habitat under a layer of covering soil approximately 1.5 cm thick, and an interval between any two pupae of 20 cm or more.

Experiment 1: Effects of Two Cultivation Environments on the Yield of *C. cicadae*

In April 2019, 150 pupae were inoculated and cultivated in sterile glass bottles for 1 week. After removing non-mummified

pupae, 120 mummified pupae were randomly divided into six groups of 20; three groups in CB and three in CS. Under both treatment conditions for 2 weeks, *C. cicadae* fruiting bodies did not grow anymore and began to produce spores, at this point, *C. cicadae* were considered mature; then we harvested all *C. cicadae* and washed any soil from CS pupae using tap water. Then, the proportion of pupae producing *C. cicadae* fruiting bodies (i.e., the number of pupae producing fruiting bodies divided by the total number) was calculated. The average proportion of pupae with fruiting bodies across all three groups was then calculated for CB and CS separately. After removing any non-mummified pupae and any *C. cicadae* damaged in the process of washing soil from CS pupae, the fresh weight, fruiting body number, length and diameter of 53 out of 55 *C. cicadae* in CB and 39 out of 57 in CS were measured. Finally, we compared the relative yield between in CB and CS by 100 cicadas i.e., 100 × the proportion of pupae producing *C. cicadae* fruiting bodies × mean fresh weight per *C. cicadae*).

Experiment 2: Effects of Two Cultivation Environments on the Bacterial Community and Metabolites of *C. cicadae*

In September 2019, 250 pupae were inoculated and cultivated in sterile glass bottles for 1 week. After removing non-mummified pupae, 200 mummified pupae were randomly divided into two groups and cultivated for 2 weeks under CB and CS treatment conditions, respectively. Since the pupae were inoculated with spore suspension, the sclerotia of nine randomly selected pupae were collected for bacterial community analysis each week, with three pupae combined in each of three replicates. Only pupae in the CB treatment condition were taken in week 1; in subsequent weeks, pupae in both CB and CS treatment conditions were taken. Samples were coded according to the week they were taken, e.g., samples from the second week were SCB_2 and SCS_2. Any soil on the CS pupae was washed off with tap water before the external mycelial layer and fruiting body were stripped off, leaving only the mummified pupae. These pupae were soaked in 75% alcohol for 2 min, then soaked in 2.5% sodium hypochlorite solution for 20 s, washed with sterile water five times, and dried with sterile tissue (Xia et al., 2015). Each pupa was crosscut in half on a clean laboratory bench, and the *C. cicadae* sclerotia were picked out with an inoculation needle and placed in sterile centrifuge tubes. Each sample was flash-frozen with liquid nitrogen, ground, and stored at −80°C until the DNA could be extracted. In the third week, the remaining *C. cicadae* in the two treatments were harvested, washed to remove soil, and then dried by dry oven at 80°C. Fruiting bodies were collected, ground and sifted through a 100 mesh screen, and used to detect the contents of five metabolites in *C. cicadae*.

Detection of Metabolites

In this study, we measured the content of adenosine, cordycepin, polysaccharides, and mannitol in *C. cicadae* and the content and type of amino acids in *C. cicadae* fruiting bodies. Amino acids were determined using the Hitachi L-8800 high speed amino acid analyzer, according to the national standard protocol *GB 5009.124-2016*. Mannitol content was determined by colorimetry according to a previously established method (San et al., 2010): 50% ethanol was used to extract mannitol using ultrasonic method, then sodium periodate solution, L-rhamnose solution and Nash reagent were used to perform the coloration reaction; absorption values were then detected at 412 nm using an ultraviolet spectrophotometer. Polysaccharide content was determined using a sulfuric acid–phenol method according to the national agricultural standard *NY/T 1676-2008*. Polysaccharides were precipitated in ethanol, and the resulting furfural derivatives of the polysaccharides were dehydrated in concentrated sulfuric acid and condensed with phenol, forming orange-red compounds with a color intensity proportional to the concentration of polysaccharides in the solution. Colorimetric quantification was carried out at 490 nm by an ultraviolet spectrophotometer. Cordycepin and adenosine were determined using high performance liquid chromatography according to national agricultural standard *NY/T 2116-2012*. The chromatographic column used in this study was the Shim-pack VP-ODS 5 micron 250 mm × 4.6 mm. The mobile phase was water and acetonitrile with 95:5 volume (v/v) ratio at a mobile speed of 1.0 mL/min. The temperature of chromatographic column was maintained at 35°C. The contents of cordycepin and adenosine were measured using a UV detector at 260 nm. We conducted three replicates for the measurement of each metabolite.

Bacterial DNA Extraction, PCR Amplification, and Sequencing

Bacterial DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek), and the concentration and purity of DNA were assessed by electrophoresis on 1.0% weight/volume agarose gels. The primers used to amplify the V4 variable region of the bacterial 16s RNA gene were: 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Zhou et al., 2020). DNA amplification was carried out using the ABI GeneAmp 9700 polymerase chain reaction (PCR) instrument. The PCR parameters were as follows: initial denaturation for 3 min at 95°C; denaturation, annealing and extension at 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C for 30 cycles; then final extension for 10 min at 72°C, then 10°C until the reaction stopped. The amplification products were sent to Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) for sequencing on the Illumina MiSeq sequencing platform.

Bioinformatics and Data Analysis

Fast Length Adjustment of SHort reads (FLASH) and Quantitative Insights Into Microbial Ecology (QIIME) software programs were used to merge and quality-filter raw reads from the original DNA fragments (Caporaso et al., 2010; Magoc and Salzberg, 2011). Effective reads were obtained by using a UCHIME algorithm to identify and remove chimeric sequences. Quality sequences were then clustered into operational taxonomic units (OTUs) with a >97% similarity cutoff value using the UPARSE algorithm (Edgar et al., 2011).

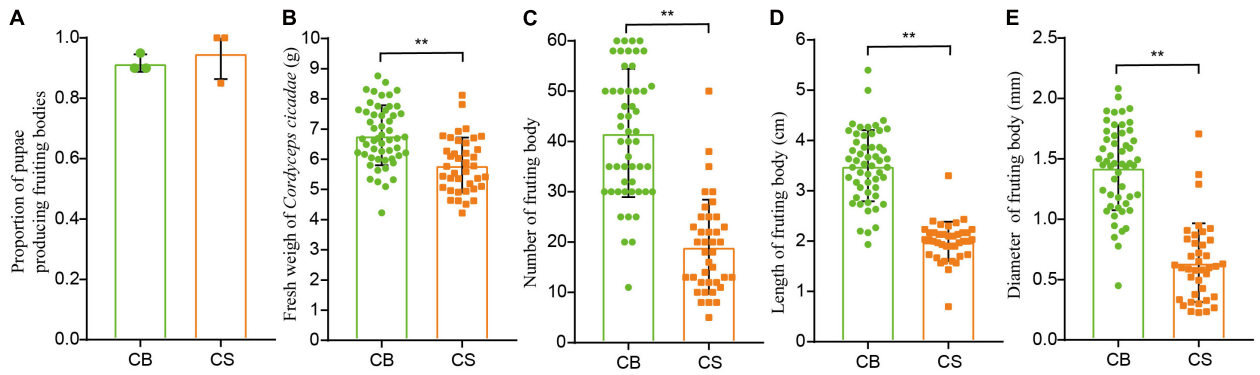

FIGURE 2 | The effects of two cultivation environments on yield measurements in *Cordyceps cicadae*. **(A)** proportion of pupae producing fruiting bodies; **(B)** fresh weight of *Cordyceps cicadae*; **(C)** number of fruiting bodies; **(D)** length of fruiting bodies; **(E)** diameter of fruiting bodies. CB, cultivation in glass bottles; CS, cultivation in the soil of a natural habitat. $^{**}p < 0.01$, according to non-parametric Mann–Whitney *U*-tests, error bars depict the standard deviation.

The Basic Logical Alignment Search Tool (BLAST) was then used to compare representative sequences for each OTU with sequences in the SILVA database to obtain the taxon of each OTU (Quast et al., 2013).

α-diversity of bacterial communities in each sample was represented by Shannon, Chao1 and Coverage indices, calculated after reads were normalized to the minimum reads (30, 665 reads) in each sample. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[1] software was used to predict the functional profile of bacterial communities using 16S ribosomal ribonucleic acid (rRNA) marker gene sequences (Langille et al., 2013). We then calculated the abundance of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and functions at the OTU level in each sample.

The Kolmogorov–Smirnov tests were used to verify the normality of data. Student's *t*-tests and non-parametric Mann–Whitney *U*-tests were used to identify differences in yield and abundance of KEGG pathways and functions between CB and CS treatment conditions that the data were normal or not, respectively. One-way analysis of variance (ANOVA) and *post hoc* comparisons were performed using Duncan's Multiple Range test to compare α-diversity among the different groups. The Spearman's rank correlation coefficient between contents of metabolites mentioned above and the top 30 most abundant bacterial genera was calculated and then visualized by the "pheatmap" package in the R software program. Visualizations of the yield index, the α-diversity index and the bacterial community composition were produced using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, United States).

RESULTS

Lower Yield of *C. cicadae* Under Natural Habitat Compared With Sterile Bottle

The proportion of pupae producing fruiting bodies was similar between CS and CB cultivation environments (92 ± 14% in CS and 95 ± 9% in CB, **Figure 2A**), indicating that *C. cicadae* can be cultivated in the soil of a natural forest habitat as in glass bottle. However, the fresh weight, number, length and diameter of fruiting bodies in CS were all significantly lower than those in CB ($p < 0.01$, **Figures 2B–E**), and the relative yield in CS (534.52g/100 cicada) was also lower than that in CB (645.05g/100 cicada, **Supplementary Table 2**), indicating that the overall growth of *C. cicadae* in CB was greater than in CS.

Contents of Metabolites in *C. cicadae* Differed Between Two Cultivation Environments

In total, 17 amino acids were detected in *C. cicadae* cultivated in both environments (**Figure 3A**). The total amino acid content of *C. cicadae* in CS (28.87 ± 0.057%) was significantly higher than in CB (26.01 ± 0.029%, $p < 0.01$, **Figure 3B**). The contents of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, cysteine, valine, methionine, isoleucine, phenylalanine, histidine, lysine, arginine, and leucine of *C. cicadae* were significantly higher in CS than in CB ($p < 0.01$). Conversely, the proline content in CS (2.51 ± 0.005%) was significantly lower than that in CB (3.04 ± 0.015%, $p < 0.01$). Cordycepin and polysaccharide contents of *C. cicadae* were slightly lower in CS than in CB. Adenosine and mannitol contents were significantly lower in CS than CB ($p < 0.01$, **Figure 3B**). These results indicate that cultivating *C. cicadae* in natural habitats increases the production of nitrogen compounds (amino acids), but decreases that of carbon compounds (polysaccharides and mannitol), and nucleosides (cordycepin and adenosine).

Bacterial Communities in Sclerotia of *C. cicadae* Were Higher Richness When Cultivated in Natural Habitat Compared With Sterile Bottle

A total of 2,931,602 raw sequences were detected. After filtering, 1,465,801 effective reads were obtained, with 30,665–92,371 in each sample. The coverage index of samples (>0.99 for all

[1]http://picrust.github.io/picrust/

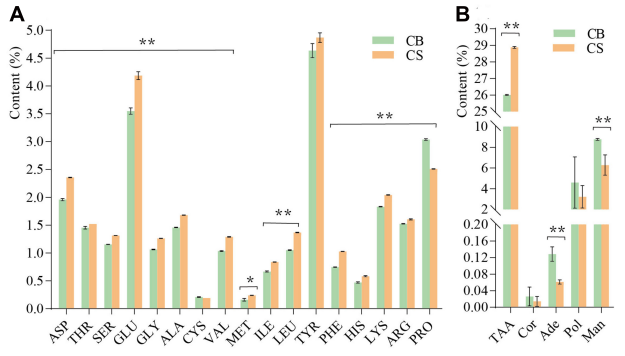

FIGURE 3 | The effects of two cultivation environments on metabolites produced by *Cordyceps cicadae*. **(A)** content of 17 amino acids; **(B)** content of five metabolites. ASP, aspartic acid; THR, threonine; SER, serine; GLU, glutamic acid; GLY, glycine; ALA, alanine; CYS, cysteine; VAL, valine; MET, methionine; ILE, isoleucine; LEU, leucine; TYR, tyrosine; PHE, phenylalanine; HIS, histidine; LYS, lysine; ARG, arginine; PRO, proline; TAA, total amino acid; Cor, cordycepin; Ade, adenosine; Pol, polysaccharide; Man, mannitol. Error bars depict the standard deviation (*$p < 0.05$, **$p < 0.01$, according to Student's *t*-tests).

samples, **Supplementary Table 3**) and the rarefaction curves (**Supplementary Figure 1**) indicated that the sampling depth of sequence data set in the current study was adequate for our analyses as the Shannon index flattens out after approximately 30,000 reads in each sample.

There was no significant difference in the Shannon diversity index of bacterial communities in the sclerotia of *C. cicadae* in each growth stage between the two environments, indicating that the bacterial community diversity was stable (**Figure 4A**). However, the Chao1 richness index in CS was higher than in CB; at week 2, the Chao1 richness index was significantly higher in SCS_2 than in SCB_2 ($p < 0.05$, **Figure 4B**). This result indicates bacterial richness is higher in *C. cicadae* cultivated in natural habitats than sterile bottle.

Bacterial Community Composition in Sclerotia of *C. cicadae* Varied Between Cultivation Environments and Growth Stages

In total, 11 phyla with a relative abundance greater than 0.1% were detected (**Figure 5A**). Proteobacteria was the dominant phylum (31.69 ± 22.74%–95.66 ± 6.09%) across all growth stages in both environments. The Firmicutes represented the dominant phylum in SCB_1, and its abundance at this time point in week 1 (67.80 ± 22.2%) was significantly higher than in week 2 or 3 under both cultivation environments (2.08 ± 1.95%–7.93 ± 1.27%, $p < 0.01$).

At the genus level (**Figure 5B**), the bacterial community composition varied between CB and CS and also across growth stages. In *C. cicadae* cultivated in sterile conditions: *Enterococcus* (37.05 ± 31.08%) and *Serratia* (26.08 ± 22.96%) were the dominant genera in SCB_1; *Achromobacter* (36.91 ± 15.24%) and *Cedecea* (33.18 ± 40.63%) were dominant in SCB_2; and *Achromobacter* (50.24 ± 46.77%) and *Serratia* (32.34 ± 56.01%) were dominant in SCB_3. In *C. cicadae* cultivated in natural habitats, *Pseudomonas* (61.80 ± 18.32%) and *Achromobacter* (80.75 ± 15.18%) were the dominant genera in SCS_2 and SCS_3, respectively. The relative abundance of *Achromobacter* gradually increased from 0.02 ± 0.012% to 80.75 ± 15.18% with the growth of *C. cicadae*, which showed significantly different relative abundance among groups ($p < 0.05$). The relative abundance of *Pseudomonas*, *Enterococcus*, *Ralstonia*, *Rhodococcus,* and *Amaricoccus* were also significantly different among groups. *Pseudomonas*, *Ralstonia*, *Rhodococcus,* and *Amaricoccus* had a significantly higher relative abundance in SCS_2 than at other stages in both environments (*Rhodococcus*, $p < 0.05$; other $p < 0.01$), while *Enterococcus* had significantly higher relative abundance in SCB_1 than at other stages in both environments ($p < 0.05$). *Cedecea* only had higher relative abundance (33.18 ± 23.46%) in SCB_2, but a low relative abundance (0.001 ± 0.00095%–1.09 ± 1.55%) at other stages in both environments (**Figure 5B**). These data show that the bacterial community composition and dominant genera in the sclerotia of *C. cicadae* vary significantly between cultivation environments and growth stages.

Predictive Functional Profiling of Bacterial Communities

The functions of bacterial communities were predicted by PICRUSt to determine the changes in relative abundance of bacterial OTUs related to functions in samples from different cultivation environments and time points (**Figure 6**). Bacteria OTUs related to metabolism had the highest relative abundance (62.80–66.33%) at the KEGG pathway Level 1, followed by environmental information processing (11.10–14.06%), genetic information processing (6.04–12.14%), human diseases (4.59–5.29%), cellular processes (2.99–4.04%), and organismal systems (1.71–2.36%).

At the KEGG pathway Level 2, bacteria OTUs with function related to carbohydrate metabolism (11.98–18.53%), global and overview maps (10.10–11.81%), Amino acid metabolism (7.47–12.58%) in metabolism of Level 1 and membrane transport (6.55–10.11%) in environmental information processing of Level 1 showed higher relative abundance than other functions. The relative abundance of bacteria OTUs related to glycan biosynthesis and metabolism and carbohydrate metabolism were significantly lower in CS (1.26 ± 0.06% and 12.42 ± 0.31%, respectively) than in CB (1.57 ± 0.29% and 14.28 ± 2.04%, respectively; both $p < 0.05$). Conversely, in CS compared with CB, the relative abundance of bacteria OTUs related to amino acid metabolism (12.08 ± 0.39% versus 10.44 ± 1.59%), xenobiotic biodegradation and metabolism (4.12 ± 0.50% versus 3.04 ± 1.02%), metabolism of terpenoids and polyketides (1.67 ± 0.03% versus 1.46 ± 0.15%), and biosynthesis of other secondary metabolites (1.60 ± 0.04% versus 1.42 ± 0.13%) were all significantly higher (all $p < 0.05$). These results suggested the relative abundance of bacteria OTUs with functions related to metabolism were significantly different between two cultivation environments, and further indicated the potential

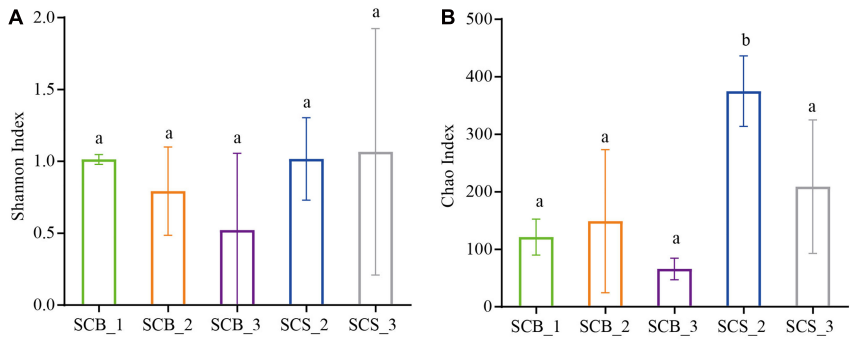

FIGURE 4 | Diversity index of bacterial communities in sclerotia of *Cordyceps cicadae* under the two cultivation environments (SCB, sclerotia of *Cordyceps cicadae* cultivating in glass bottles; SCS, sclerotia of *Cordyceps cicadae* cultivating in the soil of a natural habitat) at 1, 2, and 3 weeks (denoted by _1, _2, and _3, respectively) after inoculation. **(A)** Shannon index of diversity; **(B)** Chao1 index of richness. Different lowercase letters indicate significant difference ($p < 0.05$). Error bars depict the standard deviation.

correlation between the changes of metabolite content and bacterial community composition in *C. cicadae* cultivated in different cultivation environments.

The Relationship Between Bacterial Community and Metabolite

To further understand the relationship between bacterial communities and metabolites contents in cultivated *C. cicadae*, we calculated the Spearman's rank correlation coefficient between the top 30 most abundant genera and the contents of 22 metabolites, including 17 amino acids (**Figure 7**). There was a significant correlation between most bacterial genera and metabolite contents, especially those of amino acids.

Total amino acids, tyrosine, arginine, lysine, phenylalanine, valine, and leucine were all significantly positively correlated with *Rhodococcus*, *Faecalibacterium*, *Enterobacter*, *Bacteroides*, *Candidatus Rhabdochlamydia*, *Brevundimonas*, *Amaricoccus*, *Ralstonia,* and *Bifidobacterium*, but negatively correlated with *Cosenzaea* (all $p < 0.05$ at least). *Pseudomonas*, *Burkholderia-Caballeronia-Paraburkholderia,* and *Acinetobacter* were all significantly correlated with total amino acids, arginine, lysine, phenylalanine, valine and leucine (all $p < 0.05$ at least). Proline were significantly positively correlated with *Serratia*, *Bacillus,* and *Enterococcus*, but negatively correlated with *Achromobacter* and *Faecalibacterium* (all $p < 0.05$ at least). There was a significant positive correlation between polysaccharide and *Cosenzaea* ($p < 0.01$). Cordycepin was significantly negatively correlated with numerous genera, including *Rhodococcus*, *Faecalibacterium*, *Enterobacter*, *Bacteroides*, *Candidatus_Rhabdochlamydia*, *Brevundimonas*, *Amaricoccus*, *Ralstonia*, *Acinetobacter*, *Bifidobacterium,* and *Lactobacillus*, but significantly positively correlated with *Lactococcus* (all $p < 0.05$ at least). Adenosine were significantly positively correlated with *Cosenzaea* ($p < 0.01$), but negatively correlated with *Rhodococcus* and *Faecalibacterium* (both $p < 0.05$). Mannitol were significantly positively correlated with *Cosenzaea* and *Enterococcus*, but significantly negatively correlated with *Rhodococcus*, *Faecalibacterium*, *Enterobacter,* and *Brevundimonas* (all $p < 0.05$ at least).

DISCUSSION

This study confirmed that it is feasible to cultivate *C. cicadae* in the soil of a natural forest habitat, but the yield was lower than that of *C. cicadae* cultivated in sterile glass bottles. Our research also showed that different growth environments appear to affect the growth, bacterial community composition and metabolite content of *C. cicadae*, and bacterial community composition was correlated with metabolites content of *C. cicadae*.

Cultivating *C. cicadae* in natural habitats could help to meet consumer demand for natural products and reduce the exploitation of wild *C. cicadae* (Huang, 2008). However, in our study, the yield of *C. cicadae* cultivated in natural habitats was markedly reduced. Some research found that changes in growth environments can result in some resources being allocated to build a defense system to facilitate growth (Feng, 2008; Simon et al., 2010); it is therefore possible that, in the soil of their natural habitats, *C. cicadae* may also use resources to build a defense system to reduce the adverse effects of natural competitors and microorganisms on their growth. This would leave fewer resources allocated to growth of *C. cicadae*, which could have resulted in the reduced yield we observed in our study.

All organisms lived in complex microbial ecosystems and formed a close relationship with diverse microorganisms; changes in the growth environment can affect the microbial community associated with the host (Mueller et al., 2020). In this study, different growth environments were shown to affect bacterial community composition and dominant bacterial genera in the sclerotia of *C. cicadae*. *Pseudomonas* was significantly enriched in sclerotia of *C. cicadae* cultivated in the soil of a natural habitat. Researches showed that *Pseudomonas* was abundant in soil (Li et al., 2013), and Mou et al. (2021) found that *Pseudomonas* was the dominant genus in the sclerotia and external mycelial cortices of wild *C. cicadae*. We thought therefore the *Pseudomonas* in *C. cicadae* cultivated in a natural habitat may originated from the soil; if this is the case, this indicates that *C. cicadae* may communicate with microorganisms in the soil within its natural habitat. *Pseudomonas* spp., regarded as broad-host-range entomopathogenic bacteria, are known to

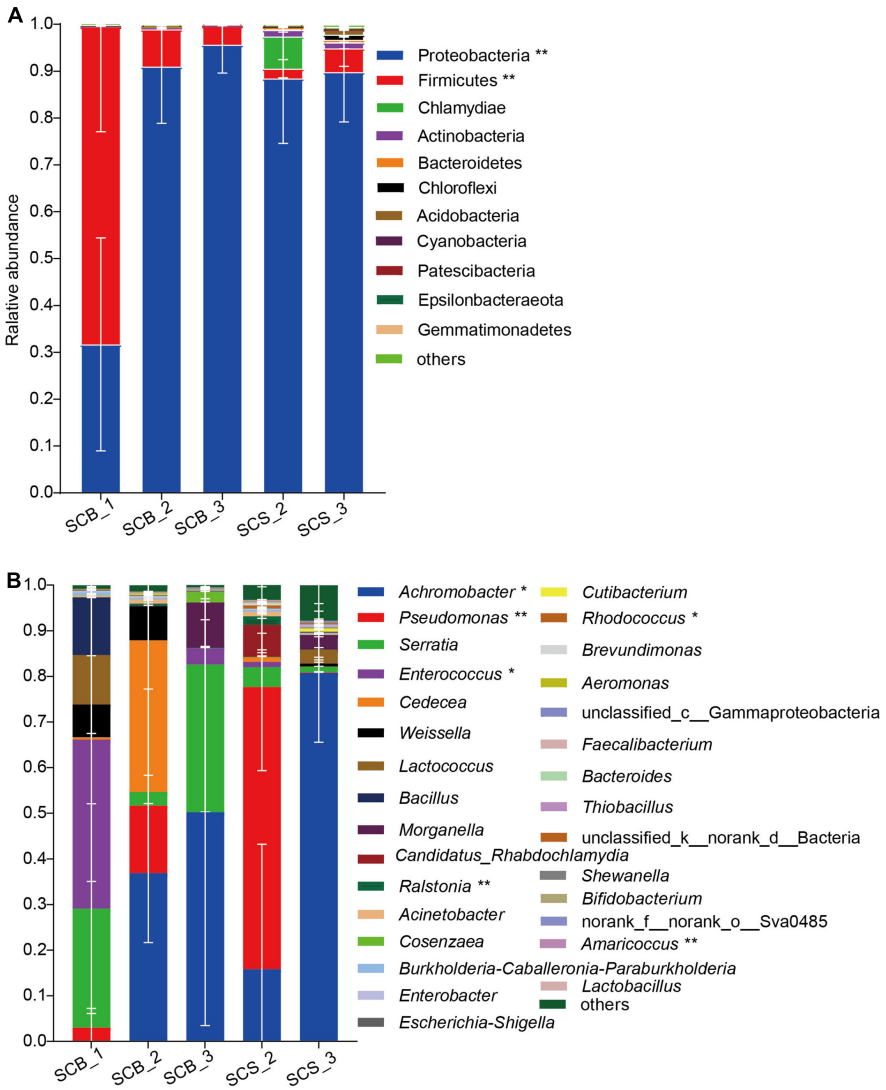

FIGURE 5 | Bacterial community composition in sclerotia of *Cordyceps cicadae* at different growth stage in both cultivation environments (explanations for abbreviations in *X* axis are shown in **Figure 4**). **(A)** Phyla with relative abundance greater than 0.1%; **(B)** Top 30 most abundant genera (* and ** denotes significance between all growth stages in both environments of $p < 0.05$ and $p < 0.01$, respectively, according to ANOVA, error bars depict the standard deviation).

exhibit insecticidal activity toward certain agricultural pests (Chen W.J. et al., 2014). This could potentially benefit the growth of *C. cicadae*, for example by promoting the mummification process, as previously observed in Chinese *Cordyceps* (Wu et al., 2020). In addition, in this study, the sclerotia of *C. cicadae* cultivated in glass bottles was rich in *Cedecea* bacteria, but this genus had a very low relative abundance in sclerotia of *C. cicadae* cultivated in natural habitats. Previous researches isolated and detected *Cedecea* in *C. cicadae* (Qu et al., 2019a,b); however, another study did not detect any *Cedecea* in sclerotia of *C. cicadae* (Mou et al., 2021), which indicated the abundance of *Cedecea* in *C. cicadae* is unstable and may be affected by the growth environment. Furthermore, in our study, bacterial community composition varied with the time point (and therefore growth stage) of *C. cicadae*, similar to previously published research (Wu et al., 2020). In particular, the relative abundance of *Enterococcus* gradually decreased with the growth of *C. cicadae*, suggesting that *I. cicadae* injected into the pupae may have inhibited some bacterial genera.

In this study, we found that the changes in relative abundance of bacteria OTUs related to some functions were consistent with changes in metabolite content: for example, the relative abundance of bacteria related to amino acid metabolism in *C. cicadae* in CS increased significantly than in CB, along with the content of total amino acids in this cultivation environment. The relative abundance of bacteria related to carbohydrate metabolism and glycan biosynthesis and metabolism in CS decreased significantly than in CB, along with the content of carbon compound (mannitol and polysaccharides) in this

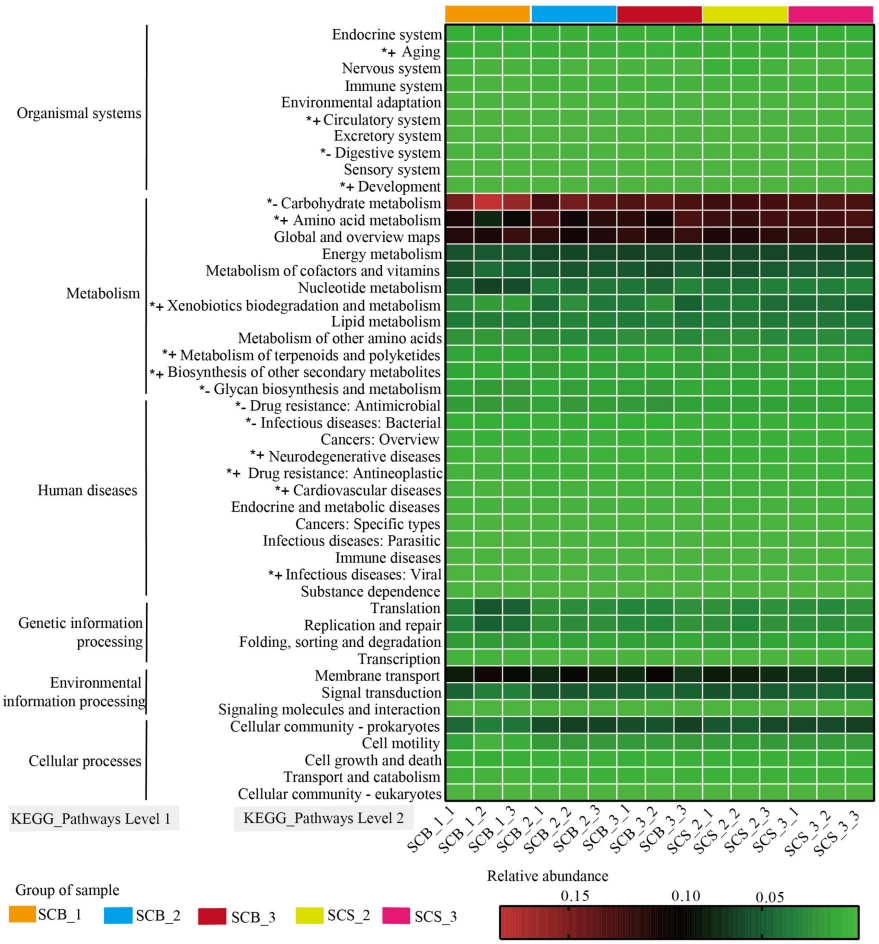

FIGURE 6 | Variation in KEGG metabolic pathways in functional bacterial communities of *Cordyceps cicadae* cultivated in different cultivation environments (SCB, sclerotia of *Cordyceps cicadae* cultivating in glass bottles; SCS, sclerotia of *Cordyceps cicadae* cultivating in the soil of a natural habitat) at 1, 2, and 3 weeks after inoculation (_1, _2, and _3, respectively). The final number in each sample label represents the replicate number of each sample (_1, _2, and _3, respectively). $^{*+}$ denotes the relative abundance of bacteria related to the function is significantly higher in CS than that in CB, $^{*-}$ represents the relative abundance of bacteria related to the function significantly lower in CS than that in CB ($p < 0.05$, according to Student's *t*-test).

cultivation environment. Researches showed culture conditions (Cheng et al., 2012; Lee et al., 2019), nutrient sources (Mao et al., 2005; Chunyu et al., 2019), different strains (Kang et al., 2017), and hosts (He Y. et al., 2019) can affect the level of metabolites, but these factors were consistent between two cultivation environment in this study. As a result, the main reason for different level of metabolite in two cultivation environment may be caused by the difference of microbial community composition and related functions (**Figures 5**, **6**). Studies on food fermentation also showed that metabolite production related closely to bacterial community composition in the progress of fermentation (He and Chung, 2020; Zang et al., 2020). Some studies also reported that endophytic microbes in *Cordyceps* spp. may affect the production of metabolites (Zhang et al., 2010; Qu et al., 2019a) and microbial co-culture can increase significantly the level of some metabolites (Akone et al., 2016; Yu et al., 2019). Therefore, further researches on determining which microbial species involve in the production of cordycepin, polysaccharide, adenosine, mannitol or amino acid and then using beneficially synthetic community or fungal-bacterial co-culture to improve the level of metabolites in *C. cicadae* are necessary. The amino acid content of edible mushrooms was known to be correlated with freshness and sweetness (Mau et al., 2001), which may explain why consumers prefer edible mushrooms grown in a natural habitat. In addition, the nitrogen compounds content of *C. cicadae* in natural soil habitats increased, while the carbon compounds decreased, which may suggest that *C. cicadae* may exchange carbon and nitrogen elements in the soil, similar function to *Metarhizium robertsii* (Behie et al., 2017; Barelli et al., 2019). We therefore speculated *C. cicadae* may exchange carbon and nitrogen elements in soil via endophytic bacteria to affect the content of carbon and nitrogen compounds in *C. cicadae*, which may potentially result in adding a branch to the carbon and nitrogen cycle in the ecosystem, entomogenous fungi *C. cicadae* is involved in carbon and nitrogen cycling in the soil.

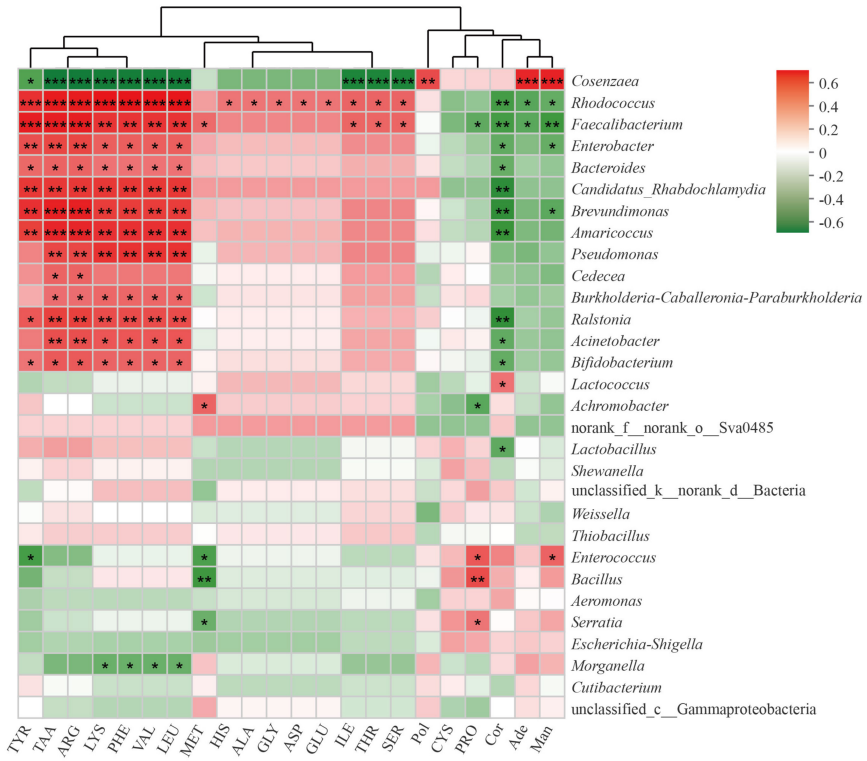

FIGURE 7 | Correlation of top 30 most abundant bacteria genera with metabolites in *Cordyceps cicadae*. The strength (Spearman's ρ value) of correlations are shown here as different shades of red (positive correlation) and green (negative correlation). Explanations for abbreviations in *X* axis are shown in **Figure 3**. *$0.01 < p \leq 0.05$; **$0.001 < p \leq 0.01$; ***$p \leq 0.001$.

Compared with the contents of metabolites in wild *C. cicadae* reported in other studies (Ge et al., 2007; Zhang et al., 2017), the total amino acids, polysaccharides and mannitol contents of *C. cicadae* in our study were higher regardless of how they were cultivated. *C. cicadae* cultivated in a natural habitat could achieve microbial communication in soil and be favored by consumer, it therefore be an alternative to the wild. Given the apparent close relationship between bacterial community composition and metabolites of *C. cicadae*, we will further isolate, test and screen microorganisms in future studies to identify which species may be beneficial to the growth and metabolite production of cultivated *C. cicadae*, so that the yield and metabolites contents could be improved by beneficial interaction between microorganisms.

CONCLUSION

Our study demonstrated for the first time to our knowledge that cultivating *C. cicadae* in natural habitat soil is feasible, but further studies are required to improve yield. This cultivation method appeared to increase nitrogenous compound (amino acid) and decrease carbonaceous compound (mannitol and polysaccharides) content, possibly through the interaction *of C. cicadae* with microorganisms in the soil. Metabolite production was found to be closely related to the bacterial community during *C. cicadae* growth; *C. cicadae* may therefore also participate in the carbon and nitrogen cycle in natural ecosystems.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA698306.

AUTHOR CONTRIBUTIONS

ZZ designed and performed the experiments, analyzed the data, and wrote the manuscript. DM designed and performed the experiments and analyzed the data. LL, WZ, and LD collected the samples and performed the experiments. XZ designed the experiments and wrote the manuscript. All authors have read and approved the submission of the manuscript.

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SUPPLEMENTARY MATERIAL

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Isolation and Identification of Dominant Bacteria From Black Soldier Fly Larvae (*Hermetia illucens*) Envisaging Practical Applications

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This study aimed to establish a representative strain collection of dominant aerobic bacteria from black soldier fly larvae (*Hermetia illucens*, BSFL). The larvae were fed either chicken feed or fiber-rich substrates to obtain a collection of BSFL-associated microorganisms. Via an approach based on only considering the highest serial dilutions of BSFL extract (to select for the most abundant strains), a total of 172 bacteria were isolated. Identification of these isolates revealed that all bacteria belonged to either the Proteobacteria (66.3%), the Firmicutes (30.2%), the Bacteroidetes (2.9%) or the Actinobacteria (0.6%). Twelve genera were collected, with the most abundantly present ones (i.e., minimally present in at least three rearing cycles) being *Enterococcus* (29.1%), *Escherichia* (22.1%), *Klebsiella* (19.8%), *Providencia* (11.6%), *Enterobacter* (7.6%), and *Morganella* (4.1%). Our collection of dominant bacteria reflects largely the bacterial profiles of BSFL already described in literature with respect to the most important phyla and genera in the gut, but some differences can be noticed depending on substrate, biotic and abiotic factors. Furthermore, this bacterial collection will be the starting point to improve *in vitro* digestion models for BSFL, to develop mock communities and to find symbionts that can be added during rearing cycles to enhance the larval performances, after functional characterization of the isolates, for instance with respect to enzymatic potential.

Keywords: black soldier fly, microbiome, fiber rich diets, isolates, culturable gut community

INTRODUCTION

The past decade has seen the rapid expansion of the industrial insect rearing sector in response to the global increasing demand in high-quality protein for human consumption (Van Huis, 2016). One of the economically most important and most promising farmed insect species is the black soldier fly (*Hermetia illucens*; Diptera: Stratiomyidae). The larvae of the black soldier fly (BSFL) have attracted the attention of many researchers during recent years as they are able

to convert low value biomass into valuable insects mass (Wang and Shelomi, 2017; Gold et al., 2018). BSFL may offer sustainable alternatives as raw materials for feed formulations and as source for biomaterials (Barragan-Fonseca et al., 2017; Almeida et al., 2020). Insects generally harbor diverse microorganisms in the gut which play pivotal roles in diverse aspects of insect physiology (Jang and Kikuchi, 2020). As a result, the gut microbial dynamics of these larvae are of specific interest to obtain insight in the insect's physiology, and further, to increase the yield of the rearing or conversion process. In recent years, the literature available on the composition of the BSFL gut microbiota, possibly in relation with its substrate and other rearing factors, increased substantially, with examples of relevant studies including those of Jeon et al. (2011); Zheng et al. (2013), Wynants et al. (2018); Jiang et al. (2019), Ao et al. (2020); Klammsteiner et al. (2020), Liu et al. (2020); Raimondi et al. (2020) and Shelomi et al. (2020). Based on the aforementioned studies, it can be concluded that a set of genera regularly occurs in the larval gut, such as *Enterococcus*, *Providencia*, *Morganella*, and *Dysgonomonas*, although relative abundances vary among and within studies. Whereas the studies mentioned have focused on the identification of members of the BSFL microbial community, information on the functions of these persistent microorganisms is still scarce.

Most of those studies on the BSFL microbiome involve culture-independent techniques (such as metataxonomics based on 16S rRNA gene sequencing) to obtain a complete view on the composition of the microbial community. However, culture-dependent methods, involving incubation, isolation and identification steps, are still necessary to obtain pure cultures needed for research purposes and for industrial applications (Prakash et al., 2013). A collection of BSFL-associated microorganisms could be useful to gain knowledge about their specific functions. In that regard, Callegari et al. (2020) obtained a bacterial collection of microorganisms from the larval gut via plating in selective liquid and solid media in order to seek for key bacteria. The authors studied the hydrolytic profiles of their isolates and selected and tested possible candidates for bacterial administration trials. In their approach, the authors focused on obtaining isolates with specific functions. However, they did not include special measures to focus on the most dominant bacteria present, which is an element that is added in our study in order to obtain a collection of dominant BSFL-associated bacteria. When considering future industrial applications, it can be important to focus on dominant strains. It is reasonable to assume that a dominant strain is more likely to colonize and function in an *in vitro* environment mimicking the BSFL gut, or the real BSFL gut, than a strain that is hardly encountered in the ecological niche of the BSFL gut.

This study aimed to establish a representative culture collection of dominant aerobic BSFL-associated microbes envisaging practical applications. Some studies suggest that the rearing substrate is an important factor shaping the gut microbiota in BSFL (Jeon et al., 2011; Bruno et al., 2019b). Therefore, we established the collection by rearing BSFL on several substrate compositions. Keeping in mind the role BSFL could play in the circular bioeconomy as a converter of organic waste streams, our focus with respect to substrate composition was on the fiber fraction. Soluble carbon sources, such as soluble starch, were not included in the study, since BSFL can produce amylase enzymes when fed substrates containing starch (Kim et al., 2011; Bonelli et al., 2019; Bruno et al., 2019a). Since bacteria that are more adapted to digest a specific compound can be expected to have a higher fitness on specific diets (De Smet et al., 2018; Galassi et al., 2021), we hypothesize that these bacteria will be more abundant in the community and concomitantly are more likely to be isolated. We selected five fiber types to enrich (each individually) substrates with. Cellulose, hemicellulose and pectin are important non-starch carbohydrates in plant material, lignin is a recalcitrant fiber in plant material as well, and in addition we included keratin as a protein fiber. These components are found in various waste streams that can serve as rearing substrate, such as (i) animal manure (Rehman et al., 2017; Masih-Das and Tao, 2018), rice straw (Zheng et al., 2012) and maize straw (Gao et al., 2019) for cellulose, hemicellulose and lignin, (ii) fruit mixtures (Meneguz et al., 2018) for pectin, and (iii) slaughterhouse waste containing discarded body parts (Gold et al., 2020a) for keratin. These substrates have a complex and variable composition, and to ensure the repeatability of our study, we preferred to use mixtures of chicken feed with the individual and (more) pure fibers for rearing the larvae, rather than rearing them on the complex and unstandardized waste streams. Isolation of microorganisms was only focused on the higher serial dilutions obtained after BSFL extraction, aiming to isolate only those strains that are abundantly present in the gut of larvae reared on the substrates used. Finally, the focus is laid on aerobic bacteria, since anaerobic bacteria would be less easy to cultivate for future industrial applications.

MATERIALS AND METHODS

Rearing and Sampling of Black Soldier Fly Larvae

The BSFL used in this study were obtained from a colony reared at laboratory scale (Thomas More University College, Geel, Belgium). **Figure 1** represents the rearing protocol used. Every cycle started at day 0 by placing 1 g of BSF eggs into a small tray placed on 200 g of nursing substrate, i.e., chicken starter feed (Startmeel voor Kuikens 259, AVEVE, Leuven, Belgium) and tap water [in an 1:1 ratio (w/v)]. The nursing substrate had a moisture content of approximately 50–60%. After 4 days, unhatched eggs were removed to ensure uniformity in larval age. At that time, the BSFL were transferred to another box (29 cm × 18 cm) to which 600 g nursing substrate [chicken starter feed and tap water in a 60:40 ratio (w/v)] was added. After 8 days, a first sample of larvae was taken and the larvae were divided over three separate containers (10 cm × 15 cm). Each box contained 100 g of new substrate (one of the fiber-rich types as described below) or chicken starter feed (=control) and approximately 500 BSFL (as determined by the average weight of three times 100 larvae), so that the density was 3.3 larvae/cm^2. From then on, 100 g substrate was administered to the larvae every 2 days until day 16. All containers were placed in a climate chamber (Pharma 600, Weiss

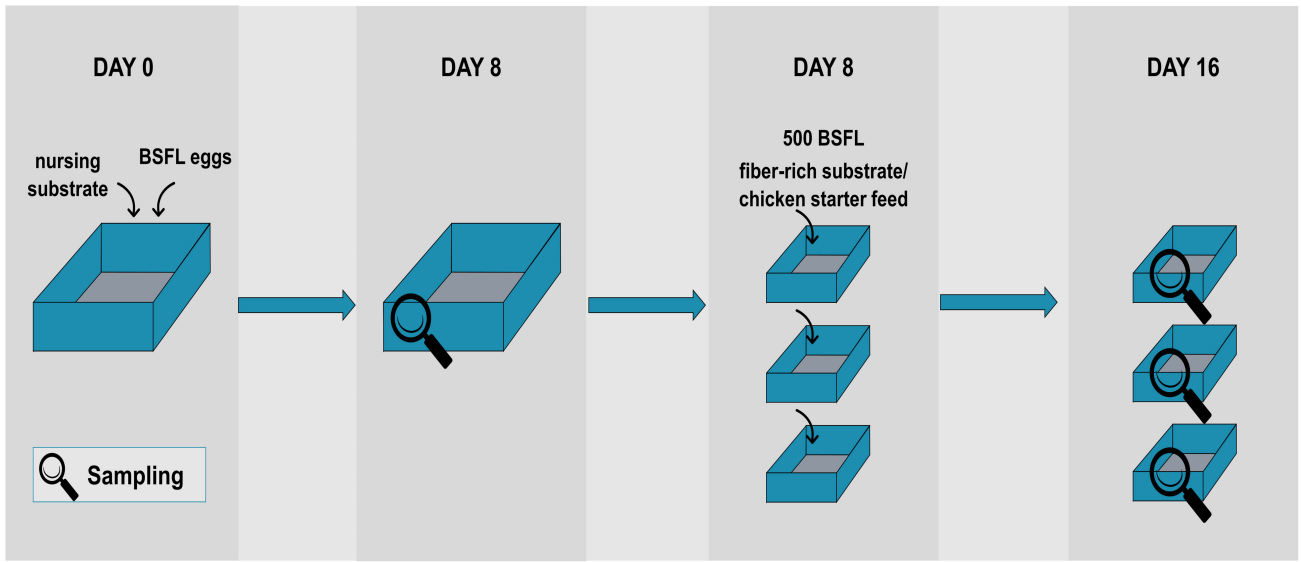

FIGURE 1 | Schematic overview of the rearing protocol used, including the sampling points.

Technik, Belgium) under controlled environmental conditions (T = 27°C, RH = 60%).

The diets given from day 8 onward were supplemented with fiber(-rich) ingredients, being either lignin (Alkaline, TCI Europe N.V., Belgium), cellulose (Alphacel, VWR International, Belgium), pectin (Pektin A, Carl Roth, Germany), feather meal rich in keratin (Pluvera, Belgium), or pre-cut straw (Stro Voorgesneden, Aveve, Belgium) as a source of hemicellulose. For each ingredient, one rearing cycle with a diet in a 70:30 ratio (w/w) of chicken feed and fiber(-rich) ingredient was conducted. In order to provide the larvae an even less digestible substrate toward a more pronounced digestion of the fiber-rich substrate, another rearing cycle was performed in a 30:70 ratio (w/w). Since the diets containing 70% pectin or pre-cut straw did not support larval growth, they were eliminated from the trial, and spelt hulls (ForFarmers, Belgium) were introduced as a source of hemicellulose (of which both ratios were executed as well). Chicken starter feed served as a control substrate and the rearing cycles using the control feed were carried out twice. The substrates were moisturized with tap water to achieve a moisture content of circa 60–70% in order to ensure efficient separation of larvae from the residue upon harvesting (Cheng et al., 2017).

Isolation of Microorganisms

For each cycle, a sample was taken at day 8 before distributing the larvae over separate containers, and another sample was taken from each of the three boxes at day 16 (i.e., in total four samples per cycle). Each sample contained about 30 g of BSFL. To eliminate the microbiota present on the exoskeleton and in this way to focus on microorganisms present in the interior of the larvae, all samples were given a rinsing disinfection treatment as described by Wynants et al. (2018). In summary, the BSFL were first washed under running tap water and then subjected to three washing steps of 1 min at 200 rpm on a laboratory shaking table (Unimax 1010, Heidolph, Germany), first once with 70% ethanol and then twice with sterile distilled water. Subsequently, the insects were aseptically blended with a home type mixer (Bosch CNHR 25) to obtain a homogenous mixture. Tenfold serial dilutions were prepared and surface-plated on Plate Count Agar (PCA, Biokar Diagnostics, Beauvais, France). Plates obtained from dilutions starting from 10^{-5} until 10^{-9} were considered, since only highly abundant microorganisms are present in those dilutions and rare or less frequent microorganisms are not. All plates were incubated aerobically at 30°C for 2–3 days. From those plates, five to ten morphologically different colonies were picked and streaked onto new PCA plates in order to obtain pure cultures. Those plates were incubated at 30°C for 24–48 h. Next, one individual colony was picked from each plate and inoculated in Luria Bertani (LB) liquid broth (10.0 g/l peptone (Biokar Diagnostics, France), 5.0 g/l yeast extract (VWR, Belgium), 5.0 g/l NaCl), and grown overnight at 30°C on a shaker (Orbital mini shaker, VWR, Belgium) at 150 rpm. Visibly turbid cultures were maintained as 25% glycerol stock at −21 and −80°C.

Identification of Isolates

By picking up colonies from plates in order to obtain isolates, it is possible that a single strain was collected more than once. To find out whether a strain occurred more than once in the collection, Random Amplification of Polymorphic DNA (RAPD) was performed to identify multiple isolates originating from the same strain. Then, unique strains were subjected to Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry for identification.

For the RAPD analysis, each isolate from the stock culture collection was grown overnight on PCA. From each plate, one individual colony was suspended in 20 µl milli-Q water. RAPD

was performed using suspended cells (i.e., no DNA extraction as described by Hilton et al., 1997) and using the universal primer GTG_5 (5′-GTG GTG GTG GTG GTG-3′). PCR was carried out in 30 μl reaction volumes containing 2 μl of PCR template, 0.7 μl primer (20 μM, IDT, Belgium), 0.1 μl DreamTaq polymerase (5 U/μl, Life Technologies, Belgium), 0.6 μl 10 nM deoxynucleotide triphosphates (dNTP's) (Thermo Fisher Scientific, Belgium), 3 μl 10 × DreamTaq Green buffer (Thermo Fisher Scientific, Belgium), and 23.6 μl milli-Q water. A negative control for which the DNA was replaced by sterile milli-Q water was included in each PCR run. After initial denaturation for 10 min at 95°C, each PCR reaction comprised 10 cycles of denaturation (45 s, 95°C), annealing (45 s, 36°C), and extension (2 min, 75°C), followed by 25 additional cycles where the annealing temperature was set at 50°C. Subsequently, post-cycle elongation was performed for 5 min at 72°C. The resulting PCR products were visualized on an 1.5% agarose gel. Identification of band fragments and comparison of the different patterns were performed using GelAnalyzer (Version 19.1 for Windows). Isolates with the same RAPD-profile were combined into the same group.

Of each group, at least one isolate was identified using MALDI-TOF mass spectrometry (Lavetan, Turnhout, Belgium). A Bacterial Test Standard (BTS), being *Escherichia coli*, and the strain *Salmonella enterica* serovar *Infantis* (LMG 18746) were included as positive control. The obtained spectrum was compared against a spectra database, i.e., MBT Compass Library (Bruker, United States of America) containing 7,854 reference spectra of 2,748 different microorganisms. The match of the obtained spectrum with spectra from this library was expressed as a log-score between 0.00 and 3.00. For accurate species identification, a log-score of minimum 2.00 is required (de Almeida Júnior et al., 2014). Isolates with a log-score below 2.00 were therefore identified via amplification and sequencing of the bacterial 16S ribosomal RNA region. Briefly, cells were dissolved in 20 μl ultra-pure water and the DNA was set free via boiling lysis (10 min at 95°C). The 16S rRNA gene was amplified using the primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-CTA CGG CTA CCT TGT TAC GA-3′) (Mapelli et al., 2013; Soldan et al., 2019). PCR fragments were partially sequenced at Eurofins Genomics using both primers. The obtained sequences were assembled using ContigExpress (Vector NTI, Invitrogen) and then aligned against the EzBioCloud database (Yoon et al., 2017).

RESULTS

Identification of the Isolates

Isolates were obtained from all fiber-rich substrates as well as the chicken feed. In total, a collection of 172 isolates was established. These isolates were ordered in 31 groups according to their RAPD-profile. **Figure 2** gives an overview of the families and genera of these organisms, which all appeared to be bacteria, and the substrate they were isolated from. All the isolates belonged to one of four phyla, being the Proteobacteria (66.3%), the Firmicutes (30.2%), the Bacteroidetes (2.9%) and the Actinobacteria (0.6%). The largest group, the Proteobacteria, encompassed the families of the Enterobacteriaceae (49.4%), the Morganellaceae (16.3%), and the Xanthomonadaceae (0.6%). The Firmicutes covered some Enterococcaceae (29.1%) and Planococcaceae (1.2%). Bacteroidetes were assigned to Sphingobacteriaceae (1.7%) and Flavobacteriaceae (1.2%). The Actinobacteria were only represented by one family, being the Micrococcaceae (0.6%).

These four phyla covered together twelve different genera. The Enterobacteriaceae were represented by the genera *Escherichia* (22.1%), *Klebsiella* (19.8%) and *Enterobacter* (7.6%). *Enterococcus* was the only genus found in the family of the Enterococcaceae, but it accounted for 29.1% of all isolates. *Providencia* (11.6%), *Morganella* (4.1%) and *Proteus* (0.6%) were the genera present from the family of the Morganellaceae. Those three families together were responsible for 94.8% of the collection. Further, isolates from the genus *Sphingobacterium* (1.7%), belonging to the Sphingobacteriaceae, *Chryseobacterium* (1.2%) from the Flavobacteriaceae, *Rummeliibacillus* (1.2%) from the Planococcaceae, *Glutamicibacter* (0.6%) from the Micrococcaceae and *Stenotrophomonas* (0.6%) from the Xanthomonadaceae were also picked up.

Distribution of the Isolates on the Different Substrates

Fiber-rich substrates containing either cellulose, hemicellulose, keratin, lignin or pectin in different ratios with chicken feed (70 and 30%), and chicken feed as such were administered to BSFL in different rearing cycles. During these cycles, bacteria were isolated on day 8 for chicken feed and on day 16 for chicken feed and the fiber-rich substrates. The majority of the isolates originated from the control group ($n = 57$ or 33.1%), the hemicellulose-rich diet ($n = 39$ or 22.7%), and the keratin-rich diet ($n = 37$ or 21.5%). A smaller fraction was collected from the cellulose-rich ($n = 18$ or 10.5%), the pectin-rich ($n = 15$ or 8.7%) and the lignin-rich substrate ($n = 6$ or 3.5%).

Figure 3 presents the relative abundance of the genera of the isolates found for each substrate. All genera of the three most abundant families found in this study, i.e., the Enterobacteriaceae, the Enterococcaceae, and the Morganellaceae, were discovered on chicken starter feed, with the exception of *Proteus*. Comparison of day 8 ($n = 31$) and day 16 ($n = 26$) shows that the obtained patterns showed some similarity, with *Klebsiella*, *Escherichia*, *Providencia* and *Enterococcus* being the genera occurring the most. On day 16, the genera *Morganella* and *Enterobacter* were isolated as well. For the 30% hemicellulose diet ($n = 20$), the only genera discovered were *Klebsiella* and *Enterococcus*, with the latter one accounting for 85% of all isolates collected from this substrate. Interestingly, no *Enterococcus* was isolated from larvae fed with substrate containing 70% hemicellulose ($n = 19$), yet *Klebsiella*, *Morganella*, *Providencia*, *Sphingobacterium* and *Proteus* were isolated. In the BSFL fed with 30% keratin *Klebsiella*, *Enterobacter*, *Escherichia*, and *Enterococcus* were present as abundant (and culturable) genera ($n = 20$). Bacteria isolated from larvae fed on the other substrate, being 70% keratin ($n = 17$), showed a high diversity. In addition to genera of the three most abundant families, *Rummeliibacillus* and

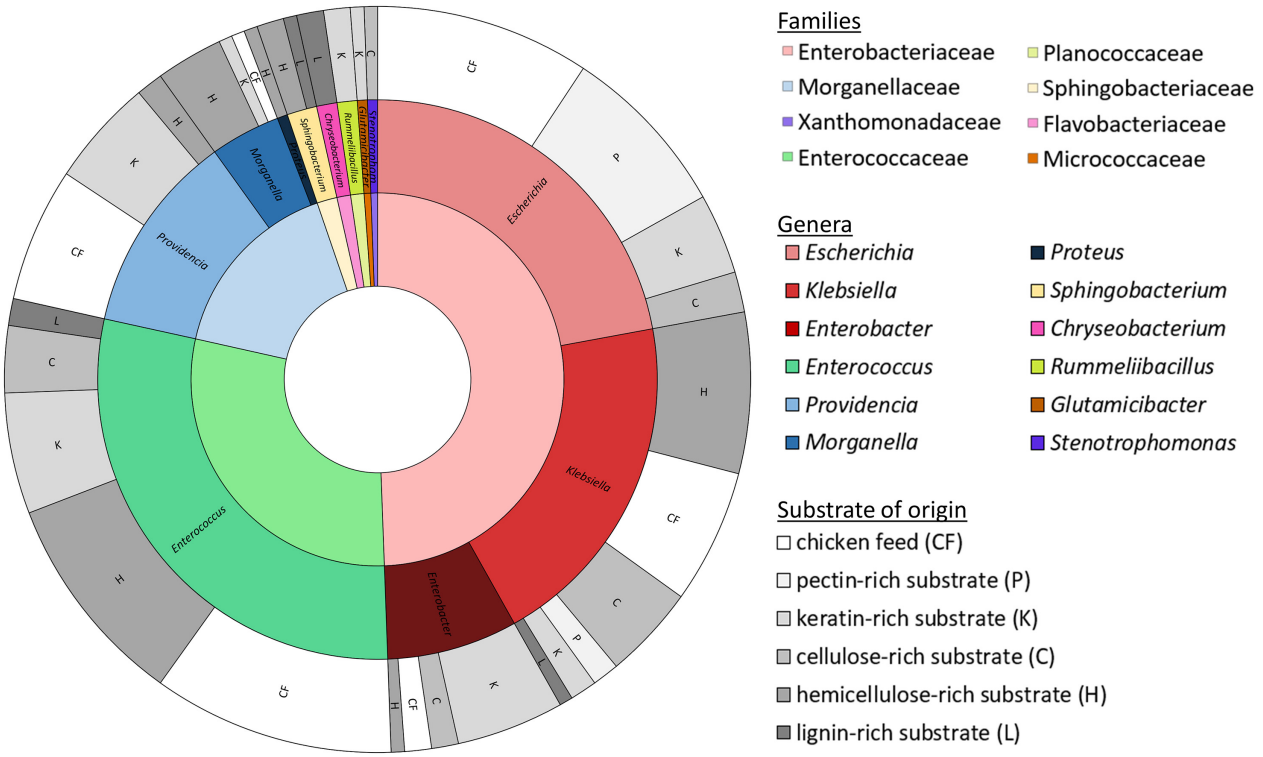

FIGURE 2 | Families (inner circle, cf. legend on the right side) and genera (middle circle, cf. legend on the right side) of the 172 identified isolates with the substrate of origin (outer circle; cf. legend on the right side).

Glutamicibacter were present in the larval interior as well. The relative abundances of the bacteria obtained from BSFL fed with 30% cellulose ($n = 13$) were quite similar to those of 30% keratin, whereas bacteria isolated from larvae fed on 70% cellulose only belonged to the genera *Klebsiella* and *Stenotrophomonas*. Nevertheless, it is important to note that only five isolates were recovered on 70% cellulose. Interestingly, no members of Morganellaceae were detected in both rearing cycles of cellulose-rich substrates. Rearing of BSFL on pectin-rich substrate has only been performed on 30% pectin ($n = 15$), as indicated earlier, and 87.0% of the isolates were from *Escherichia*. For the lignin-based diet containing 30% lignin, no isolates were obtained using the protocol described in this study. In another study in our group and connected to this work, using selective plates and not just considering the highest dilutions, isolates were obtained, but these data are still to be published. For the lignin-based diet containing 70% lignin, only six isolates were recovered, and they belonged to three genera, being *Enterococcus*, *Sphingobacterium* and *Chryseobacterium*.

From all genera found in this study, *Klebsiella* and *Enterococcus* were present in most of the rearing cycles (**Figure 3**), indicating a high general abundance of these genera in the gut of BSFL, i.e., an abundance which is independent from the diet. In contrast, *Rummeliibacillus*, *Glutamicibacter*, *Chryseobacterium*, *Proteus*, *Stenotrophomonas* and *Sphingobacterium* were found in only one or two rearing cycles. Identification on species level is given in **Supplementary Table 1**. For isolates identified via amplification of the 16S rRNA gene, the top-hit taxon is given.

DISCUSSION

Representativeness of the Collection

The microbiome of BSFL has been described in several recent studies. In order to enable the comparison of our results with those already available, an overview of the literature covering the BSFL microbiome composition is given in **Table 1**. An important distinction has to be made between research using culture-independent methods (such as metataxonomics based on 16S rRNA gene sequencing) and research, such as ours, where the culturable fraction is investigated. Culture-independent methods may yield a more comprehensive and complete view on the composition of the microbial community, since also Viable But Non-Culturable (VBNC) strains are included (Oliver, 1993). However, strains usable for additional research and industrial applications need to be culturable. Hence, an approach based on isolation and culturing strains still plays a crucial role (Prakash et al., 2013). Further, it can be noted that plates were not incubated anaerobically and that part of the microbiota, containing the strictly anaerobic organisms, is missed in this way. Strictly anaerobic bacteria were not considered in this study, due to the applications envisioned for the collection. Organisms that would have to be cultured in a strictly anaerobic way would be

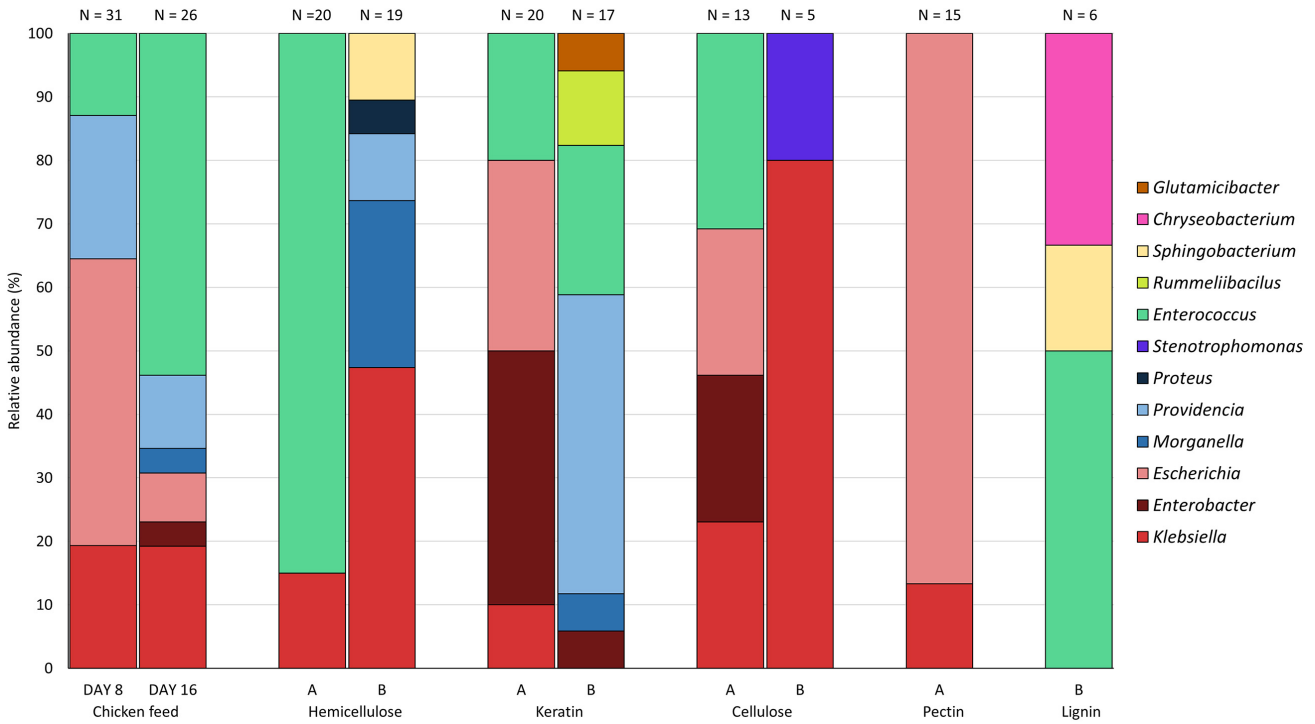

FIGURE 3 | Relative abundance of the genera isolated from black soldier fly larvae fed on chicken feed and various fiber-rich diets (A = 70% chicken feed and 30% fiber; B = 30% chicken feed and 70% fiber). Isolates from larvae fed on chicken feed were obtained at days 8 and 16, whereas isolates from fiber-rich substrate were only obtained at day 16. N is the number of isolates obtained from larvae fed on the specific fiber-rich substrate.

difficult to handle in large scale applications and would involve additional costs over (facultatively) aerobic ones.

Most authors of recent studies dissected the larval midgut in order to get more precise information on the intestinal microbiota, but due to the labor-intensive step of dissecting, this approach reduces the number of larval guts on which their results are based. For instance, Shelomi et al. (2020) only examined three larval guts per rearing cycle. Therefore, it was decided to use a more representative number of larvae in this study, to increase the relevance of the collection as a representation of the intestinal microbiota. For this reason, the larval midgut was not dissected, but larvae were rather disinfected to remove microorganisms at the external surfaces. The drawback of this approach, however, is that it cannot be claimed in our study that it focusses on the gut microbiome only. Indeed, it cannot be excluded that other organs or tissues in the BSFL interior also contain microorganisms, but this is largely unexplored.

It can be observed in **Table 1** that most studies use culture-independent methodologies. Even though only a minor fraction of a community unveiled by sequencing methods is culturable (Alain and Querellou, 2009; Prakash et al., 2013), the bacterial profile found within these culture-independent studies is to some extent comparable to that found via our approach. Overall, these studies reported that the most representative phyla are the Proteobacteria, the Firmicutes, the Bacteroidetes and to a lesser extent the Actinobacteria (Jeon et al., 2011; Zheng et al., 2013; Wynants et al., 2018; Bruno et al., 2019b; Jiang et al., 2019; Zhan et al., 2019; Ao et al., 2020; Klammsteiner et al., 2020; Liu et al., 2020, 2021; Raimondi et al., 2020; Shelomi et al., 2020; Galassi et al., 2021; Wu et al., 2021). In our collection, these four phyla are also represented: the Proteobacteria (66.3%), the Firmicutes (30.2%), the Bacteroidetes (2.9%) and the Actinobacteria (0.6%). Within these phyla, isolates from twelve genera were collected, with the dominant ones being *Enterococcus* (29.1%), *Escherichia* (22.1%), *Klebsiella* (19.8%), *Providencia* (11.6%), *Enterobacter* (7.6%), and *Morganella* (4.1%). Interestingly, these genera can also be found frequently and abundantly in culture-independent studies as well. More specifically, the genus *Enterococcus*, isolated the most in our study, has also been found in most studies shown in **Table 1**, indicating an occurrence independently of substrate and other factors. The same conclusions can be drawn for the genera *Providencia* and *Morganella*. Interestingly, *Enterococcus* sp., *Morganella* sp., and *Providencia* sp. were all found to be omnipresent in three industrial and four laboratory rearing cycles in the study of Wynants et al. (2018) and in three sets of larval groups fed on different diets in the study of Jeon et al. (2011). In addition, *Providencia* spp. have been detected throughout all life stages of BSF (Zheng et al., 2013), indicating that the genus is transmitted in a vertical way, i.e., from parents to offspring over different cycles. Even though not mentioned often in **Table 1**, the genera *Klebsiella* (Jeon et al., 2011; Ao et al., 2020; Callegari et al., 2020; Cifuentes et al., 2020), *Escherichia* (Jeon et al., 2011; Callegari et al., 2020),

and *Enterobacter* (Callegari et al., 2020; Cifuentes et al., 2020) were found in the interior of BSFL as well. Some genera, being *Sphingobacterium* (1.7%), *Rummeliibacillus* (1.2%), *Chryseobacterium* (1.2%), *Glutamicibacter* (0.6%), *Proteus* (0.6%), and *Stenotrophomonas* (0.6%), were only discovered once or twice in this study. To the author's knowledge, it is the first time that the genera *Chryseobacterium* and *Glutamicibacter* are linked to the interior of BSFL, whereas the other aforementioned isolates have been identified in BSFL previously. For example, the genera *Sphingobacterium* (Zheng et al., 2013; Wynants et al., 2018; Bruno et al., 2019b), *Rummeliibacillus* (Zheng et al., 2013), *Proteus* (Jeon et al., 2011; Zheng et al., 2013; Ao et al., 2020; Cifuentes et al., 2020; Khamis et al., 2020; Raimondi et al., 2020) and *Stenotrophomonas* (Zheng et al., 2013; Callegari et al., 2020; Cifuentes et al., 2020) have been recovered in larvae.

Until now, only one other in-depth study on the culturable section of the BSFL gut has been performed (Callegari et al., 2020). In that study, the larval guts were dissected, but their collection is based on only four larval guts whereas in this study, a much larger (and likely more representative) number of larvae were used. In addition, Callegari et al. plated on enriched and selective media in search of bacteria with specific functions. Although the authors investigated the culturable fraction of the larval gut in a specific and different manner, their bacterial collection showed some similarities to ours. In fact, all the dominant genera described above were isolated, just as *Proteus* and *Stenotrophomonas*, but they found other genera, such as *Acinetobacter*, *Pseudomonas*, and *Micrococcus*, associated with BSFL as well.

Although many resemblances are noted between bacterial profiles of BSFL described in literature and our isolates, there are still some notable differences. For instance, *Dysgonomonas* spp. were not recovered in our study, whereas the genus has been found frequently in other work (Zheng et al., 2013; Cai et al., 2018; Wynants et al., 2018; Jiang et al., 2019; Zhan et al., 2019; Ao et al., 2020; Cifuentes et al., 2020; Khamis et al., 2020; Klammsteiner et al., 2020; Shelomi et al., 2020), even as main members of the gut community (Zheng et al., 2013; Cai et al., 2018; Cifuentes et al., 2020; Klammsteiner et al., 2020; Shelomi et al., 2020). The same applies to the genera *Lactobacillus*, *Acinetobacter,* and *Bacillus* (Wynants et al., 2018; Jiang et al., 2019; Callegari et al., 2020; Cifuentes et al., 2020; Khamis et al., 2020; Raimondi et al., 2020). This may be attributed to differences in the culture-independent and -dependent approach, as pointed out earlier.

Existence of a Core Microbiome

A predominant core community has often been identified in different insect species, affecting the health and survival of the insect itself (Engel and Moran, 2013). As to BSFL, there is no general consensus on whether they contain a core microbiota and if so, which members belong to it. In our study, most of the larval groups fed on different diets share several genera, yet in other proportions. Genera that were found in at least three rearing cycles include *Enterococcus*, *Escherichia*, *Klebsiella*, *Providencia*, *Enterobacter*, and *Morganella*. As stated earlier, these genera are frequently found in the larval interior and might thus be regarded as core members of the gut community. Indeed, most studies described previously in **Table 1** have linked a recurring set of dominant genera to the gut of *H. illucens*, independently of rearing procedures. Most of these presumed core microbiomes include at least one of the genera *Enterococcus*, *Dysgonomonas*, *Providencia*, *Morganella*, *Klebsiella*, *Enterobacter*, and/or *Actinomyces*, yet with varying abundances (Jeon et al., 2011; Wynants et al., 2018; Ao et al., 2020; Cifuentes et al., 2020; Klammsteiner et al., 2020; Shelomi et al., 2020). These results are in agreement with our findings that those genera are possibly persistent regardless of the feed used. Interestingly, the composition of those presumed core microbiomes differs between studies in terms of number of genera and their abundancies. For instance, *Dysgonomonas*, *Porhyromonas*, and *Parabacteroides* have been assigned to the gut core microbiome (Shelomi et al., 2020), whereas in another study, none of these genera were considered to be a part of it (Ao et al., 2020). Due to these differences, it has been suggested that core bacterial microbiomes of *H. illucens* vary with location and feed (De Smet et al., 2018; Shelomi et al., 2020). Furthermore, the gut microbiota might be influenced by a so-called "house flora," i.e., species typically present in the rearing environment (crates, production site, equipment, . . .) (Wynants et al., 2018), but to the author's knowledge, no reports exist that document the house flora of BSFL production facilities.

In line with this discussion, the influences of the substrate, biotic [such as BSF strain (Khamis et al., 2020)] and abiotic factors (such as temperature) on shaping the gut microbiota are not clear yet. In this respect, so far substrate has been investigated the most as possible influencing factor. For instance, Bruno et al. (2019b) investigated the effect of different substrates on the microbiota of BSFL, including the different regions of the midgut. They concluded that the substrate played a major role in the composition of the microbiota. In another study, the least complex diet, i.e., cooked rice, resulted in the least diverse intestinal microflora, followed by calf forage and food waste (Jeon et al., 2011). On the other hand, no clear overlap between the community composition of the substrates and that of the BSFL fed on it was observed in other studies (Wynants et al., 2018; Cifuentes et al., 2020; Shelomi et al., 2020), suggesting that diet does not play a key role in shaping the BSFL's gut microbiome. In addition, the effect of consecutive rearing cycles and inter-batch variability should still be deciphered, as highlighted by Wynants et al. (2018). Further, environmental conditions, such as temperature (Raimondi et al., 2020), can also influence the microbiota composition. Additionally, very little is currently known about the impact of the different regions of the larval midgut on the microbiome of BSFL. A higher bacterial load, yet coinciding with a lower microbial diversity, was noticed in the posterior tract compared to the anterior region, suggesting that the bacterial community becomes less diverse along the digestive tract and consequently that the core microbiome is site-dependent (Bruno et al., 2019b). Unraveling these factors will add relevant knowledge about the bacterial microbiota of BSFL. Based on our results and those of others, there is an indication that the gut of BSFL shows common

TABLE 1 | Summary of literature data (from 2011 onward) involving an assessment of the composition of the microbiota of BSFL, either in a culture-dependent (D) or in a culture-independent (I) way.

		Main genera[a]																			
Study authors and year of publication	**D/I**	*Actinomyces*	*Bacillus*	*Bacteroides*	*Campylobacter*	*Clostridium*	*Corynebacterium*	*Dysgonomonas*	***Enterococcus***	*Ignatzschineria*	***Klebsiella***	*Lactobacillus*	*Miniimonas*	***Morganella***	*Parabacteroides*	***Proteus***	***Providencia***	*Pseudomonas*	***Sphingobacterium***	***Stenotrophomonas***	*Vagococcus*
Jeon et al. (2011)	I and D								X		X			X		X					
Zheng et al. (2013)	I			X				X									X		X	X	
Cai et al. (2018)[b]	I	X							X	X	X					X	X				
Wynants et al. (2018)	I								X					X			X	X			
Bruno et al. (2019b)[c]	I					X	X	X	X					X		X	X	X	X		X
Jiang et al. (2019)[d]	I		X					X	X			X									
Ao et al. (2020)	I	X				X			X	X	X			X		X	X				X
Callegari et al. (2020)	D								X		X			X			X	X		X	
Cifuentes et al. (2020)	I	X	X			X		X	X		X			X			X			X	
Khamis et al. (2020)	I					X		X	X	X		X		X			X			X	
Klammsteiner et al. (2020)	I	X						X	X					X							
Raimondi et al. (2020)	I		X								X						X				
Shelomi et al. (2020)	I and D							X							X						
Wu et al. (2020)[b]	I				X										X						
Galassi et al. (2021)	I				X		X	X	X		X			X			X		X		
Liu et al. (2021)	I							X					X	X							
Wu et al. (2021)[b]	I			X	X			X					X								

Studies were found via Pubmed using the following search terms: Hermetia illucens, black soldier fly larvae, microbiota, bacterial community, bacterial diversity and gut. Studies are presented in the order of online publication. Genera that are considered as main genera in at least two studies are given. Genera that are isolated in our study are in bold.

[a] *Main genera are genera that were described as "core/common members" of the larval gut, and/or were omnipresent (supplementary information not included) in all rearing cycles or life stages, and/or were found with a relative abundance of minimum 6%.*

[b] *Only genera of the initial group (no addition of tetracycline or heavy metals) were considered.*

[c] *Based on supplementary data.*

[d] *Based on a figure in the paper.*

phyla and genera as the main constituents in the gut (considered over its complete length), but some variation on this core can occur depending on feed, location, and possible other factors (such as BSF strain).

Possible Applications of the Culturable Gut Bacteria

The collection of isolates obtained offers an interesting basis for research and industrial applications. Applying the potential of these isolates in different fields might lead to promising innovations. For our established collection of 172 bacteria, we consider three developmental prospects.

The first development is situated at research level. A current challenge in the emerging insect sector is the fact that process performance can vary considerably when administering different biowaste streams to BSFL (Gold et al., 2018). Gold et al. (2020b) attempted to asses in a direct and cost-effective way the possibility of various biowastes to be processed by BSFL by predicting the biowaste conversion performance using *in vitro* digestion models. In these models, three digestion phases are distinguished and in each phase the diet is diluted with a digestion fluid and incubated for a specific time. The pH of the digestion fluid is adjusted and various enzymes are added during the digestion steps (Brodkorb et al., 2019; Gold et al., 2020b). Comparison of their *in vitro* and *in vivo* results showed that the model was able to broadly distinguish between the best and the worst performing diets, but several major discrepancies were observed as well. Adding microorganisms in the digestion step to simulate the midgut microbiota may offer a next step in optimalization of *in vitro* models, covering a broader range of biological processes taking place in the gut than in the current model. For instance, microbially produced enzymes may partially digest certain components present in the feed (Gold et al., 2020b). Since our collection was established from the interior—and presumably mainly the gut—of BSFL and our isolates indeed reflect the microbial communities of the BSFL gut described in literature, they can be used as inoculum for *in vitro* digestion models for BSFL and thus to increase the reliability of the model in a standardized and repeatable way, creating a strong tool for future research.

Further, next generation sequencing (NGS) has been intensively used in recent studies to increase our understanding of BSFL microbial communities. During sequencing, in addition to a negative control, a positive control can be used, consisting of a known collection of microbial strains, or a known quantity of DNA from multiple microorganisms in known abundances. Such positive control is called a "mock community". Adding a mock community to a sequencing run can validate whether the expected abundances are recovered, and thus it can improve the quality of the sequencing results (Nguyen et al., 2015). A diverse mock community is preferred, meaning that it consists of a wide range of taxonomic groups (Flynn et al., 2015; Nguyen et al., 2015). Recently, a mock community comprised of representatives of 374 insect species has been assembled (Braukmann et al., 2019). Since our collection consists of the main phyla and genera associated with *H. illucens*, a proper mock community for culture-independent studies on BSFL can now be designed, as is subject of our ongoing research.

Finally, microorganisms could be administered during industrial rearing cycles as symbionts in order to boost the larval performances on difficult to digest substrates. Indeed, some rearing substrates, such as dairy manure (Rehman et al., 2017) and rice straw (Zheng et al., 2012; Manurung et al., 2016), are hard to valorize by BSFL due to their high cellulose, hemicellulose and/or lignin content. If insect rearing facilities could successfully feed these difficult to digest streams to their larvae, production costs of the larvae could be lower (due to a lower feed cost) and at the same time insect production could take even a better position in a circular economy. In the development of this application, it is necessary to investigate the properties with respect to the enzyme production of the isolates. This presents another part of our ongoing research on the collection of BSFL isolates obtained. Several examples can be found in literature of successful application of bacteria in BSFL rearing, where enzyme production is considered to be (at least one) of the mechanisms by which the added cultures support growth of and/or conversion by BSFL. In this way, Jiang et al. (2019) demonstrated the importance of certain gut bacteria in BSFL during vermicomposting: *Enterococcus* and *Providencia* (also isolated in our study) were abundantly present in the process. The authors suggested that their influence might be related to carbohydrate-active enzymes they produce, and to the hydrogen, nitrogen and sulfur metabolisms they possess. In another study, the presence of *Providencia* has been strongly correlated with the total nitrogen content of manure fed to BSFL, but also with the crude fat content. Hence, *Providencia* was suggested playing a role in protein and lipid conversion in the gut (Ao et al., 2020) and this hypothesis was strengthened by the study of Callegari et al. (2020). Here, most bacteria that were isolated from the BSFL gut and that were able to degrade uric acid, belonged to the genus *Providencia*. In addition, pectinolytic activity has been related to *Klebsiella* spp. and *Stenotrophomonas* spp. strains associated with BSFL (Callegari et al., 2020). *Klebsiella* spp. were also found in larvae fed the pectin-rich diet in our study, but not exclusively in larvae fed on this type of diet. That may indicate that *Klebsiella* spp. can fulfill other functional roles in the gut of BSFL as well. It has already been proven repeatedly that the addition of bacteria during rearing of BSFL can increase larval performance (Yu et al., 2011; Rehman et al., 2019; Somroo et al., 2019; Callegari et al., 2020). The addition of *Bacillus licheniformis* HI169 has resulted in positive enhancement of larval growth (Callegari et al., 2020). Indeed, that strain has different hydrolytic activities, such as degradation of cellulose, starch and uric acid. Since our larvae were challenged with difficult-to-digest substrates, it can be hypothesized that bacteria with functional properties had a competitive advantage compared to other microorganisms (De Smet et al., 2018) and might thus lead to promising results regarding larval performance. Less frequently occurring isolates from our collection were mainly found in BSFL fed with the highest ratio of fiber-rich ingredient to chicken feed, indicating that these bacteria are more adapted to digest this type of fiber. It would thus be interesting to focus in future studies on these less frequently occurring isolates.

CONCLUSION

A representative collection of dominant aerobic bacteria isolated from the interior of BSFL was obtained in this present study. Even though only a small fraction of a community unveiled by sequencing methods is culturable (Alain and Querellou, 2009; Prakash et al., 2013), this collection of 172 isolates was mainly constituted of members that have already been described to be frequently found in the interior of BSFL by a number of studies mainly using culture-independent strategies. This indicates that core members of the larval gut microbiota were isolated in this study and that the methodology of using only high dilutions during the isolation process indeed leads to the isolation of the dominant microorganisms. In total, twelve genera were collected with the main ones being *Enterococcus* (29.1%), *Escherichia* (22.1%), *Klebsiella* (19.8%), *Providencia* (11.6%), *Enterobacter* (7.6%), and *Morganella* (4.1%). Taking into account our work and that of others, the microbiota of BSFL seems to consist of common members, but their presence and abundance may vary depending on diet, biotic and abiotic factors. Still, the influence of factors such as the stages in rearing and the location in the gut needs more research.

The microorganisms isolated in this study are an interesting tool for various future research applications. First, *in vitro* digestion models for BSFL can be improved by the addition of isolates from our collection during the digestion step. Secondly, a mock community specifically designed for sequencing the microbiota of BSFL can be developed using our bacterial collection. Lastly, the collection potentially contains isolates that can be added as probiotic symbiont during rearing to enhance larval performance such as growth and/or biowaste conversion.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

EG, LV, JD, and LV contributed to the conception and the design of the study. LF performed most work on rearing the insects, being assisted by EG. EG and LV performed the work on isolating bacterial strains. EG and JD performed the identification of the collection. EG wrote the first draft of the manuscript. LV wrote certain sections of the manuscript and did the proofreading of the first version. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.665546/full#supplementary-material

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Experimental Warming Reduces Survival, Cold Tolerance, and Gut Prokaryotic Diversity of the Eastern Subterranean Termite, *Reticulitermes flavipes* (Kollar)

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Understanding the effects of environmental disturbances on insects is crucial in predicting the impact of climate change on their distribution, abundance, and ecology. As microbial symbionts are known to play an integral role in a diversity of functions within the insect host, research examining how organisms adapt to environmental fluctuations should include their associated microbiota. In this study, subterranean termites [*Reticulitermes flavipes* (Kollar)] were exposed to three different temperature treatments characterized as low (15°C), medium (27°C), and high (35°C). Results suggested that pre-exposure to cold allowed termites to stay active longer in decreasing temperatures but caused termites to freeze at higher temperatures. High temperature exposure had the most deleterious effects on termites with a significant reduction in termite survival as well as reduced ability to withstand cold stress. The microbial community of high temperature exposed termites also showed a reduction in bacterial richness and decreased relative abundance of Spirochaetes, Elusimicrobia, and methanogenic Euryarchaeota. Our results indicate a potential link between gut bacterial symbionts and termite's physiological response to environmental changes and highlight the need to consider microbial symbionts in studies relating to insect thermosensitivity.

Keywords: thermal stress, insect microbial symbiosis, temperature tolerance, insect microbiome, climate change

INTRODUCTION

Temperature is a major factor influencing the distribution of many, if not all, living organisms (Doucet et al., 2009). Ecological studies characterizing species distribution and dispersal patterns rely heavily on external climactic variables which can be used to prepare risk assessments for invasive species or to identify geographic range limits for beneficial organisms. Current predictions of global climate change suggest increases in the frequency and magnitude of temperature fluctuations (Deser et al., 2012) which will directly impact seasonality, distribution, survival, and overall fitness of both beneficial and pestiferous insect species (Wernegreen, 2012;

Sinclair et al., 2015; Alford et al., 2018). Climate change models also predict increases in freeze-thaw cycles corresponding to reduced snow cover, which would have a direct impact on the microenvironment of many soil-dwelling insects (Sinclair, 2001). Thus, studies examining temperature tolerance and the mechanisms that contribute to this tolerance are becoming increasingly important, especially if we are to estimate how insect populations might respond in the future. As poikilotherms, insects are particularly susceptible to fluctuations in temperature which has led to the evolution of various thermal tolerance strategies to maintain internal homeostasis and preserve metabolic functions (Kokou et al., 2018). The level of vulnerability to fluctuations in environmental conditions (e.g., increased exposure to thermal stress) for various insect species has been linked with the underlying genetic and phenotypic plasticity present in these organisms, which play a direct role in mediating host physiological response (Macke et al., 2017; Kokou et al., 2018).

The diversity of mechanisms used by insects to acclimate to novel environments or shifts in environmental conditions, include examples of behavioral modifications (e.g., habitat selection, temporal activity), physiological changes (e.g., production of enzymes, antioxidants, proteins, lipids, carbohydrates, trehalose, etc.), and/or phenotypic alterations (e.g., reproduction, diapause, seasonal phenology) (Kingsolver and Huey, 1998; Huey et al., 2003; Danks, 2005; Clark and Worland, 2008; Huey, 2010; Lacey et al., 2010). There is also growing evidence linking insect-associated microorganisms with thermal tolerance, specifically the contributions of gut microbiota as members of this community are closely tied to overall host fitness (Ferguson et al., 2018; Zhang et al., 2019; Sepulveda and Moeller, 2020; Jaramillo and Castañeda, 2021). Some of the early studies on the role of microorganisms in cold tolerance focused on interannual shifts associated with phenology. For example, in certain freeze-tolerant insects, the presence of ice nucleating bacteria was found to regulate freezing in preparation for overwintering (Strong-Gunderson et al., 1990; Lee et al., 1991, 1993; Wernegreen, 2012; Moghadam et al., 2018). Conversely, in non-freeze-tolerant insects, these ice nucleating bacteria may be excreted in preparation for cold temperatures to prevent freezing (Lee et al., 1996). Gut microbiota can also indirectly impact thermal tolerance by shifting the relative abundance of certain microbial lineages to those that facilitate a physiological response that promotes tolerance to thermal stress (Sepulveda and Moeller, 2020). Ferguson et al. (2018), for example, found seasonal shifts in gut microbiota in crickets (*Gryllus veletis* Alexander and Bigelow) to be linked to increased freeze tolerance and immunity. Raza et al. (2020) showed gut microbiota in tephritid fruit flies [*Bactrocera dorsalis* (Hendel)] contributed to resistance to low temperature stress by stimulating the arginine and proline metabolism pathway in the insect host, leading to elevated levels of certain cryoprotectant transcripts and metabolites.

However, while associations with certain microbial taxa can foster phenotypic plasticity that increases tolerance to thermal stress, maintaining these microbial associations has the potential to be evolutionarily costly. Alterations to the gut microbial community could modify the functional relationships between gut microbiota and the insect host, leading to a shift in their contribution to other host functions and physiological responses (Sepulveda and Moeller, 2020). This can have a deleterious impact on host fitness if the result is selection against microbial symbionts involved in metabolism, nutrient acquisition, resistance to pathogens, host growth/development, or reproduction (Sepulveda and Moeller, 2020). In fact, it has been suggested that primary symbionts of certain insect species (i.e., microorganisms that perform specific, essential functions that influence host fitness), can actually constrain, rather than facilitate, thermal tolerance (Wernegreen, 2012; Zhang et al., 2019). Wernegreen (2012) hypothesized that because symbionts have co-evolved with their host, they are physiologically and genetically constrained, and thus more susceptible to environmental fluctuations. Some researchers have demonstrated a negative effect of temperature exposure on primary bacterial symbionts. Prado et al. (2010) found a significant reduction in bacterial symbionts in the guts of two stinkbug species (*Acrosternum hilare* and *Murgantia histrionica*) after high temperature exposure (30°C). Montllor et al. (2002) found guts of heat-stressed pea aphids to have a reduced number of bacteriocytes which house their obligate primary symbiont, *Buchnera*. However, the degree to which these symbionts act to constrain host thermal tolerance is still very much unknown. It is likely that microbially- mediated thermal tolerance can occur when there is enough flexibility in microbial diversity to respond to shifting environmental conditions, while also maintaining the core microbial community required for host functioning (Macke et al., 2017).

Perhaps one of the most studied insect-symbiont relationships is that of wood-feeding termites and their gut symbionts, which are essential for breakdown of lignocellulosic materials (Breznak and Brune, 1994; Radek, 1999; Brune, 2014). In North America, subterranean termites in the family Rhinotermitidae are responsible for the majority of economic costs associated with damage to wooden products and structures (Su and Scheffrahn, 2000). Members of Rhinotermitidae are broadly distributed in both temperate and tropical regions, although individual termite species and populations tend to thrive within relatively narrow temperature ranges and under specific environmental conditions (Esenther, 1969; Davis and Kamble, 1994). As microbial symbionts have been credited as significant drivers of species evolution and diversification in other social insects (Mueller et al., 2011), there is the potential for termite-associated microorganisms to influence thermal tolerance plasticity and constrain or expand termite geographical distributions.

A number of researchers dating back to the 1920s have examined the effect of temperature on termites (Esenther, 1969; Haverty and Nutting, 1974; Sponsler and Appel, 1991; Davis and Kamble, 1994; Cabrera and Kamble, 2001; Hu and Appel, 2004; Hu and Song, 2007; Gautam and Henderson, 2011), as well as on their symbiotic gut protozoa (Cleveland, 1923, 1924; Cook and Smith, 1942; Mannesmann, 1969; Smythe and Williams, 1972; Belitz and Waller, 1998; Cabrera and Kamble, 2004). Cleveland (1924) showed that all gut protozoa were killed after 24-h incubation at 36°C in *Reticulitermes flavipes* (Kollar).

Smythe and Williams (1972) observed no effect on protozoa at 29°C and report the highest tolerable temperature range for *R. flavipes* survival to be between 31.5 and 33°C. Despite this abundance of research relating to the effect of temperature on protozoa, the effect on gut bacteria has been mostly ignored. One exception to this is a study of termite cold tolerance, where Cabrera and Kamble (2004) found the supercooling point (SCP) to be the lowest in *R. flavipes* workers treated with antibiotics, and hypothesized that symbionts (either protists or bacteria) may be acting as ice nucleators in the termite hindgut.

In this work we examine the effect of temperature acclimation on shifts in the microbial community of subterranean termite guts as well as in termite-manipulated nest/soil. Soil material was included in this work as it is the physical substrate used by termites in construction of tunnels and galleries in the nest. It is comprised of termite feces and salivary secretions which bind the nest together and thus, can potentially provide insight into the fraction of the termite gut microbiome that might be evacuated under certain temperature conditions. The primary objectives of this study were to: (1) examine the physiological performance of eastern subterranean termites, *R. flavipes*, after prolonged exposure to three different temperature conditions using measurements of feeding activity, mortality, and cold tolerance [SCP and critical thermal minimum (CTmin)], (2) evaluate how an increase or decrease in temperature affects the microbial community of the termite gut and associated nest material, and (3) examine how these microbial shifts relate to cold tolerance. These objectives aim to gain a better understanding of temperature induced physiological and microbiological shifts of the termite gut microbial community and their potential functional role in termite thermal tolerance.

MATERIALS AND METHODS

Experimental Set-Up and Temperature Treatment

Termite experiments were performed in sterile plastic Petri dishes containing sterile soil (10 g), and sterile DI water (1 mL). A southern yellow pine wood block (*Pinus* spp. mix – *P. echinata* Mill., *P. elliottii* Engelm., *P. taeda* L.) (40 mm × 25 mm × 2 mm) which was conditioned at 27°C/30% relative humidity (RH), weighed and autoclave-sterilized, was included in each dish as a food source. A total of 100 *R. flavipes* workers from a laboratory colony were added to each dish which was then sealed with parafilm to maintain moisture levels (test start March 2018). These laboratory termites were comprised of a compilation of individuals collected in corrugated cardboard traps from Janesville, Wisconsin between the months of May and November 2017. Termites were maintained under constant conditions in an environmental chamber (27°C, 80% RH) prior to use. Termites were exposed for 4-weeks to one of three temperatures characterized as low (15°C), medium (27°C), or high (35°C), with five replicate dishes per temperature treatment. These temperatures were selected based on experimental ranges used by previous researchers that elicited physiological effects in laboratory termite tests.

Termite Guts and Soil Samples

After 4-weeks, soil material (0.25 g) manipulated by the termites (i.e., soil pasted to the side of the container by termites using salivary secretions and feces) was sterilely collected in duplicate from each of the petri dishes into sterile 2 mL microcentrifuge tubes. Pine wood feeder blocks from each dish were brushed free of debris, re-conditioned to a constant weight and re-weighed to determine the amount of termite feeding at each temperature treatment. Termites from each dish were counted to determine mortality. Individual termites were then randomly selected from each of the five replicate containers for each temperature group for use in cold tolerance assays (n = 31 termites per treatment group). Another set of individual termites were randomly selected for 16S rRNA amplicon sequencing. These samples consisted of 20 termites from each of the five replicate containers for all treatment groups, with the exception of one of the five test replicates belonging to the high temperature set as all termites were dead at the end of the test period. Preparation of these samples for sequencing involved first sacrificing termites by freezing (∼10 min. at 0°C) followed by a surface rinse in 70% EtOH. Gut dissections were done by pinching the tip of the last abdominal segment and gently pulling until the guts separated from the rest of the abdomen. Guts were pooled so that each sample consisted of 20 termite guts per replicate container (i.e., five tubes of 20 guts per temperature group, except for high temperature samples which had four tubes of 20 guts because of termite mortality). In addition to these experimental samples, extracted guts were pooled from 20 termite workers collected directly from a field site in Janesville, Wisconsin at 11 time points across late spring, summer and early winter seasons for comparison to lab termites used in the temperature exposure tests.

DNA Extraction and 16S rRNA Amplicon Sequencing

Termite guts were extracted directly into ZR bashing bead lysis tubes (Zymo Research, Irvine, CA, United States) and a mix of 600 μl of tissue and cell lysis solution and 2 μl Proteinase K from the MasterPure Complete DNA and RNA Purification Kit (Epicenter-Lucigen, Middleton, WI, United States) was added to each sample tube. All tubes were then vortexed vigorously for 2 min. Hereafter, DNA extraction methods followed the Epicenter-Lucigen total nucleic acids purification protocol. DNA was extracted from 0.25 g of collected soil material using the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, United States) following the provided protocol. Both termite gut and soil DNA was quantified using the Qubit Fluorometer (HS-assay kit; Invitrogen, Carlsbad, CA, United States) and diluted to 20 ng/μl.

Polymerase chain reaction (PCR) was done using tagged MiSeq primers targeting the V4 region of the 16S rRNA gene (primers: forward – GTGCCAGCMGCCGCGGTAA; reverse – GGACTACHVGGGTWTCTAAT) (Kozich et al., 2013). Reaction mixtures for each sample (24 μl) included 12 μl KAPA HiFi HotStart ReadyMix (2X) (KAPA Biosystems, Boston, MA, United States), 1.5 μl NanoPure water, 1 μl forward primer

(10 μM), 1 μl reverse primer (10 μM), and 8 μl diluted DNA (20 ng/μl). All samples were run in triplicate under the following conditions: initial denaturation at 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final elongation step at 72°C for 5 min. A non-template control reaction was included and submitted for sequencing.

Amplicon libraries were sequenced with the paired-end Illumina MiSeq platform at the University of Wisconsin-Madison Biotechnology Center. Reads were preprocessed, assembled, aligned, and classified using the mothur pipeline (Schloss et al., 2009). Classification was based on the Silva SEED release 132 database. Operational taxonomic units (OTUs) were defined using a 99% similarity threshold. A heatmap was built using *Z*-score normalized data was calculated as $Z = (x-u)/d$, where x is the relative abundance of a taxa, u is the mean relative abundance of a taxa across all the samples and, d is the standard deviation across all the samples. A phylogenetic tree of representative sequences (250 bp) of the top OTUs across all samples was performed in the MEGA v7 using the maximum likelihood method with 500 bootstrap replicates, assuming a General Time Reversible (GTR) substitution model. Using the OTU table with data not normalized or rarefied, OTU richness was estimated using Chao-1 index (Chao and Chiu, 2016), and non-metric multidimensional scaling (nMDS) and permutational analysis of variance (PERMANOVA) were used to quantify differences among samples and assess statistical significance, using PRIMER-e v 7.0.13. Differentially abundant OTUs were identified using MaAsLin2 (Microbiome Multivariable Associations with Linear Models) (Mallick et al., 2021).

Thermal Tolerance Tests

Termites used for this study originated from a population of *R. flavipes* along the northern range of its distribution where they are likely limited by cold seasonal conditions. Thus, two cold tolerance assays, SCP and CTmin, were selected as the physiological measures of thermal tolerance.

Supercooling Point

Two worker termites (3rd or 4th instar) were placed into each of eight 1.5 ml microcentrifuge tubes (n = 16 per treatment group). *T*-type thermocouples were wrapped near the base with cotton rounds and pushed in, next to the termites at the bottom of the tube to measure termite temperature. The other end of the thermocouple was inserted into an 8-channel TC-08 Data Logger (Pico Technology, Tyler, TX, United States) set to record temperature of all eight channels, at a sampling interval of 2 s. Tubes were then inserted into a floating tube rack and carefully placed into the refrigerated water bath (Grant Instruments, Cambridge, United Kingdom) containing 50:50 propylene glycol to water. The water bath was controlled using an external circulating pump and accompanying LabWise software, which was programmed for an initial 10-min acclimation step at 20°C followed by a decrease in temperature at a rate of 0.5°C/min down to 5°C, then at 0.2°C/min until it reached −15°C. Measurements of SCP were determined by a spike in temperature as measured by the data logger, representing the temperature at which freezing was initiated.

Critical Thermal Minimum

A custom aluminum cooling block was connected to a refrigerated water bath (Grant Instruments, Cambridge, United Kingdom) with insulated plastic tubes, allowing for cycling of the 50:50 propylene glycol to water between the water bath and aluminum block. Water bath temperature was controlled using an external circulating pump and accompanying LabWise software, programmed for an initial 10-min acclimation step at 20°C followed by decreasing temperatures at a rate of 0.2°C/min down to −15°C. Groups of three termite workers (3rd or 4th instar) were added to five of the six small culture plates attached to the center of the aluminum cooling block with thermal conducting tape (n = 15 per treatment group). Each culture plate had a small hole drilled in the lid to prevent condensation and so that a *T*-type thermocouple could be inserted for accurate temperature measurements. Temperature was recorded using a TC-08 Data Logger (Pico Technology, Tyler, TX, United States) in one of the six culture plates. Measurements of CTmin were determined as the temperature at which termites displayed a lack of righting ability after being gently flipped over with a paint brush, indicating that they could no longer coordinate their muscle movements. Once CTmin was reached, the termite was removed from the test dish into a 12-well plate and allowed to recover at room temperature (note: if termites did not recover, the CTmin threshold was passed and the data from that termite was deemed invalid).

RESULTS AND DISCUSSION

Effect on the Termite

Survival and Feeding

During the 4-week test, termites in the low temperature group consumed significantly less of the pine wood feeder block compared to termites in the other two temperature treatment groups (**Figure 1A**). Termite survival was comparable between the low and medium temperature groups but was significantly lower in termites exposed to the high temperature treatment, with 100% mortality in one of the five test containers (**Figure 1B**). These results suggest that while termites ate less at lower temperatures, this did not negatively impact survival. Termites in the high temperature group consumed slightly less wood material than the medium temperature termites, which was probably the result of increased mortality. Since wood consumption was not significantly lower however, it is likely that termite mortality occurred toward the end of the test period.

Cold Tolerance

Data from cold tolerance tests, SCP and CTmin are shown in **Figure 2**. These tests showed a significantly higher CTmin (mean 15.6°C) in high temperature exposed termites, indicating increased susceptibility to colder temperature. Termites acclimated at low temperature had a significantly lower CTmin (mean 5.7°C), but a significant increase in SCP (mean −5.9°C) compared to termites acclimated at the medium (mean CTmin 7.3°C/ SCP −8.4°C) or high (mean SCP −7.6°C) temperatures. This suggests that pre-exposure to cold allowed termites to stay

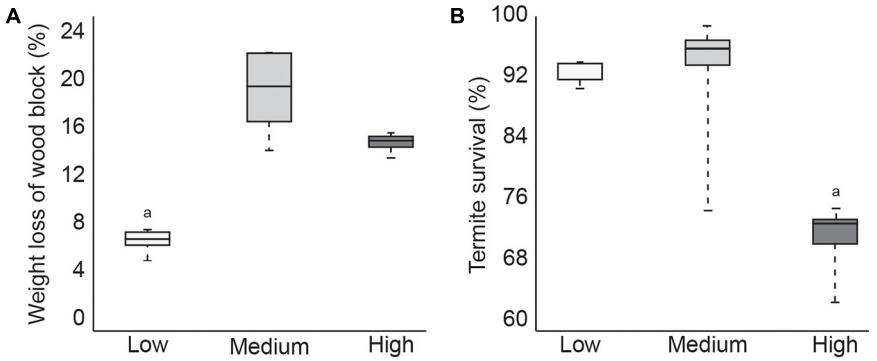

FIGURE 1 | Boxplot showing average1pc amount of feeding **(A)** and average termite survival **(B)** after 4 weeks at low, medium, or high temperatures ($p < 0.05$).

active longer in decreasing temperatures but caused termites to freeze more easily. These CTmin results might be indicative of cold tolerance adaptability in these northern termite populations by allowing for foraging and nest maintenance activities to continue into the colder, winter months. However, additional research is needed to confirm this hypothesis.

The higher SCP values recorded from the low temperature group suggest an increase in freezing susceptibility. However, this is not altogether unexpected as termites are thought to be freeze-intolerant/freeze avoidant insects (Mail, 1930; Esenther, 1969; Cabrera and Kamble, 2001, 2004; Hu and Song, 2007; Clarke et al., 2013) and SCP represents a lower limit of survival that may be dependent on multiple physiological factors (Renault et al., 2002; Lee, 2010). Results from this study are supported by those from a similar study of *R. flavipes* cold tolerance where they showed an increase in SCP after pre-exposure to 10°C, but concluded that lowering SCP is not likely a factor in cold acclimation as termites are not freeze tolerant insects (Davis and Kamble, 1994). While our results reinforce this conclusion, the reason for higher SCP values after cold acclimation remains unknown but may relate to the abundance of ice-nucleating agents in the termite gut. Other freeze-intolerant insect species have been shown to reduce ice nucleating bacteria and/or microbially produced compounds (e.g., calcium carbonate, potassium phosphate, uric acid, certain amino acids, proteins, steroids) which lead to freezing injury (Vonnegut, 1949; Head, 1961; Strong-Gunderson et al., 1990; Kawahara, 2002; Clark and Worland, 2008; Lee, 2010). When ice nucleators are present, they promote freezing at higher temperatures, which serves to limit ice formation to extracellular fluids in freeze tolerant species (Neven et al., 1989; Duman et al., 2010). Thus, high SCP in low temperature groups might be indicative of the presence of ice nucleating microorganisms that either increased during the cold acclimation period or are normally excreted seasonally in field termites in preparation for winter. Accordingly, lower SCP values in the other two temperature groups may be the result of reduction or shift in gut microbiota that would otherwise act as ice nucleating agents. Cabrera and Kamble (2004) showed antibiotic-treated *R. flavipes* workers to have the lowest SCP values and suggest this may be the result of removing ice-nucleating microorganisms. The significance of these results remains to be determined but could hint at one possible transition by which freeze intolerant insects, such as termites, may evolve to become freeze tolerant. Studies of field populations that examine seasonal shifts in gut microbiota and associated nest material across their geographical range are needed to better understand how termite-microbe interactions might be contributing to thermal tolerance.

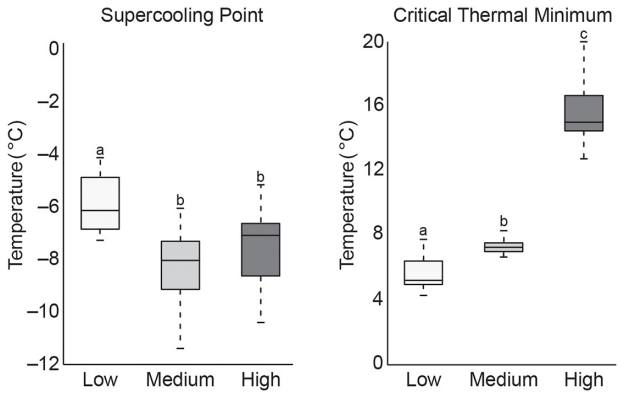

FIGURE 2 | Boxplot of SCP and CTmin values of termite workers after 4 weeks of exposure to low, medium, or high temperatures (treatments that do not share the same letter are significantly different $p \leq 0.05$).

Effect on the Termite Gut

Similarity and Diversity: Gut Microbial Community

Relative abundance of bacterial phyla from each of the test groups and from field collected gut samples are shown in **Figure 3**. The microbial community of field collected *R. flavipes* guts were dominated by Spirochaetes, followed by Firmicutes, Bacteroidetes, Elusimicrobia, and Proteobacteria, which is comparable to results from other studies that characterize the core microbiota of *Reticulitermes* spp. guts (Ohkuma and Brune, 2011; Boucias et al., 2013; Brune, 2014; Benjamino and Graf, 2016). This community seems to stay relatively constant across sampling dates. Within the temperature treatment groups, the

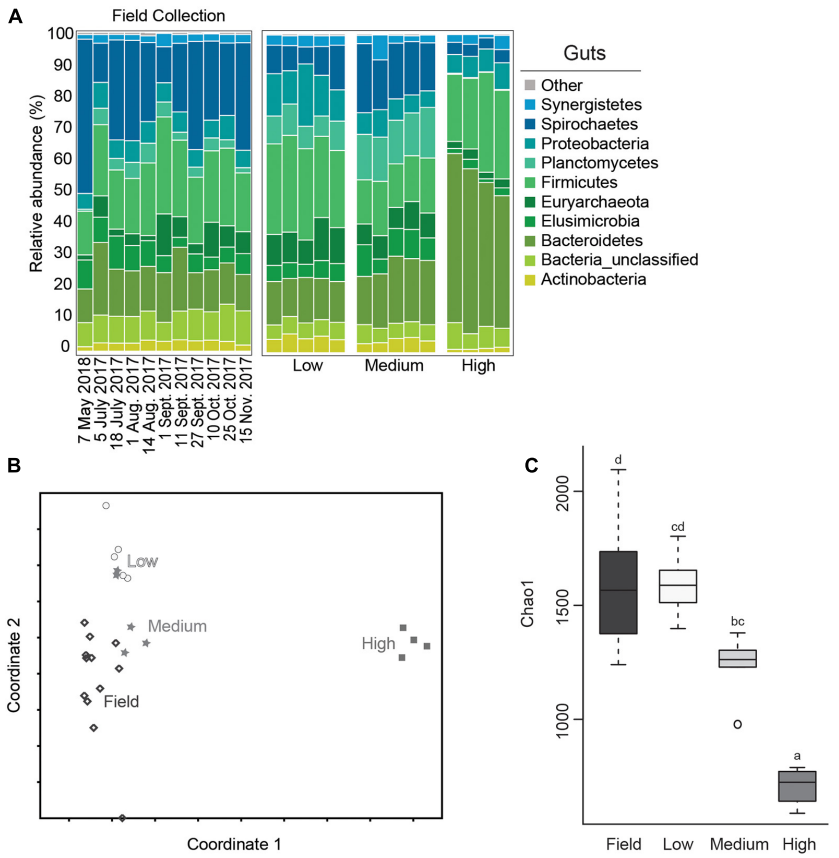

FIGURE 3 | Average relative abundance (%) of bacterial phyla from field collected termite guts and after temperature exposure **(A)**; Non-metric Multidimensional Scaling (NMDS) plot based on the Bray-Curtis distance of the bacterial communities of termite guts after temperature treatment and from field collected samples **(B)**; boxplot showing estimated microbial diversity based on Chao-1 for guts from field collected samples and those exposed to temperature treatments **(C)** (treatments that do not share the same letter are significantly different $p \leq 0.05$).

microbial community of guts from the medium temperature groups most closely resembled those of field populations at the phyla level although they did show a small reduction in the relative abundance of Spirochaetes and an increase in Planctomycetes (**Figures 3A,B**). This is important as the medium temperature value selected for this study is the same as the temperature setting used for maintaining termite populations in the lab. This suggests that there is some shift in gut microbiota in laboratory termite colonies compared to those directly from the field, but not nearly to the degree observed with low or high temperature treatments.

The gut microbial community composition was significantly more similar within treatments than between treatment groups (**Tables 1**, **2** and **Figure 3**). However, termite guts from the medium and low temperature groups showed more similarity to each other, and to those collected directly from the field, than termite guts from the high temperature group. This suggests that the high temperature treatment caused the most dramatic shift in the overall microbial community of the termite gut, specifically an increased relative abundance of Bacteroidetes to nearly 50% of the gut community composition (**Table 1** and **Figure 3A**). For the distribution of all OTUs across samples see **Supplementary Table 1**. Examination of the top 20 OTUs from gut samples of all temperature treatment groups, showed four OTUs to be members of Bacteroidetes, three of which were increased in high temperature exposed termites (**Figure 4**). Two belong to Dysgonomonadaceae (OTUs: 00020, 00005), a family that has recently been shown to contain ectosymbionts of intestinal nematodes in wood-feeding cockroaches (Murakami et al., 2019), although their significance in this study remains to be determined. Additionally, Chao-1 richness was significantly reduced in guts from the high temperature group compared to those from field samples or from the other two temperature groups, suggesting decreased species diversity overall (**Figure 3C**). Other studies have also shown the gut of poikilotherms to have lower bacterial diversity after exposure to heat stress (Bestion et al., 2017; Fontaine et al., 2018). Studies in bivalves (Abele et al., 2002) and lepidopterans (Cui et al., 2011) have demonstrated that exposure to thermal stress can lead to an increase in the production of reactive oxygen species. Therefore, one possible explanation for the observed lower bacterial diversity under high temperatures is that different species of gut symbionts show different levels of tolerance to these types of intrinsic factors produced by the host

TABLE 1 | Average Bray-Curtis similarity (%) of the bacterial communities within and between groups.

Average similarity	Treatment	Soil	Guts
Within	Field	–	58.4
	Low	42.9	63.7
	Medium	52.2	64.7
	High	49.0	64.6
Between	Field-Low	–	50.2
	Field-Medium	–	50.0
	Field-High	–	30.4
	Low-Medium	27.5	57.2
	Low-High	25.0	34.7
	Medium-High	46.7	40.0

TABLE 2 | Pair-wise PERMANOVA results for Bray-Curtis similarities of the bacterial communities from the termite guts and termite-manipulated soil materials after temperature treatment (low, medium, or high) and from field collected samples.

	Comparison groups	*t*	*P*-value
Termite guts	Field, High	3.5506	0.001
	Field, Low	2.1921	0.002
	Field, Medium	2.2398	0.002
	High, Low	3.3492	0.01
	High, Medium	3.0541	0.006
	Low, Medium	1.7686	0.008
Soil	High, Low	2.2329	0.009
	High, Medium	1.3191	0.006
	Low, Medium	2.3433	0.008

(e.g., reactive oxygen species, hormones, heat-shock proteins), all of which could result in changes in microbiome structure (Obata et al., 2018).

Results from this study also suggest a potential role of thermal stress in shaping the prokaryotic community associated with termite–protist symbionts. Phylogenetically lower termites maintain a diversity of gut protists that are essential for breakdown of lignocellulosic materials in wood-feeding termites, and thus essential for termite survival (Breznak and Brune, 1994; Hongoh, 2011; Brune, 2014). Here, high temperature treated groups showed reduced relative abundance of phyla that contain a number of protist-associated species, including Spirochaetes, Elusimicrobia, and Euryarchaeota. Within the Spirochaetes, species of *Treponema* represent members of the core microbial community in guts of *Reticulitermes* spp. where they are thought to either serve as CO_2 reducing acetogens or contribute to fixation of atmospheric nitrogen (N_2) (Noda et al., 2003; Benjamino and Graf, 2016). Examination of the 20 most abundant OTUs from all groups, four were identified as *Treponema* spp. (OTUs: 00004, 00016, 00023, and 00025) all of which were reduced in the high temperature treatment group (**Figure 4**). Decreased relative abundance of specific OTUs after high temperature exposure was also observed in Elusimicrobia candidate genus *Endomicrobium* (OTU00022) which are known cytoplasmic protist endosymbionts (Stingl et al., 2005) and in a phylum of methanogenic archaea, Euryarchaeota *Methanobrevibacter* (OTU00008). Members of Euryarchaeota have been identified from the guts of all extant families of termites (Purdy, 2007), and have mostly been found as ecto- and endosymbionts of termite gut protozoa although some have been shown to be associated with the gut wall (Ohkuma et al., 2006). Species of *Methanobrevibacter* specifically have been repeatedly identified from termite guts where they are suggested to utilize H_2 and CO_2 (Tokura et al., 2000). Thus, these data suggest that high temperatures likely have a negative impact termite gut protozoa, which agrees with results from Smythe and Williams (1972) who showed reductions in symbiotic protists (particularly those in the genera *Pyrsonympha* and *Dinenympha*) in guts of subterranean termites held at temperatures between 30 and 31.5°C. One possible explanation for this is that heat stress induced production of reactive oxygen species would negatively affect flagellate gut protists that require a strict anaerobic environment for survival. As the external and internal surfaces of these flagellate protists have been shown to serve as attachment sites for numerous gut bacteria, a loss of protozoa would result in a loss of habitat for these bacterial species. Overall, the relative abundance of 802 OTUs were found to be significantly different in the termite guts from the different treatments (**Supplementary Table 2**). These results support the hypothesis that the deleterious effects from high temperature exposure on termite survival may be indirectly related to the thermosensitivity of the microbial symbionts of the termite host.

Guts from low temperature treatment groups showed a reduction in the relative abundance of Spirochaetes and an increase in the relative abundance of Firmicutes, Lachnospiraceae (OTU 00018), in particular. The reduction in Spirochaetes could be related to decreased feeding at low temperature suggesting that less energy is being utilized for nutrient acquisition perhaps in favor of microbial associations linked to an increase in cold-tolerance. Low temperature gut samples also showed a dramatic shift in Proteobacteria, having nearly twice the relative abundance of Proteobacteria compared to the other temperature groups or field samples, specifically *Acinetobacter* (OTU00013), *Desulfovibrio* (OTU00028), Betaproteobacteriales (OTU00012, OTU00029), and *Enterobacter* (OUT00001) (**Figure 4**). These data agree with a study examining seasonal shifts in the gut microbiome of spring field crickets that also showed an increase in the relative abundance of Proteobacteria after cold temperature exposure (Ferguson et al., 2018). This association of Proteobacteria and cold temperature exposure may correspond to the known ice nucleating activity of certain members of this phylum (e.g., *Pseudomonas* spp., *Enterobacter* spp., *Xanthomonas* spp.) (Lee et al., 1991, 1993, 1995). Therefore, the increased relative abundance of Proteobacteria in termite guts from the low temperature group may relate to cold tolerance, specifically the increase in SCP in termites pre-exposed to cold.

Effect on Termite-Manipulated Soil

Similarity and Diversity: Soil Microbial Community

Microbial diversity and average relative abundance of bacterial phyla from termite-manipulated soil material are shown in **Figure 5**. It is important to note that as soil materials used in

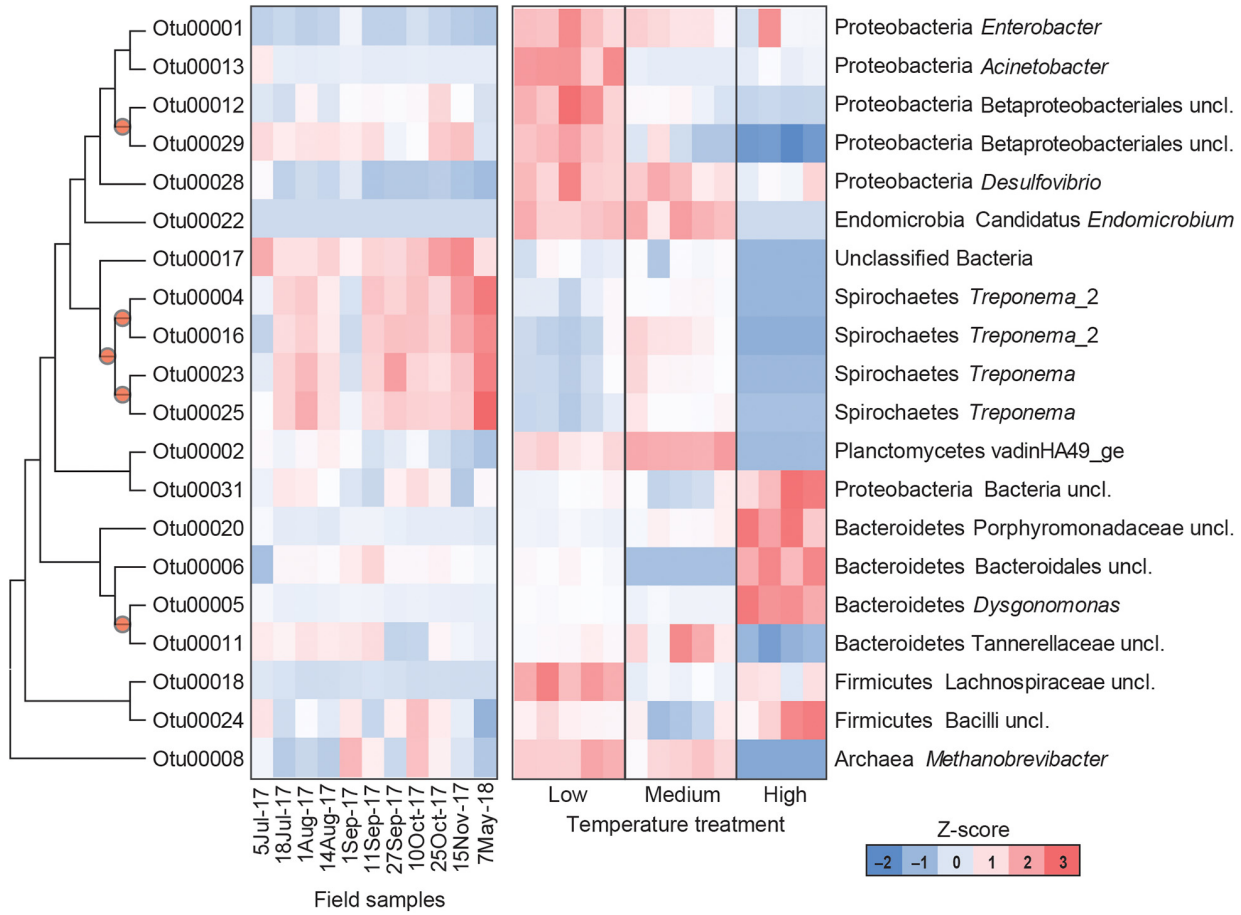

FIGURE 4 | Heatmap representing the relative abundance of the top 20 OTUs from field collected termite guts (left) and termite guts after low, medium, or high temperature exposure (right). Columns represent different samples, and rows represent *Z*-score normalized abundance of OTUs and are clustered based on a maximum likelihood phylogenetic tree of OTU representative sequences. All OTUs shown in this figure are differentially abundant among treatments [MaAsLin, $p < 0.001$, maximum false discovery rate (significance threshold) < 0.05]. Nodes with bootstrap values > 0.7 are marked with a circle.

this study were autoclaved prior to testing, the soil microbial community presented here is more representative of organisms associated with the termite cuticle or shed from the gut during tunneling/nest building activity than the microbial community composition under natural conditions. Further experiments using non-sterile soil, as well as field studies, would be needed to address how the soil microbial community shifts in the termite nest under natural conditions where they would be subjected to more gradual changes in environmental conditions as well as competition from other soil microorganisms. Therefore, results from this work should be considered, at least in part, in terms of how they compare to data from termite gut samples under the different temperature conditions.

In this study, soil samples from the low temperature group showed lower microbial diversity compared to the other temperature treatments based on Chao-1 (**Figure 5** and **Table 1**). Comparisons of soil samples showed a clear shift in the microbial community after low temperature treatment compared to the distinct, but more similar communities from the high and medium temperature samples (**Figures 5A,B** and **Tables 1**, **2**). A predominance of Proteobacteria and Bacteroidetes occurred in all soil samples, although the latter was reduced in soil from the low temperature group. As observed in termite gut samples, the relative abundance of Proteobacteria was also highest in soil samples from the low temperature groups. Soil material from the high temperature group had an increased abundance of Planctomyctes and Actinobacteria, which is contrary to the decreased abundance of these phyla observed in the corresponding termite gut samples.

In total, the relative abundance of 181 OTUs were found to be significantly different in the soil samples of the different treatments (**Supplementary Table 2**). Among the OTUs associated with the high temperature soil samples, were OTU00313, Actinobacteria – *Kitasatospora*, and OTU01388, an unclassified member of Firmicutes, order Clostridiales. Many members of both Actinobacteria and Firmicutes are able to form spores that can survive autoclaving (Hirvelä-Koski, 2004; Otte et al., 2018). Therefore, we hypothesize that spores of these bacteria may have been present in the soil before autoclaving and that high temperature treatment was favorable for spore

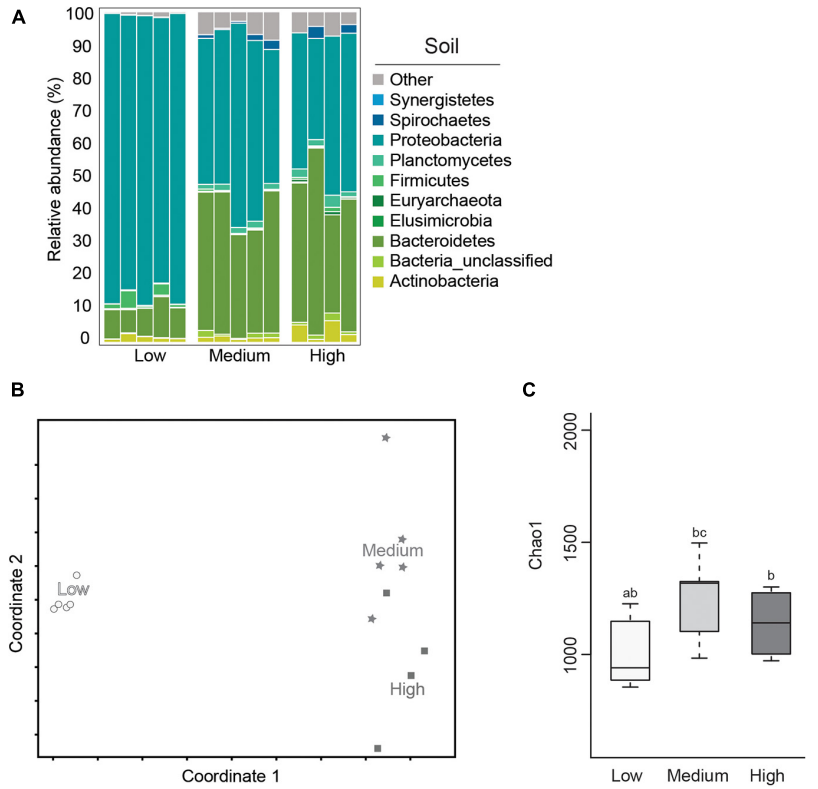

FIGURE 5 | Average relative abundance (%) of bacterial phyla **(A)**; Non-metric Multidimensional Scaling (NMDS) plot based on the Bray–Curtis distance of the bacterial communities **(B)**; and boxplot showing estimated microbial diversity based on Chao-1 **(C)**, for termite-manipulated soil materials after temperature treatment (treatments that do not share the same letter are significantly different $p \leq 0.05$).

germination. This is corroborated by the fact that these OTUs were virtually absent in termite gut samples (**Supplementary Table 2**). Two Proteobacteria [*Enterobacter* (OTU00001) and *Acinetobacter* (OTU00013)] dominated the low temperature soil samples and were also associated with low temperature guts. These results are in alignment with a number of recently published studies that have noted shifts in the relative abundance of bacterial taxa belonging to Proteobacteria associated with thermal stress in a variety of host species (Sepulveda and Moeller, 2020). Further studies are needed however, to determine the relationship between members of Proteobacteria and cold tolerance in subterranean termites.

Implications for Cold-Tolerance in Northern Termite Colonies

It has been suggested that northern populations of subterranean termites exhibit differences in reproductive biology, colony formation, and dispersal compared to their southern counterparts (Esenther, 1969). However, the various factors that contribute to these differences remain unknown. Many northern termite colonies are thought to have been introduced initially by way of infested wood materials, with subsequent colony growth occurring primarily through secondary reproductives rather than winged alates (Esenther, 1969; Raffoul et al., 2011). Initially, this led to assumptions that these populations would have lower genetic diversity caused by geographical isolation and from a lack of immigration or emigration. Previous work, however, showed Wisconsin termite populations to have higher amounts of within colony genetic variation than expected, suggesting that intraspecific genetic variation is likely to be a major factor in successful colony establishment in northern climates (Arango et al., 2015). It has been hypothesized that there is a threshold relating to genetic diversity, below which termite colonies are not able to persist (DeHeer and Vargo, 2006). Further studies are needed to examine the possible effect of host genotype on associated gut microbial diversity in relation to thermal tolerance. This information could lead to a better understanding how termite colonies become established along their northern range and the factors that subsequently support colony persistence and growth. For example, cold winter temperatures is often identified as a potential factor effecting termite dispersal by promoting caste differentiation into secondary reproductives over winged alates (Esenther, 1969; Arango et al., 2015). As gut microbiota have been shown to foster phenotypic plasticity (Macke et al., 2017), it is possible that this observed variation in termite reproductive biology may be linked with, or facilitated by, members of the gut microbial community. Studies examining gut microbial diversity in combination with cold tolerance physiology between termite castes may provide some insight into this phenomenon.

CONCLUSION

In this study, we found shifts in environmental temperature can cause substantial changes in the microbial community of *R. flavipes* guts as well as in associated soil/nest materials, particularly among members of Proteobacteria, Bacteroidetes, and Firmicutes. Our results suggest that northern termite populations might be more vulnerable to high heat exposure through microbial community loss/shifts, compared to low temperature. Thus, it is conceivable that *R. flavipes* activity might decline in warmer, southern regions (assuming similarity in gut microbial structure), and northern range edges might expand. However, the opposite may be true if southern termite populations were to be tested in a similar manner. Using an acclimation based approach, we were not yet able to establish a cause-effect relationship between these findings, and suggest that more studies are needed to investigate how the termite gut microbiota can modulate the host's physiological acclimation to temperature changes and vice-versa. Future work should include field studies examining shifts in gut-microbe communities from termite populations across seasonal and geographical gradients as they may lead to a better understanding of the potential contribution of gut microbiota in adaptive phenotypic plasticity under more ecologically relevant conditions. Additionally, research is needed to determine how changes to gut microbiota might impact overall host fitness by evaluating shifts in microbial community functionality. Specifically, how disruptions to the termite gut microbiome effects other essential processes (e.g., metabolism/nutrient acquisition, resistance to pathogens, growth/development, and reproduction).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Center for Biotechnology Information (NCBI) BioProject, https://www.ncbi.nlm.nih.gov/bioproject/, PRJNA680366.

AUTHOR CONTRIBUTIONS

RA, SS, CC, and CC-S: conceptualization, methodology, and draft revision and editing. RA and CC-S: investigation, analysis, and writing and draft preparation. All authors have approved the publication of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.632715/full#supplementary-material

Supplementary Table 1 | Distribution of OTUs for all samples across treatment and sample types.

Supplementary Table 2 | Differentially abundant OTUs based on MaAsLin2 analysis.

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Interactions Between Tsetse Endosymbionts and *Glossina pallidipes* Salivary Gland Hypertrophy Virus in *Glossina* Hosts

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Tsetse flies are the sole cyclic vector for trypanosomosis, the causative agent for human African trypanosomosis or sleeping sickness and African animal trypanosomosis or nagana. Tsetse population control is the most efficient strategy for animal trypanosomosis control. Among all tsetse control methods, the Sterile Insect Technique (SIT) is one of the most powerful control tactics to suppress or eradicate tsetse flies. However, one of the challenges for the implementation of SIT is the mass production of target species. Tsetse flies have a highly regulated and defined microbial fauna composed of three bacterial symbionts (*Wigglesworthia, Sodalis* and *Wolbachia*) and a pathogenic *Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV) which causes reproduction alterations such as testicular degeneration and ovarian abnormalities with reduced fertility and fecundity. Interactions between symbionts and GpSGHV might affect the performance of the insect host. In the present study, we assessed the possible impact of GpSGHV on the prevalence of tsetse endosymbionts under laboratory conditions to decipher the bidirectional interactions on six *Glossina* laboratory species. The results indicate that tsetse symbiont densities increased over time in tsetse colonies with no clear impact of the GpSGHV infection on symbionts density. However, a positive correlation between the GpSGHV and *Sodalis* density was observed in *Glossina fuscipes* species. In contrast, a negative correlation between the GpSGHV density and symbionts density was observed in the other taxa. It is worth noting that the lowest *Wigglesworthia* density was observed in *G. pallidipes*, the species which suffers most from GpSGHV infection. In conclusion, the interactions between GpSGHV infection and tsetse symbiont infections seems complicated and affected by the host and the infection density of the GpSGHV and tsetse symbionts.

Keywords: ***Hytrosaviridae*, tsetse microbiota, virus transmission, *Wigglesworthia*, *Sodalis*, *Wolbachia***

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INTRODUCTION

Tsetse flies (Diptera: Glossinidae) are medically and agriculturally important vectors of trypanosomes, the causative agents of human African trypanosomosis or sleeping sickness and African animal trypanosomosis or nagana in some 37 countries throughout Sub-Saharan Africa (Leak, 1998; Simarro et al., 2003). The presence of tsetse and trypanosomes are considered as one of the most important roots of hunger and poverty in humans, hindering the adoption of more productive livestock (Dyck et al., 2021; Feldmann et al., 2021). No effective vaccines are available for disease control and existing nagana drugs are a significant financial burden for livestock owners. It is hard to manage the disease in humans by treating with trypanocidal drugs due to drug toxicity and the extended treatment times required, and resistance by the animal trypanosomes to the available drugs is common (Aksoy and Rio, 2005). Conversely, disease vector eradication using vector control methods is cheap and effective for the sustainable management of the diseases (Leak, 1998; Simarro et al., 2003). Among available vector control methods (Jordan, 1974; Thompson et al., 1991; Green, 1994), the sterile insect technique (SIT) is considered very powerful for the sustainable management of the disease as part of area-wide integrated pest management (Vreysen et al., 2000; Hendrichs et al., 2007). SIT is based on the mass production, radiation-based sterilization and release of sterile insects over a target area to suppress or locally eliminate a target insect pest population (Dyck et al., 2021).

A prerequisite for large-scale SIT applications is the sustainable production and maintenance of large numbers of high-quality insects of the target species to use during the implementation of SIT (Vreysen et al., 2000). However, this may sometimes be quite challenging due to the presence of pathogens and parasites such as the salivary gland hypertrophy virus (GpSGHV) (Abd-Alla et al., 2021).

Despite the extensive research on the ecology, physiology, genetics, and reproductive biology of tsetse flies that has been carried out over the past years, there is little knowledge about the interactions between the GpSGHV, the tsetse symbionts and the host in *Glossina* species other than *G. pallidipes* (Boucias et al., 2013). Therefore, it is crucial to investigate this tripartite interaction in order to improve the production and the quality of the mass reared insects that are to be used for SIT applications.

Tsetse flies are known to harbor a unique bacterial community mainly consisting of the obligate *Wigglesworthia glossinidia*, the commensal *Sodalis glossinidius,* and the widespread symbiont *Wolbachia pipientis* (hereafter *Wolbachia*) (Aksoy, 2000; Aksoy et al., 2008; Wang et al., 2013; Doudoumis et al., 2017). The prevalence of these symbionts in natural populations of different tsetse species may vary and some individuals may only carry the primary symbiont *Wigglesworthia glossinidia* (hereafter *Wigglesworthia*) (Lindh and Lehane, 2011). *Wigglesworthia*, a member of the *Entrobacteriaceae* family, is an obligate mutualistic bacterium, primarily residing intracellularly within the bacteriome organ in the anterior midgut of tsetse (Aksoy, 1995) and extracellularly in the mother's milk gland secretion (Attardo et al., 2008; Belda et al., 2010). Its symbiotic role is crucial in immunity as well as in the metabolic provisioning of vitamins and other nutrients to the hematophagous insect host which are either lacking or are contained in low amounts in vertebrate blood (International Glossina Genome Initiative, 2014; Attardo et al., 2019). Elimination of *Wigglesworthia* from tsetse results in significantly reduced host fecundity due to the loss of these vitamins while its presence during the immature stage is vital for immune system development in adults (Weiss et al., 2011).

The facultative commensal *Sodalis glossinidius* (hereafter *Sodalis*; family *Enterobacteriaceae*) is found both intracellularly and extracellularly in many different tissues in tsetse, e.g., hemolymph, midgut, and milk glands (Attardo et al., 2008; Belda et al., 2010). The *Sodalis* genome sequence shows reduced coding capacity with a large number of fragmented predicted protein-coding sequences and pseudogenes that are non-functional. Therefore, *Sodalis* cannot survive outside its host and has transitioned from a free-living form to a mutualistic life cycle (Toh et al., 2006). Even though its elimination has been reported to reduce host longevity and there are reports which suggest that *Sodalis* may render tsetse flies susceptible to trypanosome infection (Welburn and Maudlin, 1992; Dale and Welburn, 2001; Dale and Moran, 2006; Geiger et al., 2007; Farikou et al., 2010), the specific role of this symbiont is still not clear in tsetse flies.

Populations of tsetse species may also be infected with another symbiont, *Wolbachia*, which is an obligatory intracellular maternally transmitted alphaproteobacterium (Cheng et al., 2000; Doudoumis et al., 2012). *Wolbachia* has been shown to affect many aspects of the biology of its hosts, including host reproduction, development, immunity and behavior (Saridaki and Bourtzis, 2010; Schneider et al., 2011). The presence of this symbiont in the tsetse fly *G. morsitans* has been associated with the induction of cytoplasmic incompatibility, a form of reproductive abnormality which is expressed as embryonic lethality when an infected male mates with an uninfected female or a female infected with a different strain of the symbiont (Alam et al., 2011; Doudoumis et al., 2013). Due to their importance, tsetse fly-symbiont interactions are being harnessed toward the development of novel approaches for vector and disease control (Aksoy, 2000; Aksoy et al., 2008; Abd-Alla et al., 2013; Doudoumis et al., 2013).

Tsetse flies can also be infected by the salivary gland hypertrophy virus that was first found in *G. pallidipes* and hence named GpSGHV. The GpSGHV belongs to the *Hytrosaviridae* family, which are rod-shaped viruses containing a single circular double-stranded DNA genome (Abd-Alla et al., 2009b; Kariithi et al., 2019). GpSGHV causes both asymptomatic (latent/persistent) and symptomatic infections (Lietze et al., 2010; Kariithi et al., 2013). Infection with GpSGHV results in salivary gland hypertrophy (SGH) syndrome which is characterized by the swelling of the salivary glands as well as abnormalities in the reproductive organs and associated reduction on the fecundity and fertility of insect hosts (Jaenson, 1978; Abd-Alla et al., 2010a, 2009a).

In natural populations, GpSGHV is transmitted from mother to progeny, either transovum or *via* infected milk glands (Jura et al., 1989; Sang et al., 1996, 1998). In laboratory conditions,

GpSGHV infection occurs horizontally via membrane feeding and vertically from mother to offspring (Abd-Alla et al., 2010b). Interestingly, *G. pallidipes* is highly susceptible to intra-hemocoelic GpSGHV injection. Although this infection route results in high viral titers ($\geq 10^9$ viral genome copies), it triggers the onset of overt SGH only in the F1 offspring, not in the injected adults. Notably, although GpSGHV has been detected in almost all *Glossina* species that have so far been PCR-screened, the occurrence of SGH symptoms is a very rare event. For example, PCR detection of GpSGHV in *G. pallidipes* shows widespread asymptomatic virus infection (up to 100%), with only 5–10% of the infected individuals developing SGH symptoms. However, symptomatic infections in *G. pallidipes* tsetse fly mass production factories may result to dramatic reductions in fecundity leading to colony collapse (Abd-Alla et al., 2011). It is unclear why some infected flies show symptomatic infection whereas others remain asymptomatically infected. Although a positive correlation was found between symptomatic SGH and increased virus copy number (which indicates an accumulation effect of the virus related to the SGH symptoms), other unknown factors related to the fly's genetic background and interaction with its microbiota (symbionts) cannot be excluded (Moran et al., 2008; Abd-Alla et al., 2009a; Ferrari and Vavre, 2011; Boucias et al., 2013; Eleftherianos et al., 2013). Therefore, it is important to analyze the interaction between the virus and the symbiotic bacteria in different tsetse species.

Based on the variable responses to the GpSGHV infection in different tsetse species and in the lack of molecular explanation, the role of tsetse microbiota in modulating GpSGHV infection was not excluded. Analyzing the interactions between GpSGHV and tsetse symbionts might reveal the mechanism behind the different responses to GpSGHV infection in different tsetse species. To date, the interactions between GpSGHV and the bacterial symbionts in the *Glossina* species remain an open question. Similar to the maternally-transmitted *Wigglesworthia*, *Sodalis* and *Wolbachia* (Rio et al., 2012), GpSGHV is trans-ovarially transmitted, and the absence of these symbionts can change the outcome of the virus infection (Boucias et al., 2013).

In the current study, we performed a series of assays to examine the levels of GpSGHV infection and its interaction with the three endosymbionts *Wigglesworthia*, *Sodalis,* and *Wolbachia* in laboratory colonies of six tsetse species. The disentangling of the potential diverse interactions among the different microbial species occurring in the six tsetse species studied is discussed in the context of developing an effective and robust mass production system of high-quality sterile tsetse flies for implementing SIT programs.

MATERIALS AND METHODS

Tsetse Samples

All the GpSGHV injection related laboratory experiments described in this study were conducted on tsetse fly colonies maintained at the Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria. The laboratory colonies of the six *Glossina* taxa used in the injection experiments originated from Uganda (*G. pallidipes*), Kenya (*G. brevipalpis*), Zimbabwe (*G. m. morsitans*), Tanzania (*G. m. centralis*), Central African Republic (*G. f. fuscipes*), and Burkina Faso (*G. p. gambiensis*).

Experimental flies were held in standard round holding cages (20 cm diameter × 5 cm height) at a density of 45–80 flies (male: female ratio of 1:3) based on the species size and maintained at 23 ± 1°C and 75–80% RH under the insectaria conditions. Experimental flies were fed on heated, defibrinated bovine blood (10–15 min; three times weekly) using the *in vitro* membrane feeding technique (Feldmann, 1994; Gooding et al., 1997).

In vivo Virus Replication in Intra-Hemocoelic Injected Adults

A pair of intact salivary glands dissected from 10-day old male *G. pallidipes* exhibiting overt salivary gland hypertrophy symptoms were used to prepare the virus inoculum as previously described (Boucias et al., 2013) with slight modifications including aseptic salivary gland dissections and use of non-filtered virus inoculum.

Virus injection was conducted in the different tsetse species as previously described by Demirbas-Uzel et al. (2018b). In brief, teneral flies were immobilized in the chiller (2–6°C; 5 min) and injected (intra-hemocoelic) with either 2 μl sterile phosphate buffered saline (PBS) or GpSGHV virus suspension. For each species, 160 flies (1:3 male:female) were injected and placed in standard holding cages at a density of 45–80 flies per cage. The experiments were conducted in 2–3 replicates for each species. After the injections, eight flies (six females and two males) were randomly selected and sampled from both virus and PBS injected fly groups at 0-, 1-, 5-, and 9-days post injection (dpi), and subsequently frozen at −20°C until further analysis. Virus density variations were measured separately for both females and males at different dpi.

TABLE 1 | PERMANOVA results table for tsetse species, time post GpSGHV injection and sex and their combinations.

Source	*Df*	SS	MS	Pseudo-F	*P* (perm)	Unique perms
Species	5	1.05E+05	20,905	47.16	**0.001**	999
Time post injection	2	46,283	23,141	52.206	**0.001**	999
Sex	1	175.58	175.58	0.3961	0.735	999
Species × time post injection	10	49,580	4,958	11.185	**0.001**	996
species × sex	5	5,705	1,141	2.5741	**0.002**	998
Time post injection × sex	2	473.7	236.85	0.53433	0.775	999
Species × time post injection × sex	10	8,456.3	845.63	1.9077	**0.005**	999
Res	216	95,746	443.27			
Total	251	3.04E+05				

Within the table, statistically significant differences (P < 0.05) can be seen in bold values in all three factors separately and in the combination of sex, species and time post injection. Perm(s) = permutation(s).

Extraction of Total DNA and PCR Amplifications

The total DNA of each sample was extracted from whole bodies of individual flies using the DNeasy tissue kit (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. The DNA extracted was eluted in 200 μl of the elution buffer. Then, 30 μl of the extracted genomic DNA from individual samples were pooled (six females in one pool and two males in another pool), and the DNA concentrations in the pooled DNA were determined using a spectrophotometer (Synergy H1 Multi-Mode Reader, BioTek, Instruments, Inc., United States). The pooled DNAs were diluted to obtain equal final DNA concentrations (4 ng/μl) and 5 μl of the diluted DNA was analyzed by qPCR on a CFX96 real time PCR detection system (Bio-Rad, Hercules, CA) as described previously (Abd-Alla et al., 2009a). The tsetse housekeeping β-*tubulin* gene was used to normalize the qPCR reactions (Boucias et al., 2013) to give the virus density. The primers and the qPCR conditions are given in **Supplementary Table 1**.

The aliquots of the DNA extracted at the time-points outlined in section "*In vivo* Virus Replication in Intra-Hemocoelic Injected Adults" were used to quantify the densities of bacterial symbionts normalized to the housekeeping β-*tubulin* gene as previously described (Boucias et al., 2013). *Sodalis, Wigglesworthia* and *Wolbachia* densities were quantified in both males and females and at different dpi by qPCR using primers that target *fliC* (Weiss et al., 2012), *thiC* (Yamada et al., 2007) and *Wolbachia* 16R rRNA genes, respectively (Demirbas-Uzel et al., 2018a). The primers and the PCR and qPCR conditions are given in **Supplementary Table 1**.

Statistical Analysis

The density of *Wigglesworthia*, *Sodalis* and *Wolbachia* in GpSGHV infected flies was evaluated in both PBS and GpSGHV injected flies at 0, 1, 5, and 9 dpi and was normalized against the average of the zero-time density by dividing the density of each reading at the targeted point by the average of the density at zero-time. Subsequently, the density of each targeted point of the flies injected with GpSGHV was normalized against the average of each time point of the PBS-injected flies. The densities of *Wigglesworthia*, *Sodalis* and *Wolbachia* in GpSGHV injected flies normalized against the PBS-injected densities were then used in the statistical analysis. The GpSGHV density data previously reported by Demirbas-Uzel et al. (2018b) was reanalyzed using the above method and compared with the tsetse symbiont density. Data were checked for normality and transformed where necessary using the Box-Cox transform $(x^{\lambda} - 1)/\lambda$. The significance of the overall differences of the virus and symbiont densities obtained from the various treatments were assessed by ANOVA (Sokal and Rohlf, 1995), after which the actual patterns of differences between the means were determined by Tukey's honestly significant difference (HSD) test. The *P*-values were calculated from the data with the significance threshold selected as 0.05.

The analyses were executed in R v4.0.2 (R Core Team, 2020) using RStudio v1.3.1056 (Baier and Neuwirth, 2007; RStudio Team, 2016) with packages ggplot2 v3.3.2.1 (Wickham, 2016), lattice v0.20-41 (Sarkar, 2008) and MASS v7.3-51.6 (Venables and Ripley, 2002). All regression analyses were conducted using the linear model (lm) for different times and different doses and coefficient factors (slope), *t* and *P*-values are presented in **Supplementary Table 2**. Overall similarities in *Wigglesworthia, Sodalis*, *Wolbachia* and GpSGHV density between tsetse species, time post injection and sex were shown using the multidimensional scaling (MDS) analysis, and the multidimensional plots as implemented in PRIMER version 7+ (Anderson, 2001; Clarke and Gorley, 2016). Permutational multivariate analysis of variance (PERMANOVA) was applied to Bray-Curtis similarity matrices to compute similarities between countries, tsetse species and infection type groups using PRIMER version 7+.

RESULTS

Impact of GpSGHV Infection on the Three Bacterial Symbionts in Different Tsetse Species

After the intra-hemocoelic injections of virus suspension into the teneral adults, all six tsetse taxa were found to be susceptible to GpSGHV infection under laboratory conditions and the virus relative density increased over time in most taxa (**Supplementary Figure 1**). After confirming the virus infection, the relative qPCR quantification of *Wigglesworthia*, *Sodalis* and *Wolbachia* densities over a 9-day experimental period revealed various densities of these symbionts across the six *Glossina* taxa (**Figure 1**).

Impact of GpSGHV on *Wigglesworthia* Density

The density of *Wigglesworthia* (*Wig*D) in virus injected flies relative to PBS injected flies varied significantly between tsetse taxa (Taxon: F = 42.855, *df* = 5.206, $P < 0.001$), with time over 9 days post injection (Time: F = 5.3002, *df* = 1.206, $P = 0.022$), as well as the interactions between sex and taxon ($F = 7.014$, *df* = 5.206, $P < 0.001$) and time and taxon ($F = 3.318$, *df* = 5.206, $P = 0.007$; **Figure 1A** and **Supplementary Table 2**). In general, *Wig*D in GpSGHV injected adults was not affected by the fly sex ($F = 0.023$, *df* = 1.206, $P = 0.881$; **Supplementary Table 2**) but a significant difference was observed between males and females in *G. brevipalpis* ($F = 9.280$ *df* = 1.52 $P = 0.003$) and *G. m. centralis* ($F = 14.781$ *df* = 1.32 $P = 0.001$; **Supplementary Figure 1A** and **Supplementary Table 3**). GpSGHV significantly reduced *Wig*D over time post injection in *G. f. fuscipes* (Coefficient = −0.165, $P = 0.001$) and *G. p. gambiensis* (Coefficient = −0.170, $P = 0.002$). In contrast, no significant change was observed in *Wig*D over time after injection in the other taxa (**Supplementary Table 4**). In general, *Wig*D was lower in *G. pallidipes* than the other tsetse species (**Figure 1**). The greatest reduction effect by GpSGHV on *Wig*D was seen in *G. f. fuscipes* ($P = 0.001$; **Figure 1A** and **Supplementary Table 4**).

Impact of GpSGHV on *Sodalis* Infection Density

Overall, GpSGHV effects on *Sodalis* density (*Sod*D) were significantly different between the six tsetse taxa ($F = 70.593$,

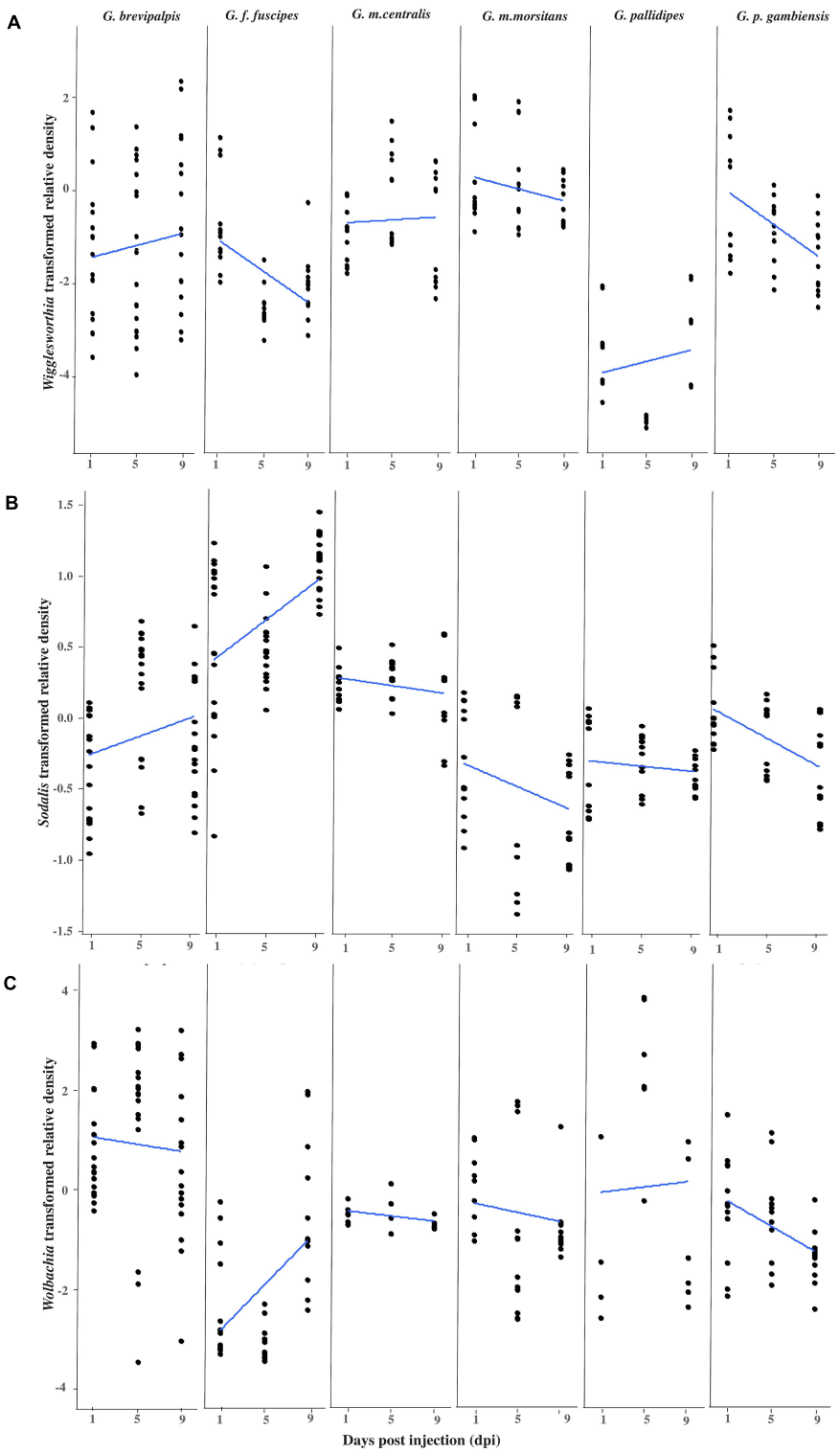

FIGURE 1 | Impact of GpSGHV infection on the density of tsetse symbionts. Transformed *Wigglesworthia* **(A)**, *Sodalis* **(B)**, and *Wolbachia* **(C)** densities for six taxa of tsetse flies injected with GpSGHV normalized against control flies. Total DNA was extracted from six females and two males at 1, 5, and 9 days post injection and used as template for qPCR. Data were normalized to the housekeeping β-*tubulin* gene, against time zero and against the control injected flies at each time point and transformed based on the Box-Cox lambda value. The linear regression line was calculated using the lm method in R.

df = 5.228, $P < 0.001$). Although no significant difference was observed between males and females ($P = 0.129$) or with time ($P = 0.350$), strong interactions between sex and time ($F = 5.819$, *df* = 1.228, $P = 0.017$), sex and taxon ($F = 4.417$, *df* = 5.228, $P < 0.001$) and time and taxon ($F = 8.778$, *df* = 5.228, $P < 0.001$) were observed (**Supplementary Table 2**).

Sodalis density was evaluated within each of the six taxa, but only in *G. f. fuscipes* did it increase significantly with time over the 9 days post injection (Coefficient = 0.071, $P < 0.001$) and reduced significantly in *G. p. gambiensis* (Coefficient = −0.050, $P < 0.001$; **Figure 1B** and **Supplementary Table 4**).

No significant difference was observed between females and males in respect to *Sod*D over all tsetse taxa ($F = 2.321$, *df* = 1.228, $P = 0.129$; **Supplementary Table 2**) but a significant difference was observed between males and females within *G. m. centralis* ($F = 32.208$, *df* = 1.32, $P < < 0.001$) and *G. p. gambiensis* ($F = 46.091$, *df* = 1.24, $P < < 0.001$; **Supplementary Table 3**).

Impact of GpSGHV on *Wolbachia* Infection Density

The difference in *Wolbachia* density (*Wol*D) was significant only between different taxa ($F = 20.868$, *df* = 5.189, $P < < 0.001$), while there was no significant difference between males and females ($P = 0.238$), between the flies from different time points post infection ($P = 0.796$) or any interaction term (**Figure 1C** and **Supplementary Table 2**). Over time, in the presence of GpSGHV infection, a significant increase in *Wol*D was observed in *G. f. fuscipes* (Coefficient = 0.224, $P = 0.004$) and a significant reduction was observed in *G. p. gambiensis* (Coefficient = −0.125, $P = 0.009$) over 9 days post injection compared to control flies. No significant changes were observed in the other taxa (**Supplementary Table 4**). No significant difference was observed between males and females when all species were analyzed together, **Supplementary Table 3**.

Interaction Between GpSGHV and Tsetse Symbionts

Comparing the GpSGHV relative density with the relative density of tsetse symbionts indicated that this multi-partite interaction seems to be complicated and varied from one tsetse species to another. Plotting the GpSGHV relative density against tsetse symbiont densities indicate a negative correlation between the GpSGHV and both *Wigglesworthia* and *Wolbachia*. The same trend was observed in *Sodalis* in most of the tested taxa except *G. fuscipes* which show that a high density of GpSGHV can be observed with high density of *Sodalis* (**Figure 2**). Analyzing the similarity between GpSGHV and tsetse symbiont densities indicated that (i) the lowest density of *Wigglesworthia* was opserved in *G. pallidipes*, (ii) the highest increase if GpSGHV density was observed in *G. m. morsitans* and *G. p. gambiensis*, (iii) the highest density of *Sodalis* was found in *G. f. fuscipes*, and (iv) the highest density of *Wolbachia* was observed in *G. brevipalpis* (**Figure 3A**). These previous statement gets more complicated when sex and time post GpSGHV injection or both of them are also taken into consideration. The lowest *Wigglesworthia* density in *G. pallidipes* was associated with a higher density of GpSGHV than the density of both *Sodalis* and *Wolbachia*. The GpSGHV density and *Sodalis* density did not

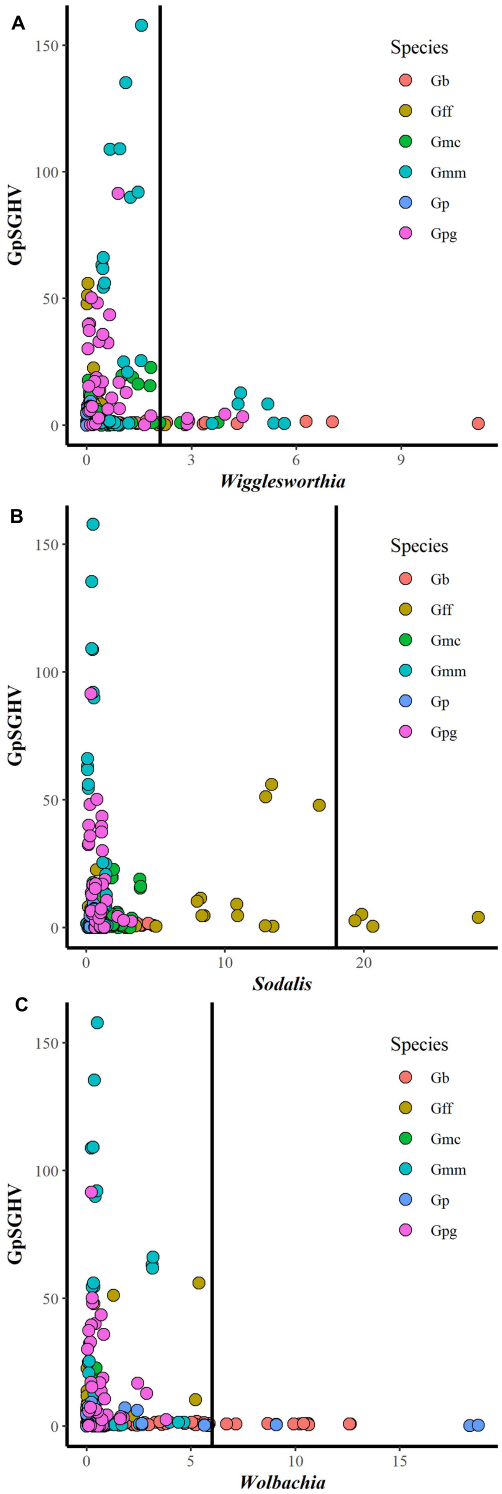

FIGURE 2 | Interaction between GpSGHV relative density and the relative density of *Wigglesworthia* **(A)**, *Sodalis* **(B),** and *Wolbachia* **(C)** in six taxa of tsetse flies. Tsetse adults were injected with GpSGHV and the density of GpSGHV and tsetse symbionts were analyzed by qPCR and the data were normalized against control flies and zero time of injection. The vertical bar indicates the tsetse symbiont relative density to limit the relative density of GpSGHV to 20.

show a visible difference between males and females, however, the density of *Wigglesworthia* and *Wolbachia* was slightly higher in males than females (**Figure 3B**). The highest density of *Sodalis* observed in *G. f. fuscipes* was not affected by the sex of the flies but was affected by the time post GpSGHV injection. The dinsity of the GpSGHV and tsetse symbionts were affected by the time post GpSGHV injection, at 1 dpi the density of GpSGHV and *Sodalis* were similar and higher than the density of *Wigglesworthia* and *Wolbachia*, however at 5 dpi the density of *Sodalis* remained unchanged but the GpSGHV density decreased and the *Wolbachia* density increased. This situation was changed at 9 dpi where the GpSGHV density increased and *Wolbachia* density decreased (**Figure 3C**). In G. morsitans, the highest GpSGHV density was associated with a low density of *Sodalis*, which was lower than *Wolbachia* density and *Wigglesworthia*. This situation was affected by the sex of the flies where the GpSGHV density was visibly higher in males than females with slightly higher density of *Wolbachia* than *Wigglesworthia*, as opposed to the females with lower GpSGHV density associated with higher density of *Wigglesworthia* than *Wolbachia*. The highest GpSGHV density was mainly observed at 5 and 9 dpi with low density of the tsetse symbiont. It is worth noting that tsetse symbiont density was not affected by the time post injection (**Figure 3C**). The highest density of *Wolbachia* observed in *G. brevipalpis* was mainly observed in the females and was not affected by the time post GpSGHV injection (**Figures 3B,D**).

The bootstrap averages analysis of the metric multidimensional scaling (MMDS) produced clusters based on the species, the time post GpSGHV injection and the sex of the tested flies (**Figure 4**). Permanova analysis indicated that the clusters observed between tsetse species or the times post injection were statistically significant (P = 0.001); however, the clusters formed based on the sex level were not statistically significant (P = 0.735). The Permanova analysis also showed that there was a significant interaction between species and time post injection (P = 0.001) and sex (P = 0.002; **Table 1**).

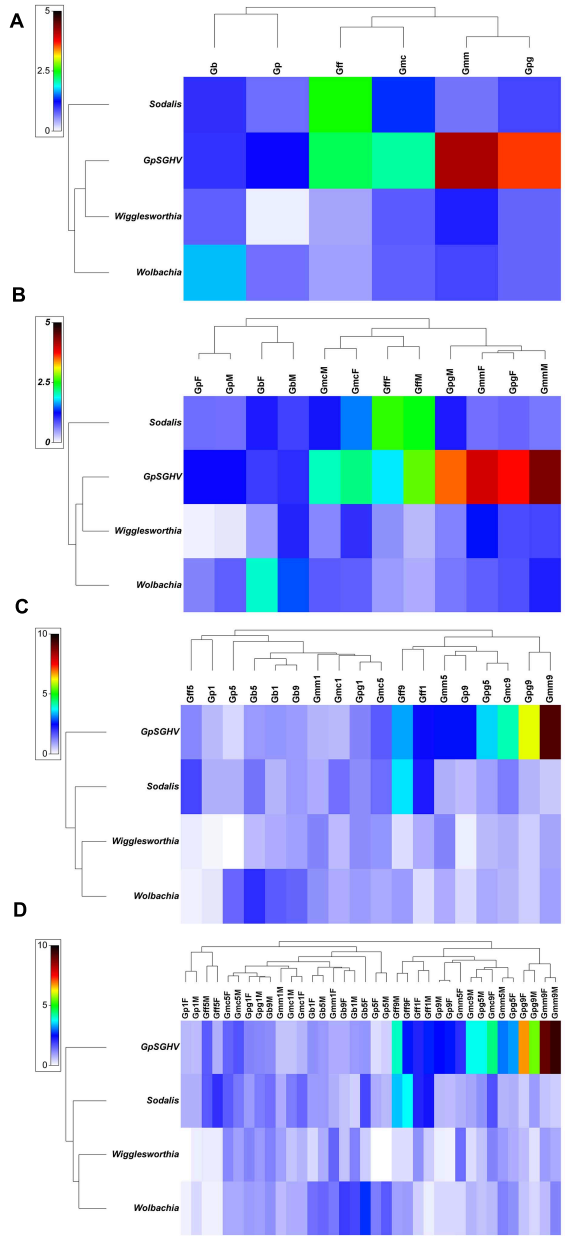

FIGURE 3 | Relative density of GpSGHV, *Wigglesworthia*, *Sodalis* and *Wolbachia* in six taxa of tsetse flies. Tsetse adults were injected with GpSGHV and the density of GpSGHV and tsetse symbionts were analyzed by qPCR and the data were normalized against control flies and zero time of injection. Data were transformed to square root and averaged based on tsetse species **(A)**, tsetse species and sex **(B)**, tsetse species and time post GpSGHV injection **(C)**, and tsetse species, time post GpSGHV injection and sex **(D)**. Gb, *Glossina brevipalpis*; Gff, *G. f. fuscipes*; Gmc, *G. m. centralis*; Gmm, *G. m. morsitans*; Gp, *G. pallidipes*; Gpg, *G. p. gambiensis*. Time post GpSGHV injection: 1, 5, and 9 dpi. F: female, M: male.

DISCUSSION

In an attempt to improve our knowledge of GpSGHV infection, we investigated in the present study the relationship between GpSGHV infection and the three main bacterial symbionts of tsetse under laboratory rearing conditions. The demonstration of the virus infection and its density was previously investigated (Demirbas-Uzel et al., 2018b) and the impact of the virus infection on tsetse symbionts and the interaction between GpSGHV and symbionts was investigated in this study. The main findings can be summarized as follows: (a) the presence of the GpSGHV in artificially infected laboratory populations affects the densities of the three bacterial symbionts in a species-dependent manner while, in general, sex does not seem to play an important role; (b) with the exception of *G. fuscipes* where the high density of the GpSGHV was found in the presence of high density of *Sodalis*, there seems to be a negative correlation between GpSGHV and tsetse symbionts in all taxa; and (c) it seems that *G. pallidipes* have the lowest *Wigglesworthia* density compared to the other taxa. These findings suggest some possible explanations for the observed host range and variation in pathogenicity of the virus.

Wigglesworthia is a key factor for tsetse productivity and was found in all tested flies in the colonized species but

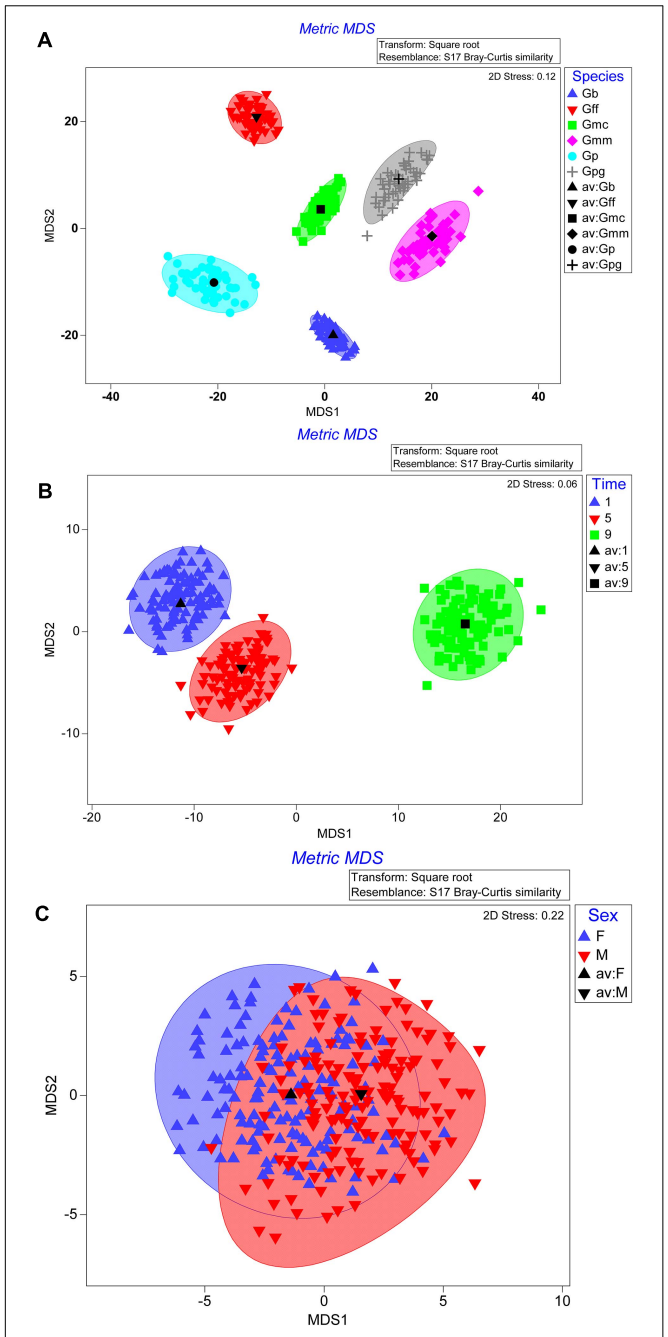

FIGURE 4 | Metric multidimensional scaling (MMDS) of GpSGHV, *Wigglesworthia, Sodalis* and *Wolbachia* relative denisty with originations from tsetse species **(A)**, time post GpSGHV injection **(B)**, or sex **(C)**.

with significant variation in the density. Similar to our results, *Wigglesworthia* has been detected in all field-collected samples that have been analyzed to date (Wang et al., 2013). Further, *Wig*Ds were found to be higher than the densities of *Sodalis* throughout all developmental stages in *G. p. gambiensis* (Soumana et al., 2013), which agrees with our results. Interestingly, in *G. f. fuscipes* and *G. g. gambiensis* infected with GpSGHV, *Wig*D was significantly reduced over time which might explain the result reported by Demirbas-Uzel et al. (2018c), which indicated a significant reduction in the flies performance in GpSGHV infected flies as compared with the control. In the same direction, the lowest *Wig*D was observed in *G. pallidipes,* a tsetse species which suffers most from the GpSGHV infection. These results support previous reports demonstrating the importance of *Wigglesworthia* for tsetse productivity and performance either by nutrition supplement or its immunological function in tsetse (Wang et al., 2009; Weiss et al., 2011). This might suggest that having low *Wig*D in *G. pallidipes* might compromise the flies' immune system making it more vulnerable for GpSGHV infection and the development of SGH symptoms. An alternative explanation could be that the presence of GpSGHV reduces available nutritional resources required for *Wigglesworthia*'s multiplication leading to lower density. Our results in *G. f. fuscipes* also support the hypothesis that GpSGHV infection is associated with a significant reduction in *Wigglesworthia* density, which leads to a significant reduction in the flies' productivity and performance (Demirbas-Uzel et al., 2018c), although SGH symptoms, similar to the ones observed in *G. pallidipes*, are not developed most likely due to low GpSGHV density (Abd-Alla et al., 2009a). The same hypothesis may be valid for *G. p. gambiensis* too; however, its validation will require the monitoring of *Wig*D in *G. f. fuscipes* and *G. p. gambiensis* for a longer period (beyond 9 dpi).

The increase in *Sodalis* densities over time in the flies injected with GpSGHV in *G. f. fuscipes* (significant increase) and *G. brevipalpis* (not significant) are in agreement with previous data reported by Demirbas-Uzel et al. (2018a), where *Sodalis* density increased in both males and females over time post emergence in a *G. m. morsitans* colony. However, the significant reduction in the *Sodalis* density in *G. p. gambiensis* and the non-significant decrease in *G. m. morsitans*, *G. m. centralis* and *G. pallidipes* contradicts the previously reported results of Demirbas-Uzel et al. (2018a). The increase of *Sodalis* density in *G. f. fuscipes* might be due to: (a) the reduction in *Wigglesworthia* density compromising the flies' immune system and providing *Sodalis* with space and biological resources for multiplication and (b) the fact that the GpSGHV did not represent a competitive factor as its density remained below the density needed to develop SGH. However, the significant decrease in *Sod*D in *G. p. gambiensis* in GpSGHV infected flies was associated with a significant decrease in *Wig*D, which might indicate a different molecular mechanism governing the interaction between the endosymbiont, the GpSGHV and the host, most likely due to the fact that the GpSGHV infection in *G. p. gambiensis* is different from that in *G. f. fuscipes* (Demirbas-Uzel et al., 2018b). This also might be due to different strains of *Sodalis*, *Wigglesworthia* or GpSGHV circulating in the different tsetse species (Doudoumis et al., 2012; Kariithi et al., 2013).

Our results on the variations in the prevalence of *Sodalis* in the *Glossina* species is in accordance with previously reported data. For instance, prevalence of *Sodalis* was reported to be 93.7, 17.5, and 1.4% in wild-caught *G. brevipalpis*, *G. m. morsitans* and *G. pallidipes*, respectively (Wang et al., 2013; Dennis et al.,

2014). Further, *Sodalis* prevalence varied widely (0–85%) across different wild-caught tsetse species (Wang et al., 2013). Although *Sodalis* lacks clearly defined functional roles in tsetse, it is interesting to note that in our study, the densities of this symbiont were significantly reduced in the virus-injected *G. p. gambiensis* compared to the controls. Another previous study showed that high *Sodalis* densities increased susceptibility of *G. morsitans* and *G. austeni* to pathogen (trypanosome) infection (Welburn et al., 1993; Dale and Welburn, 2001). In view of these previous findings, our results showing higher densities of *Sodalis* in virus-injected *G. f. fuscipes* could indicate an association between *Sodalis* and GpSGHV, however, the lack of increase in *Sod*D in *G. pallidipes* did not support some thoughts that *Sodalis* favors the establishment of GpSGHV infections in tsetse. The positive correlation between *Sodalis* and GpSGHV density in *G. f. fuscipes* only might be due to a different *Sodalis* strain circulating in *G. f. fuscipes* that coexists with GpSGHV at high density. In addition, the possibility that the GpSGHV injection induced a latent infection of a different strain of SGHV circulating in *G. f. fuscipes* should not be excluded.

*Wol*D varied significantly between tsetse taxa and the GpSGHV infection lead to a significant increase in *Wol*D in *G. f. fuscipes* and a significant reduction in *G. p. gambiensis*, whilst there was no significant alteration in other tsetse taxa. The molecular mechanism governing the dynamics of *Wolbachia* infection might be the same as for *Sodalis* (outlined above). These results confirm that tsetse flies, although they share many aspects of their biology, remain different in their interaction with bacterial symbionts and pathogens and the molecular mechanism for each species should be elucidated. Moreover, the interactions between symbionts, pathogens and their host are complex and remain to be uncovered.

Furthermore, *Wol*D was reported to differ not only between different tsetse host species but also between different populations within the same tsetse species (Alam et al., 2012; Doudoumis et al., 2012). Notably, in a previous investigation on the prevalence and co-infection dynamics among *Wolbachia,* GpSGHV and trypanosomes in wild-caught *G. f. fuscipes* (Alam et al., 2012), two findings were reported. First, in agreement with our findings, *Wolbachia* densities in wild *G. f. fuscipes* were at least 20-fold lower than in lab-bred *G. m. morsitans*. Since we found *Wolbachia* in all tested taxa, our results and the previous results imply that lab-bred flies may have higher *Wolbachia* densities than wild flies. The second finding was the negative correlation between the prevalence of GpSGHV infections and *Wolbachia* densities; our results appeared to agree with this since the high GpSGHV infections in *G. f. fuscipes* were accompanied by low *Wolbachia* titers over the 9 day period (except 0 dpi where the symbiont density were unexpectedly high).

The screening for coinfection with GpSGHV and each of the tsetse symbionts (*Wigglesworthia, Sodalis* and *Wolbachia)* in laboratory reared tsetse flies clearly indicated that the virus infection density in the tested tsetse species are independent. However, it seems that the virus infection might be controlled by tsetse symbionts regardless of tsetse taxa. Although coinfection can exist with low densities of both tsetse symbionts and GpSGHV, this does not exclude an antagonistic effect at higher density. Our data clearly show the absence of GpSGHV infection with high relative density in the presence of high density *Wigglesworthia* and *Wolbachia* infection. Moreover, a trend was also observed in *Sodalis* with the exception of the case of *G. f. fuscipes* where a high density of both GpSGHV and *Sodalis* was found. This might demonstrate the negative impact of *Wolbachia* on GpSGHV infection, in agreement with previous reports suggesting that the negative impact of *Wolbachia* on insect viruses is density dependent (Lu et al., 2012; Parry et al., 2019). The negative effect of *Wolbachia* on different RNA viruses has been well documented in mosquitoes and *Drosophila* (Hedges et al., 2008; Teixeira et al., 2008; Johnson, 2015) and this may be achieved by: (a) reducing virus replication and limiting virus transmission (Mousson et al., 2012); (b) supressing the expression of the DNA methyltransferase gene using a host microRNA to regulate its transcripts (Zhang et al., 2013); (c) activating antimicrobial peptides—defensins and cecropins (Pan et al., 2012) or (d) mediating antiviral activity, autophagy and iron metabolism and cholesterol and competition for the host cell (Rainey et al., 2014). In addition, there are several reports indicating that *Wolbachia* might enhance virus infection for both RNA and DNA viruses (Graham et al., 2012; Dodson et al., 2014). The above-mentioned mechanism might explain the possible antagonistic effect between *Wolbachia* and GpSGHV infection, however, this does not explain the observed antagonistic effect between *Wigglesworthis* and *Sodalis* (in most tsetse taxa) and GpSGHV infection. One possible mechanism is the competition for nutritional resources. This mechanism might also explain the observed dominance of one bacterium over other tsetse symbionts.

Taken together, available data show that the insect-virus-symbiont interactions are very delicate and complex, which in most cases only leave room for speculations on the mechanisms that are involved. In addition, the molecular dialogue between the tsetse host, bacterial symbionts, pathogens and parasites seems to be species specific and should not be a subject for broad generalization. In this context, the competition for nutritional resources between the symbionts and GpSGHV, as well as the potential presence and role of multiple strains of symbionts and GpSGHV circulating in different tsetse species, need to be considered. The coinfection of *Wigglesworthia, Sodalis*, *Wolbachia* and GpSGHV in laboratory reared flies seems to indicate an antagonistic effect at higher density, but in the light of the complicated situation observed in laboratory flies after GpSGHV infection, the impact of other elements, i.e., other virus infection or symbionts such as *Spiroplasma* etc., also needs to be investigated. In addition, the interactions of tsetse symbionts and GpSGHV in wild collected samples need to be analyzed. The lack of comprehensive and integrated considerations of tsetse physiology and behavior after GpSGHV infection has greatly limited our understanding of what appears to be evolutionarily significant host-virus-symbiont interactions and their underlying mechanisms. Further investigations are required to explore this topic.

CONCLUSION

In summary, the results of the current study show that injecting the GpSGHV into tsetse flies leads to variable responses in *Wigglesworthia, Sodalis* and *Wolbachia* dependent on the taxon. This indicates that the interaction and molecular dialogue between the host and its symbionts, parasites and pathogens is a complicated and species-specific process. At high infection density *Wigglesworthia*, *Sodalis* (in most tsetse taxa except *G. f. fuscipes*) and *Wolbachia* was associated with relatively low GpSGHV density. The low density of *Wigglesworthia* in *G. pallidipes* might explain the severe impact of GpSGHV infection on this species. As tsetse symbionts are more prevalent in colonized tsetse flies and their density may increase over time, this might help tsetse species avoid GpSGHV infection and thereby ensure sustainable production of sterile males for the implementation of SIT programs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/X15PQF.

AUTHOR CONTRIBUTIONS

GD-U performed the experiments, analyzed the data, and drafted the manuscript. AA and VD performed the experiments and critically revised the manuscript. AP performed statistical analysis and critically revised the manuscript. GT critically revised the manuscript. KB conceived the study, designed the experiments, interpreted the data, contributed to the drafting, and critically revised the manuscript. AA-A conceived the study, designed the experiments, interpreted the data, and draft the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.653880/full#supplementary-material

Supplementary Figure 1 | Relative density of GpSGHV in six taxa of tsetse flies over days post injection. Transformed GpSGHV density for six taxa of tsetse flies injected with GpSGHV normalized against control flies. Total DNA was extracted from six females and two males at 1, 5, and 9 days post injection and used as template for qPCR. Data were normalized to the housekeeping β-*tubulin* gene, against time zero and against the control injected flies at each time point and transformed based on the Box-Cox lambda value. The linear regression line was calculated using the lm method in R.

Supplementary Figure 2 | Heatmap indicating the relative density of tsetse endosymbionts *Wigglesworthia* **(A)**, *Sodalis* **(B),** and *Wolbachia* **(C)** between males and females in adult tsetse flies of six species injected with GpSGHV.

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Gut Bacterial Diversity in Different Life Cycle Stages of *Adelphocoris suturalis* (Hemiptera: Miridae)

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Bacteria and insects have a mutually beneficial symbiotic relationship. Bacteria participate in several physiological processes such as reproduction, metabolism, and detoxification of the host. *Adelphocoris suturalis* is considered a pest by the agricultural industry and is now a major pest in cotton, posing a serious threat to agricultural production. As with many insects, various microbes live inside *A. suturalis*. However, the microbial composition and diversity of its life cycle have not been well-studied. To identify the species and community structure of symbiotic bacteria in *A. suturalis*, we used the HiSeq platform to perform high-throughput sequencing of the V3–V4 region in the 16S rRNA of symbiotic bacteria found in *A. suturalis* throughout its life stages. Our results demonstrated that younger nymphs (1st and 2nd instar nymphs) have higher species richness. Proteobacteria (87.06%) and Firmicutes (9.43%) were the dominant phyla of *A. suturalis*. At the genus level, *Erwinia* (28.98%), *Staphylococcus* (5.69%), and *Acinetobacter* (4.54%) were the dominant bacteria. We found that the relative abundance of *Erwinia* was very stable during the whole developmental stage. On the contrary, the relative abundance of *Staphylococcus*, *Acinetobacter*, *Pseudomonas*, and *Corynebacterium* showed significant dynamic changes at different developmental stages. Functional prediction of symbiotic bacteria mainly focuses on metabolic pathways. Our findings document symbiotic bacteria across the life cycle of *A. suturalis*, as well as differences in both the composition and richness in nymph and adult symbiotic bacteria. Our analysis of the bacteria in *A. suturalis* provides important information for the development of novel biological control strategies.

Keywords: 16S rRNA, microbial composition, symbiotic bacteria, pest control, life stages

INTRODUCTION

Insects are found almost everywhere in the world. Most insects carry symbiotic microorganisms that are involved in the life cycle processes of the host (Yong et al., 2017; Zhao et al., 2019). There is an interactive relationship between insects and symbiotic bacteria, which play an important role in the health, survival, and behavior of the host (Dillon and Dillon, 2004;

Kikuchi et al., 2007; Moran et al., 2008; Santos-Garcia et al., 2017). However, the vast majority of symbiotic microorganisms are concentrated in the intestines of insects, where they act as key regulators of the insect host's multiple lifestyles (including diet and ecological niche; Gupta and Nair, 2020). Studies have shown that symbiotic bacteria in the intestinal tract of insects can promote host food consumption and digestion; provide immunity and protection against various predators, pathogens, and parasites; control the success rate of host mating and reproduction; provide essential amino acids, metabolic compounds, and nutrients (Russell et al., 2014; Douglas, 2015; Arbuthnott et al., 2016; Wielkopolan and Obrępalska-Stęplowska, 2016; Engl and Kaltenpoth, 2018; Grenier and Leulier, 2020; Horak et al., 2020). In a recent study, the main roles of insect gut bacteria were demonstrated to be providing essential nutrients, followed by digestion and detoxification (Jing et al., 2020). Therefore, insects are highly dependent on intestinal symbiotic bacteria to complete their own growth and development. In short, a very complex and interesting relationship is formed between intestinal symbiotic bacteria and their hosts.

Hemiptera insects have piercing and sucking mouthparts, which not only can directly suck plant juice and kill crops, but also cause serious economic losses by spreading plant viruses. They are notorious agricultural pests (Wang et al., 2015; Liu et al., 2018; Qin et al., 2018). *Adelphocoris suturalis* was originally a minor pest of cotton, but the widespread application of *Bacillus thuringiensis* (Bt) cotton and the reduction of broad-spectrum insecticides has made it a major problem in cotton-growing areas in China (Wu et al., 2008; Lu and Wu, 2011; Luo et al., 2017a). Bt plants can effectively control Lepidopteran pests and can significantly reduce the use of chemical pesticides that are often required in conventional planting systems (Wu et al., 2008; Wang et al., 2018). A major challenge in planting Bt crops to control pests is that insects may evolve resistance to Bt (Wu et al., 2008). *Adelphocoris suturalis* is a typical omnivorous insect, which regularly feeds on crop plants (e.g., cotton and garden pea), other insects like aphids, and occasionally its weaker siblings (Lu and Wu, 2011; Luo et al., 2021). Additionally, *A. suturalis* also has the characteristic of high liquidity (Wu et al., 2010), resulting in poor chemical controls. At present, chemical pesticides such as organophosphates and pyrethroids are widely used in China to control these insects (Zhen et al., 2016). The question of whether these insects are resistant or not needs to be resolved urgently. There is increasing evidence that there is a link between symbiotic bacteria in the insect gut and the evolution of drug resistance (Broderick et al., 2006; Kikuchi et al., 2012; Engel and Moran, 2013; Xia et al., 2013). In addition, the omnivorous nature of *A.suturalis* increases the difficulty of pest control. Previous studies have reported that some symbiotic bacteria help insect hosts form new feeding habits, thus expanding food sources and enhancing the adaptability of insects to the environment (Lu et al., 2001; Douglas, 2009), which may also increase the difficulty of *A.suturalis* control. There are currently few studies examining the symbiotic bacteria of *A. suturalis*, making the number and species of the symbiotic bacteria in *A. suturalis* uncertain (Luo et al., 2021; Ma et al., 2021). Therefore, it is necessary to investigate the distribution of symbiotic bacteria at different stages of *A. suturalis*, which will provide a framework for exploring the function of symbiotic bacteria and pest control in *A. suturalis*.

In this study, the bacterial community composition and relative abundance of 1st instar to 5th instar *A. suturalis* nymphs and 1, 6, and 9 days male and female adults of *A. suturalis* were investigated *via* high-throughput Illumina sequencing of the 16S rRNA gene. To identify new pest control strategies, we explored the cooperative coevolution of *A. suturalis* and its symbiotic bacteria to better understand how *A. suturalis* relates to the symbiotic bacteria community structure, to examine the symbiotic bacteria related to *A. suturalis*, and to provide a theoretical basis for revealing a series of principles such as its resistance regulation mechanism and feeding characteristics.

MATERIALS AND METHODS

Insect Rearing and Maintenance

The *A. suturalis* used in this study were collected from the field in Wuhan (Hubei Province, China). *Adelphocoris suturalis* strains were maintained in climate chambers (75 ± 5% relative humidity, 26 ± 2°C temperature and a 16:8 h, light:dark cycle) and fed green beans and a 5% sugar solution (Lu et al., 2008). *Adelphocoris suturalis* started feeding cotton aphid from the third instar nymph. Cotton aphids are reared on non-transgenic cotton seedlings, living in the same environment as *A. suturalis*.

Sampling and DNA Extraction

DNA was extracted from whole insects (1st instar nymph, 2nd instar nymph, 3rd instar nymph, 4th instar nymph, 5th instar nymph, and 1, 6, and 9 days male and female adults) using MagPure Stool DNA KF kit B (Magen, China) according to the manufacturer's instructions. Six biological replicates were set for samples at each developmental stage (females and males were counted as two treatments). Insects were rinsed three times in distilled sterile water prior to DNA extraction (without soaking in ethanol). DNA was quantified using a Qubit Fluorometer with a Qubit dsDNA BR Assay kit (Invitrogen, United States) and the quality was checked by performing an aliquot on 1% agarose gel.

Library Construction

The variable regions V3–V4 of the bacterial 16S rRNA gene were amplified with the degenerate PCR primers 341F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTA CHVGGGTWTCTAAT-3'). Both forward and reverse primers were tagged with Illumina adapter, pad, and linker sequences. PCR enrichment was performed in a 50 μl reaction containing a 30 ng template, a fusion PCR primer, and a PCR master mix. PCR cycling conditions were as follows: 94°C for 3 min, 30 cycles of 94°C for 30 s, 56°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products were purified with AmpureXP beads and eluted in an Elution buffer. Libraries were qualified by the Agilent 2100 bioanalyzer (Agilent, United States). The validated libraries were used for sequencing

on an Illumina HiSeq platform (BGI, Shenzhen, China) according to standard Illumina procedures, and generated 2 × 300 bp paired-end reads. The sequences obtained in this study were deposited in the GenBank short-read archive (SRA), accession number PRJNA662509.

Sequencing and Bioinformatics Analysis

The data obtained from independent sequencing were analyzed separately. Samples were marked as follows: ZL1: 1st instar nymph; ZL2: 2nd instar nymph; ZL3: 3rd instar nymph; ZL4: 4th instar nymph; ZL5: 5th instar nymph; ZM1D: adult male eclosion at 1 day; ZF1D: adult female eclosion at 1 day; ZM6D: adult male eclosion at 6 days; ZF6D: adult female eclosion at 6 days; ZM9D: adult male eclosion at 9 days; and ZF9D: adult female eclosion at 9 days.

Raw reads were filtered to remove adaptors and low-quality and ambiguous bases, and paired-end reads were added to tags using the Fast Length Adjustment of Short reads program (FLASH, v1.2.11; Magoc and Salzberg, 2011) to obtain the tags. The tags were clustered into operational taxonomic units (OTUs) with a cutoff value of 97% using UPARSE software (v7.0.1090; Edgar, 2013) and we used UCHIME (v4.2.40) and Gold database for chimera sequence alignment and detection (Edgar et al., 2011). OTU representative sequences were then taxonomically classified using the Ribosomal Database Project (RDP) Classifier v.2.2 with a minimum confidence threshold of 0.6, and trained on the Greengenes database v201305 by QIIME v1.8.0 (Caporaso et al., 2010). The USEARCH_global (Edgar, 2010) was used to compare all tags to obtain an OTU statistical abundance table for each sample. Alpha and beta diversity were estimated by MOTHUR (v1.31.2; Schloss et al., 2009) and QIIME (v1.8.0; Caporaso et al., 2010), respectively, at the OTU level. Principal component analysis (PCA) in OTUs was plotted with the R package "ade4." The difference in alpha diversity among groups was compared using Kruskal-Test, with values of $p \leq 0.05$ considered statistically significant (*, $0.01 < p \leq 0.05$; **, $0.001 < p \leq 0.01$; ***, $p \leq 0.001$). We used PICRUSt to obtain the KO corresponding to the OTU through the greengene ID corresponding to each OTU, and calculated the abundance of the KO from the sum of the abundances of the OTU corresponding to the KO. We calculated the abundance of each functional category based on the information in the KEGG database and the OTU abundance information. In addition, PICRUSt was used to obtain the levels of metabolic pathway information, and the abundance of each level was obtained.

Phylogenetic Analysis of the *Erwinia* and *Acinetobacter*

In order to explore the phylogenetic relationship of the two most abundant bacterial genera, we compared the *Erwinia* and *Acinetobacter* DNA sequences obtained by high-throughput sequencing in the NCBI nucleotide (nr) database. Six 16S rRNA fragments belonging to *Erwinia* and 16 16S rRNA fragments belonging to *Acinetobacter* were downloaded from GenBank to construct a phylogenetic tree. The phylogenetic tree analysis of 249 base pairs was carried out. Using MEGA7.0, the phylogenetic tree was constructed by Neighbor-joining method (1,000 bootstraps).

RESULTS

General Description of 16S rRNA Gene Sequencing Results

The bacteria of *A. suturalis* were analyzed by Illumina HiSeq *via* the sequencing of the 16S rRNA gene. We obtained a total of 800,836 raw reads and 707,474 clean reads, with an average length of 296 bp. Based on 97% species similarity, we clustered the spliced tags into OTU. The number of OTUs at each developmental stage is detailed in **Table 1**. We constructed dilution curves for Ace, Chao1, Shannon, Simpson, Good's Coverage and Observed species, which demonstrated the quality and credibility of sequencing quantity (**Supplementary Figure 1**). Good's coverage of all samples was above 99%, indicating that our sequencing results were sufficient to fully estimate the diversity of *A. suturalis* bacterial community (**Table 1**).

Nymphal Microbiota

Nymphs had higher species richness in ZL1 and ZL2 periods, and were significantly higher than other periods (**Table 1**; **Figure 1A**). Proteobacteria and Firmicutes were the dominant

TABLE 1 | 16S rRNA gene sequencing data.

Sample	Number of reads	Mean length	Number of OTUs	Chao1	ACE	Shannon	Simpson	Good's coverage
ZL1	64,316	296.50	589	200.42	204.28	2.46	0.20	0.99
ZL2	64,317	294.83	483	163.68	168.73	1.99	0.24	0.99
ZL3	64,288	296.33	162	61.75	66.73	0.90	0.60	0.99
ZL4	64,325	296.67	147	60.47	63.19	1.02	0.53	0.99
ZL5	64,338	296.83	151	61.28	68.76	1.04	0.52	0.99
ZM1D	64,291	295.67	103	47.67	48.84	0.82	0.54	0.99
ZF1D	64,299	296.83	98	45.81	47.63	0.80	0.59	0.99
ZM6D	64,286	297.33	127	63.06	69.44	1.21	0.41	0.99
ZF6D	64,313	296.67	130	64.41	66.10	1.23	0.39	0.99
ZM9D	64,300	295.67	114	60.38	64.43	1.17	0.41	0.99
ZF9D	64,401	296.67	118	61.75	65.94	1.26	0.38	0.99

ZL1: 1st instar nymph; ZL2: 2st instar nymph; ZL3: 3st instar nymph; ZL4: 4st instar nymph; ZL5: 5st instar nymph; ZM1D: adult male eclosion for 1 day; ZF1D: adult female eclosion for 1 day; ZM6D: adult male eclosion for 6 days; ZF6D: adult female eclosion for 6 days; ZM9D: adult male eclosion for 9 days; and ZF9D: adult female eclosion for 9 days.

phyla during the nymphal stage, and their relative abundances were 85.57% (average value across all of the samples at nymphal stage) and 11.34%, respectively (**Figure 1B**). We compared the changes of *A. suturalis* nymphs in different developmental stages at the genus level. As the nymphs grew, the microbial community changed significantly. There were 63 common OTUs classifications in the five developmental stages of nymphs, and each developmental stage had characteristic OTUs (**Figure 1C**). *Erwinia* (17.65%) (average value across all of the samples at the nymphal stage), *Staphylococcus* (9.83%), and *Acinetobacter* (9.83%) were the top three genera in relative abundance during the nymphal stages. *Erwinia* was the dominant genus of bacteria throughout the nymph period, and the microbial community was relatively stable without significant dynamic changes. However, the relative abundance of *Staphylococcus, Acinetobacter, Pseudomonas*, and *Corynebacterium* changed significantly (**Figure 1D**). The relative abundance of *Staphylococcus* increased significantly, the lowest in the ZL1 (4.98%) stage, and the highest in the ZL5 (13.98%) stage. The trends for *Acinetobacter, Pseudomonas*, and *Corynebacterium* were opposite to those of *Staphylococcus*. *Acinetobacter* had the highest abundance in the ZL1 (24.69%) stage, and then gradually decreased (**Figure 1D**).

Adult Microbiota

As in the nymph stage, the Proteobacteria (88.31%; average value across all of the samples at adult stage) and Firmicutes (7.84%) were the dominant phyla in the adult stage, and the species richness of each developmental stage was similar (**Figure 1B**). Similarly, we analyzed the bacterial communities of *A. suturalis* adults at different developmental stages at the genus level. *Erwinia* (38.42%; average value across all of the samples at adult stage) and *Lactococcus* (5.53%) were the dominant genus in the entire adult stage and their relative abundance was relatively stable, without significant dynamic changes (**Figure 2A**). Interestingly, although the relative abundance of *Corynebacterium* was low, it was significantly increased, reaching the highest in ZF9D (5.41%) during the adult stage. Compared with females and males, only *Staphylococcus* had a significant difference on the 6th day of adult development. The relative abundance of *Staphylococcus* ZM6D (6.90%) was

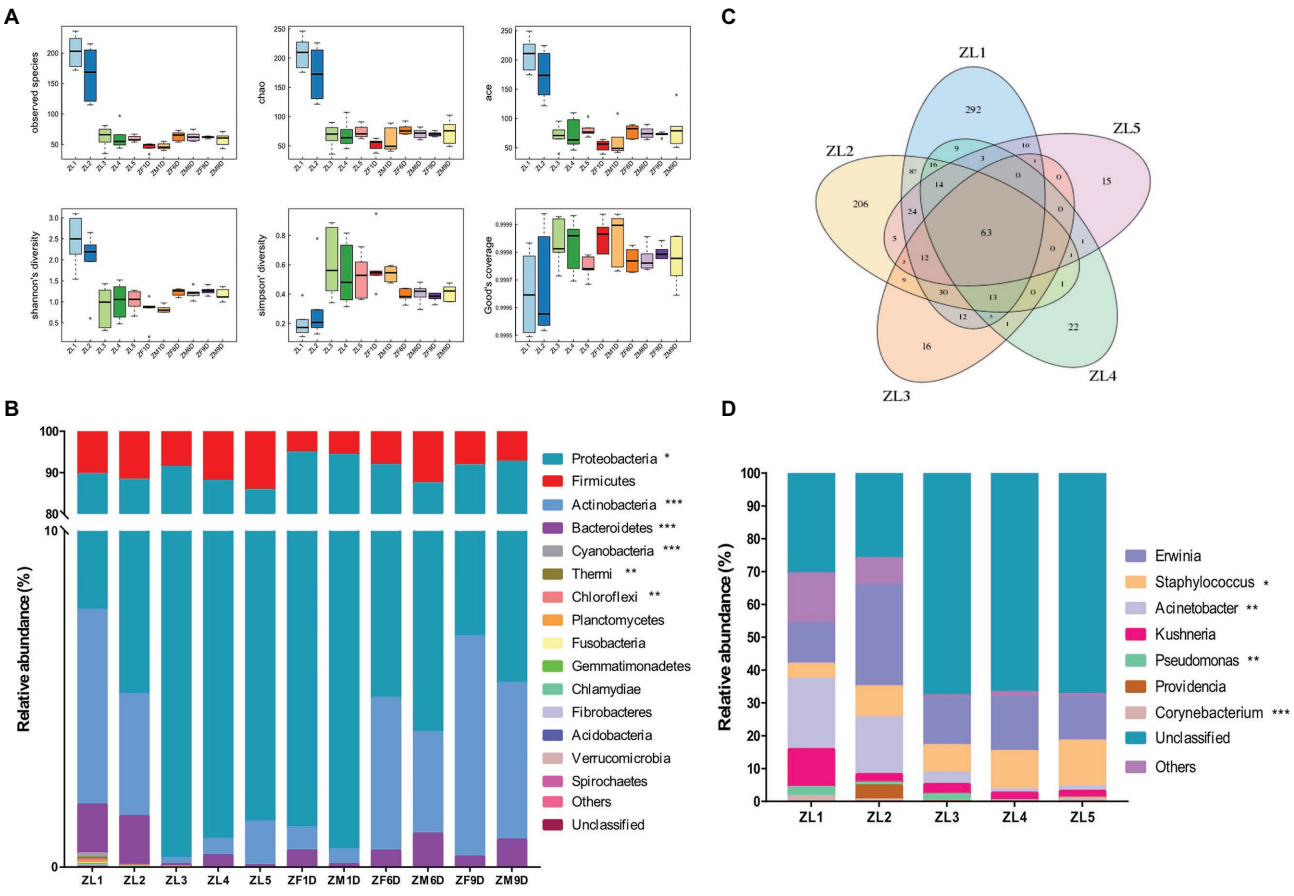

FIGURE 1 | Bacterial community dynamics among different developmental stages in *Adelphocoris suturalis*. **(A)** Boxplot of α-diversity measured by the six indexs. **(B)** Relative abundance of bacteria communities at the phylum level in different groups. **(C)** Venn diagram showing operational taxonomic unit (OTU) classification in nymphal period. **(D)** Relative abundance of bacteria communities at the genus level in nymph stages. (Bacteria with relative abundance lower than 0.5% in all samples were all merged into others. Kruskal-Wallis test, $*0.01 < p \leq 0.05$, $**0.001 < p \leq 0.01$, and $***p \leq 0.001$).

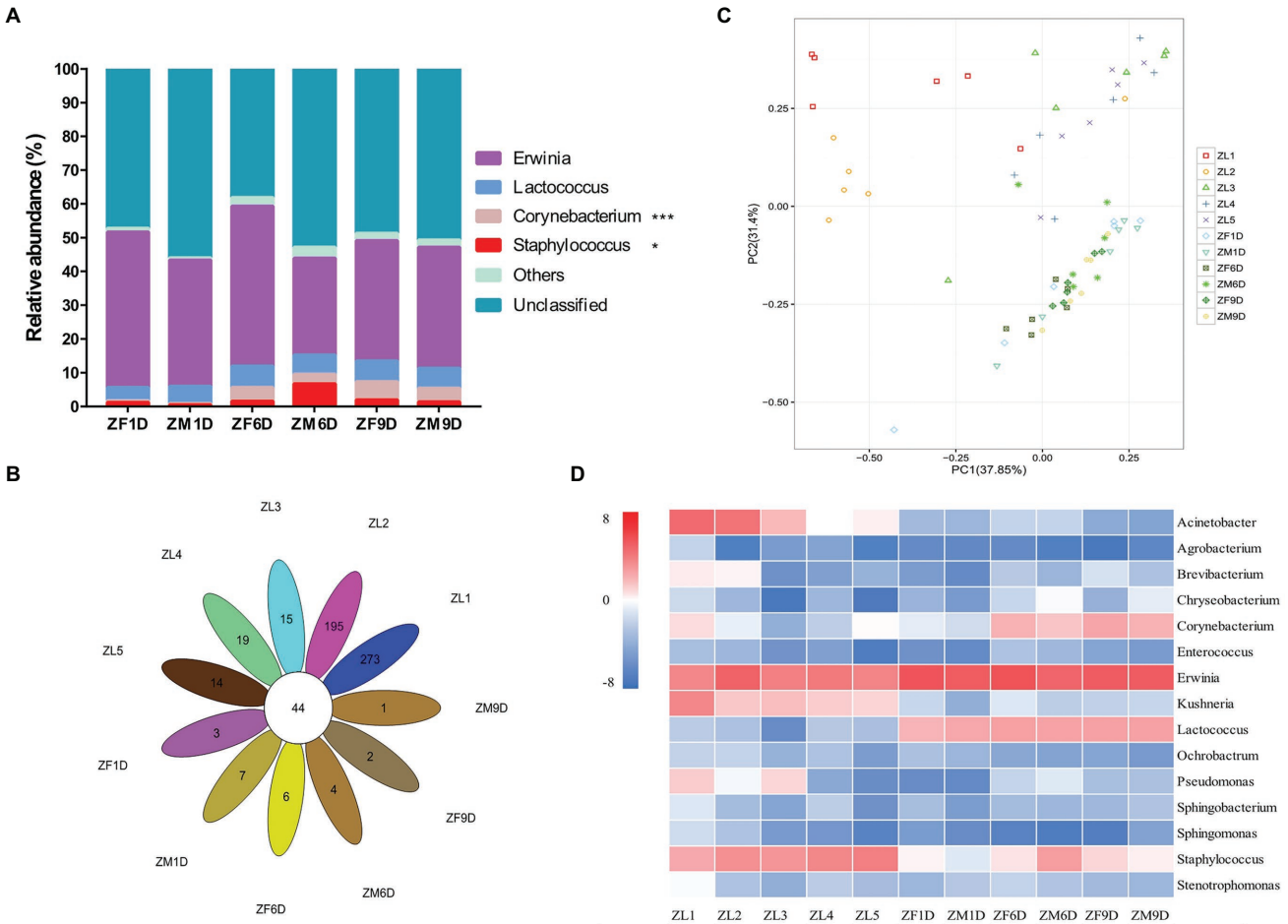

FIGURE 2 | Bacterial community dynamics among different developmental stages in *A. suturalis*. **(A)** Relative abundance of bacteria communities at the genus level in adult stages. **(B)** Core-Pan OTU presents the common and unique OTU of all samples in petal diagram. **(C)** Difference of OTU types in different developmental stages based on Principal Components Analysis (PCA). **(D)** Heat map analysis of the top 15 microbial populations with relative abundance at different developmental stages. The data represented by color in the figure is represented by log2(relative abundance). Bacteria with relative abundance lower than 0.5% in all samples were all merged into others (Kruskal-Wallis test, $*0.01 < p \leq 0.05$ and $***p \leq 0.001$).

significantly higher than that of ZF6D (1.55%), and there was no significant difference in other periods (**Figure 2A**).

Comparisons of Bacterial Communities From Different Life Stages

Species richness of ZL1 and ZL2 was the highest (**Figure 1A**), with 44 common OTU species in all samples, and the unique OTU species of ZL1 and ZL2 was also the highest (**Figure 2B**). PCA analysis demonstrated that the OTUs of *A. suturalis* in different development stages showed dispersion and aggregation. The degree of dispersioncan show whether the sample composition under the same conditions is similar (**Figure 2C**). Each point on the PCA graph represented a stage of development; the closer the distance, the more similar the composition.

At the phylum level, taxonomic analysis of all samples showed that Proteobacteria was the most prevalent phylum. Among all samples, the top three phyla with the highest relative abundance were Proteobacteria, Firmicutes, and Actinobacteria, accounting for 87.06, 9.43, and 2.8% (average value across all of the samples), respectively. Proteobacteria had the highest abundance in adult stage compared with nymph stage, with the highest relative abundance in ZM1D (93.77%) stage of adult stage (**Figure 1B**). Throughout the different development stages of *A. suturalis*, the relative abundance of Proteobacteria increased significantly in the nymph stage, and in ZL3 (91.15%) the relative abundance of the period was the highest. After the first day of adult emergence, the relative abundance of the Proteobacteria showed a decreasing trend (**Figure 1B**). Compared with Proteobacteria, the change trend of Actinobacteria was the opposite. The relative abundance of Actinobacteria decreased significantly in the nymph stage, and then increased significantly from the first day of adult emergence. The relative abundance of Proteobacteria and Actinobacteria at 6 days (Proteobacteria, ZF6D: 86.86%, ZM6D: 83.39%; Actinobacteria, ZF6D: 4.55%,

and ZM6D: 3.01%) and 9 days (Proteobacteria, ZF9D: 84.92%, ZM9D: 87.17%; Actinobacteria, ZF9D: 6.57%, and ZM9D: 4.67%) of adult stage was similar to that at nymph ZL1 (Proteobacteria: 81.97%; Actinobacteria: 5.80%) stage. Firmicutes were also ubiquitous in each developmental stage, and their relative abundance was relatively stable without significant change.

We selected the bacterial genera with the top 15 abundance ratios and drew heat maps based on their relative abundance at different developmental stages (**Figure 2D**; **Supplementary Table 1**). From the overall distribution of the microbial community at different developmental stages of *A. suturalis*, *Erwinia* (28.98%; average value across all of the samples) was still the dominant genus (**Figure 2D**; **Supplementary Table 1**). *Acinetobacter*, *Kushneria*, and *Staphylococcus* were relatively abundant in the nymph stage, and the number was very small in the adult stage. The abundance ratio of *Lactococcus* during the adult stage was significantly higher than that of the nymph, and the relative abundance during the nymph stage was all below 0.3% (**Figure 2D**).

Function Prediction and Phylogenetic Relationship Analysis

Based on the predicted results of KEGG function, we showed the pathway abundance at two levels (level 1 and level 2). In level 1, metabolism accounted for about 41.61–47.01% at each developmental stage, followed by environmental information processing (16.46–21.61%) and genetic information processing (15.02–16.02%; **Figure 3A**). In level 2, we showed the richness of the top 20 pathways, and the other pathways are classified as Others (**Figure 3B**). The relative abundance of membrane transport (13.89–18.64%) was the highest at different developmental stages. In addition, pathways related to metabolism account for the vast majority, and carbohydrate metabolism, amino acid metabolism, energy metabolism, metabolism of cofactors and vitamins, nucleotide metabolism, lipid metabolism were abundantly enriched. The phylogenetic tree indicated the developmental relationship between *Erwinia* and *Acinetobacter*, the two important genera in our study, and the more closely related genera (**Figure 4**).

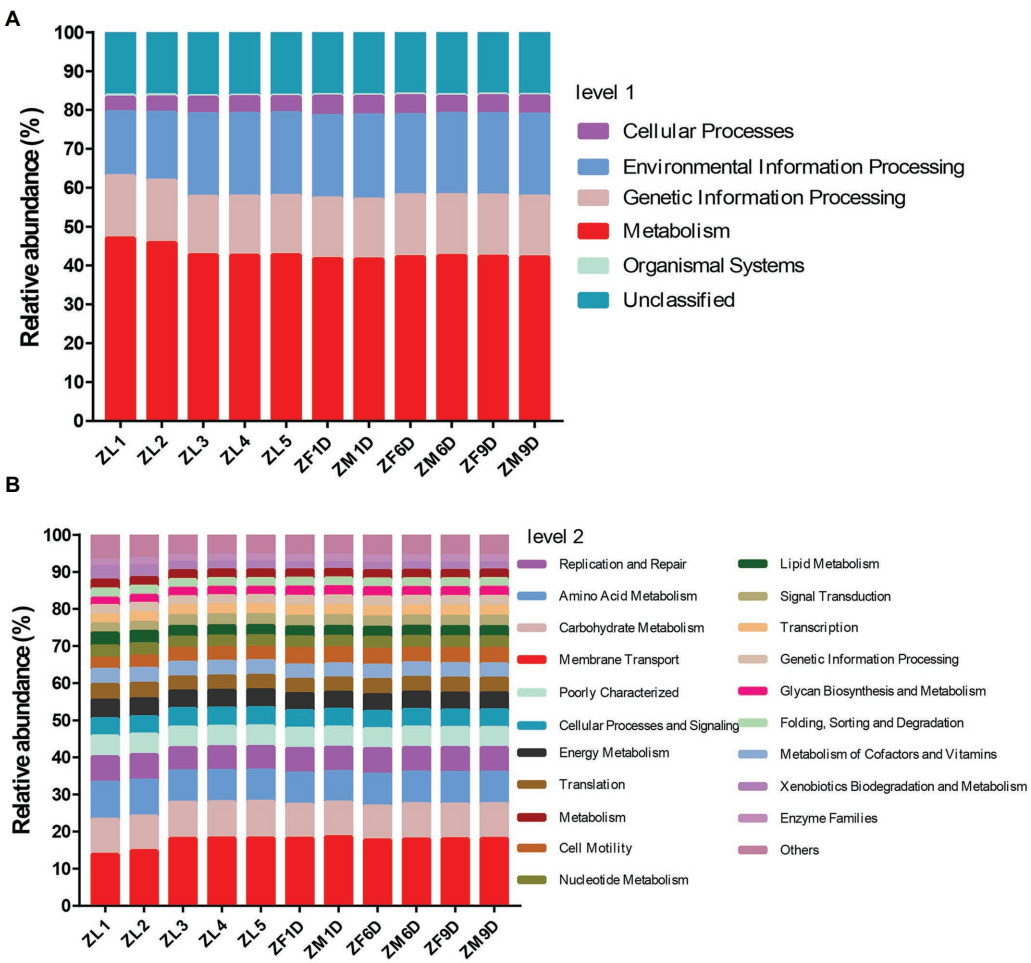

FIGURE 3 | Prediction of functional pathway abundance at different developmental stages. **(A)** Function prediction based on Level 1. **(B)** Function prediction based on Level 2. In level 2, pathways ranked below 20 in total relative abundance were classified as "Others."

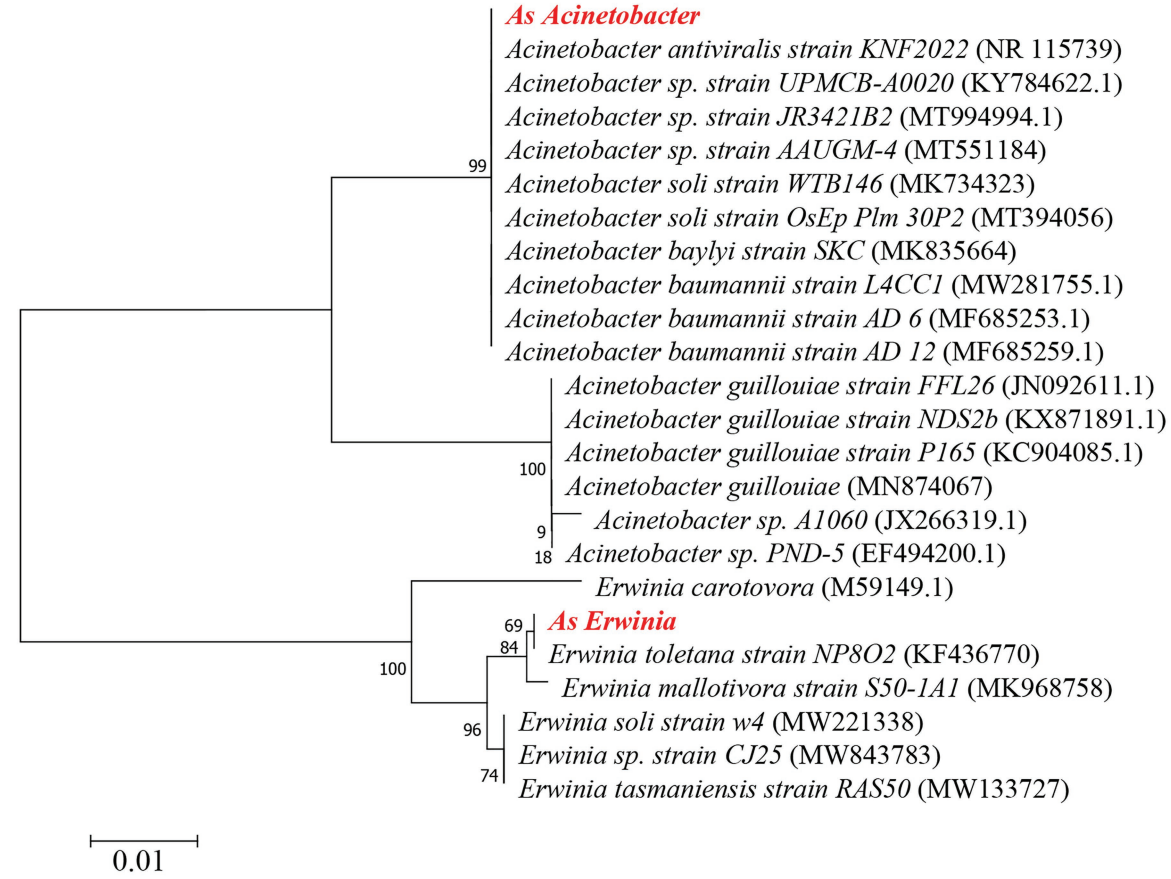

FIGURE 4 | Phylogenetic analysis of *Erwinia* and *Acinetobacter*. *As Erwinia*: *A. suturalis Erwinia*, *As Acinetobacter*: *A. suturalis Acinetobacter*. The phylogenetic tree was made by MEGA7.0 software and constructed by the Neighbor-joining method. The number in parentheses indicates the GeneBank accession number of the 16S rRNA gene sequence.

DISCUSSION

Most insects contain a large number of symbiotic bacteria (Bosch and McFall-Ngai, 2011; Herzner et al., 2013; Ruffner et al., 2013; Marchesi et al., 2016). Although the microbial diversity of insects has been extensively studied, most of the research has focused on insect gut microbes (Roh et al., 2008; Hulcr et al., 2012; Salem et al., 2013), and there are few reports on the dynamic changes of microbial diversity and species richness in different developmental stages of insects. However, the vast majority of insect symbiotic bacteria are concentrated in the intestinal tract, which is rich in symbiotic bacterial communities (Gupta and Nair, 2020). Similarly, although our study detected the microbial community of the entire insect body, intestinal microbes accounted for almost all of them (Campbell et al., 2018; Karimi et al., 2019; Rossitto De Marchi and Smith, 2020). These bacteria and host insects have formed an interdependent relationship during the co-evolution process (Gao et al., 2019). However, we have not found any endosymbionts in *A. suturalis*, although endosymbionts have been widely reported in other Hemipterans (Campbell et al., 2018; Karimi et al., 2019; Rossitto De Marchi and Smith, 2020).

Interestingly, it can be observed from the aspect of microbial diversity that the first and second instar microbial community diversity of newly hatched nymphs is the highest, and its unique OTU species are also abundant. From the third instar nymph, the diversity of the microbial community began to decline. It is well known that bacterial diversities vary from field collected to lab-reared (Rani et al., 2009) as well as across different geographical regions (Zouache et al., 2011). In addition, the composition and diversity of bacteria are affected by the *in vitro* environment (Zouache et al., 2011) and artificial feed (Priya et al., 2012). It has been confirmed in *Helicoverpa armigera* and *Lymantria dispar* that food sources have a great influence on the microbial diversity of insects (Broderick et al., 2004; Priya et al., 2012). *Adelphocoris suturalis* began to prey on cotton aphid from the third instar nymph stage, which may be one of the reasons for the changes in microbial diversity, or it may be that the nymphal microbial community becomes highly simplified through the development of the host insect. Starting from the third instar nymph stage, the alpha diversity decreased significantly (**Figure 1A**), and diet is the best explanation for this effect. This is consistent with the results of recent studies on the influence of *A. suturalis*

diet on microbial diversity (Luo et al., 2021). An increase in nutrient richness (such as protein quality) may lead to a decrease in alpha diversity, although refined diets are also associated with increased species richness (Erkosar et al., 2018; Kešnerová et al., 2020). PCA analysis also confirmed the difference of species diversity mentioned above. During the transition period from fifth instar nymph to adult, there was no significant change in alpha diversity, because *A.suturalis* is an incomplete metamorphosis insect. Nymphs and adults have small changes in their living environment, feeding habits, and food sources, which lead to changes in the intestinal bacterial community (Engel and Moran, 2013; Hammer et al., 2017; Luo et al., 2021). Complete metamorphosis involves complex structural changes, which leads to significant changes in microbial diversity from egg to adult (Chen et al., 2016; Hammer et al., 2017; Zhao et al., 2019).

We also found that Proteobacteria and Firmicutes dominated across the entire life cycle, which was similar to findings in other Hemipteran insects (Husseneder et al., 2017; Lim and Ab Majid, 2020), each of which contained a different proportion of microbes depending on the species or sample (**Figure 1B**). Similar results have been found in other insects (Chandler et al., 2011; Colman et al., 2012; Engel et al., 2012). These phyla are often listed as the most abundant bacterial communities associated with insect taxa (Colman et al., 2012; Thomas et al., 2013; Yun et al., 2014; Kim et al., 2017). Firmicutes and Proteobacteria are key to maintaining the growth and development of insects during the metabolism of secondary metabolites in host plants (Dillon and Charnley, 2002).

Erwinia in the phylum Proteobacteria is a genus of dominant bacteria in the nymphal and adult stages, a member of the Gram-negative Enterobacteriaceae family (Basset et al., 2000), and is a type of intestinal bacteria. Throughout the development cycle of *A. suturalis*, the *Erwinia* population was stable and continuous, with high abundance, indicating that *Erwinia* plays a lasting symbiotic role in the growth, development, and survival of *A. suturalis*. *Erwinia* can metabolize most sources of nitrogen, sulfur, and phosphorus (Friedl et al., 2008), and is thus an important microbial species in the intestinal tract of insects. *Adelphocoris suturalis* is a highly omnivorous insect (Luo et al., 2017b). *Erwinia* can enhance the adaptability of insect hosts to their plant hosts by regulating the diet of insect hosts (De Vries et al., 2004), which is critical for omnivorous insects. *Erwinia* have strong metabolic ability, which plays an important role in the digestion of food and body development of *A. suturalis*. Interestingly, *Erwinia* can secrete a variety of cell wall degrading enzymes, causing potato black leg disease, soft rot, fusarium wilt, and other plant diseases (Whitehead et al., 2002; Grenier et al., 2006). *Drosophila melanogaster* has been reported to be an important media for *Erwinia carotovora* (Vieira et al., 2020). The Hemiptera insect *Creontiades signatus* is a vector for the transmission of bacterial pathogenic bacteria *Serratia marcescens* (Bizio; Enterobacteriales: Enterobacteriaceae) that rots cotton seeds and bolls (Glover et al., 2020). Further research is needed to determine whether *A.suturalis* is a vector for the bacteria. *Acinetobacter* has a very high abundance in the nymph stage, but extremely low abundance in the adult stage. The vast majority of *Acinetobacter* bacteria have strong drug resistance (Li et al., 2021). In China, due to the large-scale planting of Bt crops, *A. suturalis* has risen from secondary pests in cotton fields to primary pests. Among them, Cry1, Cry2, and Cry9 toxins have been reported to show high insecticidal activity against lepidopteran pests (Palma et al., 2014; Silva et al., 2015). Once ingested by the susceptible insect larvae, these cry proteins (present in the form of protoxin) are proteolytically processed by midgut proteases to the active toxin that subsequently binds to specific protein receptors of the midgut epithelium leading to cell disruption and eventual death of the insect larvae (Pardo-López et al., 2013). The introduction of the intestinal isolate *Acinetobacter guillouiae* into *Plutella xylostella* significantly enhances its sensitivity to Bt Cry1Ac protoxin, and *Acinetobacter* plays an important role in the immune response of insects (Li et al., 2021). During the nymph period, its ability to resist the stimulation of external agents is relatively weak. It requires the support of symbiotic bacteria in the body to defend against unfavorable pesticide environments and Bt crops. We analyzed the developmental relationship of *Erwinia* and *Acinetobacter* with the aforementioned *E. carotovora* and *A. guillouiae* through phylogenetic tree development. *As Erwinia* and *Erwinia toletana strain NP8O2* are the most closely related in evolution. At the same time, *As Erwinia* and *E. carotovora* have 94.47% identity. Although *As Acinetobacter* and *A. guillouiae* are not on the same branch, they have 94.86% identity. *Staphylococcus* and *Lactococcus* are the two genera with the highest relative abundance in Firmicutes. The content of *Staphylococcus* increased significantly in the nymph stage, and the relative abundance was the highest in ZL5 (13.98%) stage, but it was scarce in adult stage. *Lactococcus* has a relatively high abundance in the adult stage and exists in large numbers in the intestines (De Jonge et al., 2020). This type of bacteria is known for fermenting complex molecular carbohydrates to produce lactic acid. *Lactococcus* is a lactobacillus of firmicutes with high abundance in both male and female adults, which can decompose sugar to produce organic acids, reduce the pH value of its environment, and defend against some acid-sensitive pathogenic bacteria (Evans and Armstrong, 2006). This ensures that *A. suturali* can obtain the nutrients it needs in the complex environment. Our functional prediction also confirmed that in different stages of development, metabolic function is the main, whether amino acid metabolism or carbohydrate metabolism, is very important for the survival of insects.

Our results showed that the dominant bacteria genera (*Erwinia*, *Acinetobacter*, *Staphylococcus*, and *Lactococcus*) of Proteobacteria and Firmicutes were mostly concentrated in the intestinal tract of insects. These bacteria played an important role in nutrient uptake and adaptability to the environment, and were directly related to the growth, development, and reproduction of the insects. We sequenced the *A. suturalis* 16S rRNA gene through Illumina HiSeq, which directly revealed the structure of the bacterial community in the life cycle of

A. suturali, predicted the biological functions of different bacterial communities, and provided a basis for further research on the role of bacteria in this and other insects. We provide a crucial theoretical basis for future research on *A. suturalis* symbiotic bacteria. These foundations can help formulate environmentally friendly management strategies for pest control and provide ideas for new pest control strategies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study cans be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA662509.

AUTHOR CONTRIBUTIONS

JC and XG: conceptualization and writing – review and editing. XG: methodology. HX, LN, and CW: software. JC: validation and funding acquisition. HX: formal analysis and writing – original draft preparation. HX and XZ: investigation. JL: resources. HX, JJ, and XG: data curation. LW and DL: visualization. KZ: supervision and project administration. All authors contributed to the article and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.670383/full#supplementary-material

Supplementary Table 1 | Relative abundance of bacteria communities at the genus level in different group (Top 15).

Supplementary Figure 1 | Alpha diversity dilution curve.

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A Case of Intragenic Recombination Dramatically Impacting the Phage WO Genetic Diversity in Gall Wasps

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The phage WO was characterized in *Wolbachia*, a strictly intracellular bacterium causing several reproductive alterations in its arthropod hosts. This study aimed to screen the presence of *Wolbachia* and phage WO in 15 gall wasp species from six provinces of southern China to investigate their diversity and prevalence patterns. A high incidence of *Wolbachia* infection was determined in the gall wasp species, with an infection rate of 86.7% (13/15). Moreover, seven species had double or multiple infections. All *Wolbachia*-infected gall wasp species were found to harbor phage WO. The gall wasp species infected with a single *Wolbachia* strain were found to harbor a single phage WO type. On the contrary, almost all species with double or multiple *Wolbachia* infections harbored a high level of phage WO diversity (ranging from three to 27 types). Six horizontal transfer events of phage WO in *Wolbachia* were found to be associated with gall wasps, which shared identical *orf7* sequences among their respective accomplices. The transfer potentially took place through gall inducers and associated inquilines infected with or without *Wolbachia*. Furthermore, 10 putative recombination events were identified from *Andricus hakonensis* and *Andricus* sp2, which harbored multiple phage WO types, suggesting that intragenic recombination was the important evolutionary force, which effectively promoted the high level of phage WO diversity associated with gall wasps.

Keywords: Cynipidae, gall wasp, horizontal transfer, multiple infections, phage WO, recombination, *Wolbachia*

INTRODUCTION

Wolbachia are maternally inherited endosymbiotic bacteria belonging to the family Anaplasmataceae that infect arthropods and filarial nematodes (Werren, 1997; Stouthamer et al., 1999). The symbiont is extremely widespread among arthropods, and probably infects about half of all terrestrial arthropod species (Hilgenboecker et al., 2008; Zug and Hammerstein, 2012; Weinert et al., 2015). *Wolbachia* manipulates its host's reproduction by inducing several phenotypes, such as cytoplasmic incompatibility, parthenogenesis, feminization of genetic males, and male-killing (Werren et al., 2008). Bacterial viruses (bacteriophages or phages) are the most abundant organisms in the biosphere and constitute a significant force in bacterial

genome evolution (Hendrix et al., 1999; Bordenstein and Wernegreen, 2004). *Wolbachia* phage particles were first observed in the *Wolbachia* infection of *Culex pipiens* by Wright et al. (1978). Subsequently, Masui et al. (2000, 2001) characterized the phage WO, a λ phage-like temperate phage, from the *Wolbachia* strain wTai, infecting *Teleogryllus taiwanemma*. They indicated that the phage WO could be either lysogenic and integrated into the *Wolbachia* chromosome, or lytic and free in the cytoplasm. As a consequence of reductive evolution, mobile DNA elements have often been shown to be rare or absent from obligate intracellular bacteria (Moran and Plague, 2004; Bordenstein and Reznikoff, 2005). However, polymerase chain reaction (PCR) amplification of the minor capsid gene *orf7* showed that the phage WO infected about 90% of supergroups A and B of *Wolbachia* from various arthropod groups (Bordenstein and Wernegreen, 2004; Gavotte et al., 2007). Moreover, nearly all sequenced *Wolbachia* genomes, except those acting as obligate mutualists, harbored prophage WO (Gavotte et al., 2007; Kent and Bordenstein, 2010; Metcalf and Bordenstein, 2012). Considering the wide distribution of *Wolbachia*, phage WO might be one of the most abundant phage lineages in arthropods.

Phage WO is believed to be a dynamic element having a significant impact on the genomic evolution of *Wolbachia* (Wu et al., 2004). As in other prokaryotes, the integration and transformation of prophage are considered major sources of *Wolbachia* lateral gene acquisition (Bordenstein et al., 2006). Phage WO can mediate lateral gene transfer between *Wolbachia* strains, regardless of whether the transferred genes originate from *Wolbachia* or other unrelated bacteria (Ishmael et al., 2009; Wang et al., 2016). They can also regulate the numbers of their host bacteria by inhibiting their replication or inducing cell lysis (Bordenstein et al., 2006). Furthermore, several studies have suggested that phage WO possibly is crucial in *Wolbachia*-induced cytoplasmic incompatibility in insect hosts (Saridaki et al., 2011; LePage et al., 2017; Shropshire et al., 2018). Mutation, recombination, and genome segment reassortment during replication might mediate genetic changes in viruses (Domingo, 2010). A phage genome can be divided into functional units or modules (each one responsible for head or tail formation, lysis, lysogeny, and so forth), which can be mixed by segment reassortment with other phages (Hatfull, 2008). Insertion sequences are frequently found in phage WO genomes and are considered to be a major factor driving these recombinations (Wu et al., 2004; Klasson et al., 2009). The nucleotide sequence of the minor capsid gene *orf7* from the wKueA1 strain of *Wolbachia* is chimeric, and the population genetic analysis has confirmed the occurrence of intragenic recombination events (Bordenstein and Wernegreen, 2004). Furthermore, based on metagenomic analysis, Bordenstein and Bordenstein (2016) demonstrated that genes with eukaryotic homology were constituents of the phage WO, implying lateral gene transfers between bacteriophage/prophage and animal genomes. However, the molecular evolution of phage WO has received far less attention compared with the impact on their bacterial host genome evolution.

Gall wasps (Cynipidae) are a phytophagous group of the superfamily Cynipoidea (Hymenoptera), which usually form structurally complex plant galls on different plant organs. They are the second most species-rich group of gall inducers after the gall midges (Diptera: Cecidomyiidae), with about 1,400 described species (Ronquist et al., 2015). In addition to true gall formers, the Cynipidae also include phytophagous inquilines, which live inside the galls of other species. Several studies have revealed *Wolbachia* infection in diverse cynipid species with high infection rates (Plantard et al., 1998; Abe and Miura, 2002; Rokas et al., 2002; Zhu et al., 2007; Yang et al., 2013; Hou et al., 2020; Zhao et al., 2021), and some gall wasp species show multiple *Wolbachia* infections (Yang et al., 2013; Hou et al., 2020). The results of Yang et al. (2013) suggested a potential possibility of plant tissue-mediated *Wolbachia* horizontal transmission between gall inducers and their associated inquilines. However, no study has reported about phage WO harboring in *Wolbachia*-infected gall wasps. The larvae of gall wasps (sometimes including associated inquilines) feed in completely closed galls. The unique living environment provides a good model to study the transmission and molecular evolution of phage WO within communities. Thus, in this study, the presence of phage WO in 15 gall wasp species collected from six provinces in southern China was detected by employing a PCR-based method with phage WO-specific gene markers so as to determine the phage WO diversity and infection patterns within *Wolbachia*-infected gall wasps. Furthermore, the effects of intragenic recombination and horizontal transmission on phage WO diversity and evolutionary dynamics were also explored.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

The galls of gall wasps were collected from six provinces in southern China during 2012–2020 (**Table 1**). The galls collected were cage-reared at room temperature in the laboratory of CSUFT. Adult gall wasps were preserved directly in 100% ethanol at −80°C within 2–7 days after emergence until DNA extraction.

Adult gall wasps were picked randomly, and total genomic DNA was extracted from each insect using the phenol-chloroform extraction method as described in a previous study (Zhu et al., 2007). The insects were washed with sterile water before DNA extraction to avoid surface contamination. The DNA was resuspended in sterile water and stored at 4°C. This study aimed to screen the quality of each genomic DNA template using nuclear ribosomal DNA internal transcribed spacer 2 gene (Partensky and Garczarek, 2011) and mitochondrial cytochrome coxidase1 (*cox1*) gene (Dyer et al., 2011) using PCR. Poor quality DNA templates were discarded.

PCR and Sequencing

The samples were first screened for *Wolbachia* infection by PCR amplification. Two primers wsp-81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and wsp-691R (5′-AAAAAT TAAACGCTACTCCA-3′) were used to amplify a portion of the *Wolbachia* surface protein (*wsp*) gene (Zhou et al., 1998). If the amplification failed, another two pairs of primers were used to verify the *Wolbachia* infection:

ftsZ-F/R for amplification of the *Wolbachia* cell division gene and 16SwolF/R for amplification of the *Wolbachia* 16S RNA gene (O'Neill et al., 1992; Jeyaprakash and Hoy, 2000). WO was screened using the primers WO-F (5′-CCCACATGAGCCAATGACGTCTG-3′) and WO-R (5′-CGTTCGCTCTGCAAGTAACTCCATTAAAAC-3′) to amplify a portion of the capsid protein gene *orf7* (Masui et al., 2000). ddH_2O was used as a blank control for all amplifications to avoid cross-contamination. The reaction mixture was composed of 1 μL of PrimeSTAR HS DNA Polymerase (Takara Biomedical Technology Co., Ltd, Dalian, China), 10 μL of buffer, 4 μL of dNTPs, 1 μL of each primer, and 2 μL of DNA with water added to achieve a total volume of 50 μL. The amplification was conducted using a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, United States). The cycling conditions were 98°C for 3 min, 35 cycles of 98°C for 10 s, 50°C–57°C for 30 s, and 72°C for 1 min.

Subsequently, 2.5 μL of the PCR products were run on a 0.8% agarose gel, and electrophoresis was performed using 1× TAE buffer. The gels were stained with GelRed and observed using a gel imaging system. The PCR products were subsequently purified using a TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver. 4.0 (Takara Biomedical Technology Co.), and the *wsp* and *orf7* gene fragments were directly sequenced from purified PCR products using PCR primers. The appearance of multiple peaks in a sample at initial sequencing was taken as an indication of multiple infections. The PCR products were then purified using a DNA gene gel extraction kit and ligated directly into the vector, following the manufacturer's protocols. For each sample, 15–40 independent positive colonies were isolated and cultured in a lysogeny broth medium fortified with ampicillin. Plasmids were extracted and partially sequenced in both directions using an ABI 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, United States) with M13F/R at Wuhan Icongene Co., Ltd.

Raw Sequence Treatments

Sequence homology analysis was first performed using the BLAST[1] program online. Genetic distances between all sequence pairs were calculated using Kimura 2-parameter distance model in MEGA 7. Sequences having greater than 1.5% nucleotide diversity in the *orf7* gene were defined as different haplotypes (Chafee et al., 2010). Different sequences were reserved and identical sequences were removed, yielding *orf7* sequences. The sequences have been deposited in GenBank under the following accession numbers: MW98182–MW980306.

Phylogenetic Analysis

The *orf7* sequences were aligned to relevant sequences previously published on NCBI[2] with ClustalW in BioEdit (Hall, 1999). Maximum likelihood (ML) was carried out to construct the phylogenetic tree using IQ-Tree 2.1.1 via the online CIPRES Science Gateway portal (Miller et al., 2010). Model selection for the ML analysis was estimated using the Model test v3.7. ML bootstrap values were generated from 1000 bootstrap replicates, under the general time-reversible (GTR) model in which the gamma distribution and invariant sites were estimated from the data (GTR + I + G).

Recombination Analysis

The individual segment alignments were analyzed using different methods described in the Recombination Detection Program (RDP5) package to detect the evidence of intragenic recombination (Heath et al., 2006). The six recombination detection methods implemented in the RDP5 program for the identification of recombinant sequences and breakpoints were as follows: 3Seq (Martin and Rybicki, 2000), BootScan/rescan recombination test (Martin et al., 2005), GENECONV (Padidam et al., 1999), MaxChi (Smith, 1992), Chimaera (Posada and Crandall, 2001), and the Siscan method (Gibbs et al., 2000). The default settings were used for all methods, and the highest acceptable *P*-value cutoff was set to 0.05.

RESULTS

Wolbachia and Phage WO Infection Patterns

The galls were collected from southern China, and adult gall wasps of 15 species were obtained. Among these, three species were inquilines (*Synergus* sp1-3), which did not make galls of their own and lived as nest parasites in the galls made by other gall-inducing hosts. Using the diagnostic PCR approach with the *wsp* gene and the phage minor capsid protein gene (*orf7*)-specific primers, a total of 770 wasps of all species obtained for *Wolbachia* and WO infections were screened. The results are listed in **Table 1**.

Furthermore, 13 out of 15 gall wasp species were infected with *Wolbachia*, and the infection rate of these species was 86.7%. The population infection rates of *Wolbachia*-infected gall wasps ranged from 25 to 100%. Among these, six species were infected with a single *Wolbachia* strain, while the other seven species had double or multiple infections. The samples were tested for *Wolbachia* infection by PCR using specific primers for the *ftsZ* and 16S RNA genes to further confirm that *Latespina jinzhaiensis* and *Cerroneuroterus* sp. were *Wolbachia*-free. The results were all negative.

All *Wolbachia*-infected gall wasp species were found to harbor phage WO. The population infection rates of phage WO ranged from 20 to 100%. Interestingly, the gall wasp species infected with a single *Wolbachia* strain (*Dryocosmus zhuili*, *Dryocosmus liui*, *Aphelomyx glanduliferae*, and *Synergus* sp1–3) were found to harbor a single phage WO type. However, almost all species (including different geographical populations) with double or multiple *Wolbachia* infections harbored diverse phage WO. *Andricus* sp1 was an exception, which had multiple *Wolbachia* infections but carried only one phage type (**Table 1**). No phage WO was detected in *L. jinzhaiensis* and *Cerroneuroterus* sp. not infected with *Wolbachia*. Although a 273-bp *orf7* sequence was obtained from six insects in the Jinzhai and Wuhan populations of *L. jinzhaiensis*, and the first 217 bp at the 3′-end shared 83% identity with the normal phage WO types, it was a non-coding pseudogene (accession no.: MW980306).

[1]https://blast.ncbi.nlm.nih.gov/Blast.cgi

[2]https://www.ncbi.nlm.nih.gov/

TABLE 1 | Sample information and infection frequency of *Wolbachia* and Phage WO in gall wasps.

Host plant	Location (code)	Latitude, longitude	Insect species	*Wolbachia* infect frequency (%)	WO infect frequency (%)	WO type number	Individuals screened
Castanea henry	Qingyuan, Zhejiang (QY)	27°73′N, 119°25′E	*Dryocosmus zhuili*	100 (single)	100	1	20
	Zhenghe, Fujian (ZH)	27°38′N, 118°86′E	*D. zhuili*	100 (single)	100	1	40
	Zhouning, Fujian (ZN)	27°21′N, 119°33′E	*D. zhuili*	100 (single)	100	1	60
Castanopsis tibetana	Yanling, Hunan (YL)	26°48′N, 114°04′E	*Dryocosmus liui*	100 (single)	100	1	30
Quercus fabri	Jinzhai, Anhui (JZ)	31°64′N, 115°97′E	*Andricus hakonensis*	100 (multiple)	100	9	16
	Changsha, Hunan (CS)	28°21′N, 112°89′E	*A. hakonensis*	100 (multiple)	100	13	40
			Synergus sp1	100 (single)	100	1	4
	Suichang, Zhejiang (SC)	28°62′N, 119°31′E	*A. hakonensis*	100 (multiple)	100	18	36
	Qingyuan, Zhejiang (QY)	27°73′, 119°25′E	*A. hakonensis*	100 (multiple)	100	7	40
	Zhouning, Fujian (ZN)	27°21′N, 119°33′E	*A. hakonensis*	100 (multiple)	100	15	48
Quercus fabri	Wuhan, Hubei (WH)	30°51′N, 114°52′E	*Andricus* sp1	100 (multiple)	100	1	12
			Synergus sp1	100 (single)	100	1	3
Quercus fabri	Jinzhai, Anhui (JZ)	31°64′N, 115°97′E	*Andricus* sp2	100 (multiple)	100	2	6
			Synergus sp1	100 (single)	100	1	3
	Taihu, Anhui (TH)	30°56′N, 116°07′E	*Andricus* sp2	100 (multiple)	100	7	8
	Wuhan, Hubei (WH)	30°51′N, 114°52′E	*Andricus* sp2	100 (multiple)	100	3	6
	Changsha, Hunan (CS)	28°00′N, 113°01′E	*Andricus* sp2	100 (multiple)	100	8	14
			Synergus sp1	100 (single)	100	1	4
Quercus fabri	Changsha, Hunan (CS)	28°00′N, 113°01′E	*Andricus* sp3	62.5 (multiple)	50	4	8
Cyclobalanopsis glauc	Yanling, Hunan (YL)	26°48′N, 114°04′E	*Plagiotrochus masudai*	100 (two)	90	3	20
Quercus fabri	Jinzhai, Anhui (JZ)	31°64′N, 115°97′E	*Aphelomyx glanduliferae*	40 (single)	40	1	20
	Shucheng, Anhui (SHC)	31°35′N, 116°91′E	*A. glanduliferae*	25 (single)	none		4
	Changsha, Hunan (CS)	28°00′N, 113°01′E	*A. glanduliferae*	50 (single)	none		2
Quercus variabili	Jinzhai, Anhui (JZ)	31°64′N, 115°97′E	*Latuspina jinzhaiensis*	none	none		40
	Taihu, Anhui (TH)	30°56′N, 116°07′E	*L. jinzhaiensis*	none	none		20
	Wuhan, Hubei (WH)	30°51′N, 114°52′E	*L. jinzhaiensis*	none	none		20
Quercus chenii	Changsha, Hunan (CS)	28°13′N, 113°00′E	*Latuspina* sp1	46.7 (multiple)	20	7	30
	Taihu, Anhui (TH)	30°56′N, 116°07′E	*Latuspina* sp1	40 (multiple)	30	5	20
Quercus variabilis	Changsha, Hunan (CS)	28°13′N, 113°00′E	*Latuspina* sp2	80 (two)	60	3	10
Quercus variabilis	Jinzhai, Anhui (JZ)	31°64′N, 115°97′E	*Cerroneuroterus* sp.	none	none		20
Quercus fabri	Jinzhai, Anhui (JZ)	31°64′N, 115°97′E	*Synergus* sp2	100 (single)	100	1	8
	Taihu, Anhui (TH)	30°56′N, 116°07′E	*Synergus* sp2	90 (single)	90	1	10
	Changsha, Hunan (CS)	28°00′N, 113°01′E	*Synergus* sp2	86.6 (single)	86.6	1	30
	Guiding, Guizhou (GD)	26°61′N, 107°23′E	*Synergus* sp2	100 (single)	100	1	6
	Qingyuan, Zhejiang (QY)	27°73′N, 119°25′E	*Synergus* sp2	80 (single)	60	1	10
	Zhouning, Fujian (ZN)	27°21′N, 119°33′E	*Synergus* sp2	75 (single)	62.5	1	16
Quercus fabri	Jinzhai, Anhui (JZ)	31°64′N, 115°97′E	*Synergus* sp3	100 (single)	100	1	24
	Taihu, Anhui (TH)	30°56′N, 116°07′E	*Synergus* sp3	100 (single)	100	1	16
	Wuhan, Hubei (WH)	30°51′N, 114°52′E	*Synergus* sp3	100 (single)	100	1	6
	Changsha, Hunan (CS)	28°00′N, 113°01′E	*Synergus* sp3	100 (single)	95	1	40

Phage WO Diversity and Typing

Phage types with similarity on *orf7* DNA sequences larger than 98.5% were defined as identical types according to a previous study (Chafee et al., 2010). For a single *Wolbachia* strain-infected wasp species (*D. zhuili*, *D. liui*, *A. glanduliferae*, and *Synergus* sp1–3) and one species *Andricus* sp1 with multiple *Wolbachia* infections, completely identical *orf7* sequences were obtained from different individuals or/and populations. They harbored only one phage WO type (**Table 1**). On the contrary, other gall wasp species infected with multiple *Wolbachia* strains harbored phage WO types with a high level of diversity (**Figure 1** and **Supplementary Figure 1**). A total of 493 *orf7* sequences were obtained from five geographical populations of *Andricus hakonensis*, which could be divided into 27 types, WOAha-1–27. Types WOAha-1, WOAha-4, WOAha-15, WOAha-21, and WOAha-26 were found from all five populations, with an abundance rate of 45.8, 9.5, 10.3, 7.3, and 13.0%, respectively. Several types were found with only one *orf7* sequence from one population (**Figure 1A**). The 318 *orf7* sequences obtained from the four populations of *Andricus* sp2 belonged to 10

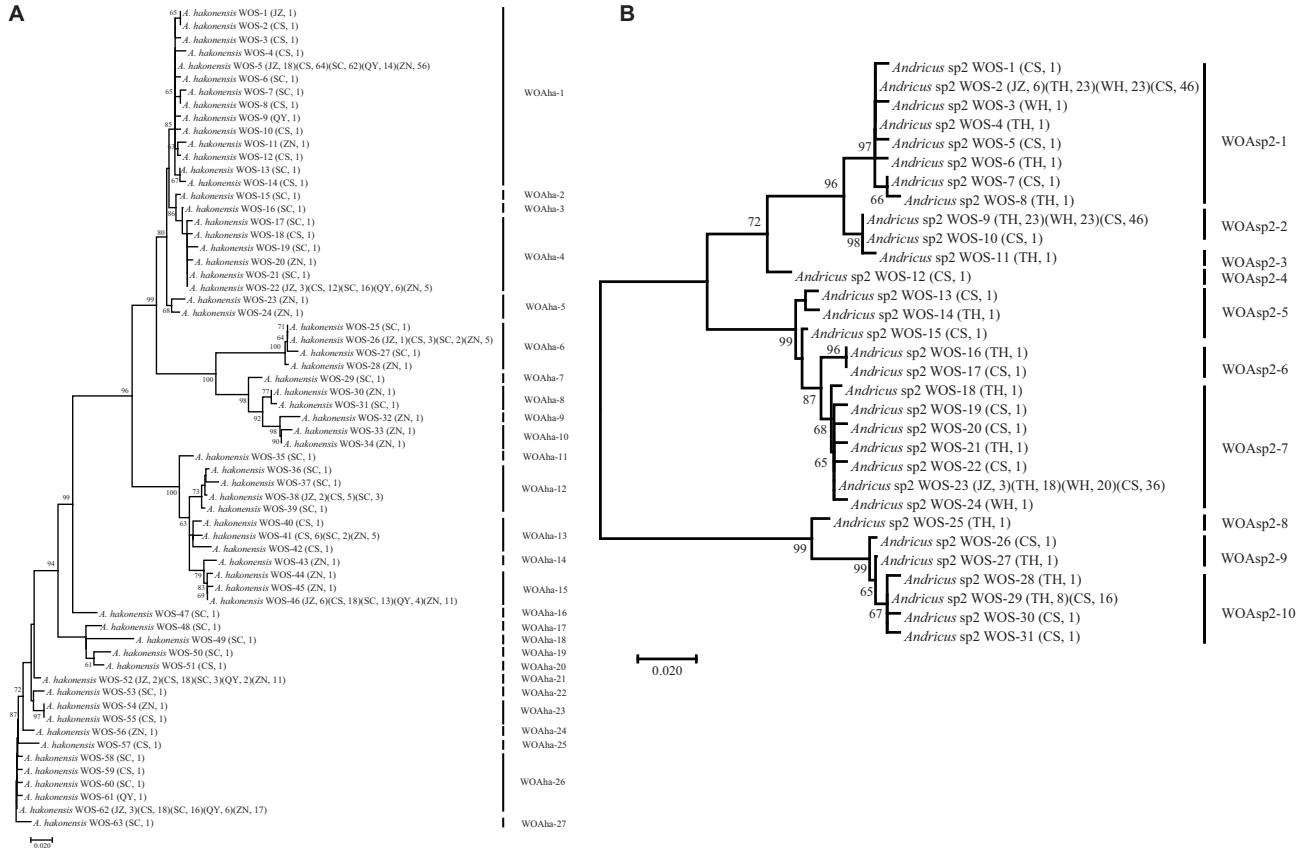

FIGURE 1 | Maximum likelihood tree for phage WO types of *Andricus hakonensis* **(A)** and *Andricus* sp2 **(B)** based on the *orf7* sequences. The letters in parentheses indicate the sampled populations shown in **Table 1**, and the numbers indicate the number of sequences obtained per population. WOS-1 refers to the serial number. Phage WO types are shown on the right. Numbers above branches are bootstrap values computed from 1,000 replications.

phage types, WOAsp2-1–10. Among these, types WOAsp2–1, WOAsp2–2, WOAsp2–7, and WOAsp2–10 were detected from two to four populations, and they accounted for 33.0, 29.2, 26.1, and 8.5% of total sequences, respectively (**Figure 1B**). Furthermore, *Plagiotrochus masudai*, *Latuspina* sp1, *Latuspina* sp2, and *Andricus* sp3 harbored three, nine, three, and four WO types, respectively (**Supplementary Figure 1**).

Phage WO Phylogeny and Horizontal Transfer

Phylogenetic reconstruction of phage WO *orf7* sequences from gall wasps and other reference insect species was performed using ML methods (**Figure 2**). According to the phylogenetic tree, the phage WO types clustered into five distinct clades, labeled as groups I–V. The average *orf7* nucleotide divergence levels within and between groups were estimated using the Jukes and Cantor method. The intragroup value was 4.7, 5.1, 7.8, 4.8, and 4.6% in groups I, II, III, IV, and V, respectively, and the intergroup value was 11.9 ± 3.2% (mean ± standard deviation) in average. The groups I–III included the representative phage WO types of their groups retrieved from the gene bank, and the phage WO types from gall wasps tested in the present survey were divided into groups I–V. Although groups IV and V contained ungrouped known types, phage WO from gall wasps could be clustered into new independent branches, which were distinct from groups I–III. Phage WO types of single type harbored in gall wasps belonged to group I (WODli, WOSsp1–3), III (WDzh), IV (WOAsp1), and V (WOAgl). Different types of multiple-infection phage WO types harbored in the same insect species might belong to different groups, for example, those from *A. hakonensis* belonged to groups I, III, IV, and V; from *P. masudai* and *Andricus* sp2 belonged to groups III, IV, and V; from *Latuspina* sp1 belonged to groups I, II, and IV; from *Latuspina* sp2 belonged to groups I, III, and IV; and from *Andricus* sp3 belonged to groups I, II, and III.

No congruence was found between phage WO and its host *Wolbachia* phylogenies, suggesting that phages did not cospeciate with their hosts. Although the phylogenetic relationship between phages and its host *Wolbachia* was not compared, this study provided direct evidence for six horizontal transmission events of phage WO types from gall wasps: (1) WOAha-10 (from *A. hakonensis*), WOSsp1(Aha) (from *Synergus* sp1, inquiline, live inside the galls of *A. hakonensis*), WOSsp1(Asp1) (from *Synergus* sp1, inquiline), WOSsp1(Asp2) (from *Synergus* sp1, inquiline, live inside the galls of *Andricus* sp2), WOSsp2 (from *Synergus*

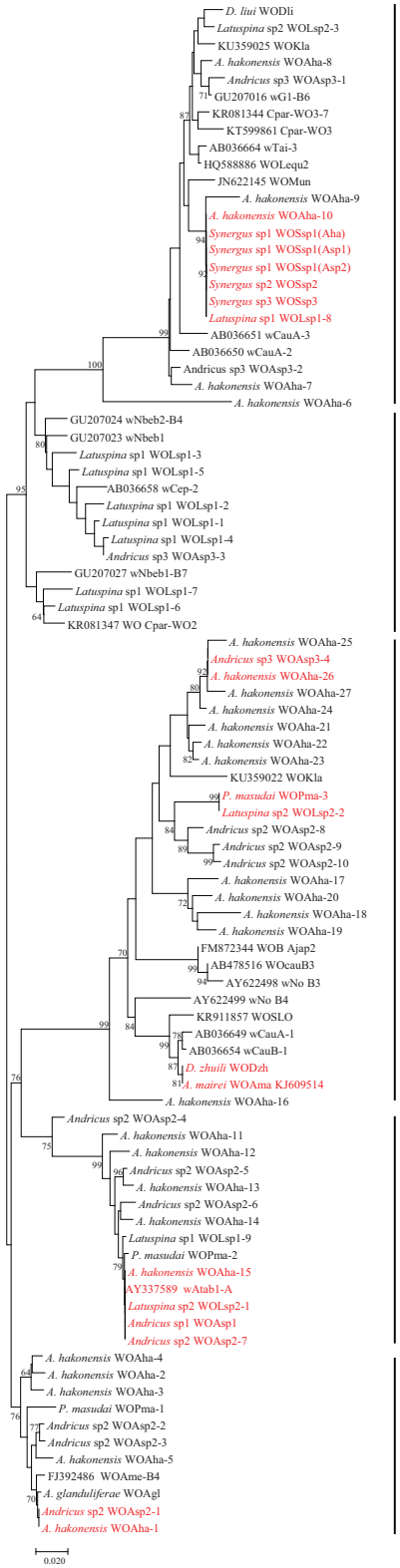

FIGURE 2 | Maximum likelihood phylogenetic tree of the phage WO *orf7* nucleotide sequences found in this study with known phage WO types. Numbers above branches are bootstrap values computed from 1,000 replications. Roman numerals on the right indicate different groups. Red font indicates identical *orf7* sequences among their respective accomplices.

sp2, inquiline), WOSsp3 (from *Synergus* sp3, inquiline), and WOLsp1-8 (from *Latuspina* sp1); (2) WOAsp3–4 (from *Andricus* sp3), and WOAha-26 (from *A. hakonensis*); (3) WOPma-3 (from *P. masudai*) and WOLsp2-2 (from *Latuspina* sp2); (4) WODzh (from *D. zhuili*) and WOAma (from *Andricus mairei*); (5) WOAha-15 (from *A. hakonensis*), WOLsp2-1 (from *Latuspina* sp2), WOAsp1 (from *Andricus* sp1), and WOAsp2–7 (from *Andricus* sp2); (6) WOAsp2–1 (from *Andricus* sp2) and WOAha-1 (from *A. hakonensis*), which shared identical *orf7* sequences among their respective accomplices (**Figure 2**).

Intragenic Recombination of *orf7*

The larvae of gall wasps fed in completely enclosed galls, and the occurrence of phage WO diversity in the closed niche provided an ideal sample for obtaining direct evidence of gene recombination. Recombination analysis of the aligned *orf7* sequences was performed using RDP5 programs to understand the extent to which recombination contributed to the diversification of phage WO in gall wasps. In this study, 10 putative recombination events were identified, resulting in new phage types, from *A. hakonensis* to *Andricus* sp2, which harbored diverse phage WOs (**Table 2**, **Figure 3** and **Supplementary Figures 2–5**).

In *A. hakonensis*, eight *orf7* gene recombination events were detected (**Table 2**). Recombinants were obtained in two ways; one major parent and one minor parent or one major parent and two minor parents were recombined into a new phage WO lineage. The former involved six recombination events, while the latter involved two. For example, recombination events between type WOAha-26 and WOAha-1 and among WOAha-20, WOAha-15, and WOAha-26 are shown in **Figure 3** (for others, see **Supplementary Figures 2–5**). Type WOAha-4 was detected as a recombinant by three of the six used methods: 3Seq ($P < 10^{-9}$), BootScan ($P < 10^{-6}$), and GENECONV ($P < 10^{-5}$). The major and minor parents were WOAha-26 and WOAha-1, and the beginning breakpoint was 116 bp. WOAha-17 was detected as a recombinant by four methods: 3Seq ($P < 10^{-9}/10^{-8}$), BootScan ($P < 10^{-8}/10^{-8}$), GENECONV ($P < 10^{-5}/10^{-7}$), and MaxChi ($P < 10^{-4}/10^{-6}$). The major parent was WOAha-20, and the minor parents were WOAha-15 and WOAha-26; the beginning breakpoint was 116 and 284 bp, respectively. In *Andricus* sp2, two phage WO recombinants were identified, WOAsp2–3 and WOAsp2–4, and their presumed parent types were WOAsp2–1 and WOAsp2–7, respectively (**Table 2**).

Almost all the parents involved in the recombination events were highly abundant phage lineages. The five phage types (WOAha-1, WOAha-4, WOAha-15, WOAha-21, and WOAha-26) harbored in *A. hakonensis* with a wide distribution and high abundance (**Figure 1A**); all participated in the recombination as parents and were the main force for the recombination. Similarly, the parents WOAsp2–1 and WOAsp2–7 of recombination were the most abundant types harbored in *Andricus* sp2 (**Figure 1B**). On the contrary, the abundance of the phage WO recombinant was lower in both *A. hakonensis* and *Andricus* sp2. In addition, WOAha-4 and WOAha-12, which were obtained by recombination, could also be used as parents of recombination

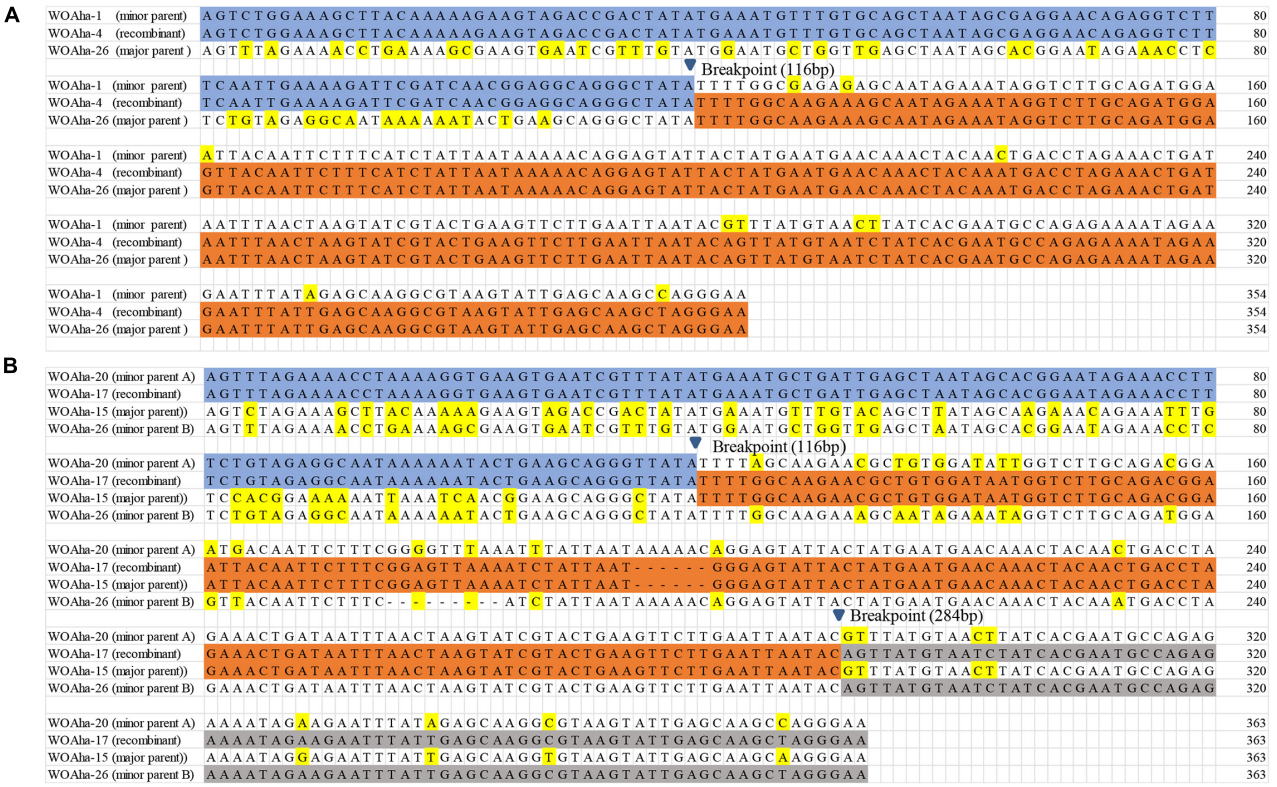

FIGURE 3 | Recombination events of the *orf7* gene **(A)** between WOAha-1 and WOAha-26 resulting in recombinant WOAha-4, and **(B)** among WOAha-20, WOAha-15, and WOAha-26 resulting in recombinant WOAha-17.

to contribute to the diversity of phages, suggesting the frequent occurrence of intragenic recombination. Furthermore, most *orf7* gene recombination events occurred between groups, including group III and V, III, and IV, IV, and V, I and V, and I and IV, except for group III, where recombination between different phage WO types of the same group was found, and almost all recombinants belonged to the same group as their major parents (**Table 2**).

DISCUSSION

Although bacteriophages have usually been proven to be rare or lacking in obligate intracellular bacteria (Moran and Plague, 2004; Bordenstein and Reznikoff, 2005), phage WO is widely distributed in various *Wolbachia*-infected insect groups (Bordenstein and Wernegreen, 2004; Gavotte et al., 2007; Kent and Bordenstein, 2010; Metcalf and Bordenstein, 2012; Wang et al., 2016; Kaushik et al., 2019). The present study demonstrated that 86.7% (13/15) gall wasp species were infected with *Wolbachia*, and all *Wolbachia*-infected gall wasps were found to harbor phage WO. From two populations of *L. jinzhaiensis*, a *Wolbachia*-free species, a 273-bp *orf7*-like non-coding pseudogene of phage WO was obtained. It might be considered as the vestige of prophage DNA remaining in the chromosomes of the host insect after a previous lateral gene transfer event, suggesting that *L. jinzhaiensis* might have been infected by *Wolbachia* carrying phage WO. Based on the phylogenetic analysis of the *orf7* sequences from the gall wasps in this study and other reference insect species, the phage WO types were divided into five groups, and each group contained the phage WO harbored in *Wolbachia*-infected gall wasps.

Multiple phage infections, where a *Wolbachia* strain displayed more than one phage type, have been reported in several *Wolbachia* strains (Chauvatcharin et al., 2006; Gavotte et al., 2007). However, most phage-infected *Wolbachia* strains display low numbers of phage types, with 85% showing only one or two different phage types (Gavotte et al., 2007; Tanaka et al., 2009). The findings of the present study indicated that a single type of phage WO was found in seven gall wasp species, which were infected with one strain of *Wolbachia*, except for *Andricus* sp1 with multiple *Wolbachia* infections. On the contrary, other six gall wasp species infected with double or multiple *Wolbachia* strains harbored diverse types of phage WOs: *A. hakonensis* with 27 types, *Andricus* sp2 with 10 types, *Latuspina* sp1 with nine types, *P. masudai* and *Latuspina* sp2 with three types, and *Andricus* sp3 with four types. The presence of multiple *Wolbachia* strains has been documented in several insect species (van Borm et al., 2001; Jamnongluk et al., 2002; Reuter and Keller, 2003; Hou et al., 2020). For gall wasps, a high level of multiple *Wolbachia* infections was found in *A. mukaigawae* and its associated inquiline *Synergus japonicus* with five and eight strains, respectively (Yang et al., 2013), and *Belonocnema*

TABLE 2 | Recombination analysis of *orf7* gene using six methods implemented RDP5 package in gall wasps harbored with multiple phage WO.

Insect	Recombinant	Major parent	Minor parent A/B	Breakpoint	Method	*P*-value
Andricus hakonensis						
	WOAha-4 (V)*	WOAha-26 (III)	WOAha-1 (V)	116	3Seq	8.61E-10
					BootScan	4.28E-07
					GENECONV	4.31E-06
	WOAha-2 (V)	WOAha-1 (V)	WOAha-26 (III)	298/334	GENECONV	4.23E-04
					BootScan	9.24E-05
	WOAha-2 (V)	WOAha-1 (V)	WOAha-12 (IV)	298/334	BootScan	1.78E-04
					3Seq	8.06E-04
	WOAha-12 (IV)	WOAha-15 (IV)	WOAha-4 (V)	298	3Seq	4.96E-07
					GENECONV	3.03E-04
					MaxChi	8.08E-04
	WOAha-12 (IV)	WOAha-15 (IV)	WOAha-26 (III)	298	3Seq	3.94E-08
					GENECONV	2.47E-05
	WOAha-23 (III)	WOAha-26 (III)	WOAha-16 (III)	298	3Seq	1.03E-05
					MaxChi	3.14E-04
	WOAha-17 (III)	WOAha-20 (III)	WOAha-15 (IV)/WOAha-26 (III)	116/298	3Seq	5.31E-10/4.36E-09
					BootScan	3.28E-09/2.36E-09
					GENECONV	4.31E-09/4.21E-08
					MaxChi	8.86E-08/1.27E-07
	WOAha-7 (I)	WOAha-8 (I)	WOAha-1 (V)/	116/334	3Seq	9.86E-10/6.79E-09
			WOAha-15 (IV)		BootScan	7.53E-09/6.28E-09
					GENECONV	8.12E-09/6.38E-08
					MaxChi	7.94E-08/5.63E-07
***Andricus* sp2**						
	WOAsp2–3 (V)	WOAsp2–7 (IV)	WOAsp2–1 (V)	161	3Seq	3.53E-05
					BootScan	5.96E-05
	WOAsp2–4 (IV)	WOAsp2–7 (IV)	WOAsp2–1 (V)	116/334	3Seq	2.48E-07
					MaxChi	5.11E-05
					Chimera	5.11E-05

**Roman numerals in parentheses refer to the group number of the phage WO type.*

treatae with four strains (Schuler et al., 2018). *Wolbachia* strains were identified by *wsp* gene genetic distance greater than 2%. *P. masudai*, *Latuspina* sp2, and *Andricus* sp3 were found to be infected with three, three, and four *Wolbachia* strains, respectively, and *A. hakonensis*, *Andricus* sp2, and *Latuspina* sp1 were infected with more than ten *Wolbachia* strains (data not shown and will be published in another study). Bacteriophages provide beneficial genes to the bacterial host (Abedon and Lejeune, 2005) or mediate the horizontal transfer of genes (Wommack and Colwell, 2000). Several reports have proven that phage WO can mediate horizontal gene transfer between *Wolbachia* strains (Ishmael et al., 2009; Wang et al., 2016). Therefore, diverse types of phage WOs harbored in the gall wasp species with a high level of multiple *Wolbachia* infections, effectively promoting the molecular evolution of host and increasing *Wolbachia* diversity through mediating the horizontal gene transmission or/and providing beneficial genes.

The absence of an evolutionary correlation between WO and *Wolbachia* phylogenies indicates that many horizontal phage WO transfers have occurred between different *Wolbachia* endosymbionts (Bordenstein and Wernegreen, 2004; Gavotte et al., 2004; Wang et al., 2016). The results of the present study suggested that an abundant horizontal transfer of phage WO in *Wolbachia* was associated with gall wasps. Prophages undergo a lytic phase capable of rupturing bacterial and eukaryotic cell membranes, and phage WO occurs in the extracellular matrix of arthropods. Thus, they might pass through the eukaryote cell wall and then initiate new infections (Masui et al., 2001; Bordenstein et al., 2006; Gavotte et al., 2007). In the closed system of galls, living organisms include gall formers, inquilines, parasitoids, *Wolabchia*, phage WO, and so forth. Gall inducer-inquiline association (Yang et al., 2013) and host-parasitoid association (Hou et al., 2020) are two known routes of horizontal transmission of *Wolbachia* in gall wasps. In three inquilines, *Synergus* sp1 (including individual insects obtained from the galls made by different gall wasps) and *Synergus* sp3 were infected with the same *Wolbachia* strain, while *Synergus* sp2 was infected with another *Wolbachia* strain (**Supplementary Figure 6**); however, they all carried the same phage WO type. The phage WO type was also detected in *A. hakonensis*. *D. zhuili*, and *A. mairei* infected with different *Wolbachia* strains (Hou et al., 2020), but they carried phage WO with identical *orf7* sequence. Therefore, it was highly likely that phage WOs were transferred in gall wasp species through gall inducer-inquiline

association (and host-parasitoid association) with or without *Wolbachia*.

Recombination occurs with both DNA and RNA viruses, and has been viewed as a means to rescue fit viral genomes from low fitness parents or a means to produce highly divergent genomes, resulting in dramatically impact evolution and epidemiology (Chetverin et al., 2005; Domingo, 2010). Bordenstein and Wernegreen (2004) confirmed the recombinogenic nature of phage WO, and, in the case of the capsid protein gene *orf7*, the recombination rate was the fastest reported rate for *Wolbachia* genome. In this study, 10 putative recombination events were identified from *A. hakonensis* and *Andricus* sp2, which harbored multiple phage WO types. The recombinant types and both parent types were all found in the same insect species. This study was novel in providing practical molecular evidence supporting *orf7* gene recombination of phage WO. These results strongly suggested that intragenic recombination was the important evolutionary force, which effectively promoted the high level of phage WO diversity associated with gall wasps (such as *A. hakonensis* and *Andricus* sp2). Furthermore, phage types in almost all groups participated in recombination, and recombination events occurred within or between groups. The phage WO phylogenetic relationship constructed using only the *orf7* gene sequence was not highly reliable due to the frequent occurrence of recombination.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, MW980182-MW980305 and MZ325445-MZ325462.

AUTHOR CONTRIBUTIONS

D-HZ and C-YS designed the study, wrote the manuscript, and performed experiments. D-HZ, C-YS, and X-HY performed the analyses. YA identified the gall wasps. YA and X-HY revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.694115/full#supplementary-material

Supplementary Figure 1 | Maximum likelihood tree for phage WO types of *Plagiotrochus masudai* **(A)**, *Latuspina* sp1 **(B)**, *Latuspina* sp2 **(C)**, and *Andricus* sp3 **(D)** based on the *orf7* sequences. The letters in parentheses indicate the sampled populations shown in **Table 1**, and the numbers indicate the number of sequences obtained per population. WOS-1 refers to the serial number. Phage WO types are shown on the right. Numbers above branches are bootstrap values computed from 1,000 replications.

Supplementary Figure 2 | Recombination events of the *orf7* gene **(A)** between WOAha-1 and WOAha-26 resulting in recombinant WOAha-2, and **(B)** among WOAha-8, WOAha-1, and WOAha-15 resulting in recombinant WOAha-7.

Supplementary Figure 3 | Recombination events of the *orf7* gene between WOAha-1 and WOAha-12 resulting in recombinant WOAha-2 **(A)**, WOAha-15 and WOAha-4 resulting in recombinant WOAha-12 **(B)**.

Supplementary Figure 4 | Recombination events of the *orf7* gene between WOAha-15 and WOAha-26 resulting in recombinant WOAha-12 **(A)**, WOAha-26 and WOAha-16 resulting in recombinant WOAha-23 **(B)**.

Supplementary Figure 5 | Recombination events of the *orf7* gene between WOAsp2–7 and WOAsp2–1 resulting in recombinant WOAsp2–4 **(A)**, WOAsp2–7 and WOAsp2–1 resulting in recombinant WOAsp2–3 **(B)**.

Supplementary Figure 6 | Maximum likelihood phylogenetic tree of the *Wolbachia wsp* nucleotide sequences with single infection found in this study (underlining) with known *Wolbachia* strains from gall wasps.

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ORIGINAL RESEARCH
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Microbiome Structure of the Aphid *Myzus persicae* (Sulzer) Is Shaped by Different Solanaceae Plant Diets

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Myzus persicae (Sulzer) is an important insect pest in agriculture that has a very broad host range. Previous research has shown that the microbiota of insects has implications for their growth, development, and environmental adaptation. So far, there is little detailed knowledge about the factors that influence and shape the microbiota of aphids. In the present study, we aimed to investigate diet-induced changes in the microbiome of *M. persicae* using high-throughput sequencing of bacterial 16S ribosomal RNA gene fragments in combination with molecular and microbiological experiments. The transfer of aphids to different plants from the Solanaceae family resulted in a substantial decrease in the abundance of the primary symbiont *Buchnera*. In parallel, a substantial increase in the abundance of *Pseudomonas* was observed; it accounted for up to 69.4% of the bacterial community in *M. persicae* guts and the attached bacteriocytes. In addition, we observed negative effects on aphid population dynamics when they were transferred to pepper plants (*Capsicum annuum* L.). The microbiome of this treatment group showed a significantly lower increase in the abundance of *Pseudomonas* when compared with the other Solanaceae plant diets, which might be related to the adaptability of the host to this diet. Molecular quantifications of bacterial genera that were substantially affected by the different diets were implemented as an additional verification of the microbiome-based observations. Complementary experiments with bacteria isolated from aphids that were fed with different plants indicated that nicotine-tolerant strains occur in Solanaceae-fed specimens, but they were not restricted to them. Overall, our mechanistic approach conducted under controlled conditions provided strong indications that the aphid microbiome shows responses to different plant diets. This knowledge could be used in the future to develop environmentally friendly methods for the control of insect pests in agriculture.

Keywords: insect microbiome, Solanaceae, *Nicotiana tabacum*, *Solanum melongena*, *Capsicum annuum*

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INTRODUCTION

Microorganisms are ubiquitous in the environment and often associated with eukaryotic hosts. During the last decades, it has been shown that microorganisms in plants and animals often fulfill important functions (Van der Ent et al., 2009; Fuchs, 2010). Individual plants and animals often harbor more than 1,000 different microbial species; some of them are crucial determinants for

their host's health and productivity because they can modulate metabolism, improve pathogen defense, and increase resource uptake (Van Der Heijden et al., 2008; Etalo et al., 2018; Alavi et al., 2020; Matsumoto et al., 2021). Insects, analogous to other animals, also have a close relationship with their microbiota; it was previously shown to have a substantial impact on insect ecology and various implications for evolutionary host development (Lewis and Lizé, 2015). Targeted studies have provided evidence that the composition of the insect microbiome is shaped by both geographic location and diet (Guo et al., 2019; Xu et al., 2019; Ma et al., 2021). Symbionts are found ubiquitously in insects among a variety of other microbes with as yet unknown functions. They can play various beneficial roles, such as promotion of growth and defense against natural enemies (Lee et al., 2017; Oliver and Perlman, 2020). Moreover, they support the adaptability of insects to adverse environmental conditions (Dunbar et al., 2007).

Aphids are sap-sucking insects that belong to the globally widespread insect order Hemiptera. Over 4,000 different aphid species were described within this order so far (Dixon et al., 1987). Therein, over 250 species are considered to be among the most destructive pests of cultivated plants (Blackman and Eastop, 1984). Aphids can negatively affect their host plants in different ways. They can damage plants *via* direct and indirect mechanisms; the latter is especially due to transmitted viruses. It is currently assumed that aphids can vector more than 200 viral diseases, which accounts for over 30% of all relevant plant viruses (Brault et al., 2010). In addition, aphids often served in the past as models to study microorganism–insect interactions (Oliver et al., 2010). The bacterial genus *Buchnera*, which includes various symbiont species that can be obligate for their hosts, was found to supply vitamins and essential amino acids that are not taken up through their plant diets, such as methionine and tryptophan (Birkle et al., 2002; Akman Gündüz and Douglas, 2009). In return, the aphid can also provide distinct compounds to its symbionts, which they cannot synthesize due to evolutionary losses of distinct biosynthetic genes (Brinza et al., 2009). Their interaction is therefore obligate and mutualistic in a way that neither partner can reproduce in the absence of the other. *Buchnera* cells are commonly distributed in the cytosol of specialized host cells around the aphid gut; they are often subjected to changes during their host's lifecycle (Simonet et al., 2016). In some insects, *Buchnera* was shown to be enriched before adulthood and to gradually decrease in adult insects (Simonet et al., 2018). In addition to the obligatory *Buchnera* symbionts, a broad range of secondary symbionts was previously discovered in aphids. Some of them are known to manipulate host reproduction, whereas others are involved in a mutualistic interplay that can increase host survival or fecundity, such as *Serratia symbiotica*, *Hamiltonella defensa*, *Regiella inseticola*, *Rickettsia*, *Rickettsiella*, *Spiroplasma*, *Wolbachia*, *Arsenophonus*, and *Fukatsuia symbiotica* (Scarborough et al., 2005; Russell and Moran, 2006; De Clerck et al., 2015; Ayoubi et al., 2020). Among aphid species, *Myzus persicae* (Sulzer) is one of the most destructive, cosmopolitan, and generalist agricultural pests. It can cause substantial damage to more than 400 plant species (Quaglia et al., 1993). Some subspecies are specifically adapted to Solanaceae plants, which are economically the third most important plant family, consisting of approximately 2,700 plant species (Olmstead and Bohs, 2006; Tapia et al., 2008). Especially the cultivation of pepper (*Capsicum annuum* L.), eggplant (*Solanum melongena* L.), and tobacco (*Nicotiana tabacum* L.) is often affected by *M. persicae*, which can substantially reduce the amount and quality of harvested plant products. Nowadays, the control of *M. persicae* is mainly based on chemical control; however, insecticides have brought serious resistance problems as a result of their extensive use. We aimed at further expanding the knowledge related to factors that shape the aphid microbiome by conducting feeding experiments under controlled conditions. We assume that this knowledge could provide an extended basis for the development of a new control strategy for *M. persicae*. Previous studies with a similar aim found potential implications of the sampling location and host plant; however, the shaping capacity of the latter on the aphid's microbiota remained to be explored in more detail (Xu et al., 2019). This especially applies to host changes with highly different secondary metabolite profiles. In the present study, we hypothesized that the microbiome of aphids undergoes significant changes as they move from an initial host that produces low levels of bioactive compounds to different plants within the Solanaceae family. We selected three plant species that were fed separately to *M. persicae* populations in a controlled environment. After rearing them on cabbage (*Brassica rapa* L. var. *pekinensis*), they were transferred to eggplant, pepper, and tobacco plants. After 2 weeks, we dissected the aphid guts to which bacteriocytes are attached and extracted the total community DNA. Following a targeted amplification of bacterial 16S ribosomal RNA (rRNA) gene fragments and high-throughput sequencing, the data were subjected to an explanatory bioinformatics approach. From a long-term point of view, our findings may facilitate the development of microbiome management approaches targeting symbiotic bacteria of aphids to minimize their destructive impact on crops.

MATERIALS AND METHODS

Myzus persicae Rearing and Sample Preparation

Plants used to feed aphids were cultivated from seeds to provide controlled conditions, especially in terms of agrochemical residues that might affect the microbiota. Cabbage seeds (*B. rapa* L. var. *pekinensis*) were obtained from Guizhou Debang Agricultural Products Co., Ltd. (Guiyang, Guizhou; location: N26°57′83.42″, E106°71′34.78″), eggplant seeds (*S. melongena L.* cv. Zilong No. 8) and pepper seeds (*C. annuum* cv. Gui Yan No. 13) from Guizhou Lifeng Zhongye Co., Ltd. (Guiyang, Guizhou, China), and tobacco seeds of the cultivar MS K326 from Yuxi Zhong Yan Seed Co., Ltd. (Yuxi, Yunnan; location: N24°19′54.32″, E102°31′44.95″). For their cultivation, soil, perlite, and vermiculite were mixed in a 3:3:1 ratio and irrigated with water after the respective seeds were added. They were kept in an incubator at 25°C, 66% relative humidity, and 16-h/8-h day/night cycles for around 10 days until the seeds started to germinate. At the seedling stage, they were transferred to

larger pots (11.5 × 9.5 cm) with the same soil mixture as mentioned earlier. The pots were then placed into mesh cages (35 × 35 × 28 cm). One plant was placed into each mesh cage with 12 replicates for each Solanaceae plant diet. For the control group, aphids were kept for the same duration on a cabbage diet in 12 additional mesh cages. When the plants reached stable growth, adult *M. persicae* aphids reared on cabbage were transferred together with cabbage leaves cut from the plants into the mesh cages with the Solanaceae plants to allow a natural transition; the cabbage leaves were subsequently removed from the containers. A complementary experiment with the same setup and six replicates per plant species was conducted to assess the population dynamics of *M. persicae* on the plant diets that were implemented. Observations and counts were made on the first day of transfer from cabbage to Solanaceae plants and continued for 14 days. The average number of aphids was obtained every day and used to construct a graph showing the population dynamics. The temperature and humidity were monitored with an EL-USB-2 device (Lascar Electronics). During the whole feeding experiment, the temperature ranged between 18.5 and 33°C, while the relative humidity ranged between 47 and 98% (**Supplementary Figure 1**). After 2 weeks on the respective diets, the adult aphids were removed for dissections. For surface sterilization, *M. persicae* was submerged in 75% ethanol for 10 s; the step was repeated in a different tube and followed by transfer into sterile water to remove the residual ethanol. Then, the aphids were placed into a phosphate-buffered saline buffer to prepare them for dissection. Disposable sterile syringe needles (0.3 × 13 mm; Zhejiang Kangdelai Medical Equipment Co., Ltd.) and tweezers (ST-11; Shenzhen Deer Fairy Technology Co., Ltd.) were used to remove the guts, to which also bacteriocytes are attached, under a stereoscope (Olympus SZ2-ILST). In the first step, the heads of the adult aphids were clamped with tweezers. Then, using sterile disposable syringe needles, the abdomens of the aphids were cut open. For each sample, 50 guts from aphids kept in the same mesh cage were collected in a sterile 1.5-ml tube. This was replicated 12 times for each plant diet that was administered in a separate mesh cage. All samples were stored on ice during the dissection process. In total, 48 composite samples, each consisting of 50 aphid guts from separate mesh cages, were obtained and stored at −80°C before total community DNA extraction. The whole experiment procedure is shown schematically in **Supplementary Figure 2**.

Total Community DNA Extractions From Aphid Guts

All gut samples were processed with a DNA extraction kit (Fast DNA SPIN Kit for soil; MP Biomedicals, Solon, OH, United States) to extract the total community DNA. The frozen samples were directly transferred into the extraction vials provided in the kit mentioned earlier to avoid contaminations during handling. Subsequently, all total community DNA extractions were conducted according to the manufacturer's protocol. The extracts were photometrically analyzed with a Nanodrop 2000 device (Thermo Fisher Scientific, Wilmington, DE, United States) to quantify the DNA and verify its quality; 12 biological replicates were obtained with sufficient DNA concentrations for the subsequent steps. The total community DNA extracts were stored at −20°C until further processing.

Barcoding and High-Throughput Sequencing of 16S Ribosomal RNA Gene Fragment Amplicons

The DNA samples mentioned earlier were sent to a sequencing company (Novogene Co., Ltd., Beijing, China) for next-generation sequencing that targeted the hypervariable region 4 (V4) of bacterial 16S rRNA genes. The samples were amplified with the primers 515f (5′GTGYCAGCMGCCGCGGTAA) and 806r (5′GGACTACHVGGGTWTCTAAT) according to the Earth Microbiome Project protocol[1] with sample-specific barcodes and Illumina sequencing adaptors. The following polymerase chain reaction (PCR) program (95°C for 5 min to denature the DNA, 30 cycles at 96°C for 60 s, 78°C for 5 s, 54°C for 60 s, 74°C for 60 s, and 10 min at 74°C for a final extension) was used for the generation of amplicons according to a method described before (Thompson et al., 2017). During the PCR amplification, specific peptide nucleic acid (PNA) oligomers were added to the PCR mix to prevent the amplification of mitochondrial (mPNA) or plastidial (pPNA) RNA from eukaryotic origin (Lundberg et al., 2013). The PCR blockers were obtained from PNA Bio Inc. (Newbury Park, CA, United States). Negative controls were included in each PCR reaction. They did not result in visible products and were thus not subjected to sequencing to avoid misassignments due to "index hopping" in low-quantity DNA samples (Van der Valk et al., 2020). High-throughput sequencing was conducted by Novogene (Beijing, China) on the Illumina PE250 platform that produces 2 × 250-bp paired-end reads.

Bioinformatic Processing of the 16S Ribosomal RNA Gene Fragment Library

The reads were assigned to samples by demultiplexing them according to their unique barcode sequences. All demultiplexed paired-end reads were imported into QIIME2 2019.10 (Bolyen et al., 2019) and quality-filtered using the q2-demux plugin followed by denoising with DADA2 (Callahan et al., 2016) (with the q2-dada2 plugin) to summarize sequence variants (SVs) and to generate a filtered feature table as well as representative sequences. The chimeras were filtered from the table, and taxonomy was assigned to amplicon sequence variants (ASVs) using the q2-feature classifier (Bokulich et al., 2018) in combination with "Greengenes 13_8 99% ASVs" reference sequences (McDonald et al., 2012). Subsequently, the determination of alpha and beta diversity was performed using the QIIME 2 core diversity metrics and group significance tests using the q2-diversity plugin (Faith, 1992) after samples were rarefied to 66,246 reads per sample. The feature table was split into four separate tables according to the sample

[1] www.earthmicrobiome.org

group (cabbage-fed, pepper-fed, eggplant-fed, and tobacco-fed). For visualization, the feature table was exported from QIIME2, and barplots were generated with a cut-off of 0.1% abundance. Significant differences in the occurrence of distinct ASVs between the control and each treatment were calculated using the R (version 4.03) package edgeR (version 3.30.3) (Robinson et al., 2010).

Verification of *Pseudomonas* and *Buchnera* Enrichment in the Samples

To confirm changes in the abundance of the genera *Pseudomonas* and *Buchnera* when *M. persicae* was fed with different plant diets, a quantitative PCR (qPCR)-based approach was used. The same samples used to generate the amplicon library were also used to quantify *Pseudomonas* and *Buchnera* in the DNA extracts. All samples were adjusted to a concentration of 2 ng/μl according to quantifications with a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, United States) to account for the differences in the extraction efficiency and subsequently used for molecular quantifications with a CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, United States). Each sample was analyzed with four technical replicates. The primer pair used for *Pseudomonas* quantification was Pse434F (5′-ACTTTAAGTTGGGAGGAAGGG-3′) and Pse665R (5′-ACACAGGAAATTCCACCACCC-3′) (Pereira et al., 2018). The primer pair used for *Buchnera* quantification was dnaK2F (5′-GATTGTCTTCGGCTGTTG-3′) and dnaK2R (5′-GTCACTCCTTTATCACTTGG-3′). In addition, the aphid's elongation factor 1α (using primers AWRT002F 5′-CTGATTGTGCTGTGCTTATTG-3′ and AWRT002R 5′-CAAGGTGAAAGCCAATAGAGC-3′) (Jiang et al., 2013) was included in the quantifications as a reference gene. The total reaction volume was 20 μl and contained 10-μl PowerUp SYBR Green Master Mix (Applied Biosystems, Vilnius, Lithuania), 1-μl DNA template, 1-μl (5-nM) forward primer, 1-μl (5-nM) reverse primer, and 7-μl sterile ddH2O. The PCR cycling conditions for *Pseudomonas* quantification included an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 25 s. The PCR cycling conditions for *Buchnera* quantification included an initial denaturation at 95°C for 5 min, followed by 45 cycles at 95°C for 20 s, 57°C for 20 s, and 72°C for 25 s. For quantification of the aphid's elongation factor 1α, the initial denaturation was conducted at 95°C for 5 min, followed by 40 cycles at 95°C for 20 s, 59°C for 20 s, and 72°C for 25 s. A melting curve (68–95°C) was obtained in the final step for all three approaches. The cycle threshold was used as a reference to validate differences in the abundance of *Pseudomonas* and *Buchnera* in *M. persicae* grown on different plant diets.

Isolation of Bacteria From Guts of *M. persicae* and Susceptibility Testing Toward Nicotine

Aphids were randomly collected from the four host plants and dissected as described earlier to obtain aphid 50 guts per sample. After grinding the guts using an automated tissue grinding device (BBI Life Sciences, Shanghai, China) for 5 s, the guts were transferred into 10% nutrient broth and incubated at 30°C on a shaker at 180 rpm/min for 12 h. Subsequently, a micropipette was used to transfer 10-μl bacterial suspension after dilution with 0.85% NaCl (10^{-1}–10^{-3} dilutions) to Petri dishes with LB agar, nutrient agar, and R2A agar. The plates were cultivated at 30°C until the appearance of bacterial colonies. For *Pseudomonas* isolations, *Pseudomonas* isolation broth was used to incubate 50 guts that were dissected from cabbage- and Solanaceae-fed aphids. Incubations were performed at 30°C on a shaker at 180 rpm/min for 12 h. Diluted suspensions (10^{-1}–10^{-3} dilutions) were then transferred to *Pseudomonas* CFC selective agar (Qingdao Hope Bio-Technology Co., Ltd.) with *Pseudomonas* CFC selective agar supplement (Qingdao Hope Bio-Technology Co., Ltd.) and to King's B medium (Qingdao Hope Bio-Technology Co., Ltd.). They were cultivated at 30°C until bacterial colonies appeared, which were subsequently purified. The bacterial genomic DNA extraction kit (Beijing Solarbio Science & Technology Co., Ltd.) was used to extract the DNA of all isolates before PCR amplification of 16S rRNA gene fragments for taxonomic identification with the primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The PCR amplification comprised an initial cycle at 95°C for 2 min, followed by 34 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 1 min, extension at 72°C for 2 min, and a final extension step at 72°C for 10 min. The length of PCR products was confirmed by 1% gel electrophoresis, and the PCR products were sent to Sangon Biotech (Shanghai, China) for Sanger sequencing. Based on the obtained 16S rRNA gene fragment sequences, phylogenetic placement was conducted. The candidate taxa for the phylogenetic analysis included all isolates from aphid guts and such with high similarities in BLAST searches within the National Center for Biotechnology Information (NCBI) nucleotide database. To construct a phylogenetic tree with those sequences, multiple alignments were performed using Clustal W (Thompson et al., 1994). Subsequently, molecular phylogenetic analyses based on neighbor-joining algorithms were created with MEGA7 (Kumar et al., 2016). Bootstrap values were calculated with 1,000 replications for the neighbor-joining method.

All isolates were additionally subjected to susceptibility testing toward nicotine, a bioactive metabolite characteristic of Solanaceae plants. Bacteria were cultivated on nutrient medium and R2A medium (for slow-growing bacteria) to obtain single colonies. Sterile toothpicks were used to transfer single colonies to the nutrient broth and liquid R2A medium, followed by incubation for 16 h at 30°C and 180 rpm/min. Subsequently, the bacterial suspensions were diluted to an optical density measured at a wavelength of 600 nm of 0.1 in a liquid R2A liquid medium. Aliquots of 5 ml were transferred into glass test tubes (18 × 180 mm) with 0, 2, 4, and 8 g/l nicotine passed through a 0.22-μm PES filter (Tianjin Keyilong Lab Equipment Co., Ltd) for sterilization. The glass test tubes were incubated in a shaker at 30°C and 160 rpm/min. The optical density measured at a wavelength of 600 nm was then recorded each 6 h for 48 h with the Multiskan GO instrument (Thermo Fisher Scientific, Vantaa, Finland) to monitor bacterial growth in a nicotine-supplemented medium.

Statistical Analyses

Statistical tests for the microbiome analyses were performed using the QIIME 2 and R studio (R version 4.03) software packages. The significance of the differences in alpha diversity was tested with the implemented Kruskal–Wallis test and for the beta diversity with the analyses of similarities test in the QIIME 2 pipeline. The R package edgeR was implemented to identify differently abundant genera between the treatments ($\log_2$ fold change > 2 and $\log_2$ fold change < -2; $P < 0.01$). The statistical significance of the differences in the qPCR data was assessed with the Kruskal–Wallis test.

RESULTS

Alpha and Beta Diversity Analyses of the Aphid Microbiome Under Different Plant Diets

After filtering chimeric, mitochondrial, and plastid sequences, the feature table was reduced from a total read count of 6,200,722 to 5,310,692, representing 20,876 features. Data normalization resulted in a read count of 66,246 per sample; a total number of 20,111 features remained in the dataset. Collapsing features on genus level resulted in 1,607 bacterial genera and 37 archaeal taxa. The Shannon index (**Figure 1A**) was used to compare the bacterial diversity among the different samples. The microbiomes of cabbage-fed aphids ($H' = 2.21 \pm 0.27$), eggplant-fed aphids ($H' = 2.41 \pm 0.28$), and tobacco-fed aphids ($H' = 3.05 \pm 0.56$) had a significantly lower alpha diversity than pepper-fed aphids ($H' = 5.88 \pm 0.78$; $P < 0.05$). There were no significant differences between the other groups ($P > 0.05$). Similar results were obtained when the number of observed ASVs (**Figure 1B**) was compared between cabbage-fed aphids (observed ASVs $= 544 \pm 80$), eggplant-fed aphids (obs. ASVs $= 560 \pm 65$), tobacco-fed aphids (obs. ASVs $= 845 \pm 108$), and pepper-fed aphids (obs. ASVs $= 1{,}163 \pm 180$). Significant differences were found when cabbage-fed aphids and eggplant-fed aphids were compared with pepper-fed aphids ($P < 0.05$), whereas no significance was observed between pepper-fed aphids and tobacco-fed aphids ($P > 0.05$). Beta diversity analyses revealed distinct clustering of sample groups based on Bray–Curtis dissimilarities (**Figure 2**). Analyses of similarities confirmed highly significant ($R = 0.5$; $P = 0.001$) differences in community composition between the cabbage-fed and the Solanaceae-fed aphids.

Assessment of the Bacterial Community Structure and Composition

At the bacterial family level, the cabbage-fed aphids were primarily colonized by *Enterobacteriaceae* (68.2%), *Moraxellaceae* (7.9%), *Erysipelotrichaceae* (5.3%), and *Rickettsiaceae* (4.7%; **Figure 3**). The prevalent bacterial genera were identified as *Buchnera* (67.7%), *Acinetobacter* (7.8%), and *Allobaculum* (5.3%). The eggplant-fed aphids were mainly colonized by the bacterial families *Pseudomonadaceae* (69.5%), *Enterobacteriaceae* (13.4%), *Oxalobacteraceae* (2.4%), and *Burkholderiaceae* (1.8%). *Pseudomonas* (69.4%), *Buchnera* (12.5%), *Ralstonia* (2.1%), and *Burkholderia* (1.7%) were the most common genera in the eggplant-fed aphids. In tobacco-fed aphids, *Pseudomonadaceae* (62.3%), *Enterobacteriaceae* (15.9%), *Oxalobacteraceae* (3.2%), and *Burkholderiaceae* (2.2%) were the prevalent taxonomic groups on the bacterial family level. *Pseudomonas* (62.2%) was the predominant genus, followed by *Buchnera* (14.6%), *Ralstonia* (3.0%), and *Burkholderia* (2.1%). In the pepper-fed aphids, *Enterobacteriaceae* (29.3%) was the most prevalent bacterial family, whereas *Pseudomonadaceae* (11.8%) and *Oxalobacteraceae* (9.3%) were less abundant, followed by *Burkholderiaceae* (5.1%). The predominant genus in pepper-fed aphids was *Buchnera* (25.5%). The second most abundant genus was *Pseudomonas* (11.7%), followed by *Ralstonia* (8.4%), *Burkholderia* (5.0%), *Enterobacteriaceae* (3.2%), *Acinetobacter* (2.3%), and *Stenotrophomonas* (1.7%). Notably, the pepper plant diet not only showed the least increase in the abundances

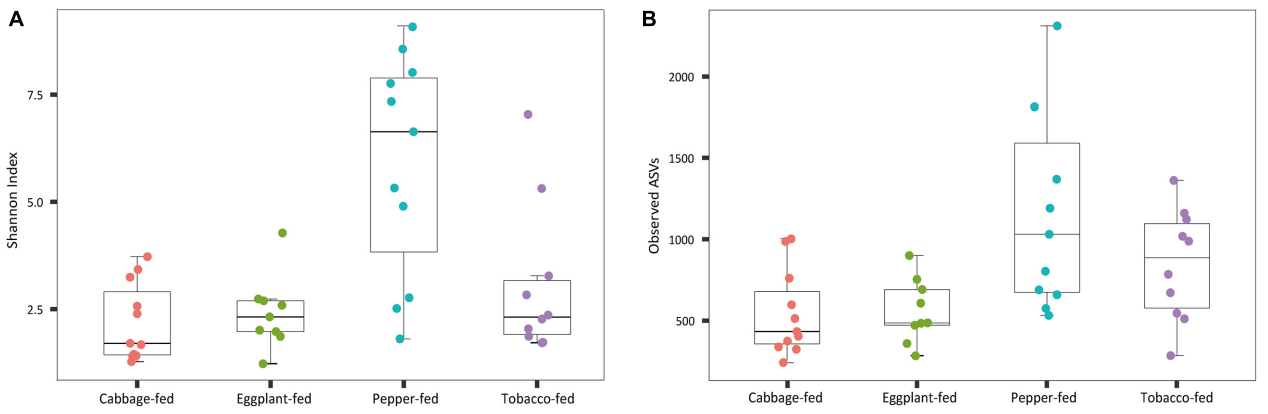

FIGURE 1 | Alpha diversity of bacterial communities in *Myzus persicae* subjected to different plant diets. Diversity was assessed within the QIIME2 pipeline on basis of Shannon index **(A)** and observed operational taxonomic units (ASVs) **(B)** in the normalized dataset. All samples were rarefied to a sequence depth of 66,246 reads before being subjected to diversity analyses.

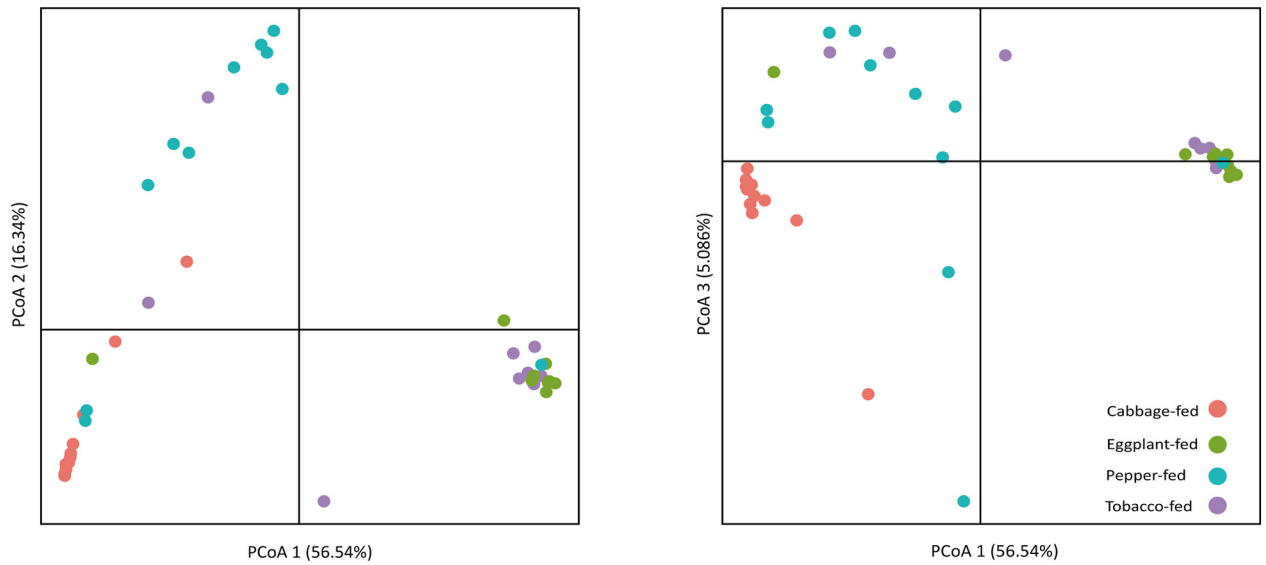

FIGURE 2 | Principal coordinates analysis (PCoA) of bacterial community structure in cabbage-fed and Solanaceae-fed aphids. Three dimensions that explain highest degree of variance were included in visualization. Percentage variance explained is indicated on respective axes. Different colors of dots indicate different sample types. Significance of differences between different bacterial communities was tested with ANOSIM in QIIME 2.

of *Pseudomonas* and the least decrease in the abundance of *Buchnera* but also other changes in the microbiome when compared with the other two Solanaceae plants. Several low-abundant members of the Gram-positive bacterial phylum *Firmicutes* increased under the pepper diet, which reflects the previously observed differences in alpha and beta diversity (**Figures 1**, **2**).

Identification of Bacteria That Were Significantly Affected by the Diet

In addition to the general analysis of the microbiome in differently fed aphids, deepening statistical analyses based on edgeR were conducted to identify differentially abundant taxa in each of the treatment groups (**Figure 4** and **Supplementary Dataset 1**). Taxa that were significantly more abundant in eggplant-fed aphids included *Pseudomonas* (12 features), *Staphylococcus* (three features), *Serratia*, *Rhodoplanes*, *Thermotoga*, *Rubellimicrobium*, *Acinetobacter,* and unassigned bacteria (12 features). Forty-six features were shown to be depleted when eggplant-fed aphids were compared with cabbage-fed aphids (**Figure 4A**). In pepper-fed aphids, *Psychrobacter*, *Phormidium*, *Comamonas*, *Sporosarcina*, *Pseudomonas*, *Ruminococcus* (three features), and unassigned bacteria (three features) were enriched. In contrast, 61 features were enriched in cabbage-fed aphids when compared with pepper-fed aphids (**Figure 4B**). In the tobacco diet group, 21 features were more abundant than in the cabbage group and assigned to *Dokdonella*, *Dok59*, *Methylibium*, *Nitrosopumilus*, *Pseudomonas* (five features), *Acinetobacter*, and unassigned bacteria (11 features). The remaining 81 features were found to be enriched in cabbage-fed aphids (**Figure 4C**). When all groups were comparatively assessed, *Pseudomonas* was found to be enriched in all Solanaceae-fed aphids, whereas 21 genera were depleted in those aphids when compared with cabbage-fed aphids. The depleted genera were assigned to *Buchnera*, *Wolbachia*, *Helicobacter*, *Acinetobacter*, *Actinoplanes*, *Adlercreutzia*, *Alicyclobacillus*, *Allobaculum,* *Chitinophaga*, *Coprococcus*, *Dyadobacter*, *Lysobacter*, *Lactobacillus*, *Microbacterium*, *Mucispirillum*, *Oscillospira*, *Paracoccus,* *Promicromonospora,* *Ruminococcus,* *Staphylococcus,* and *Streptomyces* (**Figure 4**).

Quantitative Polymerase Chain Reaction Confirmed Relative Enrichment of *Pseudomonas* and Depletion of *Buchnera*

As a complementary approach to the marker gene sequencing analyses, the abundance of *Pseudomonas* and *Buchnera* was confirmed by qPCR targeting these taxa in the total community DNA extracts (**Figure 5**). The conducted Kruskal–Wallis test for the cycle thresholds indicated that *Pseudomonas* was significantly increased in eggplant-fed aphids (Cq = 25.9 ± 0.6) and tobacco-fed aphids (Cq = 25.4 ± 0.8; $P < 0.01$) when compared with cabbage fed aphids (Cq = 30.1 ± 0.1). However, the difference between pepper-fed aphids (Cq = 29.8 ± 0.5) and cabbage-fed aphids (Cq = 30.1 ± 0.4; $P = 0.7903$) was not significant. *Buchnera* showed a significant decline in eggplant-fed aphids (Cq = 33.9 ± 0.8), pepper-fed aphids (Cq = 32.2 ± 0.6), and tobacco-fed aphids (Cq = 31.9 ± 0.7; $P < 0.01$). The overall results reflected the observations of the microbiome analyses, especially in the substantial reduction of *Buchnera* in Solanaceae-fed aphids. A complementary quantification of the aphid's elongation factor 1α in total community DNA extracts indicated that the host–microbe DNA proportion was similar among

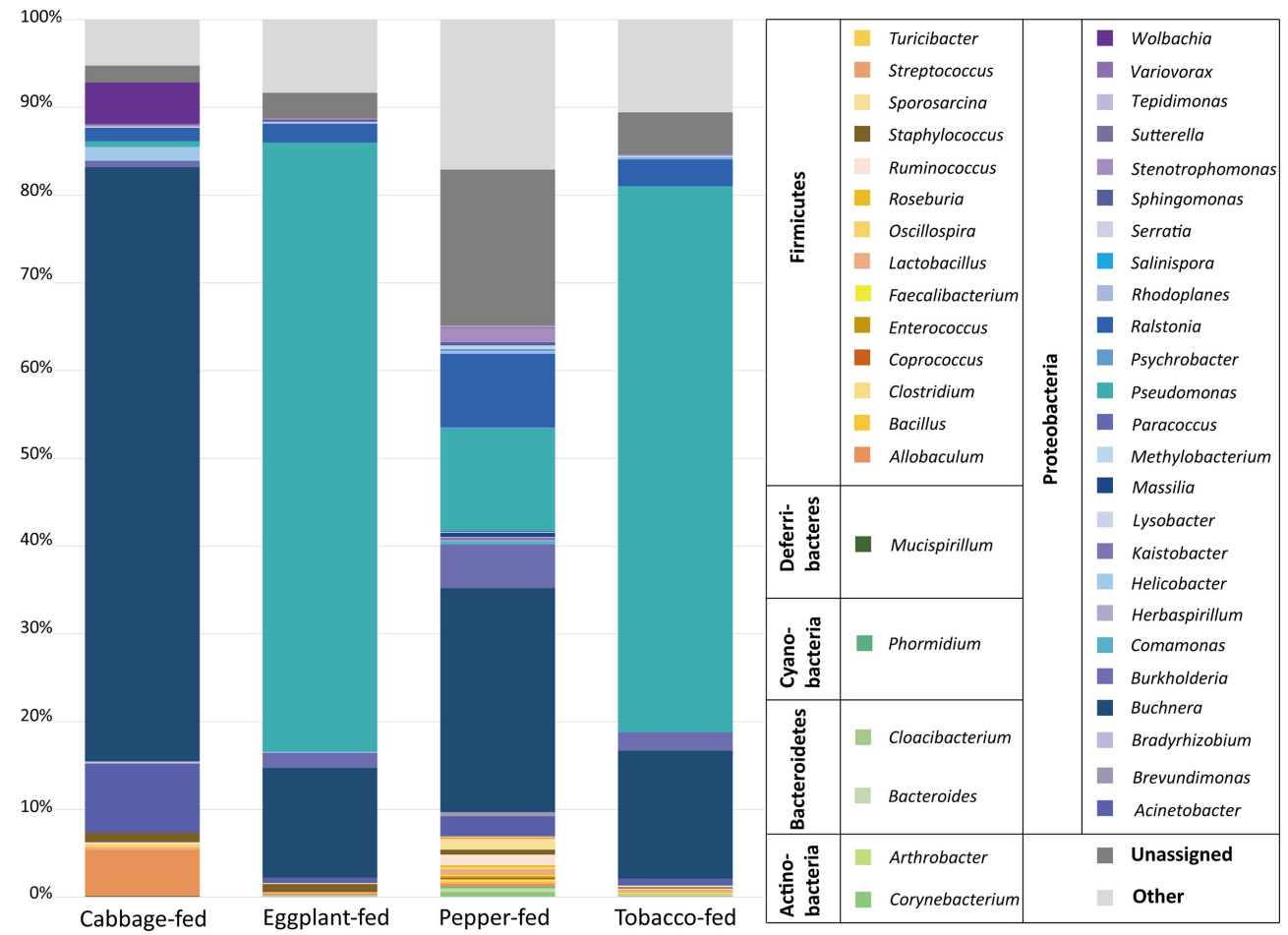

FIGURE 3 | Bacterial community structure of different sample types. Taxonomic classification of highly abundant (>0.1%) members of aphid microbiome on different diets. Taxa with a lower abundance than the implemented threshold were summarized as "other." Bacterial community structure was visualized up to genus level for each sample type. In addition, all taxa were grouped on bacterial phylum level in legend for better differentiation.

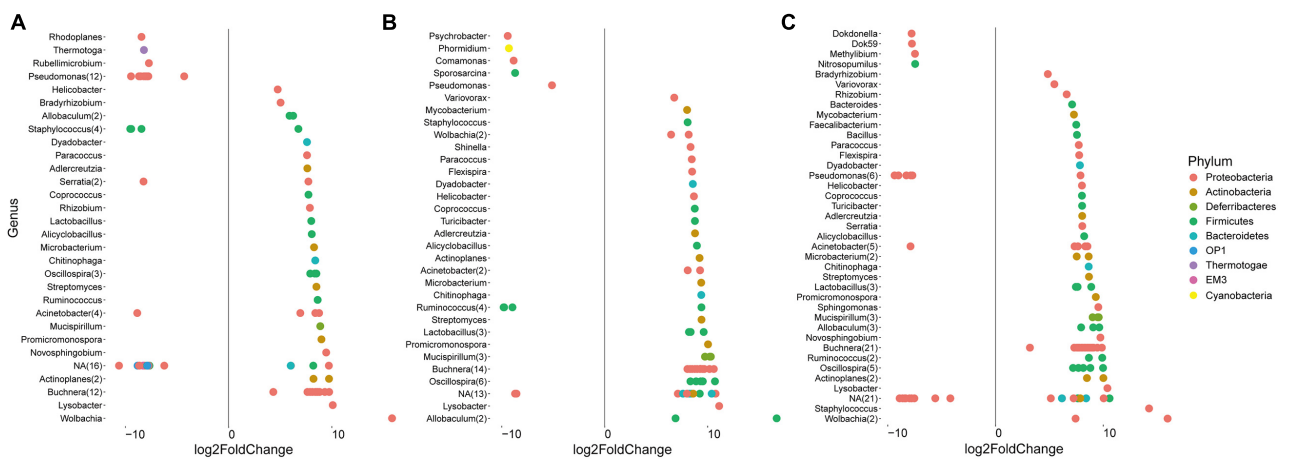

FIGURE 4 | Identification of amplicon sequencing variants (ASVs) that differ significantly in their relative abundance among treatments. Pairwise comparisons were conducted for Solanaceae-fed aphids and cabbage-fed aphids using edegR tool ($P < 0.01$). **(A)** Comparison of cabbage-fed aphids with eggplant-fed aphids; **(B)** comparison of cabbage-fed with pepper-fed aphids; **(C)** comparison of cabbage-fed aphids with tobacco-fed aphids. Taxonomy of ASVs was assigned at bacterial genus level whenever it was possible.

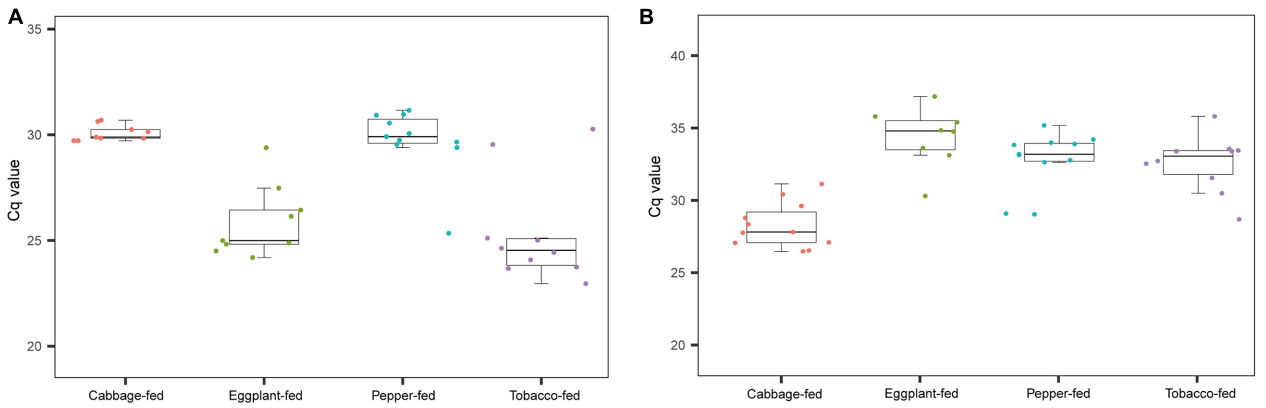

FIGURE 5 | Complementary molecular quantifications of changes in relative abundance of *Pseudomonas* and *Buchnera* in aphids on different plant diets. Enrichment of *Pseudomonas* and depletion of *Buchnera* in Solanaceae-fed aphids was confirmed with a complementary qPCR-based approach and genus-specific detection primers. **(A)** Cq values of *Pseudomonas* in gut of *M. persicae* fed with different plants; **(B)** Cq values of *Buchnera* fed with different plants.

all sample groups (**Supplementary Figure 3**). Complementary analyses of *M. persicae* population dynamics on different hosts indicated that it was only negatively affected following the transition to pepper plants (**Supplementary Figure 4**).

Isolation of Bacteria From *M. persicae* and Their Phylogenetic Placement

To obtain complementary insights into the observations of the microbiome analysis, isolation of gut bacteria on different cultivation media was conducted. Guts of *M. persicae* aphids feeding on cabbage, eggplant, pepper, and tobacco were dissected, and a total of 16 isolates was obtained using LB agar, nutrient agar, R2A agar, *Pseudomonas* CFC selective agar, and King's B medium. Five bacterial strains were isolated from aphids reared on the cabbage diet and assigned to *Curtobacterium citreum*, *Brevibacterium sediminis*, *Microbacterium esteraromaticum*, *Microbacterium proteolyticum*, and *Pseudomonas reactans*. Six strains were isolated from aphids fed with tobacco and assigned to *B. sediminis*, *Methylorubrum aminovorans*, *M. esteraromaticum*, *Exiguobacterium indicum*, *Pseudomonas brenneri*, and *P. reactans*. Three strains that were respectively assigned to *Microbacterium paraoxydans*, *P. reactans*, and *P. brenneri* were isolated from aphids fed with eggplant. *Bacillus indicus* and *P. reactans* were isolated from aphids that were fed with pepper. The isolates were used for phylogenetic placements based on their 16S rRNA gene fragments (**Figure 6**). All isolates showed a high similarity to sequences deposited in the NCBI nucleotide database, indicating that the cultivable fraction of the *M. persicae* microbiome consists of bacteria that were previously described.

Susceptibility Testing of Bacterial Isolates Toward Nicotine

Isolated bacteria from guts of *M. persicae* growing on four plants diets were subjected to specific susceptibility tests. To assess the susceptibility of bacteria toward nicotine, their growth in medium with 0, 2, 4, and 8 g/l nicotine was monitored for 48 h (**Supplementary Figure 5**). According to the results of a two-factor analysis of variance, the growth rate of all bacterial was significantly reduced with an increasing concentration of nicotine or even completely restrained ($P < 0.01$). *M. aminovorans* strain T2 was not able to grow in any medium supplemented with nicotine. *C. citreum* strain C3, *M. proteolyticum* strain C4, *P. reactans* strain C-P, *E. indicum* strain T4, *P. brenneri* strain T-P1, *P. brenneri* strain T-P2, *M. paraoxydans* strain E2, *P. reactans* strain E-P1, *P. brenneri* strain E-P2, and *P. reactans* strain P-P were not able to grow in medium supplemented with 8 g/l nicotine. Isolates that were able to grow in medium supplemented with 8 g/l nicotine included the *B. sediminis* strain C1 and *M. esteraromaticum* strain C2 that were obtained from cabbage-fed aphids, two isolates from tobacco-fed aphids (*B. sediminis* strain T1 and *M. esteraromaticum* T3), and the *B. indicus* strain P that was isolated from pepper-fed aphids.

DISCUSSION

By assessing the changes in the bacterial community of *M. persicae* fed with three Solanaceae plants (eggplant, pepper, and tobacco) and cabbage as a reference treatment, we could mechanistically confirm that the microbiome of aphids is influenced by the plant diet. This is in line with recent findings of Xu et al. (2019) that assessed the microbiome of *Aphis gossypii* (Hemiptera: Aphididae) and found clear indications that it was affected by its host plants. In contrast, previous field studies conducted with *M. persicae* feeding on different plants did not show significant differences in the aphid's microbiome composition (Xu S. et al., 2021). This is most likely due to the described migration of the aphids. In the present study, this factor was excluded by conducting feeding experiments under controlled conditions. The present study provides indications that the microbiome of *M. persicae* can be substantially shaped by the host plant and that these changes take place within a short period. More in-depth studies will be needed in the future to assess whether these changes are temporary or whether they

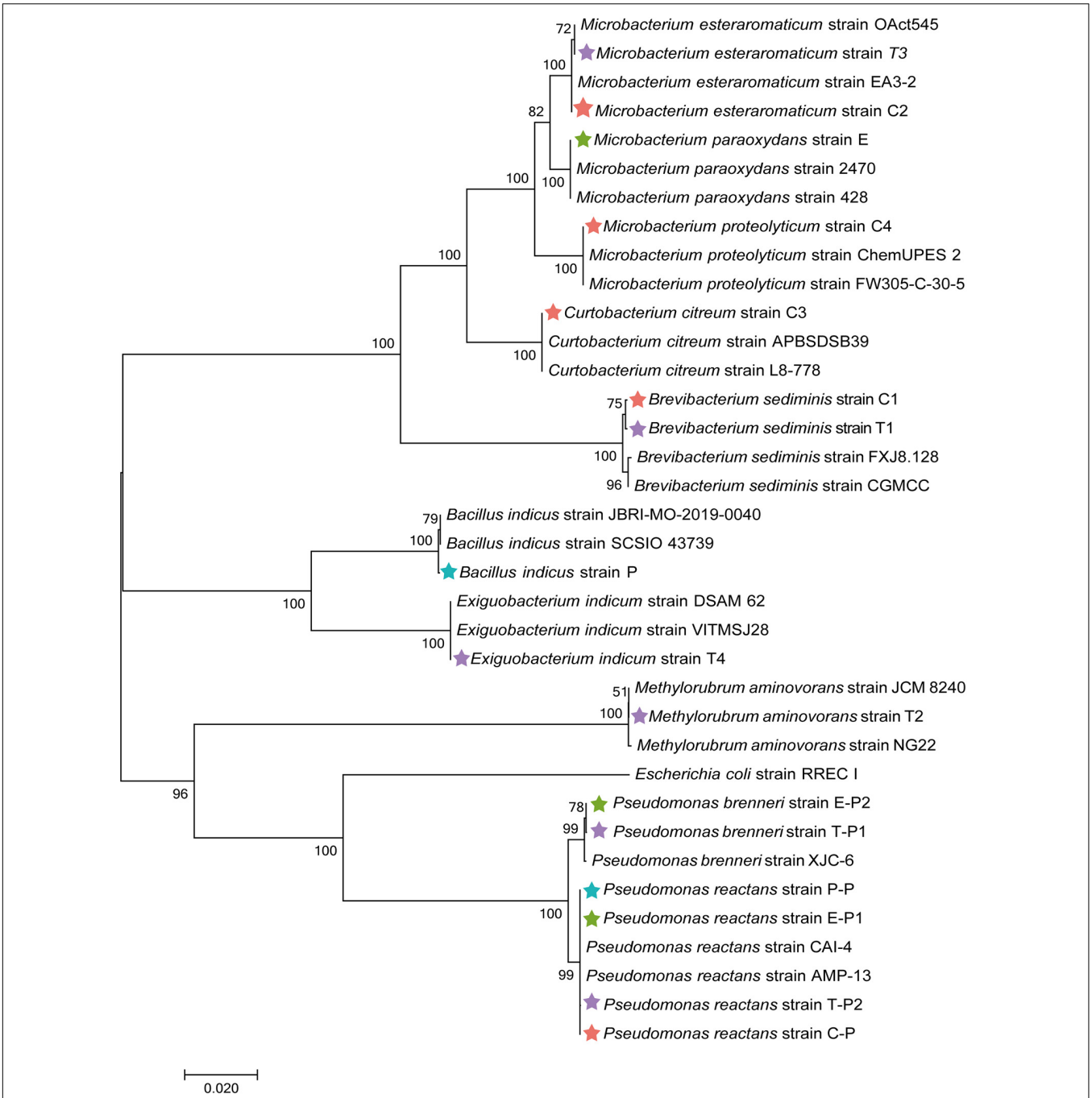

FIGURE 6 | Phylogenetic placement of isolated bacteria based on their 16S rRNA gene fragment sequences. Neighbor-joining method in MEGA7 with 1,000 bootstrapping replications was used to generate phylogenetic tree. Highly matching sequences of bacteria from public nucleotide databases were included; *Escherichia coli* RREC I was used as an outgroup. Isolates from cabbage-fed aphids are labeled with red stars, eggplant-fed aphids with green stars, pepper-fed aphids with blue stars, and tobacco-fed aphids with purple stars.

manifest in subsequent generations. For this purpose, targeted studies could be conducted in which aphids are transferred back to the original host plant, followed by microbiome monitoring.

Overall, we found that even if the aphids were fed with different plants, *Buchnera* still remained as the most abundant primary symbiont in *M. persicae* guts and the attached bacteriocytes; however, it was substantially reduced after the transfer to Solanaceae hosts. *Buchnera* is known for its importance as the primary symbiont of various insects and is commonly inherited from mother to offspring (Baumann et al., 1995). We hypothesize that *Buchnera* was substantially reduced after the transition of aphids to the three Solanaceae hosts either

because the plants' secondary metabolites had a negative effect on it or they provided more suitable conditions for its competitors. This observation was confirmed by complementary qPCR analyses, which showed analogous trends when compared with the microbiome results. The implications of nutrient provision by primary symbionts of insects are well known (Moran et al., 2003; Akman Gündüz and Douglas, 2009). It can therefore be assumed that a temporally reduced occurrence of the primary symbiont, at least in the gut and the attached bacteriocytes, does not negatively affect the host. Interestingly, pepper-fed aphids showed a different bacterial community composition than the other two Solanaceae-fed aphids. The most evident difference was a lower increase of the genus *Pseudomonas* represented by one ASV, whereas the other two Solanaceae-fed aphids showed higher increases represented by multiple ASVs. Moreover, *Pseudomonas* was found to occur at very low abundances in cabbage-fed aphids (0.6%), but it substantially increased (maximum observed abundance: 69.4%) after the transition to Solanaceae-based diets. Complementary qPCR-based analyses confirmed a substantial enrichment in tobacco-fed and eggplant-fed aphids, whereas the aphids subjected to the pepper diet showed a comparatively lower enrichment of quantifiable *Pseudomonas* sequences in the total community DNA extracts. This is in line with the microbiome profiles that were generated with the high-throughput sequencing approach. Some *Pseudomonas* species are known to be pathogens (Flury et al., 2016), whereas others have beneficial traits, and some are even commercialized for certain applications in agriculture (Kupferschmied et al., 2013). In addition, various *Pseudomonas* species are associated with crop plants and can occur in different tissues and the plant rhizosphere. Some were found to be important elicitors of induced systemic resistance in plants (Kloepper et al., 2004). When plants are under attack by pathogens or insect pests, they can recruit beneficial pseudomonads for their defense (Lee et al., 2012). A previous study indicated that certain plant-beneficial *Pseudomonas* strains could improve the growth of feeding aphids under certain conditions (Blubaugh et al., 2018). The substantial enrichment of *Pseudomonas* in *M. persicae* observed in the present study may be partially due to uptake from host plants. We could also show that some pseudomonads are naturally occurring in the aphid's gut, although at substantially lower abundances if they are not subjected to a Solanaceae-based diet. Further future studies will be required to determine the proportion of bacteria transferred from the host plant to the feeding aphids. They will require additional sequencing of the plant leaf microbiome before exposure to aphids. A study by Stavrinides et al. (2009) showed that *Pseudomonas syringae* could use pea aphids as hosts and vectors to infect other plants. *Pseudomonas* was generally shown to be a highly efficient colonizer of insect hosts (Lindeberg et al., 2008). In a recent study, it has been shown that certain members of this genus can change from a commensal to a pathogenic lifestyle in the gut of insects (Xu L. et al., 2021). Our findings indicate that *Pseudomonas* may play a role in the adaption of aphids to new host plants. This observation is reinforced by our complementary analysis of population dynamics, which revealed that only bell pepper plants in which the smallest increases in *Pseudomonas* abundance were found negatively affected aphids. The microbiome of aphids fed with pepper plants was also characterized by an increase of low-abundant members of the bacterial phylum *Firmicutes*. The genera *Sporosarcina* and *Ruminococcus* have significantly enriched these aphids. Members of the genus *Sporosarcina* are occurring in the environment and are known for their bioremediation potential (Achal et al., 2012), whereas *Ruminococcus* is mainly associated with the gut system of mammals (La Reau et al., 2016). Less is known about their potential roles in insects. The underlying mechanisms of the diet-induced microbiome changes and the potentially linked implications for host fitness remain to be explored in more detail in the future because they may provide an exploitable basis for the control of *M. persicae* and other insect pests. In addition to the increased abundance of *Pseudomonas* and decrease of *Buchnera* in Solanaceae-fed aphids, another bacterium that is commonly associated with insects was also subjected to changes. *Wolbachia* is generally known to be less abundant in aphids than the predominant *Buchnera* but can also occur as a symbiont (Augustinos et al., 2011). In the present study, *Wolbachia* followed the same trend as *Buchnera*; a substantial decrease in its relative abundance was observed after the aphid's transition to Solanaceae plants. Based on the observations of this study, it seems that this reduction also does not negatively affect the host.

As a complementary approach to the bioinformatics analyses, we also aimed to assess parts of the cultivable fraction in the present study to explore potential links to their tolerance of a bioactive metabolite common in the Solanaceae plant family. Nicotine has strong insecticidal properties and is used in different formulations for plant protection (Casanova et al., 2002). It was previously observed that *M. persicae* could adapt to high nicotine concentrations in host plants (Ramsey et al., 2014). Although certain genes are associated with detoxification processes in the aphid, other mechanisms remained unexplored. To assess the potential roles of facultative symbionts isolated from *M. persicae* guts, we conducted tolerance assays with nicotine. Nicotine is generally known to have toxic and pharmacological properties and thus affects a wide range of organisms, including bacteria (Hossain and Salehuddin, 2013; Vieira et al., 2013). We could show that several bacterial strains isolated from aphid guts displayed a high nicotine tolerance under laboratory conditions. Various facultative symbionts are not required for aphid survival, reproduction, and invasion, but they can still play important roles in their host (Oliver et al., 2010). For instance, facultative symbionts can affect the aphid's adaptability to plants, and they can improve nutrient uptake of their host among various other protective properties (Russell and Moran, 2006; Oliver et al., 2010; Tsuchida et al., 2010; Gauthier et al., 2015). The susceptibility tests toward nicotine showed that the isolated bacteria tolerated the toxic metabolite to different extents. With the increasing nicotine concentrations, the growth rate of all tested bacteria decreased. *M. aminovorans* (basionym: *M. aminovorans*) strain T2 was an exception; it was already inhibited by low nicotine concentrations, although it was isolated from aphids feeding on tobacco. Methylobacteria are nonspore-forming bacteria and form Gram-negative rods that can utilize methanol and often other small hydrocarbons for their growth Urakami et al. (1993). They are commonly found

in the atmosphere, soil, and on plant leaves in close spatial proximity to stomata, where some can produce plant-growth-promoting substances (Holland, 1997; Green and Ardley, 2018). We assume that *M. aminovorans* T2 occurred in the aphid gut without having any implications for the host's adaptability to the host change. Among the other tested isolates, *B. sediminis*, *M. esteraromaticum,* and *B. indicus* strains were shown to tolerate nicotine at up to 8 g/l, but their growth rates were substantially reduced at this concentration. These isolates might be important facultative symbionts that facilitate the adaptation of *M. persicae* to Solanaceae-based diets. Interestingly, *P. brenneri* T-P1 and *P. reactans* T-P2, which were both isolated from tobacco-fed aphids, also tolerated nicotine well to a concentration of 4 g/l. This might be sufficient to facilitate the aphid's proliferation on the Solanaceae plant diets, but the detailed implications of pseudomonads under these conditions remain to be further explored.

CONCLUSION

In summary, our microbiome-guided assessment of bacterial communities in aphids subjected to different diets has shown that a transition to Solanaceae host plants resulted in substantial adaptations of their microbiome. The most evident response was the significant reduction of the primary symbiont *Buchnera* and the simultaneous increase of *Pseudomonas*. Complementary analyses revealed that facultative symbionts might facilitate the aphid's transition to secondary-metabolite-rich hosts. If the implications are confirmed, then future biotechnological developments could make use of the findings by harnessing pseudomonads that can thrive in insect guts but negatively affect the fitness and propagation of their hosts.

DATA AVAILABILITY STATEMENT

The 16S rRNA gene fragment amplicon library was deposited in European Nucleotide Archive (ENA; accession number: PRJEB42912) and the 16S rRNA gene sequences of the isolated bacteria were deposited in NCBI Genbank (accession numbers MW564208–MW564219 and MW699619–MW699622).

AUTHOR CONTRIBUTIONS

TC and HY conceived the idea and developed the study design. BH performed all laboratory experiments under the supervision of TC, HY, and XC. TC and BH performed the bioinformatic analyses, interpreted the data, and prepared the final visualizations. HY and XC provided valuable inputs related to insect rearing and physiology. TC, HY, and BH wrote the manuscript. All authors reviewed the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.667257/full#supplementary-material

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Spider Mites Singly Infected With Either *Wolbachia* or *Spiroplasma* Have Reduced Thermal Tolerance

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Heritable symbionts play an essential role in many aspects of host ecology in a temperature-dependent manner. However, how temperature impacts the host and their interaction with endosymbionts remains largely unknown. Here, we investigated the impact of moderate (20°C) and high (30 and 35°C) temperatures on symbioses between the spider mite *Tetranychus truncatus* and two maternally inherited endosymbionts (*Wolbachia* and *Spiroplasma*). We found that the thermal tolerance of mites (as measured by survival after heat exposure) was lower for mites that were singly infected with either *Wolbachia* or *Spiroplasma* than it was for co-infected or uninfected mites. Although a relatively high temperature (30°C) is thought to promote bacterial replication, rearing at high temperature (35°C) resulted in losses of *Wolbachia* and particularly *Spiroplasma*. Exposing the mites to 20°C reduced the density and transmission of *Spiroplasma* but not *Wolbachia*. The four spider mite strains tested differed in the numbers of heat shock genes (*Hsps*) induced under moderate or high temperature exposure. In thermal preference (Tp) assays, the two *Wolbachia*-infected spider mite strains preferred a lower temperature than strains without *Wolbachia*. Our results show that endosymbiont-mediated spider mite responses to temperature stress are complex, involving a combination of changing endosymbiont infection patterns, altered thermoregulatory behavior, and transcription responses.

Keywords: *Wolbachia*, *Spiroplasma*, *Tetranychus truncatus*, thermal tolerance, thermal preference

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INTRODUCTION

Temperature has a substantial impact on a wide range of ecologically important traits in many ectothermic organisms (Colinet et al., 2015; Sgro et al., 2016; Ma et al., 2021) and their symbiotic microbiota (Kiers et al., 2010). Heritable bacterial symbionts, which are pervasive in many arthropods, affect the host's response to abiotic stressors, including temperature (Lemoine et al., 2020). Bacterial endosymbionts can both mediate and constrain host adaptation to temperature

extremes (Corbin et al., 2017). For example, the obligate bacterial endosymbiont, *Buchnera,* limits the thermal tolerance of its aphid hosts and thus represents an "Achilles' heel" of their thermal response (Dunbar et al., 2007; Zhang et al., 2019), while the facultative endosymbiont *Serratia symbiotica* increases aphid survival or reproduction under heat exposure likely through releasing metabolites (Montllor et al., 2002; Burke et al., 2010). Some strains of *Wolbachia* can increase *Drosophila melanogaster*'s thermotolerance by accelerating dopamine metabolism (Gruntenko et al., 2017), while *Hamiltonella* infections in whitefly also confer a fitness advantage under heat that may involve induction of the expression of host-produced stress genes (Brumin et al., 2011). Thermal effects mediated by symbionts may therefore affect host distribution as well as responses to ongoing changing climate.

Conversely, the persistence and stability of the bacterial symbionts themselves can be affected by temperature stress (Wernegreen, 2012), which can impact facultative endosymbiont dynamics through influencing their density, transmission, and phenotypic effects (Renoz et al., 2019). For instance, heat stress from exposure to high temperatures (>30°C) reduces *Wolbachia* density in various species, including *Aedes aegypti* (Ross et al., 2020), *D. simulans* (Hoffmann et al., 1986), and *Nasonia vitripennis* (Bordenstein and Bordenstein, 2011), while in aphids it affects densities of the nutritional obligate symbiont *Buchnera* (Zhang et al., 2019). Reduced *Wolbachia* density results in weaker cytoplasmic incompatibility (CI) which assists endosymbionts like *Wolbachia* to spread in populations, and reduced endosymbiont density can also lead to maternal transmission failure (Ross et al., 2017). Other host species such as *Acyrthosiphon pisum,* that have a complex mixture of symbionts, can show a recovery of their obligate symbionts after heat stress that depends on their facultative symbionts (Heyworth et al., 2020). Thus, impacts of thermal conditions on symbionts may vary depending on the nature of the stress, host species, and symbiont community.

The spider mite, *Tetranychus truncatus*, is a dominant mite species in China (Jin et al., 2018) and is frequently associated with multiple endosymbionts including *Wolbachia* and *Spiroplasma* (Zhu et al., 2018). Both these symbionts are maternally inherited and can co-infect the same *T. truncatus* host. *Wolbachia* induces incomplete CI in spider mites (Yang et al., 2020). A comprehensive survey of endosymbionts in the natural population of *T. truncatus* has shown that the incidence of endosymbionts is associated with environmental factors. In particular, *Wolbachia* infection rates increase at localities with higher annual mean temperatures (Zhu et al., 2018). This is in contrary to high temperatures negatively impacting *Wolbachia* transmission and CI strength in the related mite T. *urticae* (van Opijnen and Breeuwer, 1999), which may be correlated with the lytic activity of phage WO (Lu et al., 2012). *Wolbachia*-induced CI phenotype and transmission are therefore temperature dependent in spider mites; however, the impact of temperature on endosymbiont interactions is not clear in *T. truncatus*.

In the present study, we investigated the impact of high and low temperatures on the survival of females from *T. truncatus* strains that were either uninfected or singly and doubly infected with *Wolbachia* and *Spiroplasma* strains. We also tested the effect of temperature stress on endosymbiont density and vertical transmission efficiency, and we explored the transcriptome to investigate possible mechanisms associated with any endosymbiont-mediated temperature responses. Finally, we tested whether infection with *Wolbachia* and *Spiroplasma* affected host thermal preference (Tp). We hypothesized that spider mites infected with different symbiont strains would display differences in thermal tolerance and Tp; in turn, symbioses of *Wolbachia* and *Spiroplasma* with *T. truncatus* would be affected by temperature stress.

MATERIALS AND METHODS

Spider Mite Strains and Rearing

Four spider mite strains with a similar genetic background were established (Yang et al., 2020; Zhu et al., 2020): strains co-infected with *Wolbachia* and *Spiroplasma* (designated as *w* + *s* +), *Wolbachia* only (*w* +), *Spiroplasma* only (*s* +), or no symbionts (*w-s-*). Symbiont-infected mite strains (*w* + *s* + , *w* +, and *s* +) were collected from Shenyang, Liaoning Province, China. A *w-s-* strain was obtained by tetracycline treatment of the *s* + strains for three generations. To eliminate potential effects of the tetracycline, we cultivated the *w-s-* strain without tetracycline treatment for 15 generations. To minimize genetic background differences between four spider mite strains, we mated infected female adults with the uninfected male adults and backcrossed female offspring for at least seven consecutive generations.

Spider mite strains were reared on leaves of the common bean (*Phaseolus vulgaris* L.) placed on a water-saturated sponge mat in a Petri dish at 25 ± 1°C and 60% relative humidity and under 16 h light: 8 h dark conditions. Prior to the experiment, the infection status was checked as by PCR (Yang et al., 2020). A schematic of the study design is given in **Supplementary Figure 1**.

Survival at Constant Temperatures

To determine survival of the four strains following different temperature treatment, we exposed 20 female adults (2-day) to either 20, 25, 30, or 35°C in an incubator and then monitored spider mite survival daily for a week using a stereo microscope. Six biological replicates for each treatment were tested.

Crossing Experiments

Wolbachia-induced incomplete CI was determined through crosses as previously described (Yang et al., 2020). To test the impact of different temperatures on the strength of CI, females without *Wolbachia* (♀: *s* + or *w-s-*) were crossed with males infected with *Wolbachia* (♂: *w* + *s* + or *w* +), producing a total of four crosses. Single females in the teleiochrysalis stage and a 1-day-old adult virgin male were placed on the same leaf disk, which was subjected to one of four temperature treatments: 20, 25, 30, or 35°C. Each cross and treatment combination was replicated 18–99 times using different leaf disks. Females were allowed to lay

eggs for 5 days while maintaining treatment temperatures, and then removed. Eggs on leaf disks were continued to be kept under their respective treatment temperatures and were checked daily to calculate hatchability.

Transmission Efficiency of *Wolbachia* and *Spiroplasma*

To examine the vertical transmission of *Wolbachia* and *Spiroplasma*, spider mites from each strain were placed on bean leaf discs (diameter ca. 3 cm) and were reared for four generations under 20, 25, 30, or 35°C. Twenty female adults (2 days old) were randomly selected from each treatment and generational combination (F1 to F4) of these cultures and used in PCR analyses. Six biological replicates were set up per strain. DNA was extracted from a single mite using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. *Wolbachia* and *Spiroplasma* infections were screened by PCR amplification using the specific primers listed in **Supplementary Table 1**. PCRs were carried out using a Veriti thermocycler (ABI Biosystems, United States) in a 25 μl volume containing 12.5 μl 2 × rapid Taq master mix (Vazyme Biotech, China), 0.5 μl primers (20 μM each), and 1 μl of DNA extract. PCR cycling parameters were 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, the annealing temperature for 45 s, and 72°C for 15 s, and then 72°C for 5 min at the end. PCRs included a positive and negative control and were run on a 1% agarose gel with ethidium bromide to visualize the product.

Densities of *Wolbachia* and *Spiroplasma*

To estimate the dynamics of *Wolbachia* and *Spiroplasma* following different temperature exposures, the ratio of the single-copy genes *16S rRNA* (*Spiroplasma*) and *wsp* (*Wolbachia*) to the *rps-18* (spider mite host) reference gene was determined by real-time qPCR (Yang et al., 2020). Spider mites subjected to the four different temperatures were sampled at 6, 12, and 24 h, as well as on the third and fifth days. For each time point, nine biological replicates were tested per treatment. For each of the biological replicates, we performed three technical replicates. Densities of *Wolbachia* and *Spiroplasma* were estimated by qPCR with the ABI QuantStudio 6 Flex (Applied Biosystems, CA, United States). The 20-μl Q-PCR reaction mixture consisted of 10 μl 2 × SYBRP remix Ex Taq (Vazyme, China), 0.4 μl 10 mmol/L of each primer, 0.4 μl 50 × ROX Reference Dye, 2 μl DNA template, and 6.8 μl H_2O in single wells of a 96-well plate (PE Applied Biosystems, CA, United States). The Q-PCR cycling conditions included one cycle (5 min at 95°C) followed by 40 cycles (10 s at 95°C and 34 s at 60°C), and finally one cycle to produce melting curves (15 s at 95°C, 1 min at 60°C, and 15 s at 95°C). The primers are listed in **Supplementary Table 1**. Standard curves were plotted using a 10-fold dilution series of the DNA samples prepared from plasmid DNA. The plasmid DNA was obtained with the pEASY-T3 vector (TransGen Biotech, Beijing, China). The quality and concentration of all purified standard DNA were measured on a Nanodrop 2000 (Thermo Scientific, MA, United States).

Transcriptome Processing and Annotation

To explore transcriptional responses to short-term high or low temperature exposure in the four spider mite strains, we sampled 2-day-old female adults with exposure to either 20, 25, 30, or 35°C for 6 h. About 100 adult female spider mites (2-day-old) were collected for each replicate and then frozen in liquid nitrogen prior to RNA extraction. Four biological replicates for each treatment were tested.

Total RNA was extracted from each sample using Trizol protocol according to manufacturer's instructions (Invitrogen, CA, United States). RNA quality was qualified and quantified using a Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, United States). Construction of cDNA libraries and subsequent sequencing using the BGIseq500 platform were conducted at BGI-Shenzhen, China. Raw data were filtered using SOAPnuke v1.5.2 by removing reads containing adapters, poly-N, and low-quality reads to obtain clean data. Different gene expression was analyzed using the DESEQ2 package (Love et al., 2014) with Q value ≤ 0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis and Gene Ontology (GO) were based on the KEGG pathway database[1] and the GO database[2].

To confirm the results of the RNA-seq analysis, the expression levels of randomly selected genes were determined by RT-qPCR. The RT-qPCR reactions were performed on an ABI QuantStudio 6 Flex Real-Time PCR System with SYBR Premix Ex Taq (Takara Bio, Kyoto, Japan). The amplification reactions were performed in a 20 μL final volume containing 10 μL of TaKaRa buffer, 7.2 μL of ddH_2O_2, 0.4 μL of DyeII (TaKaRa), 0.2 μL of forward primer (5 mM) and reverse primer (5 mM), and 2 μL of cDNA first-strand template. Thermal cycling conditions were as follows: 95°C for 10 min, then 40 cycles of 5 s at 95°C, and 34 s at 60°C. There were three technical replicates for each sample. Primer sequences were designed using Primer Premier 6.0, and are listed in **Supplementary Table 1**. Expression levels for each gene were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Thermal Preference Assays

Temperature preference assays were performed using a custom-built thermal gradient apparatus consisting of a 500-mm-long aluminum bar along which a temperature gradient (10–40°C) created with water baths at the ends. Six grooves were etched along the aluminum bar which allowed mites to move freely up or down the gradient without interacting with each other (**Supplementary Figure 2**). All assays were conducted in a room with a constant temperature of 25°C and constant 40% humidity. For each data point, six individuals of the same strain were, respectively, transferred to the center point of each groove in the apparatus (25°C) and allowed to move freely along its groove for 30 min. The temperatures at the positions of rest for the six spider mites after 30 min, as measured with K-type thermocouples, were used to calculate a mean preferred temperature. In total, more

[1]http://www.genome.jp/kegg/

[2]http://www.geneontology.org/

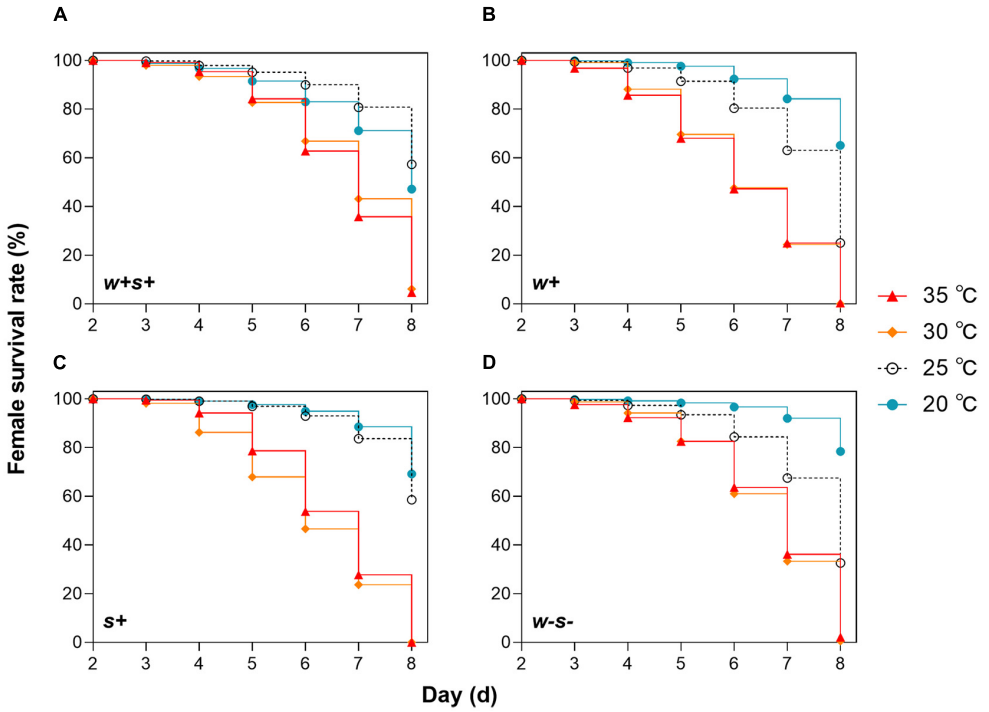

FIGURE 1 | Survival rates of four spider mite strains over 8 days under different temperatures. **(A)** *Wolbachia* and *Spiroplasma* co-infected strains (*w+s+*), **(B)** singly *Wolbachia*-infected strains (*w+*), **(C)** singly *Spiroplasma*-infected (*s+*), and **(D)** uninfected stains (*w-s-*).

than 282 individuals from each spider mite strain were used to test host Tp.

Statistical Analysis

All statistical analyses were carried out in R ver 3.3.1 or GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, United States). Log-rank (Mantel–Cox) tests were used to compare the survival proportions of the spider mite strains under different temperatures. We also compared differences in survival rate between each treatment at the same time point using two-way ANOVAs with multiple comparisons. We used either ANOVAs or Mann–Whitney tests to compare endosymbionts densities, egg hatch proportions, and frequencies of endosymbiont infection, depending on whether data were normally distributed or deviated from normality based on Kolmogorov–Smirnov tests. We conducted a non-parametric Kruskal–Wallis test to compare the Tp values. Differences were considered significant at $p < 0.05$ for all analyses.

RESULTS

Singly Infected *Wolbachia* or *Spiroplasma* Strains Have Lower Fitness Than a Co-infected Strain at Higher Temperatures

We first compared female survival for mites reared under either 20, 25, 30, or 35°C. The survival rate of four spider mite strains was significantly ($p < 0.0001$) affected by temperature (log-rank test, $w + s +$: $\chi^2 = 311.2$, $df = 3$; $w +$: $\chi^2 = 660.8$, $df = 3$; $s +$: $\chi^2 = 885.0$, $df = 3$; *w-s-*: $\chi^2 = 522.5$, $df = 3$; **Figure 1**). After 2–4 days, the female survival rates of co-infected strains were not significantly different at 25, 30, and 35°C, but after 4 days, the female survival rate of the other three strains was significantly lower at the higher temperatures (30 and 35°C) than at 25°C (**Figure 1**). This suggests that co-infection with *Wolbachia* and *Spiroplasma* delayed high temperature effects on the host. Moreover, when the four mite strains were reared at 30 and 35°C, the female survival rate after 5 days was sharply lower in the singly infected strains than in the co-infected and uninfected strains (**Figure 1**). These results indicate that spider mites singly infected with *Wolbachia* or *Spiroplasma* are sensitive to high temperatures. In contrast, female survival rate did not differ significantly between 20 and 25°C for either co-infected or *Spiroplasma*-infected strains at any time point. However, survival did sharply decrease at 25°C in *Wolbachia*-infected and uninfected strains after 5 days (**Figure 1**). Spider mites infected with *Spiroplasma* may therefore have a higher fitness at the intermediate temperature.

Higher and Lower Temperatures Modify the CI Strength of *Wolbachia*

Consistent with previous results (Yang et al., 2020), we found that *Wolbachia* induced incomplete CI, resulting in 20–30% offspring mortality when mites were reared at 25°C. Embryos

showed aborted development when CI occurred (**Figure 2A**). Exposure to a high temperature (35°C) resulted in a significant decrease in the mean egg hatch rate in the cross between *Spiroplasma*-infected females and co-infected or *Wolbachia*-infected mites compared to those same crosses at 25°C (Mann–Whitney test, $w + s + ♂ \times s + ♀$: 35°C vs. 25°C, $p = 0.0025$; $w + ♂ \times s + ♀$: 35 vs. 25°C, $p = 0.0002$) (**Figure 2B**). The hatch proportion significantly declined at the lower temperature in the cross between co-infected males and uninfected females at 20°C compared to those at 25°C, from 77.78% at 25°C to 69.39% at 20°C (Mann–Whitney test: $p = 0.0004$). For the crosses between uninfected females and *Wolbachia*-infected males, there was no significant difference in the hatch proportion of offspring exposed to either 20, 25, 30, or 35°C (**Figure 2B**).

Higher Temperatures Affect Endosymbiont Titer in Female Hosts

We measured the effect of temperature stress on *Wolbachia* and *Spiroplasma* density in female hosts. *Wolbachia* titers in co-infected strains exposed to 35°C were significantly higher than in these strains at 25°C at days 1, 3, and 5, but no significant difference between strains was found at 20 and 25°C at all time points (**Figure 3A**). In contrast to co-infected strains, *Wolbachia* titers in *Wolbachia* singly infected strains at either 20 and 35°C were significantly lower than those at 30 or 25°C at day 3 (**Figure 3B**). *Spiroplasma* densities in both co-infected and *Spiroplasma*-infected strains exposed to either 30 or 35°C were significantly higher than those strains at either 20 or 25°C at day 0.5 (**Figures 3C,D**). *Spiroplasma* densities in co-infected strains fluctuated at later time points under high and low temperatures, while there were no significant differences in *Spiroplasma* densities in singly infected strains among temperature treatments at days 1, 3, and 5 (**Figures 3C,D**). The average titer and the shifts in titer with age varied across spider mite strains.

High Temperatures Reduce Infection Frequency of Both *Wolbachia* and *Spiroplasma*

We tested the *Wolbachia* or *Spiroplasma* infection frequency in co-infected and singly infected strains over four generations when mites were held at 20, 25, 30, or 35°C. At 25°C, the normal rearing temperature for host spider mites, both *Wolbachia* and *Spiroplasma* were stably maintained with 100% prevalence in either co-infected or singly infected strains through successive host generations (**Figures 4A–D**). However, *Spiroplasma* was lost in both co-infected and singly infected strains when mites were reared at 35°C for one generation (**Figures 4C,D**). The prevalence of *Wolbachia* in singly infected strains sharply decreased from 97.5% in the F1s to 14.2% in the F2s and the infection was nearly lost in later generations, but in co-infected mites, the reduction at 35°C was less (**Figures 4A,B**). At 30°C, *Wolbachia* maintained nearly 100% prevalence in both co-infected and singly infected strains. In contrast, the prevalence

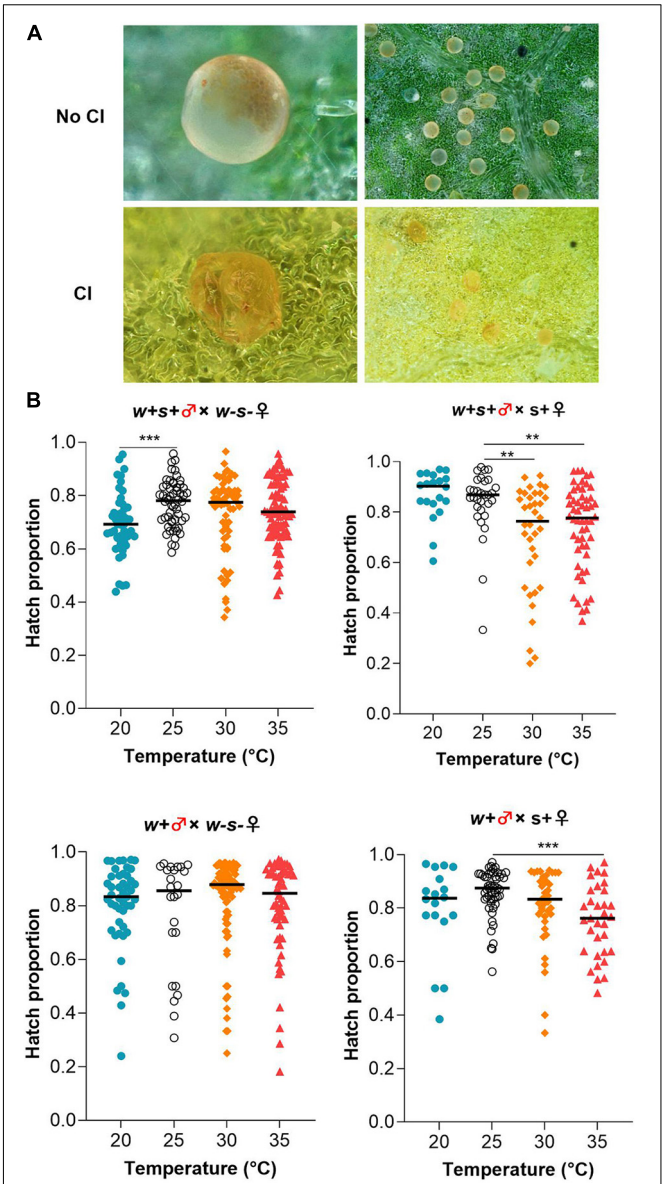

FIGURE 2 | Effect of low (20°C) and high (30 or 35°C) temperatures on *Wolbachia*-induced CI. **(A)** Visual differences in normal eggs (upper panels) and stunted eggs from CI (lower panels). **(B)** Egg hatch rates in four crosses between different spider mite strains at either kept under different temperatures. Horizontal bars indicate the medians based on $n = 18–99$ per treatment. Mann–Whitney test, $^{**}p < 0.01$, $^{***}p < 0.001$.

of *Spiroplasma* in singly infected strains decreased from 90.8% in the F1s to 55.8% in the F2s, while the prevalence of *Spiroplasma* in co-infested strains fluctuated across the four generations (**Figures 4C,D**). These results suggest that high temperature reduces vertical transmission efficiency of *Wolbachia* or *Spiroplasma* in all spider mite strains with varying degrees, and *Spiroplasma* is more sensitive to high temperatures than *Wolbachia*. Exposure to a cooler temperature (20°C) did not affect the infection frequencies of *Wolbachia* and *Spiroplasma* in singly infected strains. However, the *Spiroplasma*

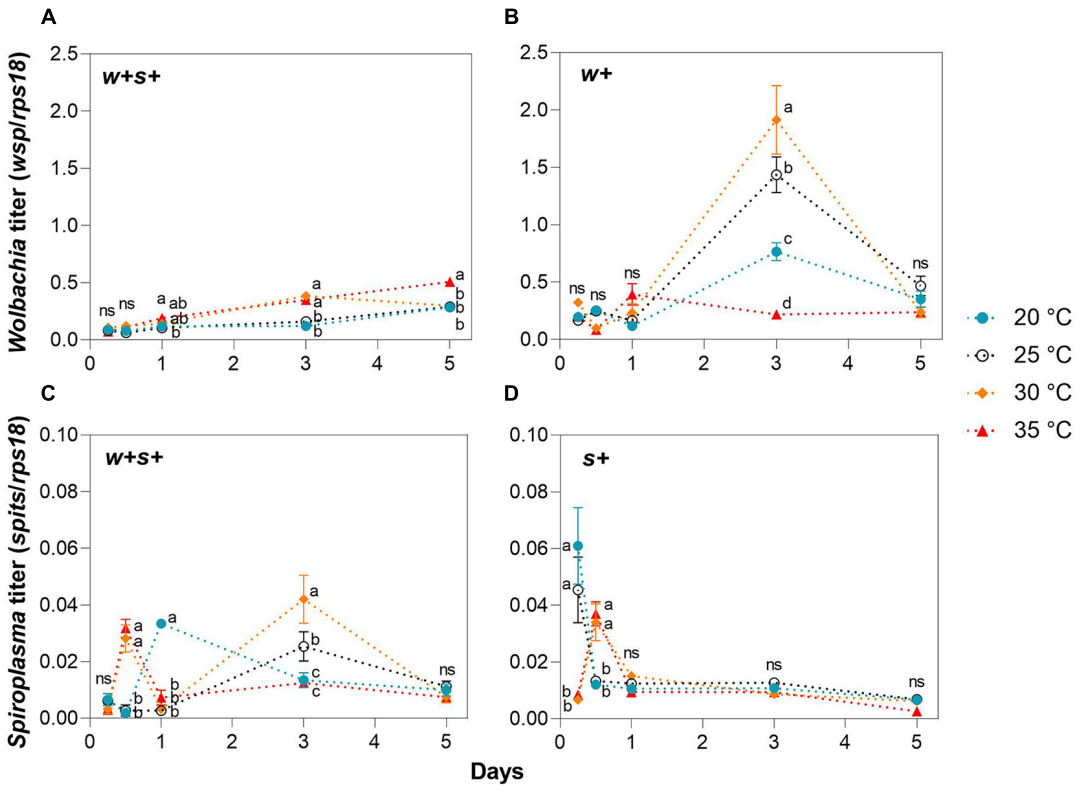

FIGURE 3 | *Wolbachia* and *Spiroplasma* densities in female adult spider mites maintained under low (20°C) and high (30 or 35°C) temperatures for up to 5 days. *Wolbachia* density in co-infected strains **(A)** and *Wolbachia*-infected strains **(B)**. *Spiroplasma* density in co-infected strains **(C)** and *Spiroplasma*-infected strains **(D)**. Data are shown as the mean ± SEM. Different letters indicate significant differences between each treatment at the same time point ($p < 0.05$; ns, no significant).

infection rate at this temperature in co-infected strains rapidly decreased from 88.3% in the F1s to a low level of 25% in the F3s, before increasing in later generations to 80.8%. The *Wolbachia* infection also varied somewhat in the co-infected strain across generations when mites were held at 20°C (**Figure 4A**).

Gene Expression Differentiation Among Four Spider Mite Strains Under High and Low Temperatures

Comparison of transcriptional responses to high and low temperatures was based on a total of 2,685.47 Mb clean reads from 63 libraries derived from four spider mite strains (**Supplementary Table 1**). Unigenes were annotated to the KEGG and GO databases (**Supplementary Figure 3**). The PCoA analysis explained 87.88% of the variance in gene expression of the four spider mite strains under different temperatures (**Figure 5A**). PCoA demonstrated that the expression patterns of the singly infected *Wolbachia* strain and singly infected *Spiroplasma* strain were closely related at all temperatures (**Figure 5A**).

Variation in gene expression between temperatures was analyzed through comparisons of 35 vs. 25°C, 30 vs. 25°C, and 20 vs. 25°C. The doubly infected strain had the fewest differentially expressed genes compared with the other three mite strains under both high and low temperature exposure (**Figure 5B**—note scale differences). For the low temperature (20°C) comparison, *Spiroplasma*-infected strains showed the most differently expressed genes (DEGs). For 35°C comparison, the number of DEGs in un-infected strains was higher than that in the other three spider mite strains when compared to the control treatments.

Among the DEGs, expression of the heat shock protein genes, including *Hsp20*, *Hsp70*, and *Hsp90*, varied in particular between the four spider mite strains. Within the *Hsp20* superfamily, a total of 11, two, and six orthologous genes were strikingly up-regulated in *Spiroplasma*-infected, *Wolbachia*-infected, and uninfected spider mite strains when spider mites were reared at 35 vs. 25°C. In contrast, two *Hsp* genes, *Hsp70* and *Hsp90*, were significantly down-regulated in *Spiroplasma*-infected strains under low temperature exposure (**Figure 5C**). We selected eight genes for RT-qPCR validation of differential expression in all samples. The results showed a concordant direction of change between the qPCR and transcriptomic analyses (**Supplementary Figure 4**).

Effects of Endosymbionts on Thermal Preference in Spider Mite

We used a custom-built thermal gradient to test whether *Wolbachia* and *Spiroplasma* affected temperature preference.

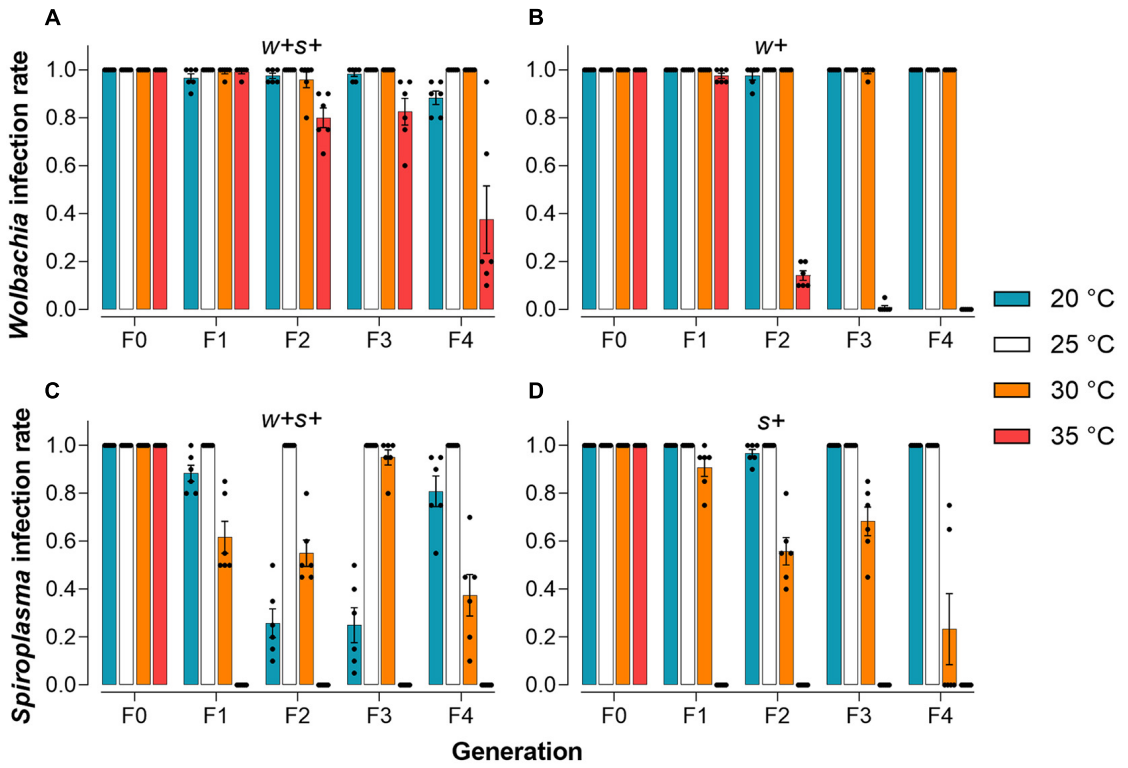

FIGURE 4 | Infection frequencies of two endosymbionts in female adult spider mites reared for four generations under different temperature conditions. Infection rate of *Wolbachia* in co-infected strains **(A)** and *Wolbachia*-infected strains **(B)**. Infection rate of *Spiroplasma* in co-infected strains **(C)** and *Spiroplasma*-infected strains **(D)**.

The four spider mite strains exhibited a large variance in Tp, falling between 11.80 and 33.40°C (**Figure 6A**). There was some overlap in Tp distributions between the four strains (**Figure 6B**). However, the preferred average temperature of *Wolbachia* and *Spiroplasma* co-infected strains (Tp = 20.74 ± 0.22°C) and *Wolbachia*-infected strains (Tp = 20.21 ± 0.20°C) were significantly lower than either *Spiroplasma*-infected (Tp = 22.31 ± 0.20°C) or uninfected strains (Tp = 22.47 ± 0.18°C) (Kruskal–Wallis statistic: 67.67, $p < 0.0001$; **Figure 6A**), representing a difference of around 2°C. The results showed that spider mites infected with *Wolbachia* preferred cooler temperatures than uninfected spider mites.

DISCUSSION

In this study, we found that thermal tolerance was lower for mites that were singly infected with either *Wolbachia* or *Spiroplasma* than for co-infected and uninfected mites (**Supplementary Table 3**). It is likely that spider mites singly infected with either *Wolbachia* or *Spiroplasma* have reduced thermal tolerance, but co-infection by the two endosymbionts confers a level of protection for their hosts. Host fitness costs associated with high-temperature stress appear to be magnified when spider mites are singly infected with either *Wolbachia* or *Spiroplasma* (Corbin et al., 2017). In contrast, the co-infecting endosymbionts may additively or synergistically confer greater tolerance in their host (Vautrin and Vavre, 2009) which may improve performance under stressful environmental conditions (Renoz et al., 2019).

While underlying mechanisms remain unclear, researchers have previously documented that endosymbionts are vulnerable to both high- and low-temperature stress and that temperature-challenged insects with a disrupted symbiotic system often show elevated mortality and other defective phenotypes (Prado et al., 2010). Symbioses of *Wolbachia* and *Spiroplasma* with *T. truncatus*, like other heritable symbioses, appear vulnerable to temperature stress (Corbin et al., 2017). High temperatures can reduce endosymbiont density and even eliminate symbionts in various insects (Doremus et al., 2019). *Wolbachia* density can be reduced and lost within three generations when *T. urticae* is reared at 35°C (van Opijnen and Breeuwer, 1999). This also appears to be the case for *T. truncatus*, where *Wolbachia* is reduced over generations when the host is reared at 35°C but remains stable at 20 and 25°C. *Spiroplasma* is sensitive to both warmer and cooler conditions in co-infected spider mites when compared to the normal rearing temperature of 25°C. A similar phenomenon has been observed in *D. nebulosa*, where *Spiroplasma* was rapidly lost at 18°C

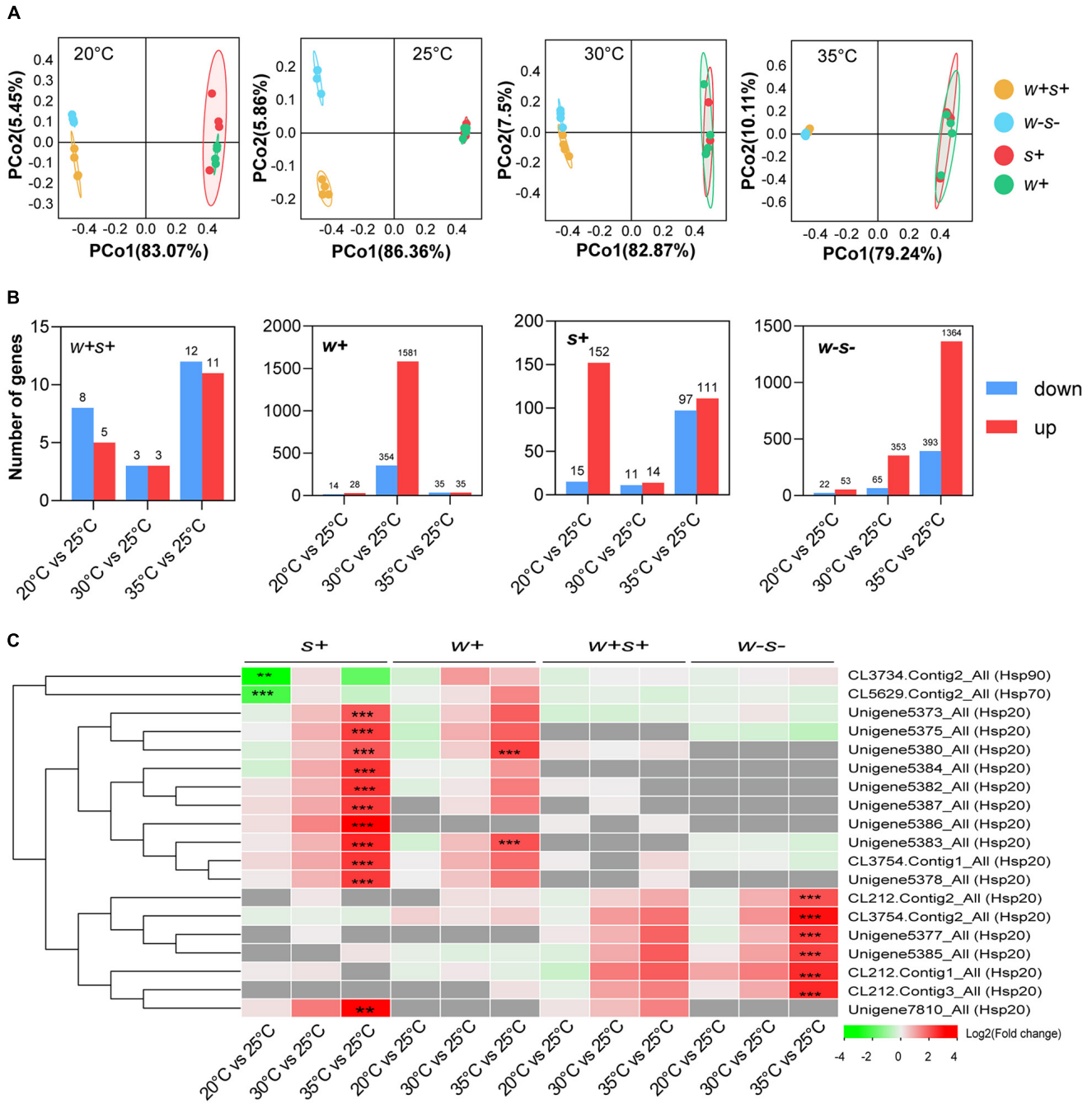

FIGURE 5 | Transcription response of four female adult spider mite strains following 6 h of different temperature exposures. **(A)** Principal components analysis (PCoA) of gene expression patterns of four spider mite strains under 20, 25, 30, and 35°C. **(B)** Number of differentially expressed genes (DEGs) with fold change > 2 and an FDR-adjusted *p*-value of < 0.05 for four spider mite strains under different temperatures. **(C)** Significantly differently expressed *Hsp* genes were analyzed by comparing each of 20, 30, and 35°C vs. the control (25°C) based on a threshold of > 2-fold change and an FDR-adjusted *p*-value of < 0.05. The gray squares in the heat map indicate that gene expression was absent. Asterisks indicate statistically significant differences (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

and gradual loss occurred at 28°C (Anbutsu et al., 2008). Taken together, these results suggest that *Wolbachia* infections in mites differ from *Spiroplasma* in their response to low- and high-temperature stress, in that (i) *Spiroplasma* are more susceptible to high temperature than *Wolbachia* in either co-infected or singly infected strains, possibly due to *Spiroplasma* density being lower than *Wolbachia* density, and (ii) *Wolbachia* are more stable under high temperatures in doubly infected strains than in singly strains, whereas *Spiroplasma* seem more cold-sensitive in

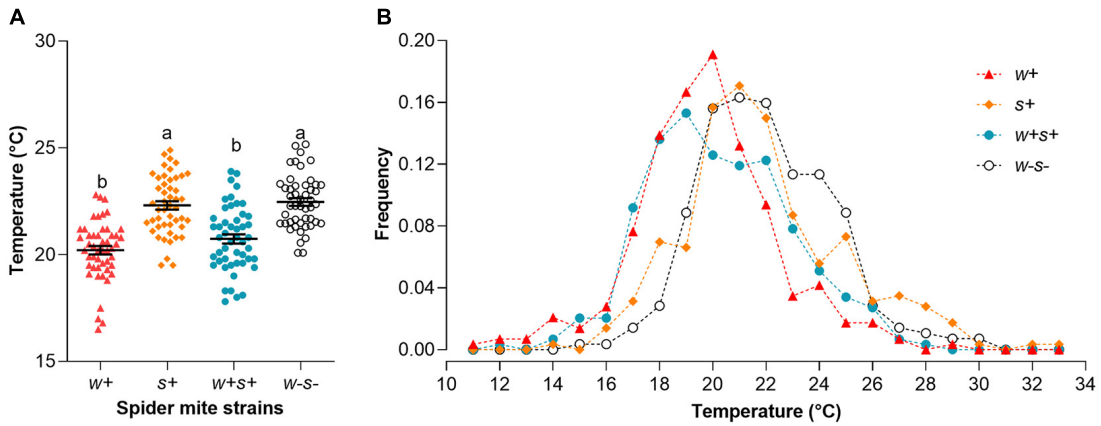

FIGURE 6 | Thermal preference (Tp) of the four spider mite strains. **(A)** Average Tp of the four spider mite strains. Each dot on the graph indicates the mean of six individuals in each replicate. Long bar indicates the mean of all dots in the strain, and error bars indicate SE. Different letters indicate significant differences between four spider mite strains ($p < 0.05$). **(B)** Relative proportions of the four spider mite strains observed at a given temperature.

doubly infected strains than in singly infected strains. These patterns are consistent with the notion that temperature affects symbiont density and transmission in different ways, depending on the symbiont, host, and nature of the co-infection (Renoz et al., 2019).

Intriguingly, at both 30 and 20°C, the frequency of *Spiroplasma* in co-infected strains decreased at the second generation and then fluctuated in later generations. When one of the co-infected endosymbionts causes CI, this helps to maintain not only the CI-causing endosymbiont but also any co-infecting endosymbiont *via* a hitchhiking effect (Engelstadter et al., 2004). *Wolbachia* induced incomplete CI in co-infected *T. truncatus* strains. Thus, the *Spiroplasma* infection which exhibited an improvement under high or low temperature in co-infected strains may be hitchhiking with co-infecting *Wolbachia* that induce CI. This also partly helps to explain why co-infections with multiple endosymbionts are common in natural populations of *T. truncatus* (Zhu et al., 2018).

Temperature-induced changes to endosymbionts densities are likely to have cascading effects. For example, high temperature decreased *Wolbachia* and *Cardinium* density and thus decreased CI levels in *N. vitripennis* and Euphorbia *suzannae* (Bordenstein and Bordenstein, 2011; Doremus et al., 2019). In contrast, *Wolbachia* quickly replicate in *D. simulans* (Clancy and Hoffmann, 1998) and *Leptopilina heterotoma* wasps (Mouton et al., 2006) at warmer temperature, and yet CI strength decreases. Thus, complex interactions between symbiont densities and temperature influence CI strength variation, and this process may involve other factors such as host behavior, development, host genetic variation (Shropshire et al., 2020), and especially activity of the CI enzyme CidB, which also varies with temperature (Beckmann et al., 2019). Here, we found that high and low temperatures may impact *Wolbachia* density and *Wolbachia* CI strength; however, there was no direct association between CI strength and *Wolbachia* density (Yang et al., 2020). In *T. urticae*, high temperature also reduced *Wolbachia* densities, but CI strength may associate with an increase in phage WO lytic activity (Lu et al., 2012). This remains to be tested in *T. truncatus*.

In various heritable symbiotic systems, the thermal sensitivity of bacterial symbionts is likely to contribute to their ability to establish and persist in natural populations (Wernegreen, 2012). *Wolbachia* frequencies are high in spider mites across China and increase at localities with higher annual mean temperatures, while *Spiroplasma* is present at relatively low frequencies and patchily distributed (Zhu et al., 2018). The temperature conditions examined in this study are relevant to natural conditions experienced by mites in the field, although conditions there are variable, and it would be worth carrying out additional experiments under fluctuating temperatures (Ross et al., 2017).

We report for the first time that *Wolbachia* modifies Tp in spider mites, which might represent a behavioral accommodation to host–symbiont interactions (Truitt et al., 2019). Recently, *Wolbachia* strains in some *Drosophila* species were shown to prefer cooler temperatures (Arnold et al., 2019; Truitt et al., 2019; Hague et al., 2020), consistent with the current results. Differences in Tp between infected and uninfected flies could result from conflicting physiological requirements of *Wolbachia* and hosts (Hague et al., 2020), and this notion is worth further examination. We speculate that, as a consequence of trade-offs in thermal adaptation and balancing selection between the symbiont and the host, *Wolbachia*-mediated host thermoregulatory behavior may affect mite thermoregulation, endosymbiont spread, and the maintenance of facultative symbioses.

The main molecular mechanisms of symbiont-mediated effects on host tolerance may involve endosymbiont-induced changes in stress-response genes (Brumin et al., 2011). Among these genes, *Hsp* genes such as *Hsp*70, *Hsp*90, and small heat shock protein (*sHsp*) have been reported to play roles in high-temperature responses (Dahlgaard et al., 1998; Chen and Wagner, 2012). These proteins are molecular chaperones that promote

correct refolding and prevent aggregation of denatured proteins in the face of a variety of stress factors, and thus may lead to host thermotolerance (King and MacRae, 2015). In the current study, the four spider mite strains differed in the expression changes of heat shock genes when exposed to different temperatures. The low number of DEGs in comparisons with the doubly infected strain suggests that co-infected strains are the least sensitive to different temperatures. In addition, *Spiroplasma* seems to interact with the relative expression of *Hsp70* and *Hsp90* at 20°C. It is not yet clear if the differences in host gene expression are a consequence of responses to temperature, temperature effects on the endosymbionts, or some combination of these factors. The few DEGs in the doubly infected strain may reflect a relatively higher tolerance of this strain to high temperatures, so that host *Hsps* were not triggered to the same extent. Alternatively, symbiont encoded genes may affect host tolerance and directly affect host gene expression. For example, mutants of the symbiont *Buchnera aphidicola* with lower expression of the heat-shock gene *ibpA* decrease heat tolerance of pea aphids (Zhang et al., 2019). Horizontal gene transfer (HGT) events between the bacteria and the host may also play a role and have recently been documented (Husnik et al., 2013; Husnik and McCutcheon, 2018). For instance, several genes horizontally transferred from diverse bacterial symbionts are known to be expressed in the host and can assist or enhance specific metabolic activities to promote host development (Manzano-Marín et al., 2020; Zhou et al., 2021), thus indirectly changing the thermal tolerance of the host. It is not clear whether HGT events between spider mites and endosymbionts produce similar effects.

CONCLUSION

In summary, we found that *Wolbachia* and *Spiroplasma* in spider mites may affect the fitness of spider mites at different temperatures. We also show that the nature of these effects depends on whether *Wolbachia* and *Spiroplasma* are present singly or together in the host. Endosymbionts may mediate host thermoregulatory behavior and influence *Hsp* expression under temperature stress. These results suggest that endosymbionts affect thermal responses of mites and raise issues about how ongoing climate change might influence the distribution of endosymbionts as well as their hosts.

DATA AVAILABILITY STATEMENT

All sequence data have been submitted to the NCBI Sequence Read Archive, BioProject accession number PRJNA717652.

AUTHOR CONTRIBUTIONS

Y-XZ, AH, and X-YH designed the research. Y-XZ, Z-RS, Y-YZ, and X-YH performed the research. Y-XZ, Z-RS, AH, and X-YH wrote and edited the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.706321/full#supplementary-material

Supplementary Figure 1 | Schematic representation of the study design. *w + s +* , *w +* , *s +*, and *w-s-* represent the spider mite strains infected with both *Wolbachia* and *Spiroplasma*, only *Wolbachia*, only *Spiroplasma*, and no endosymbionts, respectively. More than 282 mites from each strain were selected to test host Tp. The others were subjected to one of the four temperature treatments (20, 25, 30, and 35°C) for various time periods, depending on the experiment they were used for: (1) To assess survival rates at different temperatures, more than 116 female adults (2 days old) of each strain, grouped as six replicates of about 20 individuals, were exposed to each temperature treatment for a week with daily monitoring. (2) After 6 h of exposure to different temperatures, about 100 mites from each strain were collected for DNA extraction and transcriptome sequencing. (3) We collected mites exposed to different temperatures after 6, 12, 24, 72, and 120 h for testing densities of *Wolbachia* and *Spiroplasma*. (4) Each strain was reared for four generations under different temperature treatments. Twenty female adults were randomly selected from each combination of temperature and generation (F1, F2, F3, and F4) of these cultures, and used for PCR to detect the infection rates of *Wolbachia* and *Spiroplasma*. (5) In the crossing experiment, single females without *Wolbachia* (♀: *s +* or *w-s-*) in the teleiochrysalis stage were crossed with an adult virgin male (1 day old) infected with *Wolbachia* (♂: *w + s +* or *w +*) to produce four crosses. Mating and egg laying (up to 5 days) were carried out under one of the four temperature treatments, resulting in 16 total combinations, with 18–99 replicates each.

Supplementary Figure 2 | (A) Schematic of the thermal gradient apparatus. **(B)** Plots showing the length of the 500 mm aluminum gradient and where temperature was recorded with K-type thermocouples. Red dots indicate spider mite entry point. **(C)** Linearity of temperature change for the different surfaces as measured with K-type thermocouples.

Supplementary Figure 3 | Gene Ontology (GO) terms **(A)** and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms **(B)** of genes identified from female *Tetranychus truncatus*.

Supplementary Figure 4 | Confirmation of gene expression by quantitative real-time PCR. The relative expression level of each gene was determined by qPCR (red) and was compared with the expression of the transcriptomic data (blue).

Supplementary Table 1 | Primers sequences. Sequences of DNA oligonucleotides used for assays described in this manuscript.

Supplementary Table 2 | Information on *de novo* assembled genes of female spider mites and annotations to different databases.

Supplementary Table 3 | Summarizes of endosymbiont infection pattern affects each of the host phenotypes.

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The Effect of Radiation on the Gut Bacteriome of *Aedes albopictus*

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The sterile insect technique (SIT) has been developed as a component of area-wide integrated pest management approaches to control the populations of *Aedes albopictus,* a mosquito vector capable of transmission of dengue, Zika and chikungunya viruses. One of the key factors for the success of SIT is the requirement of high biological quality sterile males, which upon their release would be able to compete with wild males for matings with wild females in the field. In insects, gut bacteriome have played a catalytic role during evolution significantly affecting several aspects of their biology and ecology. Given the importance of gut-associated bacterial species for the overall ecological fitness and biological quality of their hosts, it is of interest to understand the effects of radiation on the gut-associated bacteriome of *Ae. albopictus*. In this study, the effect of radiation on the composition and density levels of the gut-associated bacterial species at the pupal stage as well as at 1- and 4-day-old males and females was studied using 16S *rRNA* gene-based next generation sequencing (NGS) and quantitative PCR (qPCR) approaches. Age, diet, sex, and radiation were shown to affect the gut-associated bacterial communities, with age having the highest impact triggering significant changes on bacterial diversity and clustering among pupae, 1- and 4-day-old adult samples. qPCR analysis revealed that the relative density levels of *Aeromonas* are higher in male samples compared to all other samples and that the irradiation triggers an increase in the density levels of both *Aeromonas* and *Elizabethkingia* in the mosquito gut at specific stages. Our results suggest that *Aeromonas* could potentially be used as probiotics to enhance protandry and sex separation in support of SIT applications against *Ae. albopictus*, while the functional role of *Elizabethkingia* in respect to oxidative stress and damage in irradiated mosquitoes needs further investigation.

Keywords: gut bacteriome, *Elizabethkingia*, *Aeromonas*, 16S *rRNA* gene, *Aedes albopictus*

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INTRODUCTION

Mosquito-borne diseases including malaria, dengue, Zika, chikungunya, West Nile, yellow fever, and Japanese encephalitis cause severe burden on public health worldwide (Tolle, 2009). The sterile insect technique (SIT) is a species-specific and environment-friendly approach, which has been proposed and currently tested for the control of mosquito populations and mosquito-borne

diseases (Lees et al., 2015; Bourtzis et al., 2016; Bouyer et al., 2020; WHO and IAEA, 2020). An important factor in SIT applications is male mating competitiveness, which is the ability of the mass-reared and radiation-sterilized males to compete with wild males for mating with wild females (Knipling, 1955; Dyck et al., 2020). Several studies have shown that mass-rearing, radiation, handling, marking, and release processes may affect male mating competitiveness and hence, the suppression efficiency of the SIT application (Helinski and Knols, 2008; Ami et al., 2010; Bellini et al., 2013; Maïga et al., 2014; Yamada et al., 2014; Cai et al., 2018; Diallo et al., 2019; Bouyer and Vreysen, 2020; Dyck et al., 2020).

Insects are known to have established diverse symbiotic associations with microbial species (particularly of bacterial origin), which have played a catalytic role during evolution significantly affecting several aspects of their biology and ecology (Dillon and Dillon, 2004; Bourtzis and Miller, 2009; Zchori-Fein and Bourtzis, 2011; Engel and Moran, 2013). During the recent years, a number of studies have characterized the gut-associated microbiota of several insect species, including pests and disease vectors, in an attempt to determine the role of microbial species (mainly bacteria) in host physiology, and also to potentially exploit them as a tool for pest and disease control (Guégan et al., 2018; Itoh et al., 2018; Strand, 2018; Deutscher et al., 2019; Huang et al., 2020; Wang et al., 2020). Due to their importance, any change in the composition or abundance of the bacterial species associated with the insect hosts may have an impact on their physiology. For example, several studies on fruit fly species, which are target for sterile insect technique applications, have shown that changes in the composition and abundance of gut-associated bacterial species may be triggered by irradiation treatments, which in turn may be related to physiological changes affecting the biological quality and the overall fitness of the host (Ami et al., 2010; Gavriel et al., 2010; Cai et al., 2018; Asimakis et al., 2019; Woruba et al., 2019).

However, the biological quality and the ecological fitness of irradiated insects can be restored through probiotic applications as has been shown in two major agricultural tephritid pest species, the Mediterranean fruit fly *Ceratitis capitata* and the Oriental fruit fly *Bactrocera dorsalis* (Niyazi et al., 2004; Ami et al., 2010; Yuval et al., 2013; Augustinos et al., 2015; Kyritsis et al., 2017; Cai et al., 2018). Ben-Ami et al. (2010) showed that, upon irradiation, the abundance of *Klebsiella oxytoca* decreased in the guts of Mediterranean fruit fly. However, the provision of *Klebsiella oxytoca* as probiotic supplement into the adult diet of irradiated medflies resulted in its successful colonization in the gut, thus enhancing the mating competitiveness and longevity of sterile males (Ami et al., 2010; Gavriel et al., 2010). Similarly, Cai et al. (2018) also found that radiation of *Bactrocera dorsalis* induced changes in the gut-associated microbiota resulting to a significant reduction in the ecological fitness. However, probiotic applications of the gut associated *Klebsiella oxytoca* (BD177 strain) were able to rescue these effects and restore the biological quality of irradiated insects.

During the last two decades, symbiotic bacteria have been harnessed for the control of mosquito vector species and mosquito-borne diseases (Flores and O'Neill, 2018; Guégan et al., 2018; Nazni et al., 2019; Zheng et al., 2019; Caputo et al., 2020; Crawford et al., 2020; Huang et al., 2020; Ryan et al., 2020). In parallel, several studies have focused on the characterization of mosquito gut-associated microbiota with an emphasis on bacterial species (for recent reviews see Guégan et al., 2018; Strand, 2018; Scolari et al., 2019). Environmental factors and food resources have been shown to play an important role in the acquisition of bacterial species from breeding sites, which in turn may determine the composition and abundance of bacterial species in the mosquito gastrointestinal tracts (Pastoris et al., 1989; Lindh et al., 2005; Rani et al., 2009; Wang et al., 2011, 2018; Minard et al., 2013a,b; Guégan et al., 2018; Chen et al., 2020; Saab et al., 2020). Mosquito host species, sex and age may also affect the structure of the gut bacteriome and the density levels of its associated bacterial species (Minard et al., 2014, 2018; Guégan et al., 2018; Mancini et al., 2018; Rosso et al., 2018; Wang et al., 2018; Chen et al., 2020). It is also worth noting that many studies have indicated that mosquito-associated bacterial species may affect both metabolism and life history traits including food digestion, supply of vitamins and amino acids, body size, oviposition site choice and egg production, longevity, sex ratio, larval development as well as virus dissemination (Chouaia et al., 2012; Mitraka et al., 2013; Sharma et al., 2013; Coon et al., 2014, 2016a,b; Muturi et al., 2016; Dickson et al., 2017; Guégan et al., 2018).

Aedes albopictus (Diptera: Culicidae) is widespread all over the world contributing to the transmission of dengue, Zika and chikungunya viruses (Gasperi et al., 2012; Weaver et al., 2018). The Insect Pest Control Laboratory of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture has been developing the SIT package as a component of area-wide integrated pest management (AW-IPM) approaches to suppress *Ae. albopictus* populations. Previous studies suggested that a radiation dose (>35 Gy) may negatively affect the longevity and mating competitiveness of *Ae. albopictus* adult males (Bellini et al., 2013; Madakacherry et al., 2014; Zhang et al., 2016; Du et al., 2019). However, and given the importance of gut-associated bacterial species for the overall ecological fitness and biological quality of their hosts, none of these studies investigated whether the radiation affects the structure of the gut-associated bacteriome, and in the case of a negative effect, whether probiotic applications could restore the ecological fitness as previously observed for other pest species, for example, the tephritid *B. dorsalis* (Cai et al., 2018). In the present study, *Ae. albopictus* pupae were exposed at a radiation dose of 40 Gy, a dose which can induce full sterility in females and up to 99% sterility in males (Balestrino et al., 2010; Yamada et al., 2014; Zhang et al., 2015a, 2016; Du et al., 2019). The effect of radiation on the composition and density levels of the gut-associated bacterial species at the pupal stage as well as at 1- and 4-day-old males and females were studied using 16S *rRNA* gene-based next generation sequencing (NGS) and quantitative PCR (qPCR) approaches. The data produced were also assessed in respect to the age and sex, and they are discussed in the context of mating competitiveness of sterile males and potential microbiota manipulations for the enhancement of SIT applications against the major mosquito vector species *Ae. albopictus*.

MATERIALS AND METHODS

Mosquito Strains and Maintenance

The experiments were conducted at the Joint FAO/IAEA Insect Pest Control Laboratory (hereafter IPCL), Seibersdorf, Austria. *Ae. albopictus* wild type strain (Guangzhou, China), known as GUA strain (Zhang et al., 2015b), at F_{13} generation was used in these experiments. Egg hatching, larvae rearing, and adult maintenance was performed as described previously (Zhang et al., 2015b). The colony was maintained under at 26 ± 1°C with a light: dark cycle of 12 h: 12 h, and 60 ± 10% relative humidity.

Irradiation Procedures and Gut Dissection

Bovine-defibrinated blood meals were preheated and then provided to GUA females 7–9 days post emergence. Two days after the blood meal, a plastic 250-ml beaker containing 100 ml sterilized deionized water and a strip of sterilized white filter paper (white creped papers IF C140, Industrial Filtro S.r.l., Cologno Monzese, Italy) was placed in the cage (30 × 30 × 30 cm, BugDorm 1, MegaView, Taichung, Taiwan, China). After maturation, eggs were hatched as described previously (Zhang et al., 2015b). Larvae were fed on modified IAEA liquid larvae diet (Balestrino et al., 2014). Male and female pupae from 24 to 36 h after pupation were separated using an improved Fay and Morlan's separator (Focks, 1980) and then were irradiated. The irradiation treatment was conducted with a Co-60 Gammacell irradiator 220 (Atomic Energy of Canada Ltd., Canada). The irradiation dose was set to 40 Gy, and the actual dose was measured with Gafchromic® MD-V3 film (Ashland, Bridgewater, New Jersey, United States) and DoseReader 02 (FWT-92D, Far West Technology, Inc., Goleta, Canada). The irradiation dose was set to 40 Gy, a dose which has been shown to induce over 99% sterility on *Ae. albopictus* males and a reduction in longevity and mating competitiveness (Balestrino et al., 2010; Zhang et al., 2015a, 2016; Du et al., 2019). Due to time and space limitation, materials for pupae and adults were reared and irradiated separately. To minimize variation, pupae and adults of the same batch were taken as control group, respectively.

After irradiation, some of the pupae were immediately dissected under the stereomicroscope to isolate the whole gut to understand how irradiation may affect the gut-associated bacterial community. The rest of the pupae were reared separately in sterilized deionized water and placed in cage for adult emergence. Non-fed early emerged adults (both males and females) were collected and their whole gut was dissected upon adult emergence (less than 24 h) in order to see whether potential changes occurred in the pupal gut-associated bacteriome due to radiation could last until the adult stage. The rest of adults were supplied with 10% sucrose solution. To study whether any potential changes in the gut-associated bacteriome due to radiation are naturally restored during the first days of the adulthood, which is important for mating competitiveness and SIT applications, the whole guts from males and females were collected on the 4th day after emergence. Ventral diverticulum and Malpighian tubules were removed from every gut sample.

Samples from 12 groups: male pupae irradiated (MPI), male pupae control (MPC), female pupae irradiated (FPI), female pupae control (FPC), early emerged males irradiated (1DMI), early emerged males control (1DMC), early emerged females irradiated (1DFI), early emerged females control (1DFC), 4 days old males irradiated (4DMI), 4 days old males control (4DMC), 4 days old females irradiated (4DFI), 4 days old females control (4DFC), were collected for dissection. The detail information on the age, diet, sex and radiation treatment of the analyzed samples is shown in **Supplementary Table 1**. Alive adults which had been anesthetized at 4°C or alive pupae were surface disinfected by dipping in 70% ethanol for 1 min, placed into sterile 1 × PBS (phosphate buffer saline) for rinsing, and then dissected in sterile PBS under a binocular microscope with sterilized needles to get whole guts.

DNA Extraction and 16S *rRNA* Gene Sequencing

Ten guts per tube were mechanically homogenized using sterile pestles in liquid nitrogen. DNA was extracted following the protocol of DNeasy Blood and Tissue Kit (QIAGEN, Germany), then the contents of two tubes were mixed thus each sample was consisting of 20 guts. DNA was concentrated to > 15 ng/μl according to the original concentration estimated by a NanoDrop 3,000 spectrophotometer and was used for next generation sequencing (NGS) and qPCR analysis. In summary, each group contained four replicates and each replicate included 20 guts.

The NGS analysis was based on the 16S *rRNA* gene. Two regions of the gene were amplified using the primers U341F (5′-CCTACGGGRSGCAGCAG-30) and 805R (50-GTGCCAGCM GCCGCGGTAA-3′) (V3-V4) and 909F (50-ACTCAAAK GAATWGACGG-30) and 1391R (5′-GACGGGCGGTGWG TRCA-3′) (V6-V8), respectively (Sogin et al., 2006; Cole et al., 2007; Eid et al., 2009; Klindworth et al., 2013). The PCRs, the preparation of the libraries and the sequencing using the Illumina MiSeq platform were performed by Macrogen (Macrogen, Seoul, Korea). The sequences have been deposited to NCBI under the accession number PRJNA682321.

Bioinformatic Analysis

De-multiplexing of the raw sequencing reads was performed followed by their conversion to FASTQ, and standard algorithms were used to trim the Illumina adapters. The sequence reads were prepared for subsequent analysis in usearch v.10 and v.11 (Edgar, 2017). The paired-end reads were assembled and trimmed by length, and the usearch -fastq_mergepairs option in usearch v.11 was used to check for errors and assess quality. Unassembled reads as well as reads which were outside the range of 400–520 bp were not included in the downstream analysis. Using the -fastq_filter in usearch v.11, the quality of the assembled sequences was further improved while unique read sequences and abundances were found with the -fastx_uniques option. The -cluster_otus command was used for the clustering of sequences into operational taxonomic units (OTUs) at 97% similarity (Edgar, 2013), while the -unoise3 option of usearch v.10 was used to remove chimeras (Edgar, 2016b). Taxonomy

was assigned against the SILVA 128 release database (Quast et al., 2012; Edgar, 2016a) using the qiime feature-classifier classify-consensus-blast command of qiime2-2020.11 with 0.97% identity (Bolyen et al., 2019; Estaki et al., 2020). The OTUs of chloroplast, Archaea and unassigned OTUs were manually removed and the taxonomy was repeated as mentioned above. No mitochondrial OTUs were detected.

Relative abundance heatmap was created with matrics display in PRIMER version 7 + and was based on the OTU table along with the taxonomy data previously obtained in order to visualize the most dominant OTUs (n = 35) in each sample group at phylum and genus levels. To test the overall statistical differences between the different factors, the bootstrap averages were calculated using the Bray Curtis resemblance matrix of the OTUs abundance results which is based on the analysis of similarity (ANOSIM) pairwise test and plotted in PRIMER version 7 + using number of bootstraps between 50 and 150 per sample.

Quantitative Analysis of the Main Gut Bacteria Groups

Based on the NGS results, the three most abundant genera, which also varied greatly among treatments, were quantified by quantitative PCR (qPCR): *Aeromonas*, *Elizabethkingia* and *Enterococcus*. The V3-V4 region of the 16S *rRNA* gene sequences of these three genera obtained from the NGS analysis were used for BLAST searches in the databases to search the most similar reference sequences. All available 16S *rRNA* gene sequences of the gut-associated bacteria of the *Ae. albopictus* GUA strain detected in our previous study (Chen et al., 2020) were also taken as reference for primer specificity. Pairs of genus-specific qPCR primers were designed for these three genera with Primer3plus[1]. Primer self-complementarity were checked with NCBI primer-blast. We also searched the Silva database to ensure that our primer pairs fit as many target sequences whereas as few untargeted ones. The *Ae. albopictus* ribosomal protein S6 (*rpS6*) gene was chosen as reference. The primers used in this analysis along with the annealing temperatures (TA) and melting temperature (TM) are presented in **Supplementary Table 2**. The amplification was performed using iQ™ SYBR® Green Supermix (Bio-Rad, United States). The reaction mixture (15 μl) consisted of 5 ng DNA template and 7.5 μl of 2 × Supermix. qPCR and 200 nM of each primer was performed with a CFX96 Touch Real-Time PCR Detection system (Bio-Rad, United States). The concentration of DNA was diluted, and 5 ng DNA was used in 15 μl reactions. Due to different T(A)s of the primers, housekeeping gene *rpS6* and target genes were put on the same wells of different plates. An initial denaturation at 95°C for 2 min was followed by 40 cycles consisting of denaturation at 95°C for 10 s, at annealing temperature for 60 s. The fluorescence collection was performed at the annealing stage. To check and confirm the quality of amplification, a melting profile was generated for the amplicon over a temperature range of 65–95°C. Melting curves for each sample were analyzed after each run to check that there was no primer dimer formation. Four biological replicates were performed for each sample. There were three technical replicates for each biological sample. Relative quantity (Mean ± SEM) of products was calculated using CFX Manager™ Software (Bio-Rad Laboratories, Inc.). The relative abundance of bacteria was determined by using the $2^{-\Delta\Delta CT}$ calculation method.

[1]http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

Statistical Analysis

Normalcy of the data was assessed by the D'Agostino-Pearson omnibus normality test of the GraphPad Prism 6.0 software. For the NGS results, alpha-diversity analysis was also performed with Qiime2 and usearch v.11. Pielou.s.evenness and Richness index were used to estimate the number of OTUs per samples whereas Shannon's and Simpson's reciprocal [1/Simpson's Index (D)] indices were used to determine species diversity. The bacterial richness and diversity indices among the samples were compared using Kruskal-Wallis test and Dunn's multiple comparisons test with the default setting or Two-tailed Mann-Whitney U-test based on different classified groups. Similarities in the structure of bacterial communities and the role of different factors such as age, sex and radiation were assessed using the metric multidimensional scaling (mMDS) plot with bootstrap averages in PRIMER version 7 + and were displayed with a Bray and Curtis matrix based on the square-root transformation of the bacterial OTU abundance data (Clarke and Gorley, 2016). The tests were based on the multivariate null hypothesis via the use of the non-parametric statistical method PERMANOVA (Anderson, 2001).

For qPCR results, Dunn's multiple comparisons test or Two-tailed Mann-Whitney U-test was used to compare the relative density of the selected bacterial species.

RESULTS

The sequence data of the two 16S *rRNA* gene regions, amplified by the primer pairs U341F/805R and 909F/1391R, were compared and the analysis indicated that there were no statistically significant differences (**Supplementary Table 3**). Based on this finding, the sequence data of these two regions were combined and used for all the downstream analysis which resulted to the data, figures and tables presented below.

Effect of Age, Sex, and Radiation on the Alpha Diversity of Gut Microbiota

The effects of age, sex and radiation on the bacterial community composition and diversity in the guts of *Ae. albopictus* GUA strain, including male and female pupae, 1- and 4- day-old male and female adult mosquitoes, were investigated by 16S *rRNA* gene sequencing. A total of 506,854 (minimum 30433–maximum 46819) reads, corresponding to 496 (minimum 30–maximum 53) OTUs, were used for analysis in this study after quality filtering of the sequencing results (**Table 1** and **Supplementary File 1**).

Among all the sequenced samples, significant difference on the bacterial community abundance was observed based on species richness indices (Kruskal-Wallis test, df = 11, X^2 = 37.15, $P < 0.01$) (**Table 1**). The samples were then classified based on

TABLE 1 | Analysis of α diversity indices of the experimental samples.

ID	No. of reads (min-max)	No. of OUTs (min-max)	Pielou.s.evenness	Species richness	Species diversity indices	
					Shannon	Simpson reciprocal
MPI	39574 (33521–46250)	53 (50–55)	0.68 ± 0.04 a	52.75 ± 1.11 a	2.70 ± 0.15 a	0.87 ± 0.02 a
MPC	38066 (34262–42291)	49 (45–56)	0.71 ± 0.06 a	49.25 ± 2.39 ac	2.77 ± 0.23 a	0.89 ± 0.04 a
FPI	30433 (25015–38219)	45 (40–49)	0.72 ± 0.07 a	45.25 ± 2.06 ad	2.75 ± 0.29 a	0.87 ± 0.03 a
FPC	36737 (31621–41777)	44 (40–48)	0.69 ± 0.09 a	44.25 ± 1.65 ae	2.64 ± 0.35 a	0.85 ± 0.06 a
1DMI	45956 (44453–47533)	42 (37–45)	0.58 ± 0.02 ac	41.72 ± 2.00 bdef	2.16 ± 0.08 ac	0.84 ± 0.01 ac
1DMC	43916 (38650–47109)	50 (46–53)	0.63 ± 0.04 a	50.00 ± 1.47 af	2.46 ± 0.18 a	0.85 ± 0.02 ac
1DFI	41387 (30377–48489)	43 (29–50)	0.65 ± 0.06 a	42.99 ± 4.75 af	2.44 ± 0.26 a	0.86 ± 0.02 a
1DFC	45642 (40658–48208)	34 (25–40)	0.51 ± 0.01 ab	33.50 ± 3.18 bf	1.80 ± 0.07 ab	0.75 ± 0.02 ab
4DMI	46819 (45618–48200)	30 (25–34)	0.39 ± 0.02 b	29.71 ± 1.89 bf	1.30 ± 0.05 b	0.61 ± 0.01 b
4DMC	45925 (42468–47963)	31 (26–38)	0.41 ± 0.01 b	30.69 ± 2.62 bef	1.40 ± 0.04 bc	0.66 ± 0.01 bc
4DFI	46604 (46000–47354)	37 (35–41)	0.40 ± 0.02 bc	37.00 ± 1.35 bdef	1.46 ± 0.08 bc	0.64 ± 0.03 b
4DFC	45797 (45098–46758)	39 (38–40)	0.37 ± 0.02 b	39.00 ± 0.41 bcd	1.37 ± 0.06 bc	0.60 ± 0.01 b

For each diversity index, Kruskal-Wallis test followed by the Dunn's multiple comparisons test, ($P < 0.05$). Significant differences are indicated by different letters based on the data presented in ***Supplementary Tables 4, 5****. Data are presented as Mean or Mean ± SEM of four biological replicates per sample.*

treatment (irradiated or non-irradiated), age (pupa, 1- and 4-day-old) and sex (male and female), and, respectively compared based on richness and Shannon indices. The results showed that there were no significant differences on bacterial richness in treatment (**Figure 1A**, Two-tailed Mann-Whitney *U*-test, $U = 296.5$, $P = 0.869$) or sex (**Figure 1C**, Two-tailed Mann-Whitney *U*-test, $U = 335$, $P = 0.337$), however, significant difference was observed on age with the highest bacterial diversity being observed in the guts of pupae and those of 4-day-old adults (**Figure 1B, Kruskal-Wallis test** and **Dunn**'s multiple comparisons test, $x^2 = 23.451$, $df = 2$, $P < 0.001$). Using the Pielou.s.evenness index for assessing the diversity along with species richness, significant differences were detected between samples ($x^2 = 37.347$, $df = 11$, $P < 0.001$).

Regarding the bacterial community diversity, significant differences were observed in the tested samples according to the Shannon (Kruskal-Wallis test, $x^2 = 37.390$, $df = 11$, $P < 0.001$) and Simpson indices (Kruskal-Wallis test, $x^2 = 35.737$, $df = 11$, $P < 0.0001$) (**Table 1**). Samples were classified as above-mentioned and comparison was performed based on Shannon index. As shown in **Figure 1E**, the bacterial diversity was highest in the guts of pupae, followed by 1- and 4-day-old adults (Kruskal-Wallis test and Dunn's multiple comparisons test, $x^2 = 34.839$, $df = 2$, $P < 0.0001$). No significant differences on bacterial diversity of guts with respect to irradiation treatment (**Figure 1D**, Two-tailed Mann-Whitney *U*-test, $U = 301.5$, $P = 0.798$) and sex (**Figure 1F**, Two-tailed Mann-Whitney *U*-test, $U = 298$, $P = 0.846$) were observed (**Supplementary File 1**).

Major Bacterial Taxa

Based on the NGS results, the most abundant gut bacteria (≥ 2%) in *Ae. albopictus* GUA strain are shown in **Figure 2**. Based on phylum, the abundance of OUTs was relatively uniform, but also showed some minor differences, in all tested samples (**Figure 2A**). *Bacteroidetes* (18.9 and 21.1%), *Firmicutes* (16.4 and 43.4%), *Proteobacteria* (55.6 and 31.7%) and *Actinobacteria* (6.9 and 2.7%), were the four most abundant phyla in pupae and 1-day-old adult mosquitoes, respectively, however, *Bacteroidetes*, with a relative abundance of up to 86.0%, was the main phylum in the guts of 4-day-old adults. These differences were also observed at the genus level. For example, *Elizabethkingia* was the genus with the highest relative abundance in 4-day-old adults while *Elizabethkingia* and *Enterococcus* were the two relatively high abundant genera in pupae and 1-day-old mosquitoes. In addition, except for female pupae, *Aeromonas* was also occurred with a high relative abundance in male pupae and 1-day-old mosquitoes. Interestingly, when compared to the corresponding non-irradiated (control) mosquitoes, the irradiated samples exhibited a higher average abundance of *Aeromonas* (**Figure 2B**). In contrast, except for 1-day-old male mosquitoes, the average abundance of *Enterococcus* in the irradiated mosquitoes was lower than their corresponding control samples (**Figure 2B**).

Effect of Age, Sex, and Radiation on the Beta Diversity of Gut Microbiota

Regarding the beta diversity of the bacterial communities, the sequenced samples were classified according to irradiation treatment (hereafter treatment), age or sex. The results clearly revealed that bacterial communities differed significantly in respect to these three independent factors (**Table 2**, PERMANOVA; Treatment: $P = 0.004$; Age: $P = 0.001$; Sex: $P = 0.001$). The metric multidimensional scaling (mMDS) showed that unique community clusters were formed separately between the irradiated and non-irradiated samples (**Figure 3A**). Similarly, the formation of distinct clusters among pupae, 1- and 4-day-old adults (**Figure 3B**) or between male and female mosquitoes (**Figure 3C**) were, respectively observed.

Strong clustering of bacterial communities was observed only when age and sex factors combined (**Table 2**, PERMANOVA; Age × Sex: $P = 0.001$). Results of mMDS confirmed the clusters (**Figures 3D–F**). However, three factor combinations did not show such a high clustering (**Table 2**, PERMANOVA,

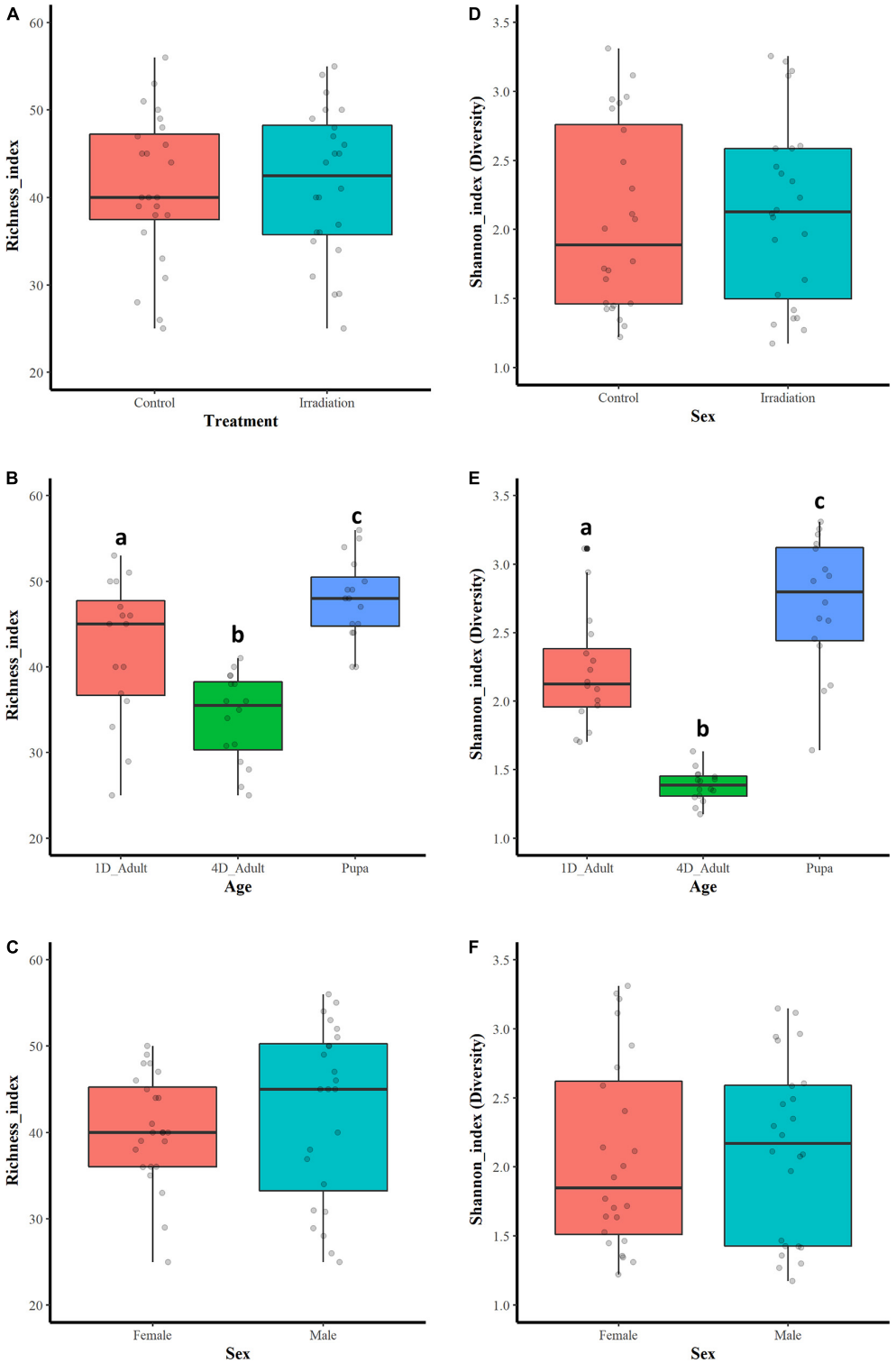

FIGURE 1 | Bacterial community richness and diversity based on the Species richness and Shannon indices. Bacterial richness **(A)** and diversity **(D)** of guts between irradiated and non-irradiated samples. Bacterial richness **(B)** and diversity **(E)** of guts from pupa, 1- and 4-day-old adults. Bacterial richness **(C)** and diversity **(F)** of guts between male and female mosquitoes. Boxes extend between the 25th and 75th percentile. A thick line denotes the median. The whiskers extend up to the most extreme values. The gray circles indicate the number of data points used in each plot. Two-tailed Mann-Whitney *U*-test was used to compare the bacterial richness and diversity indices in treatment or sex groups. Kruskal-Wallis test and Dunn's multiple comparisons test were used to compare the bacterial richness and diversity indices in age groups. Significant differences are indicated by different letters.

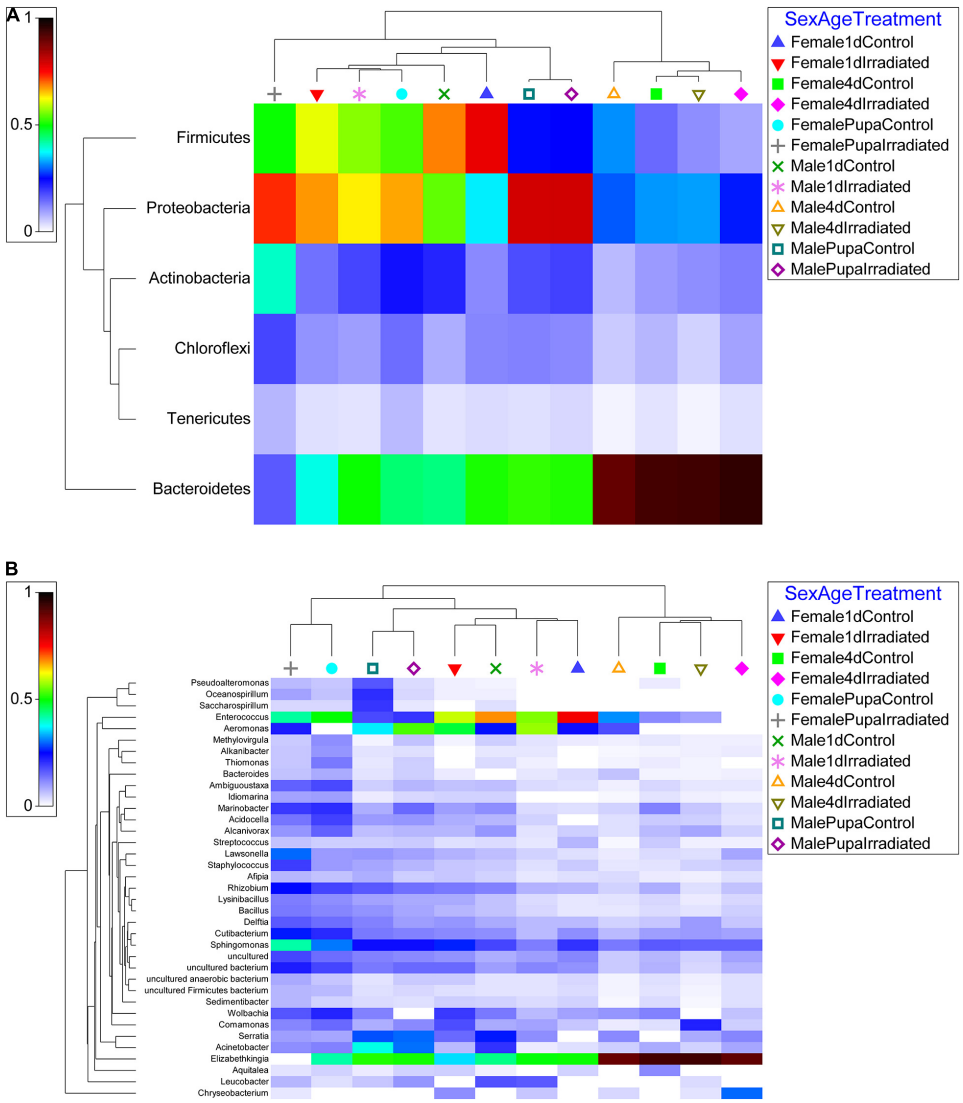

FIGURE 2 | Relative abundance heatmap of the most dominant phylum and genus of all samples examined. **(A)** Phylum level. **(B)** Genus level. OTUs abundance results from qiime2 analysis were transformed with square root transformation and averaged based on the number of replicates per sample. The resemblance matrix was conducted with Bray Curtis similarity of the OTUs abundance results.

$P = 0.156$). In addition, the combination between Age and Sex factors or Treatment and Sex factors also did not present high clustering (**Table 2**, PERMANOVA, Treatment × Age: $P = 0.219$, Treatment × Sex: $P = 0.060$).

Relative Abundance of *Aeromonas*, *Elizabethkingia*, and *Enterococcus*

Based on qPCR results, the relative abundance of the three taxa is presented in **Figure 4**. In general, all three bacterial taxa showed clear and distinct patterns among different developmental stages, ages, and sexes. The relative abundance of *Aeromonas* in the midgut of pupae, with or without irradiation, showed no significant difference compared to either 1- or 4-day-old adult mosquito for both males and females (Dunn's multiple comparisons test, $P > 0.05$) except for the female pupae which exhibited a lower density than 1-day-old female adults (Dunn's multiple comparisons test, $P < 0.05$). Interestingly, irradiated 1-day-old adult mosquitoes had a higher Aeromonas density than in 4-day-old mosquitoes (Dunn's multiple comparisons test, $P < 0.05$), but this pattern was not observed in the control groups (Dunn's multiple comparisons test, $P > 0.05$). One-day-old adult mosquitoes showed no significant difference regarding *Elizabethkingia* abundance compared to either pupae or 4-day-old males and females, irrespective to irradiation treatment (Dunn's multiple comparisons test, $P > 0.05$). However, 4-day-old adults had a higher *Elizabethkingia* density than their respective pupae (Dunn's multiple comparisons test, $P < 0.05$). No significant difference on *Enterococcus* density was observed among pupae, 1- and 4-day-old female adults, regardless

TABLE 2 | PERMANOVA table of results for all three factors and their combinations for genera level abundance.

	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Treatment	1	1898.8	1898.8	3.8904	**0.004**	998
Age	2	24,882	12,441	25.49	**0.001**	999
Sex	1	3,086	3,086	6.3228	**0.001**	998
Treatment × Age	2	1277.2	638.62	1.3084	0.219	998
Treatment × Sex	1	1063.8	1063.8	2.1795	0.06	998
Age × Sex	2	3156.7	1578.4	3.2338	**0.001**	999
Treatment × Age × Sex	2	1416.1	708.04	1.4507	0.156	999
Res	36	17,571	488.08			
Total	47	54,351				

Twelve samples were studied with four biological replicates each. Within the table, statistically significant differences ($P < 0.05$) can be seen in bold values in all three factors separately and in the combination of age and sex. Perm(s) = permutations.

of irradiation treatment (Dunn's multiple comparisons test, $P > 0.05$). In respect to male mosquitoes, 1-day-old male adults exhibited higher *Enterococcus* density than pupae (Dunn's multiple comparisons test, $P < 0.05$). Four-day-old males showed no significant difference compared to either pupae or 1-day-old males (Dunn's multiple comparisons test, $P > 0.05$). When combined the data from same sex, males had a higher *Aeromonas* density than females in both treatment and control groups (Two-tailed Mann-Whitney *U*-test, $P < 0.05$), but this was not observed for *Elizabethkingia* (Two-tailed Mann-Whitney *U*-test, $P > 0.05$) or *Enterococcus* (Two-tailed Mann-Whitney *U*-test, $P > 0.05$).

The effect of irradiation on the abundance of the three bacterial taxa was compared to the respective control groups of the same sex and developmental stage/age. Increased *Aeromonas* density was only observed in the irradiated 1-day-old adult males when compared to non-irradiated ones (Two-tailed Mann-Whitney *U*-test, $P < 0.05$). Similar results were found in 4-day-old irradiated adults which had higher *Elizabethkingia* density than the controls in both males and females (Two-tailed Mann-Whitney *U*-test, $P < 0.05$). No significant difference was observed on the *Enterococcus* density between irradiated and non-irradiated samples (Two-tailed Mann-Whitney *U*-test, $P > 0.05$). Detailed information about the statistical analysis is shown in **Supplementary Tables 4**, **5**.

DISCUSSION

Sterile insect technique (SIT), as a component of area-wide integrated pest management (AW-IPM) programmes, has been successfully applied to suppress or even locally eradicate insect populations of several major agricultural and livestock pest species during the last seventy years (Vreysen et al., 2007; Dyck et al., 2021). Based on these successful applications, recent efforts have focused on the development and validation of the SIT package to suppress populations of mosquito disease vectors including *Ae. albopictus* and *Ae. aegypti* (Lees et al., 2015; Bourtzis et al., 2016; Bouyer and Vreysen, 2020). Indeed, several small-scale field trials have provided quite encouraging results suggesting that SIT has the potential to suppress *Aedes* mosquito populations (Bellini et al., 2013; Kittayapong et al., 2018, 2019; Zheng et al., 2019). One of the key elements for the successful application of SIT to suppress populations of insect pests and disease vectors, including mosquitoes, is the production of high-quality sterile males (Bouyer and Vreysen, 2020; Dyck et al., 2021).

It has been shown that mass rearing, handling, transport, release and sterilization may affect the male mating competitiveness (Bellini et al., 2013; Madakacherry et al., 2014; Maïga et al., 2014; Yamada et al., 2014; Diallo et al., 2019; Du et al., 2019; Zheng et al., 2019; Bouyer and Vreysen, 2020; Dyck et al., 2021). In addition, several studies have suggested that changes in the male mating competitiveness may be associated with radiation-induced changes in the gut-associated bacteriome of SIT target species (Ami et al., 2010; Gavriel et al., 2010; Cai et al., 2018; Asimakis et al., 2019; Woruba et al., 2019). Using 16S *rRNA* gene-based sequencing approaches, the present study investigated the potential effect of age, sex and radiation on the gut-associated bacteriome of the laboratory-reared *Ae. albopictus* GUA strain. Our results clearly showed that all three factors can affect the gut-associated bacterial communities with age having the highest impact triggering significant changes on bacterial diversity and clustering among pupae, 1- and 4-day-old adult samples. It is also worth noting that the qPCR results revealed that the relative density levels of *Aeromonas* are higher in male samples compared to all other samples and that the irradiation triggers an increase in the density levels of both *Aeromonas* and *Elizabethkingia* in the mosquito gut at specific stages.

Previous studies on fruit flies have clearly shown that age plays an important role in shaping their gut-associated bacteriome (Ami et al., 2010; Hamden et al., 2013; Augustinos et al., 2015; Malacrinò et al., 2018). The present study confirmed that age is a critical factor in shaping the bacterial communities in *Ae. albopictus* guts too (**Figures 3C,D**). *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were found to be the four main phyla in pupae and 1-day-old adult mosquitoes while *Bacteroidetes* was detected as the main phylum in 4-day-old adults (**Figure 2A**). However, age-dependent differences may be reflecting the distinct feeding behaviors during different developmental stages. In Diptera insects, once larva becomes pupa, it does not feed anymore until adult emergence (Truman, 2019). This indicates that all the metabolic activities taking place

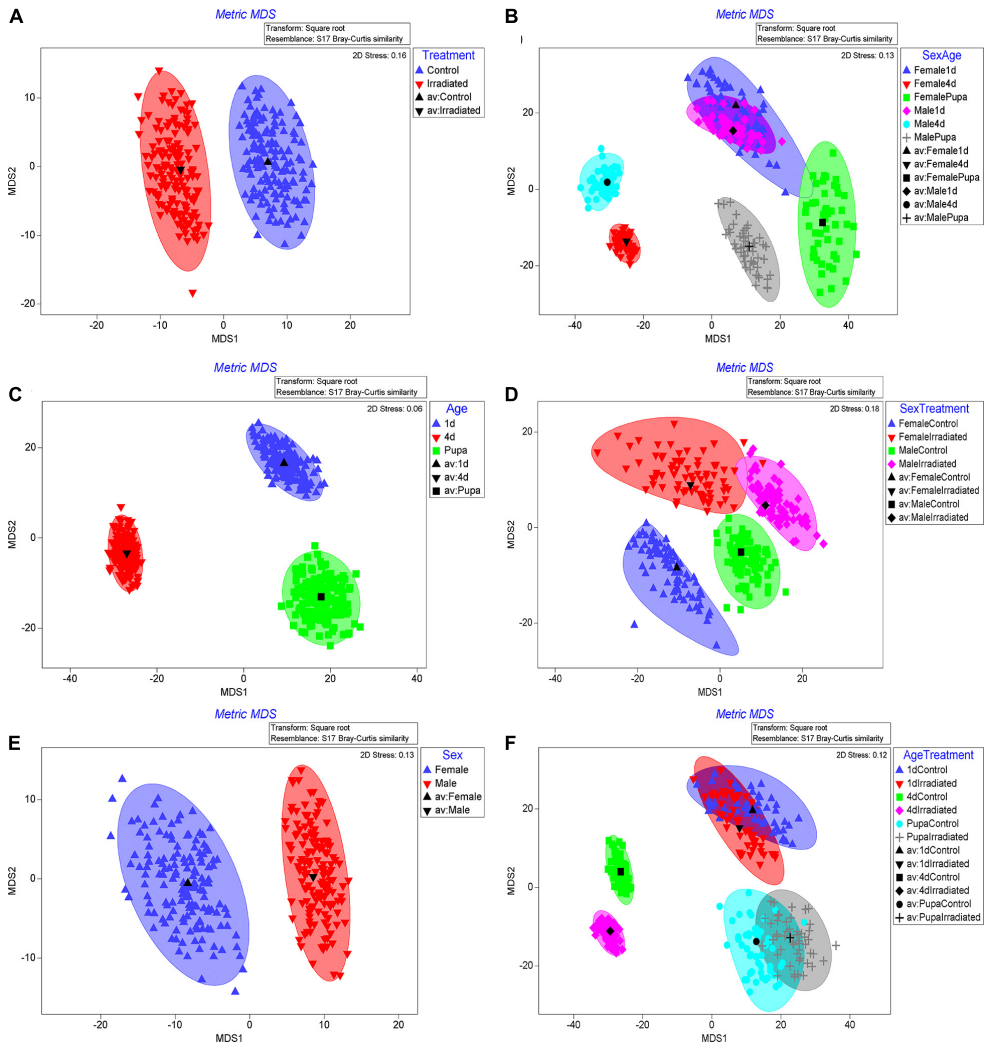

FIGURE 3 | Bootstrap averages with Metric multidimensional scaling (mMDS) of bacterial communities based on relative abundances of OTUs originating from treatment, age or sex groups **(A–C)** or combinations of them **(D–F)**. OTUs abundance results from qiime2 analysis were transformed with square root transformation and the resemblance matrix was conducted with Bray Curtis similarity. Bootstrap averages were analyzed with 5 **(D,F)**, 75 **(E)** and 150 **(A–C)** bootstraps per sample indicated by the number of points in the plots.

during the pupal stage are supported from nutrients produced through gut bacterial-mediated decomposition of larva-acquired intestinal contents. It is interesting to note that during the dissection of mosquito pupal guts, partial remnants of intestinal contents were observed in some samples and this may explain why higher bacterial diversity was observed at the pupal stage compared to adults (**Figure 1E**). Teneral mosquito adults (<24 h old) do not feed and their guts are usually empty indicating that the intestinal contents have been digested after eclosion. Four-day-old adult mosquitoes are only provided with sugar solution which may explain why teneral adults may present lower bacteria diversity compared to pupae (**Figure 1E**). It is noteworthy that *Elizabethkingia* is the most abundant bacterium in 4-day-old adults (**Figure 2B**), which is in line with previous studies showing that this bacterial species is mainly involved in the digestion of blood and sugar (Wang et al., 2011; Chen et al., 2015, 2020).

Sex may also affect the composition of mosquito gut-associated bacteriomes and differences have mainly been attributed to different food sources and nutrients (Minard et al., 2013a, 2014; Mancini et al., 2018). For blood-sucking insects such as mosquitoes, females require animal blood, which contains essential nutrients for the maturation of eggs (Dodd, 1995; Barrozo, 2019). Intestinal bacterial species are involved in the digestion of blood to produce these essential nutrients and this may explain why the abundance of key bacterial species increases after a blood meal (Strand, 2018). Unlike females, males usually feed only on honey or sugar throughout their entire lifetime. Previous studies have documented clear differences between the bacterial communities associated with male and female mosquitoes (Minard et al., 2018; Rosso et al., 2018; Wang et al., 2018; Chen et al., 2020). The present study also confirmed the different structure of the gut-associated

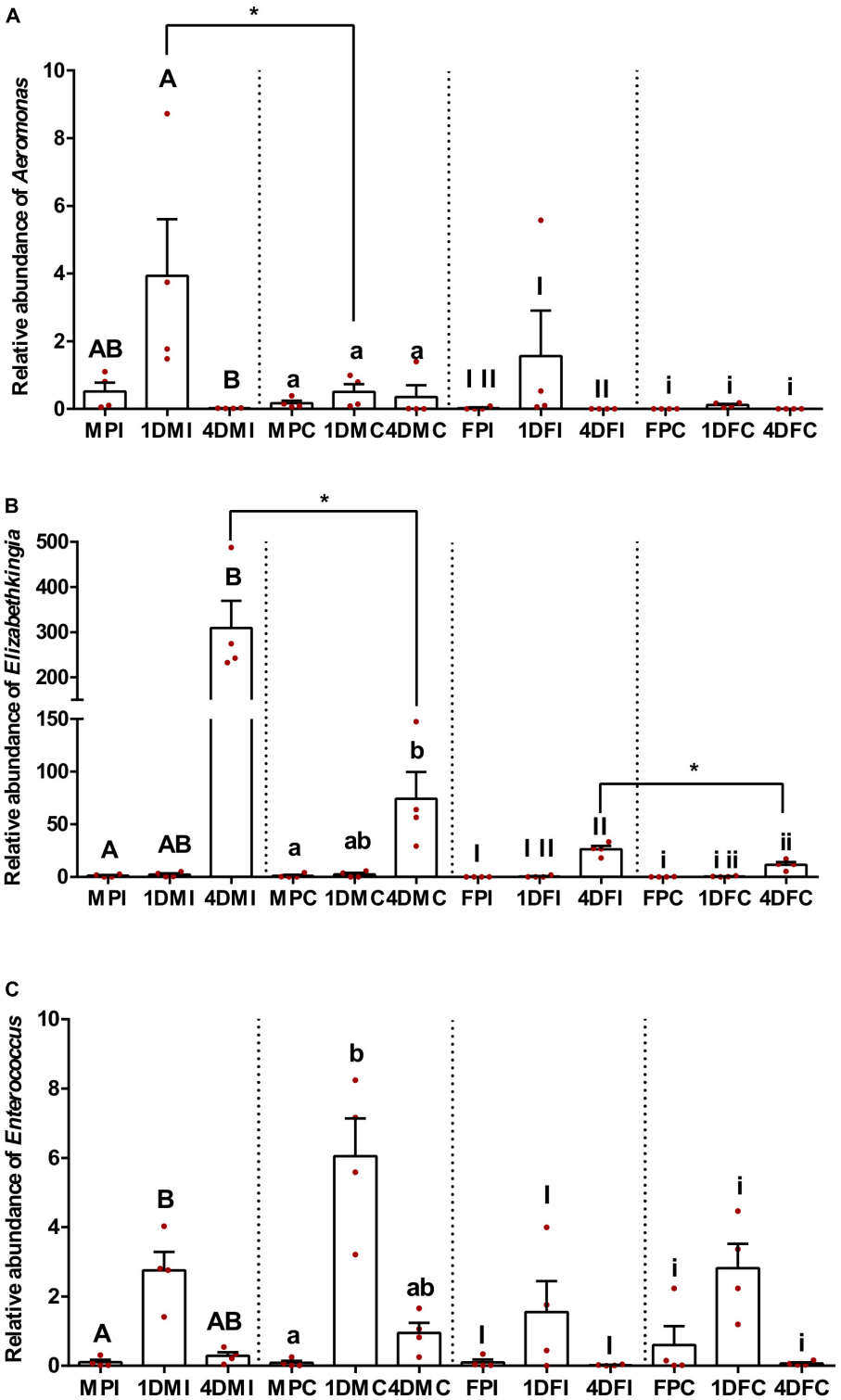

FIGURE 4 | Relative abundance of three major bacterial groups based on qPCR. **(A)** *Aeromonas*; **(B)** *Elizabethkingia*; **(C)** *Enterococcus*. Dots represent biological replicates, each one as the mean of three technical replicates. Relative abundance data ($n = 4$ for each sample, Mean ± S.E.M) are presented relative to the housekeeping gene *rps6*. Within **(A–C)** samples of the same sex and irradiation/control, values followed by different lowercase letters or capital letters or Roman numbers were statistically different using Kruskal-Wallis test and Dunn's multiple comparisons test ($P < 0.05$). Two-tailed Mann-Whitney *U*-test was performed between the irradiated and control samples of the same developmental stage/age/sex. Only the significantly different results are shown in the figures. * indicates $P < 0.05$.

bacterial communities present in *Ae. albopictus* male and female mosquitoes (**Figure 3C**).

As in many insect species, *Ae. albopictus* is characterized by the phenomenon of protandry, male mosquitoes develop faster than female ones (Zhang et al., 2015b). In addition, it has been shown that *Aeromonas*, as well as *Klebsiella* and yeast, can accelerate the larval development in the mosquito species *Culex pipiens* (Díaz-Nieto et al., 2016). Interestingly, our study showed that *Aeromonas* bacteria are present in higher densities in *Ae. albopictus* males compared to females in both irradiation and control groups, while no difference was observed in respect to *Elizabethkingia* and *Enterococcus* taxa (**Supplementary Table 2**). These results suggest that *Aeromonas* maybe a contributing factor for the different developmental rate observed in *Ae. albopictus* males and females (**Supplementary Table 2**). Further experimental work is needed to test this hypothesis and see whether *Aeromonas* could be used as probiotic to further enhance the protandry phenomena in *Ae. albopictus* thus enhancing sex separation efficiency and male recovery in support of SIT applications against this major vector species.

Several studies have shown that radiation may affect the diversity and abundance of gut-associated bacteria in insects (Ami et al., 2010; Lauzon and Potter, 2012; Yuval et al., 2013; Cai et al., 2018; Asimakis et al., 2019; Woruba et al., 2019). However, our results suggest that irradiation of *Ae. albopictus* pupae does not affect the overall abundance and diversity of the gut-associated bacterial community (**Table 2** and **Figures 1A,D**), but it rather triggers a shift to its actual composition (**Table 2** and **Figure 3**). It is worth noting that 4-day-old non-irradiated mosquitoes have significant higher abundance of *Elizabethkingia* when compared to their irradiated counterparts (**Supplementary Table 2**), whereas this difference is not observed in pupae and 1-day-old mosquitoes. The *Elizabethkingia*'s genome is properly equipped to metabolize sugars present in the host intestine (Kukutla et al., 2014). This has been confirmed by an increase of *Elizabethkingia*'s density levels in the intestine of 14-day-old male *Ae. albopictus* adults, which have been fed on sugar solution only, by more than 30 times when compared to 1-day-old males (Chen et al., 2020). In addition, *Elizabethkingia* encodes an antioxidant protein HemS (heme-degrading protein), which is known to reduce the oxidative damage during blood digestion (Kukutla et al., 2014). Previous studies have shown that irradiation increases the abundance of oxygen free radicals in the intestine of insects causing oxidative damages (Zaghloul et al., 2013; Ali et al., 2017; Wang et al., 2019). Based on this observation, the higher abundance of *Elizabethkingia* detected in the irradiated adults in the present study may indicate that irradiated mosquitoes use this bacterium to remove the oxygen free radicals to reduce any potential oxidative damage. In addition, the genome of *Elizabethkingia* encodes for several hemolysins, and with their hemolytic activity this bacterial species can participate in the digestion of red blood cells (Kukutla et al., 2014). The sharp increase of the density levels of this bacterium in the guts of female mosquitoes after a blood meal is also in agreement with this (Chen et al., 2020).

In conclusion, the effects of age (pupa, 1- and 4-day-old adults), sex (female and male) and radiation on the gut-associated bacterial species of lab-reared *Ae. albopictus* were investigated in the present study using 16S *rRNA* gene-based next generation sequencing approaches. The results showed that age, perhaps in conjunction with the diet, is a key factor that can shape the diversity and structure of the mosquito gut-associated bacteriome. In addition, sex and radiation may also affect the bacteria community, even though no significant impact was observed on richness and diversity. In addition, our data suggested that *Aeromonas* could potentially be used as probiotics to enhance protandry and sex separation in support of SIT applications against *Aedes albopictus*, while the functional role of *Elizabethkingia* in respect to oxidative stress and damage in irradiated mosquitoes needs further investigation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA682321 and https://dataverse.harvard.edu/dataset.xhtml?persistentId=, doi: 10.7910/DVN/HEL3PS.

AUTHOR CONTRIBUTIONS

DZ and SC performed the experiments, analyzed the data, and drafted the manuscript. AA-A performed the bioinformatic analysis, interpreted the data, contributed to the drafting, and critical revision of the manuscript. KB conceived the study, designed the experiments, interpreted the data, contributed to the drafting, and critically revised the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.671699/full#supplementary-material

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Wolbachia Strain *w*Gri From the Tea Geometrid Moth *Ectropis grisescens* Contributes to Its Host's Fecundity

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Members of the *Wolbachia* genus manipulate insect–host reproduction and are the most abundant bacterial endosymbionts of insects. The tea Geometrid moth *Ectropis grisescens* (Warren) (Lepidoptera: Geometridae) is the most devastating insect pest of tea plants [*Camellia sinensis* (L.) O. Kuntze] in China. However, limited data on the diversity, typing, or phenotypes of *Wolbachia* in *E. grisescens* are available. Here, we used a culture-independent method to compare the gut bacteria of *E. grisescens* and other tea Geometridae moths. The results showed that the composition of core gut bacteria in larvae of the three Geometridae moth species was similar, except for the presence of *Wolbachia*. Moreover, *Wolbachia* was also present in adult female *E. grisescens* samples. A *Wolbachia* strain was isolated from *E. grisescens* and designated as *w*Gri. Comparative analyses showed that this strain shared multilocus sequence types and *Wolbachia* surface protein hypervariable region profiles with cytoplasmic incompatibility (CI)-inducing strains in supergroup B; however, the *w*Gri-associated phenotypes were undetermined. A reciprocal cross analysis showed that *Wolbachia*-uninfected females mated with infected males resulted in 100% embryo mortality (0% eggs hatched per female). Eggs produced by mating between uninfected males and infected females hatched normally. These findings indicated that *w*Gri induces strong unidirectional CI in *E. grisescens*. Additionally, compared with uninfected females, *Wolbachia*-infected females produced approximately 30–40% more eggs. Together, these results show that this *Wolbachia* strain induces reproductive CI in *E. grisescens* and enhances the fecundity of its female host. We also demonstrated that *w*Gri potential influences reproductive communication between *E. grisescens* and *Ectropis obliqua* through CI.

Keywords: cytoplasmic incompatibility, culture-independent, mutualism, reciprocal cross, multilocus sequence types

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INTRODUCTION

Wolbachia are Gram-negative bacteria that were first identified in the oophoron of *Culex pipiens*. Their prevalence in arthropods and nematodes worldwide and stunning arsenal of parasitic and mutualistic adaptations (Zug and Hammerstein, 2012). It has been recently estimated that ∼50% of arthropod and several filarial nematode species harbor *Wolbachia* (Weinert et al., 2015; Lefoulon et al., 2016). Moreover, *Wolbachia* is estimated to infect 40–60% of insect species (Mateos et al., 2020). In fact, *Wolbachia* is the most common and widespread facultative symbiont of insects

(Zug and Hammerstein, 2015). As parasites, *Wolbachia* manipulate insect–host reproduction in various ways, *via* cytoplasmic incompatibility (CI), male-killing, feminization, and parthenogenesis, in order to facilitate their maternal transmission (Yen and Barr, 1971; Rousset et al., 1992). Of these, CI is the most prevalent host phenotype caused by the presence of *Wolbachia* (Stouthamer et al., 1999). In contrast to *Wolbachia*'s well-established reproductive role, recent years have extensive evidence that *Wolbachia* can benefit their arthropod host as mutualists (Teixeira et al., 2008; Brownlie et al., 2009; Nikoh et al., 2014; Zug and Hammerstein, 2015). In some insects, *Wolbachia* strains function as mutualists to provide benefits to the host, including supplementing essential nutrients, protection of the host against pathogens, increasing successful egg development, and enhancing female insect host fecundity (Dedeine et al., 2001; Martijn and Ellers, 2008; Hosokawa et al., 2010; Iturbe-Ormaetxe et al., 2011; Guo et al., 2018). For example, *Wolbachia* provides riboflavin (vitamin B_2) to all its insect host, and *Wolbachia*'s riboflavin provisioning certainly contributes to growth, survival, and reproduction in its insect host (Moriyama et al., 2015). For instance, in the two planthopper species *Nilaparvata lugens* and *Laodelphax striatellus*, intracellular *Wolbachia* strains *w*Lug and *w*StriCN (both in supergroup B) provide biotin and riboflavin to enhance reproduction of their female hosts (Ju et al., 2020). Accurate identification of *Wolbachia* strains is a fundamental component of all *Wolbachia*-related research. However, *Wolbachia* cannot be cultured outside host cells, and identification of *Wolbachia* strain is solely based on molecular typing.

The genus *Wolbachia* is currently divided into at least 17 possible phylogenetic supergroups, named A–F, H–Q, and S (O'Neill et al., 1992; Werren et al., 1995; Bandi et al., 1998; Ros et al., 2009; Lefoulon et al., 2020). Supergroups A and B are primarily found in arthropods and often function as reproductive parasites that manipulate host reproduction to increase their own spread through the matriline (Kaur et al., 2021). An earlier phylogenetic analysis based on *Wolbachia* surface protein (*wsp*) gene sequences further divided the A and B supergroups into 12 groups (Mel, AlbA, Pip, Uni, Riv, Pap, Aus, Ori, Dei, Haw, Con, and Mors) (Zhou et al., 1998). Later, seven more groups were added (Kue, Dro, Sib, Kay, Div, For, and Vul) (van Meer et al., 1999). However, the *wsp* gene in different strains of *Wolbachia* has undergone extensive recombination (Werren and Bartos, 2001). Thus, this single-locus identification system is unreliable as a research tool. A multilocus sequence typing (MLST) system was developed to type *Wolbachia* strains of diverse origins (Baldo et al., 2006). This system is based on five conserved genes (*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*) and four WSP hypervariable regions (HVRs): HVR1 (52–84 aa), HVR2 (85–134 aa), HVR3 (135–185 aa), and HVR4 (186–222 aa). Data generated using the five gene-based MLST system have been deposited in a web-accessible database[1] that facilitates the storage, management, and analysis of sequence data and isolate information.

[1]http://pubmlst.org/wolbachia/

Tea plant [*Camellia sinensis* (L.) O. Kuntze], a perennial evergreen woody plant, is one of the most important economic crops in almost 30 countries such as China, Japan, India, Kenya, and Indonesia. The young leaves and buds are processed into a variety of tea, including green, white, yellow, oolong, black, and dark tea, which is a popularly daily beverage (Jiang et al., 2019). Tea has numerous health and medical benefits for humans due to its many characteristic secondary plant metabolites, including catechins, caffeine, polyphenols, and theanine (Too et al., 2015; Ji et al., 2016). Tea production plays a role in the development of agricultural economy in China. Unfortunately, leaf herbivory on tea plants by tea Geometridae moths can cause severe yield loss and quality damage for tea.

Tea Geometridae moths are the most destructive chewing insects for tea plant by feeding on the young tea leaves in China (Zhang, 2001). Among them, *Ectropis grisescens* (Warren) (Lepidoptera: Geometridae, Ennominae) is the most serious Geometridae pest of tea in China because of its wide distribution and destructive nature (Zhang et al., 2019; Pan et al., 2021). This pest infests thousands of hectares of tea plants per year, severely reducing tea plant growth and negatively impacting tea production (Jiang et al., 2014). Compared with *Ectropis obliqua* and *Scopula subpunctaria*, the other main Geometridae pests of tea, *E. grisescens*, has a wider distribution and stronger fecundity (Chen L. L. et al., 2020). Little is known about why *E. grisescens* is the most harmful pest in tea gardens, and there is no explanation from the perspective of insect symbionts. Recently, extensive evidence shows that insect symbionts do affect essential physiological functions in their lepidoptera host, including degrading complex dietary polymers, supplementing essential nutrients, overcoming plant anti-herbivore defenses, or strengthening of immune responses for protection against pathogens (Paniagua et al., 2018). A study reported that mundticin KS (bacteriocin) produced by gut bacteria *Enterococcus mundtii* inhibits pathogen colonization in its host *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Shao et al., 2017). Besides, Lei et al. (2020) found that *Wolbachia* reduce the susceptibility of striped stem borer, *Chilo suppressalis* (Lepidoptera: Crambidae), to two insecticides (fipronil and avermectin).

TABLE 1 | Collection information for larvae samples.

Insect	Sampling locality	Latitude/longitude	Sampling time
Ectropis grisescens	Luan, Anhui, China	31.47°N, 116.93°E	September 2016
Ectropis grisescens	Xishui, Hubei, China	30.28°N, 113.23°E	September 2020
Ectropis grisescens	Xinchang, Zhejiang, China	29.49°N, 120.99°E	June 2020
Ectropis grisescens	Nanping, Fujian, China	27.65°N, 117.98°E	September 2020
Ectropis grisescens	Menghai, Yunnan, China	24.98°N, 102.72°E	May 2020
Ectropis obliqua	Xuancheng, Anhui, China	31.12°N, 119.18°E	September 2020
Scopula subpunctaria	Xuancheng, Anhui, China	31.12°N, 119.18°E	September 2020

In a previous study, we detected *Wolbachia* in the gut of *E. grisescens* larvae (Zhang et al., 2019). However, the *Wolbachia* strain in *E. grisescens* was not typed, and its resulting phenotypes were not elucidated. Here, we use a culture-independent method to investigate and compare the diversity and richness of gut bacterial communities among three species of Geometridae moths and found that the presence of *Wolbachia* was the most obvious difference. MLST- and wsp-based phylogenetic analyses revealed that the *Wolbachia* from different populations of *E. grisescens* had the same *Wolbachia*, which is from supergroup B. Reciprocal cross analyses revealed that *Wolbachia* enhances the fecundity of *E. grisescens*. This may be one of the reasons why its host *E. grisescens* is the most damaging leaf-eating tea pest in China.

TABLE 2 | Primers used for PCR amplification and sequencing.

Gene	Primer	Sequence (5′-3′)	Annealing *T* (°C)
gatB	gatB_F1	GAKTTAAAYCGYGCAGGBGTT	54
	gatB_R1	TGGYAAYTCRGGYAAAGATGA	
coxA	coxA_F1	TTGGRGCRATYAACTTTATAG	55
	coxA_R1	CTAAAGACTTTKACRCCAGT	
hcpA	hcpA_F1	GAAATARCAGTTGCTGCAAA	55
	hcpA_R1	GAAAGTYRAGCAAGYTCTG	
ftsZ	ftsZ_F1	ATYATGGARCATATAAARGATAG	50
	ftsZ_R1	TCRAGYAATGGATTRGATAT	
fbpA	fbpA_F1	GCTGCTCCRCTTGGYWTGAT	53
	fbpA_R1	CCRCCAGARAAAAYYACTATTC	

MATERIALS AND METHODS

Insect Collection and Preservation

The larvae were collected from tea gardens in the five main tea-producing provinces of China (**Table 1**) and were maintained at the State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, Hefei, China (31.86°N, 117.27°E). The collected larvae were reared on tea leaves in transparent boxes in a controlled climate room (22°C ± 1°C; relative humidity 75% ± 10%; 16-h light:8-h dark photoperiod). The tea leaves used in the experiments were cut from branches of tea plants using scissors and inserted into floral foam for storage.

Sample Preparation and DNA Extraction

For Geometrid larvae, 10 fifth instar larvae were pooled to provide a biological replicate and five biological replicates were established per group. For *E. grisescens* adults, five female adults were pooled to provide a biological replicate and five biological replicates were established per group. Note that the DNA used for *Wolbachia*-typing was extracted from single female moth.

Larvae were surface-sterilized by dipping in 70% ethanol for 15 s and then rinsing twice with sterile water for 15 s each time. Dissecting scissors were used to cut laterally behind the head capsule, and the gut was removed from the cuticle with larval forceps. The whole gut including the gut contents was collected and placed in a 2.0-ml micro-centrifuge tube for DNA extraction.

Wings were removed from female moths, and other tissues were surface-sterilized by dipping in 70% ethanol for 15 s, followed by rinsing twice with sterile water for 15 s each time. The tissues were then ground for 2 min and placed in a 2.0-ml micro-centrifuge tube for DNA extraction.

Total genomic DNA was extracted from samples using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The quality of the extracted DNA was assessed by electrophoresis on a 1.2% (w/v) agarose gel. The genomic DNA extracted from bacteria in the larvae gut was subjected to amplification and sequencing.

The Genomic DNA extracted from bacteria in adult female moths was subjected to metagenomic and *Wolbachia*-typing analyses.

Amplification and Sequencing of the V3–V4 Region of the 16S rRNA Gene

Gut bacteria from three Geometridae larvae were analyzed by sequencing the V3–V4 region of the 16S ribosomal RNA gene (16S rRNA), using the Illumina NovaSeq platform.

Genomic DNA samples were subjected to PCRs for amplification of the V3–V4 regions of the 16S rRNA with the universal primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). All PCR reactions consisted of 15 μl of Phusion ®High-Fidelity PCR Master Mix (New England Biolabs, Beverly, MA, United States), 2 μM forward and reverse primers, and 10 ng of template DNA. The thermal cycling conditions were as follows: initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, and final extension at 72°C for 5 min. Then, the mixture of PCR products was purified with a Gel Extraction Kit (Qiagen).

After PCR amplification, the samples were sequenced on the Illumina NovaSeq platform (Illumina, San Diego, CA, United States) and 250-bp paired-end reads were generated. All sequences are available as SRA files at the National Center for Biotechnology Information Sequence Read Archive database (NCBI-SRA) under bioProject PRJNA720281 (SRA accession numbers: SAMN18644841-843).

Bioinformatics and Statistical Analysis

The sequences were analyzed using the QIIME software package and used to compare the relative abundance of bacterial taxa (Caporaso et al., 2010b). Operational taxonomic units (OUTs) were assigned at 97% similarity cutoff using UCLUST version 1.2.22 (Edgar, 2010). The representative sequence (the sequence with the highest relative abundance) of each OTU was selected to build the overall OTU table. The taxonomic classification of each microbial OTU was assigned by Ribosomal Database Project classifier PyNast using SILVA and UNITE as bacterial 16S rRNA databases (Caporaso et al., 2010a). Abundance values of OTUs were normalized using a standard value for the sample with the least sequences. Subsequent analyses of alpha diversity were performed using this normalized output.

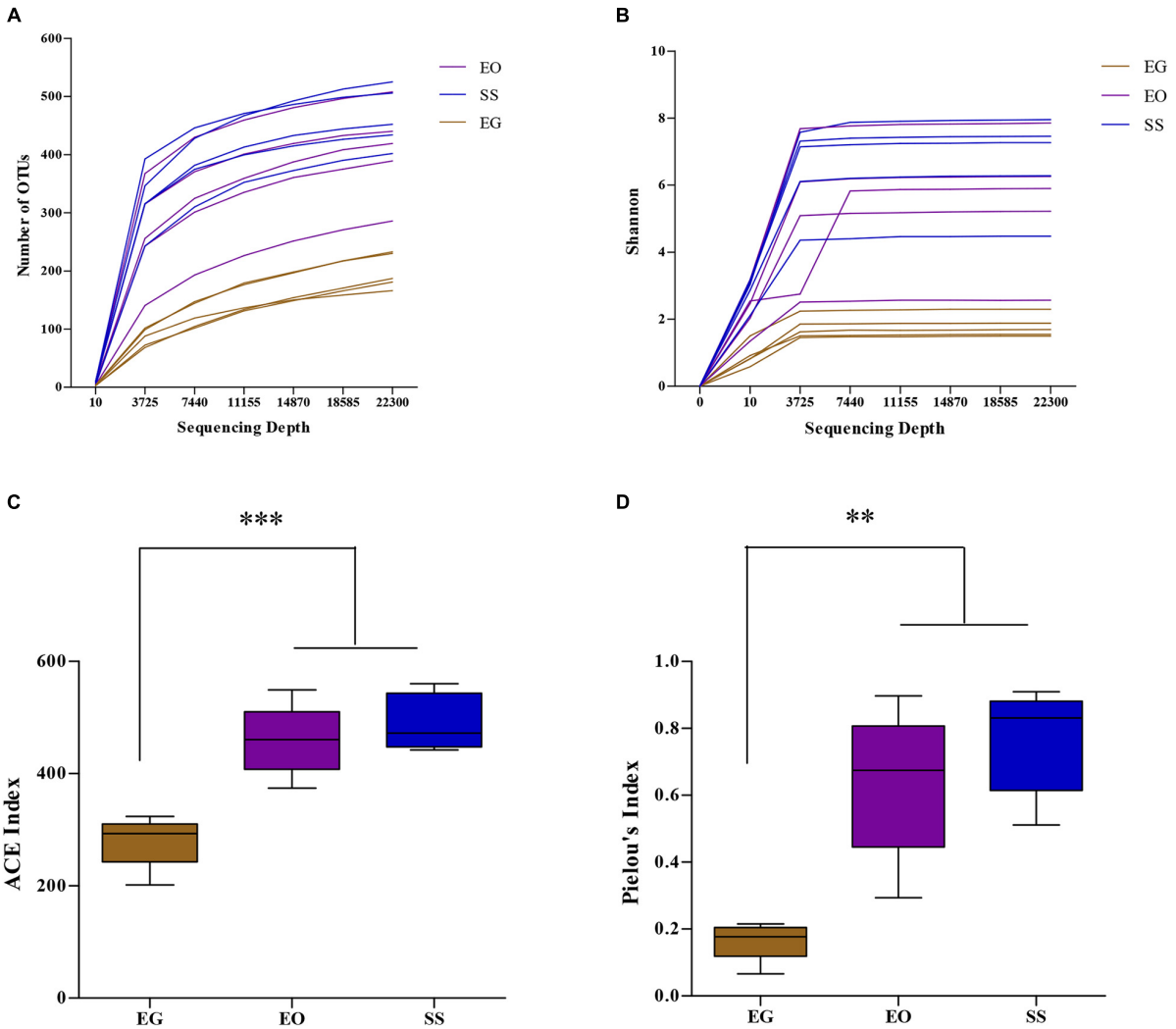

FIGURE 1 | Rarefaction curves, based on number of operational taxonomic units (OTUs) observed **(A)**, and Shannon index **(B)**, and the alpha-diversity, based on ACE index **(C)**, and Pielou's index **(D)**, of the microbial community in the larval gut of *E. grisescens*, *E. obliqua*, and *S. subpunctaria* (EG, EO, and SS, respectively). Significant differences were detected by unpaired two-tailed *t*-test; $^{**}p < 0.001$; $^{***}p < 0.0001$.

The alpha- and beta-diversity indexes were calculated using QIIME V1.7.0. The alpha-diversity indexes were the Pielou evenness index (*J*) and the ACE richness index (Shannon and Weaver, 1949; Pielou, 1966; Chao et al., 1993). Rarefaction curves and Shannon index were used to verify the quality and depth of sampling. Principal coordinate analysis (PCoA) with weighted and unweighted UniFrac distance metrics was performed to detect differences in microbial community structures among the three Geometridae larvae (Lozupone and Knight, 2005). Analysis of similarity (ANOSIM) was carried out based on Bray–Curtis dissimilarity to assess the difference of microbial community structures (Clarke, 1993). Microbes that showed significant differences in relative abundance among different groups were identified using Metastats software[2] with false discovery rate (FDR) (Paulson et al., 2011).

[2]http://metastats.cbcb.umd.edu/

Metagenomic Analysis of Endosymbionts in *E. grisescens* Female Moths

The DNA sample was fragmented by sonication to produce 350-bp fragments; then, these DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. Finally, PCR products were purified using the AMPure XP system. The libraries were analyzed to determine size distribution with an Agilent2100 Bioanalyzer and quantified using real-time PCR.

Clustering of the index-coded samples was performed using the cBot Cluster Generation System according to the manufacturer's instructions. After cluster generation, the libraries were sequenced on the Illumina NovaSeq 6000 platform and paired-end reads were generated.

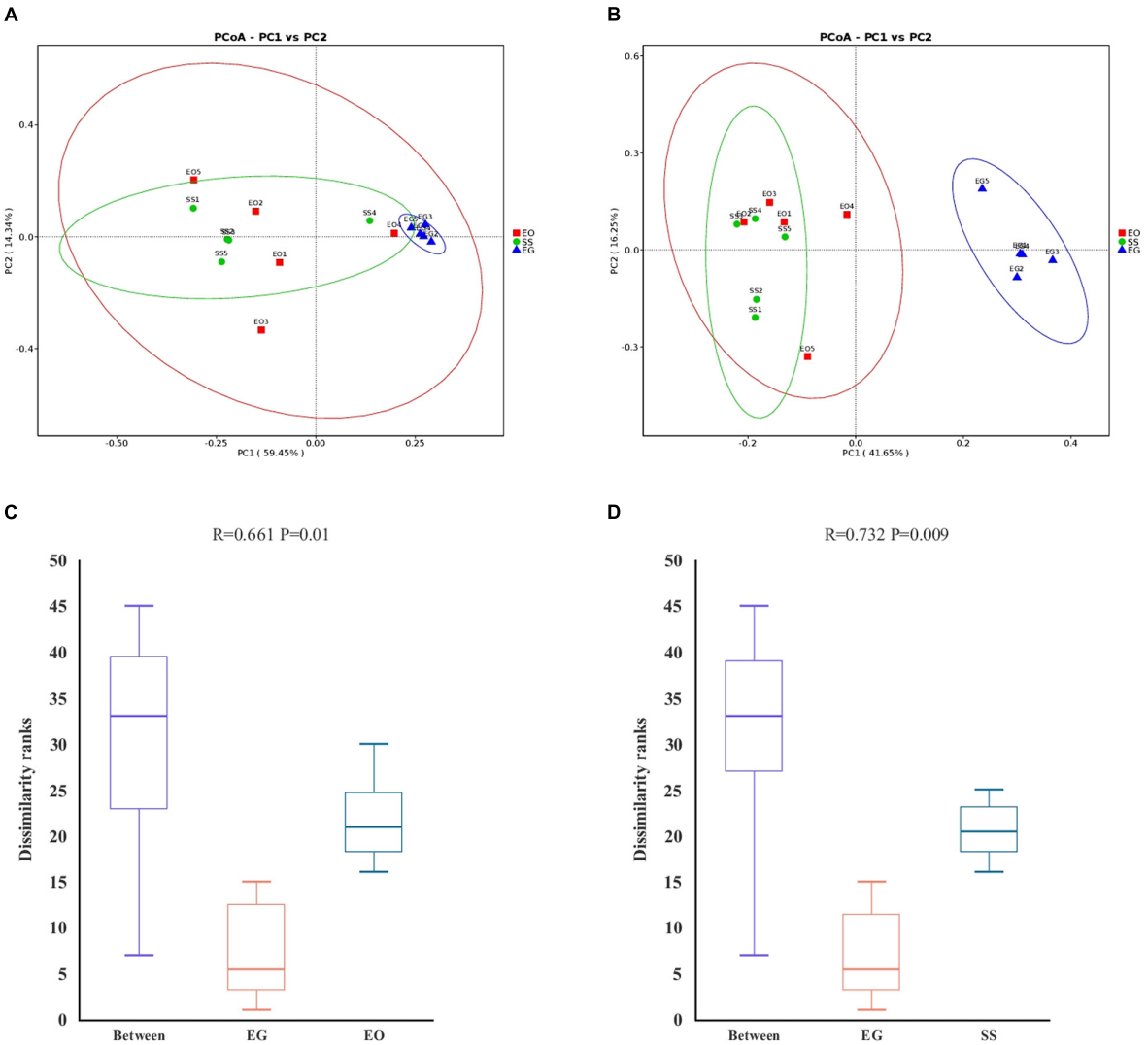

FIGURE 2 | Principal component and non-parametric test analysis of bacterial diversity. Principal coordinate analysis (PCoA) of bacteria using distance based on weighted **(A)** and unweighted **(B)** UniFrac values. Non-parametric test (ANOSIM) of bacterial diversity in EG compared with EO **(C)** and SS **(D)**. EG, EO, and SS refer to *E. grisescens*, *E. obliqua*, and *S. subpunctaria*, respectively.

The raw data obtained from the Illumina NovaSeq sequencing platform using Readfq (V8)[3] were processed to obtain clean data for subsequent analyses. The processing steps removed reads containing low-quality bases (default quality threshold value $\leq$ 38, default length 40 bp), reads containing a certain proportion of N (unspecified) bases (default length 10 bp), and reads containing adapter sequences (default length 15 bp). The raw data have been submitted to the NCBI-SRA under bioProject PRJNA720281 (SRA accession number: SAMN18644841).

Considering the possibility that samples may be polluted with host DNA, the clean data were used in BLAST searches against the host database using Bowtie2.2.4 software (Bowtie2.2.4)[4] to filter reads of host origin. The parameters were as follows : –end-to-end, –sensitive, -I 200, -X 400.

Due to serious host pollution, we use a read-based (mapping) approach for metagenomic analysis. Species annotations were based on clean data and the RPKM method was used to calculate abundance. The MetaPhalAn2 method was used to analyze bacterial diversity (Segata et al., 2012).

Cloning and Sequencing of *wsp* and MLST Genes

The WSP locus was amplified using the *wsp* primers (*wsp*_F1: 5′-GTCCAATARSTGATGARGAAAC-3′ and *wsp*_R1: 5′-CYGCACCAAYAGYRCTRTAAA-3′) to confirm single infections. The PCR reactions were performed in 25-μl final volumes containing 9.7 μl of ddH_2O, 12.5 μl of Premix Taq

[3]https://github.com/cjfields/readfq

[4]http://bowtie-bio.sourceforge.net/bowtie2/index.shtml

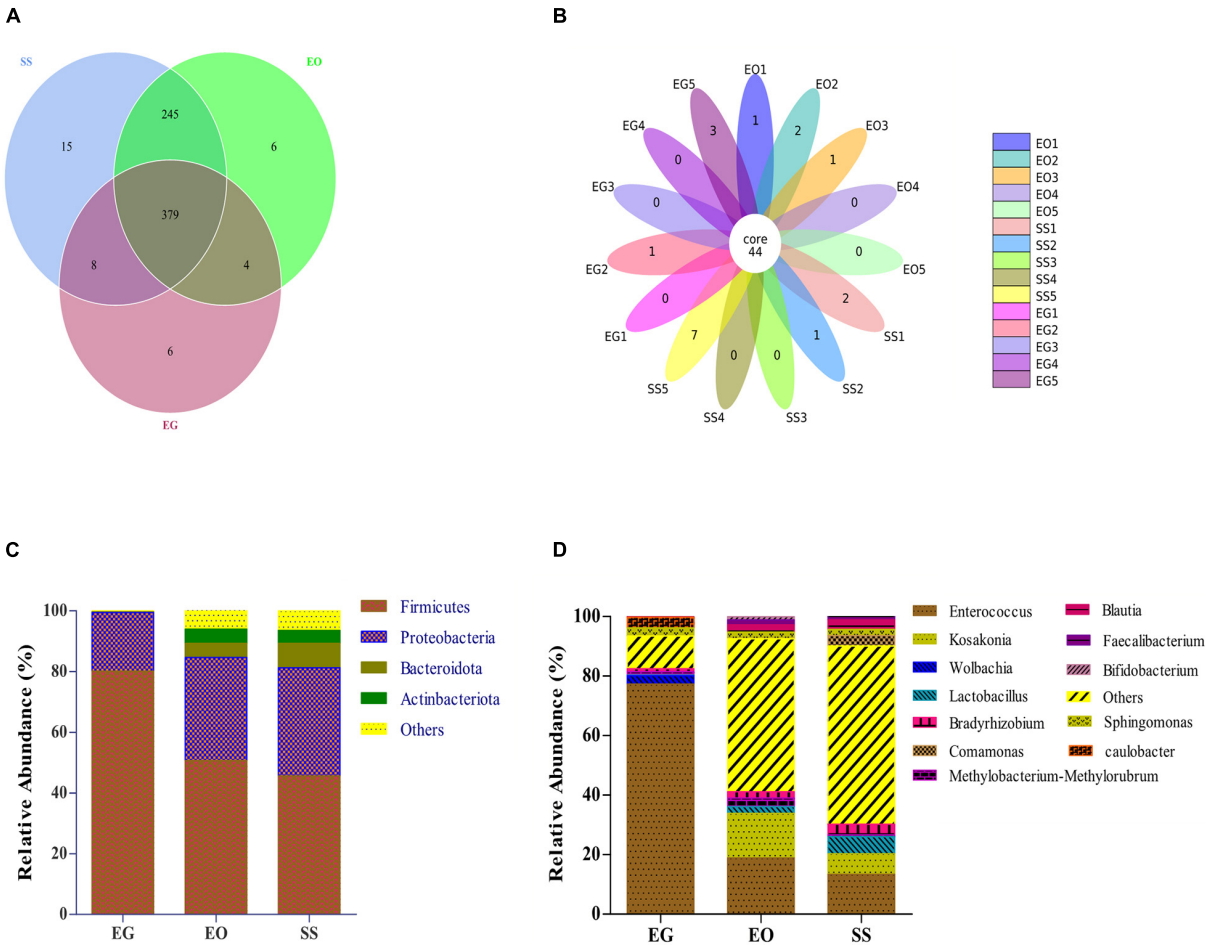

FIGURE 3 | Venn diagram **(A)** and petal diagram **(B)** of operational taxonomic units (OTUs) in EG, EO, and SS. Relative abundances are shown for bacteria with relative abundance of >1% in larval gut of at least one species at the phylum level **(C)** and three species at the genus level **(D)**. EG, EO, and SS refer to *E. grisescens*, *E. obliqua*, and *S. subpunctaria*, respectively.

(LA Taq Version 2.0, TaKaRa, Dalian, China), 0.8 μl of DNA template, and 1 μl of each primer (10 μM). The thermal cycling conditions were as follows: 95°C for 3 min; 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 90 s; 72°C for 10 min. The products were stored at 4°C.

The MLST loci were amplified in accordance with previously published protocols (see text footnote 1). The PCR reactions were performed in 25-μl final volumes containing 9.7 μl of ddH_2O, 12.5 μl of Premix Taq (LA Taq Version 2.0, TaKaRa), 0.8 μl of DNA template, and 1 μl of each primer (10 μM). The thermal cycling conditions were as follows: 94°C for 4 min; 35 cycles of 94°C for 30 s, appropriate T_m (**Table 2**) for 45 s and 72°C for 90 s; 72°C for 10 min. The products were stored at 4°C.

Each sample was mixed with the same volume of 1× loading buffer containing SYBR green and then separated by electrophoresis on 1.2% (v/v) agarose gels for amplicon detection. All the PCR products were sequenced by Sangon Biotech (Shanghai, China). The sequences of all products were used in BLAST or MLST analyses at the GenBank and PubMLST databases, respectively.

Wolbachia Strain Typing Based on *wsp* Gene

The amplified *wsp* gene sequences were subjected to BLAST analysis at the GenBank database. A list of *Wolbachia* isolates, including those reported by Zhou et al. (1998) and van Meer et al. (1999) and those with *wsp* gene sequences in GenBank, was created (**Supplementary Table 1**). These isolates were used as references to classify the type of *Wolbachia* strain isolated from *E. grisescens*.

A phylogenetic tree was constructed using the *Wolbachia wsp* genes and the maximum likelihood (ML) approach implemented in MEGA X. The substitution model was Tamura 3-parameter and gamma distributed (G) with 1000 bootstrap replications.

MLST, WSP HVR Profiling, and Phylogenetic Analyses

The sequences of *gatB*, *coxA*, *hcpA*, *ftsZ*, *fbpA*, and *wsp* were submitted to the PubMLST database for sequence typing to generate MLST allelic profiles and WSP HVR profiles. A list of

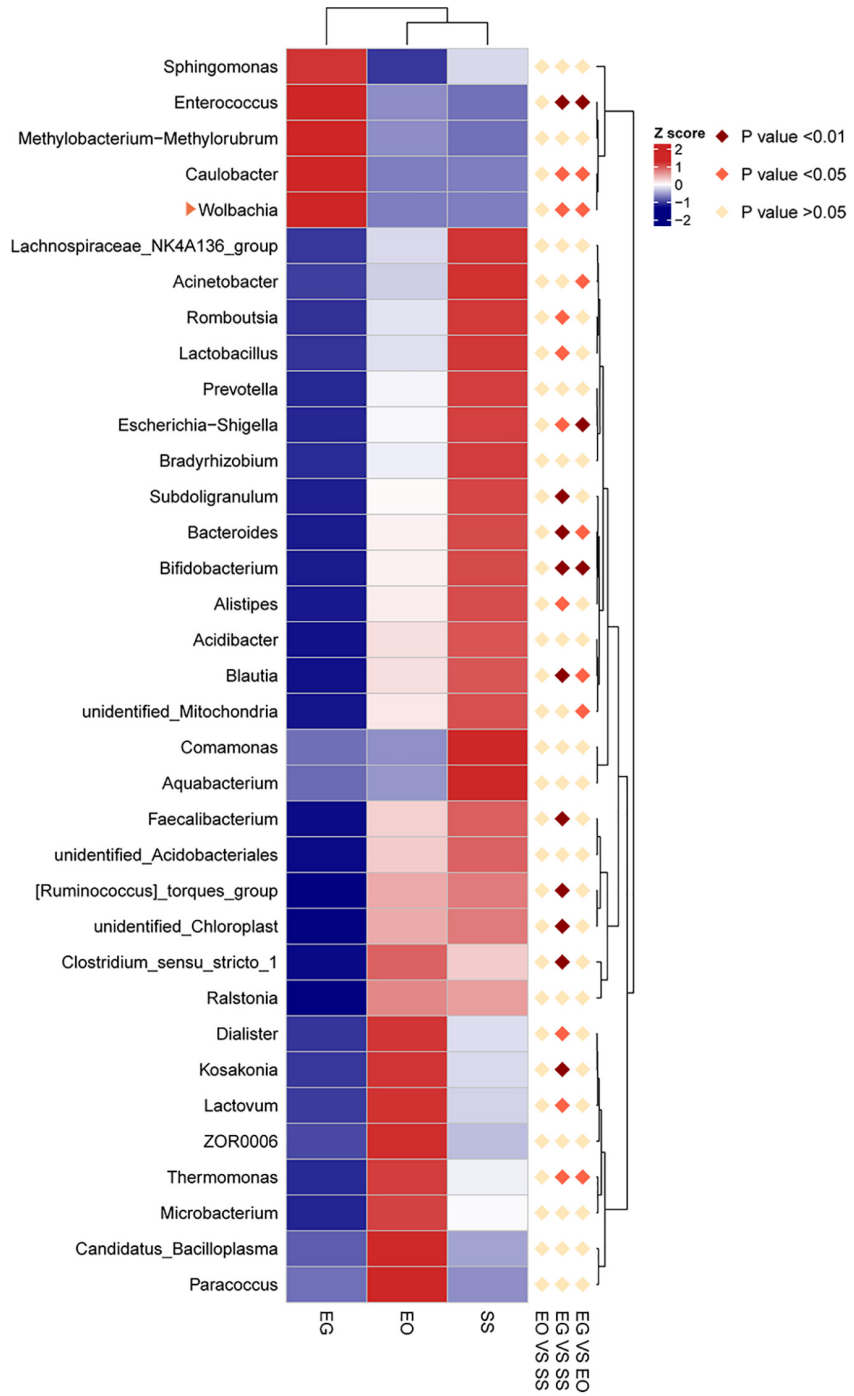

FIGURE 4 | Hierarchical clustering analysis of three groups based on the relative abundance of the top 30 microbiota genera identified in EG, EO, and SS. EG, EO, and SS refer to *E. grisescens*, *E. obliqua*, and *S. subpunctaria*, respectively.

Wolbachia isolates was generated by searching PubMLST and downloading isolate sequences from GenBank (**Supplementary Table 2**). Isolates with a complete set of MLST and HVR profiles, as well as phenotypic data, were selected. These isolates had been used by Baldo et al. (2006) to establish the MLST typing system for *Wolbachia*. The ML trees were constructed using the concatenated MLST and *wsp* sequences, independently, in MEGA X.

The alignments of concatenated MLST alleles and *wsp* alleles were generated using ClustalW with 1000 bootstrap iterations. For phylogenetic reconstructions, the nucleotide substitution model was determined by the BIC value calculated by MEGAX

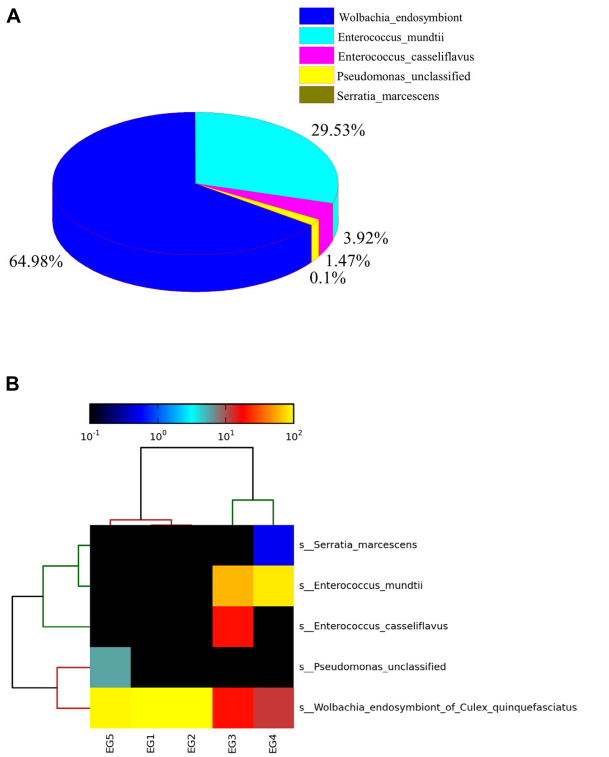

FIGURE 5 | Relative abundances of microbiota species in *E. grisescens* female moths **(A)**. Hierarchical clustering analysis of five samples identified in *E. grisescens* female moths **(B)**.

software. The best-fit substitution models were as follows: GTR + G for MLST and T92 + G for *wsp*. The sequences of the *wsp* gene and the five genes used for MLST of the *Wolbachia* strain from *E. grisescens* have been deposited in the GenBank database (Accession number: MW630082-MW630111).

Influence of *Wolbachia* on *E. grisescens* Phenotypes

To analyze the effects of *Wolbachia* on its host *E. grisescens*, the fecundity (egg production) of infected (W^+) and uninfected (W^-) insects was recorded. All the insects used in the experiments were confirmed to be infected with *Wolbachia* by amplifying the *wsp* gene. *E. grisescens* was collected from Lu'an, Anhui Province, and successfully subcultured for more than 40 generations.

The F1 generation of W^+ *E. grisescens*, as larvae, were reared on tea leaves until they reached the third instar stage. Then, 140 healthy third-instar larvae were divided into two groups of 70 larvae, which were placed individually in 90-mm plastic Petri dishes. Each dish contained food for the insects and had a tight lid. The control group was fed an agar-based insect diet, and the antibiotic group was fed the insect diet supplemented with 300 μg/ml each of rifampicin, gentamicin, penicillin, and streptomycin (Zhang et al., 2019). The insect diet comprised 60 ml of sterile water, 20 g of tea powder, and 2 g of wheat

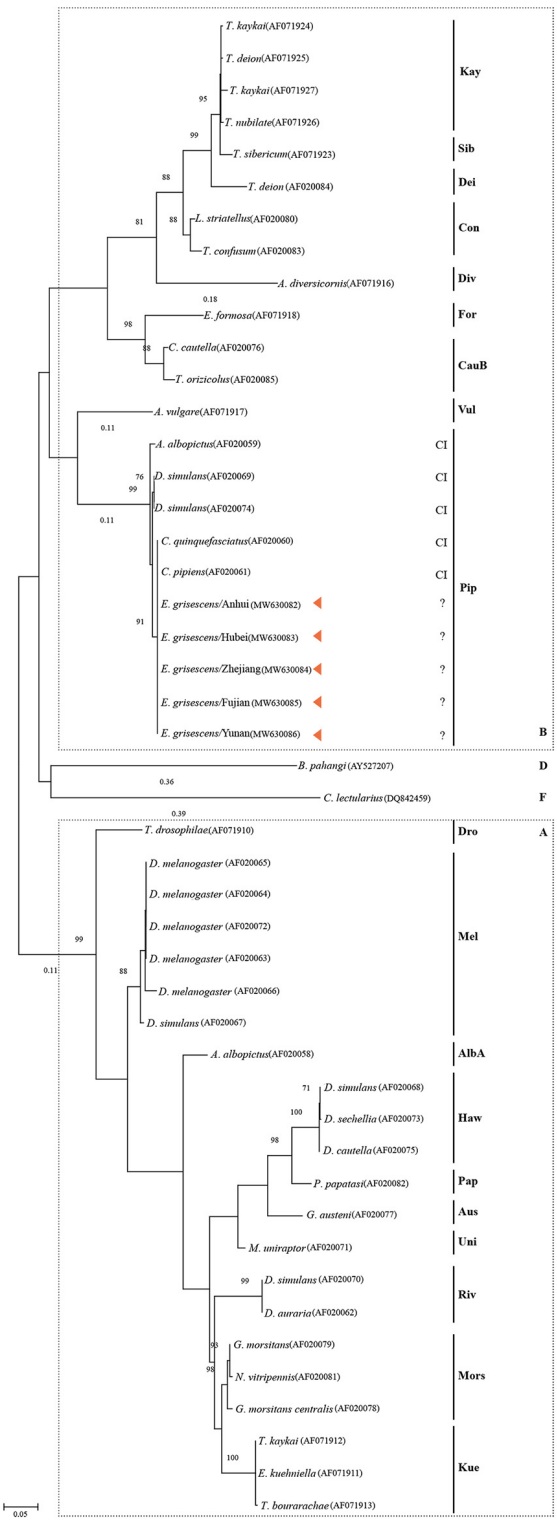

FIGURE 6 | Phylogenetic tree of *Wolbachia* based on the *wsp* sequences. The tree has been constructed by the Maximum likelihood method. The name of the host arthropod species is listed in **Supplementary Table 1**. Bar represents amino acid substitutions per site. Bootstrap values are shown at each node. CI, cytoplasmic incompatibility; A, supergroup A; B, supergroup B; D, supergroup D; F, supergroup F.

TABLE 3 | MLST and WSP HVR profiles of *Wolbachia* strains.

Strain	MLST						WSP				
	gatB	*coxA*	*hcpA*	*ftsZ*	*fbpA*	ST	HVR1	HVR2	HVR3	HVR4	*wsp*
*w*GriAH	39	14	40	36	4	41	10	8	10	8	10
*w*GriHB	39	14	40	36	4	41	10	8	10	8	10
*w*GriZJ	39	14	40	36	4	41	10	8	10	8	10
*w*GriYN	39	14	40	36	4	41	10	8	10	8	10
*w*Gri FJ	39	14	40	36	4	41	10	8	10	8	10

agar. Fresh tea leaves were collected, freeze-dried, and ground to make the tea powder.

To ensure that the effects on reproduction were due to the absence of *Wolbachia* and not due to antibiotics, the same antibiotics were used to treat *E. obliqua* and *S. subpunctaria*.

Reciprocal Cross Analysis

Ectropis obliqua and *E. grisescens* are two closely related moth species. Reciprocal cross analyses were conducted to determine whether *Wolbachia* affects reproduction between these two moths. *E. grisescens* was collected from Luan, Anhui Province, and successfully subcultured for more than 40 generations. *E. obliqua* was collected from Xuancheng, Anhui Province, and successfully subcultured for more than three generations.

We selected newly emerged moths of *E. obliqua* and *E. grisescens* for reciprocal crosses. The egg hatching rate was counted.

Data Analyses

GraphPad Prism 7.0, R, or Origin 9.0 was used for statistical analyses and to construct figures.

RESULTS

Composition and Diversity of Gut Bacteria in Larvae of Three Tea Geometridae

The Illumina NovaSeq sequencing of the bacterial 16S rRNA amplicons from larvae of *E. grisescens* (EG), *E. obliqua* (EO), and *S. subpunctaria* (SS) yielded 951,717 raw reads in total. After quality filtering and read merging, a total of 876,703 high-quality sequences remained for bacteria. Rarefaction curves (**Figure 1A**) and Shannon index (**Figure 1B**) clearly demonstrated that the sampling efforts were adequate to fully represent the richness of the gut communities analyzed.

The OTU richness (ACE index) of the gut bacteria of EG (280.04 ± 41.54) differed from those of EO (458.75 ± 55.42; $t = 6.91$; df = 7; $p < 0.0001$) and SS (490.30 ± 45.15; $t = 6.91$; df = 7; $p < 0.0001$) (**Figure 1C**). The OTU evenness (Pielou index; J) of the gut bacteria of EG ($J = 0.16 \pm 0.05$) differed from those of EO ($J = 0.64 \pm 0.19$; $t = 4.62$; df = 8; $p < 0.001$) and SS ($J = 0.76 \pm 0.14$; $t = 7.98$; df = 8; $p < 0.0001$) (**Figure 1D**). Thus, the OTU richness and evenness of the gut bacteria were lowest in *E. grisescens* among the three species analyzed.

The PCoA analysis using the distances based on weighted and unweighted UniFrac showed that the gut bacteria communities of *E. grisescens* larvae were the most distinctive (**Figures 2A,B**). A non-parametric test (ANOSIM), which is based on Bray–Curtis dissimilarity, indicated that the gut bacterial beta-diversity of EG was significantly different from those of EO ($R = 0.664$; $p < 0.05$) and SS ($R = 0.732$; $p < 0.001$) (**Figures 2C,D**). These results showed that the gut bacterial beta-diversity of *E. grisescens* larvae was significantly distinctive compared with those of *E. obliqua* and *S. subpunctaria* larvae.

As shown in the Venn diagram in **Figure 3A**, 379 OTUs were shared by all groups, and accounted for 95.47, 59.78, and 58.58% of the total OTUs in EG, EO, and SS, respectively. The petal diagram in **Figure 3B** shows that a total of 44 OTUs were shared among all samples. The relative abundance of these 44 OTUs in EG, EO, and SS was 83.41% ± 6.95%, 60.01% ± 11.02%, and 65.46% ± 13.21%, respectively (**Supplementary Figure 1**). The alpha- and beta-diversity indexes were lowest for bacterial community in the gut of *E. grisescens* larvae, but overall, the composition of core gut bacteria in the three geometrids was similar.

The dominant bacterial phyla in each group were Firmicutes (45.64% ± 12.25% to 80.13% ± 8.63%) and Proteobacteria (19.26% ± 4.22% to 33.92% ± 4.22%) (**Figure 3C**). The dominant bacterial genera in EG were *Enterococcus* (77.29% ± 16.53%), *Caulobacter* (3.58% ± 1.6%), and *Wolbachia* (2.8% ± 1.52%). The dominant bacterial genera in EO and SS were *Enterococcus* (13.40% ± 5.51% to 18.8% ± 6.20%), *Kosakonia* (6.87% ± 2.33% to 14.99% ± 4.16%), and *Lactobacillus* (2.27% ± 2.33% to 5.74% ± 4.16%) (**Figure 3D**). These results showed that *Enterococcus* was the dominant gut bacterial genus in tea Geometridae larvae.

The heat map of the top 35 abundant gut bacteria illustrates differences in microbial community structures at the genus level (**Figure 4**). The relative abundance of *Caulobacter* and *Wolbachia* in *E. grisescens* was significantly different from that in *E. obliqua* and *S. subpunctaria* as determined by MetaStat analysis. Among these three bacterial species, *Wolbachia* was only detected in *E. grisescens* larvae, with a relative abundance of up to 7.6% (**Supplementary Figure 2**).

Composition of Microbial Communities in Adult Female Moths of *E. grisescens*

The Illumina NovaSeq sequencing of bacteria from female adults of *E. grisescens* yielded about 6.33–6.73 G raw data per sample. After quality filtering and read merging, a total of 6.32–6.73 G clean data remained per sample. After removing the host's genomic data, only 0.32–0.39 G data per sample remained for analysis. We also used a read-based (mapping) approach for metagenomic analysis.

The dominant bacterial species in EG female moths were *Wolbachia* endosymbiont (64.98%) and *E. mundtii* (29.35%) (**Figure 5A**). *Wolbachia* spp. were detected in all five EG samples and were the most abundant bacteria in EG1, EG2, and EG5 (**Figure 5B**).

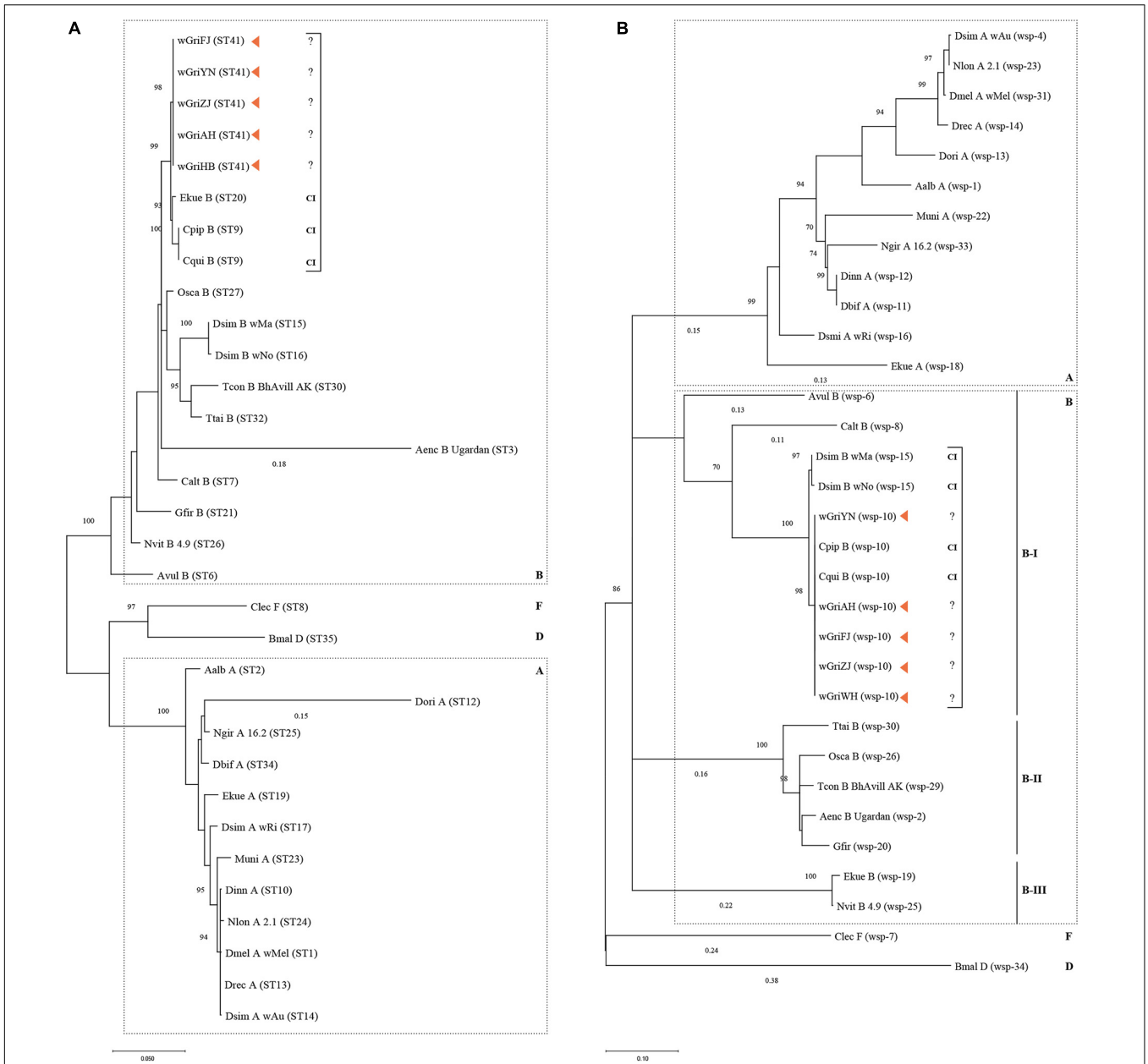

FIGURE 7 | Phylogenetic analysis based on *Wolbachia* MLST and *wsp* sequences. Maximum likelihood trees constructed using the MLST allelic **(A)** and WSP HVR **(B)** profiles. For strain names, see **Supplementary Table 2**. Bar represents amino acid substitutions per site. Bootstrap values are shown at each node. CI, cytoplasmic incompatibility; A, supergroup A; B, supergroup B; D, supergroup D; F, supergroup F.

Molecular Characterization of Native *Wolbachia* Strain

Next, molecular typing analyses were conducted to identify the *Wolbachia* strain in *E. grisescens*. Five *Wolbachia* strains were isolated from *E. grisescens* collected from Anhui, Hubei, Zhejiang, Fujian, and Yunnan (the five representative tea-producing provinces in China) and were designated as *w*GriAH, *w*GriHB, *w*GriZJ, *w*GriFJ, and *w*GriYN, respectively.

Figure 6 shows the tree based on the general data set using the ML implementation after bootstrapping 1000 times. The topology shows the division of *Wolbachia* into four supergroups, A, B, D, and F. *w*GriAH, *w*GriHB, *w*GriZJ, *w*GriFJ, and *w*GriYN were in supergroup B, subgroup Pip. All the reference members of this group are CI-inducing strains (**Figure 6**).

In the MLST typing analyses, *w*GriAH, *w*GriHB, *w*GriZJ, *w*GriFJ, and *w*GriYN had the same allelic profile and HVR profiles. The allelic profile for *gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA* was 39, 14, 40, 36, and 4 (ST-41), respectively, and the HVR profiles for HVR1, 2, 3, and 4 were 10, 8, 10, and 8 (*wsp*-10), respectively (**Table 3**).

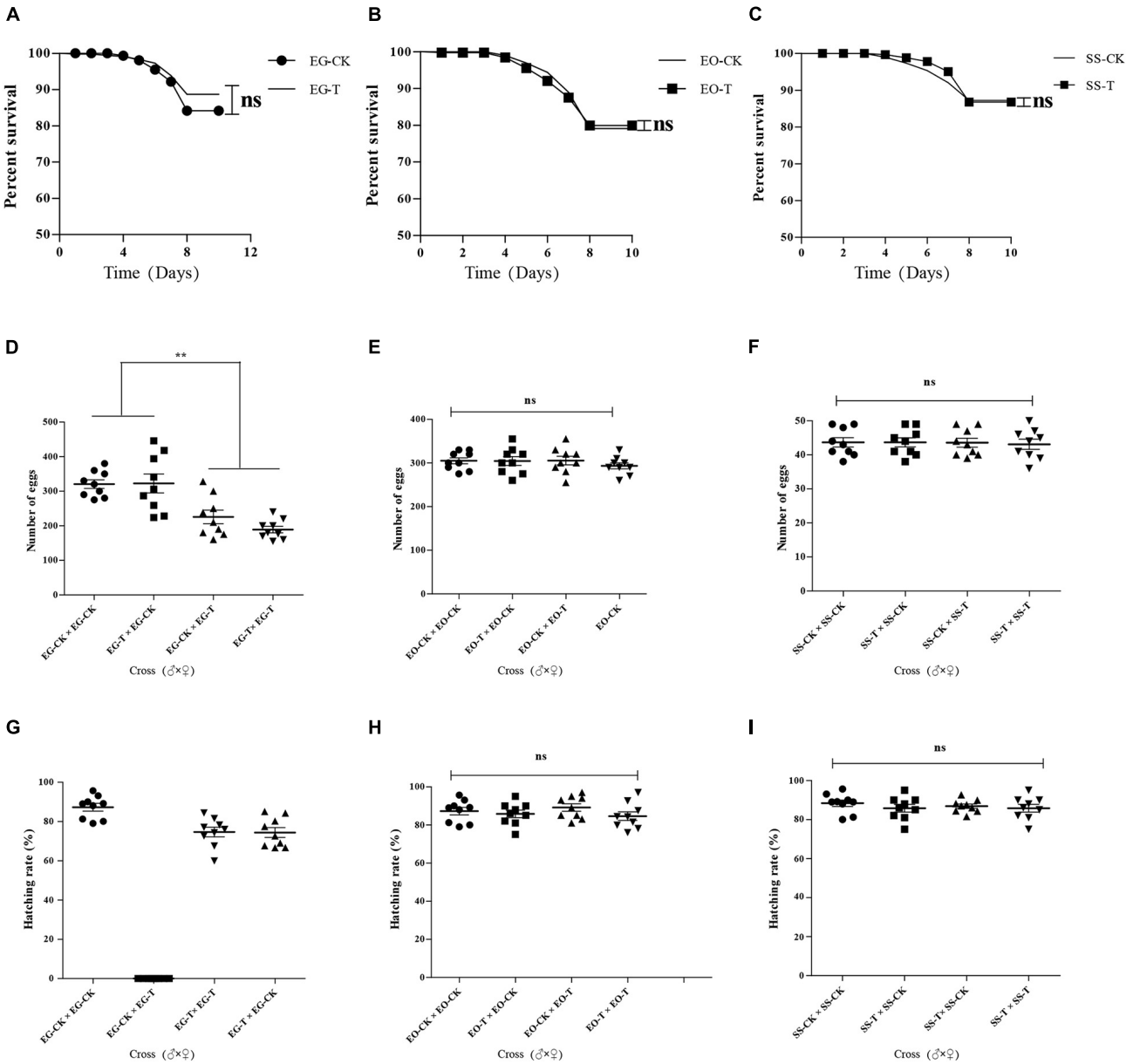

FIGURE 8 | Effect of antibiotic on the survival rates of EG **(A)**, EO **(B)**, and SS **(C)**; fecundity of EG **(D)**, EO **(E)**, and SS **(F)**; and fertility of EG **(G)**, EO **(H)**, and SS **(I)**. EG, EO, and SS refer to *E. grisescens*, *E. obliqua*, and *S. subpunctaria*, respectively. Significant differences were detected by unpaired two-tailed *t*-test; ns: no significant difference; $^{**}p < 0.001$.

The MLST-based ML tree separated the isolates into three major clusters: A, B, and D + F (**Figure 7A**). Within supergroup B, *w*GriAH, *w*GriHB, *w*GriZJ, *w*GriFJ, and *w*GriYN, as one subclade, formed a cluster with Ekue_B, Cpip_B, and Cqui_B, all of which are CI-inducing strains. Differently, the HVR-based ML tree separated the isolates into five major clusters: A, B-I, B-II, and B-III, and a cluster containing D and F (**Figure 7B**). *w*GriAH, *w*GriHB, *w*GriZJ, *w*GriFJ, and *w*GriYN formed a subclade with Cpip_B and Cqui_B and placed in B-I. All the available reference *Wolbachia* strains shared the same HVR and had CI-inducing phenotypes (**Figure 7B**).

Based on this, *Wolbachia* isolates *w*GriAH, *w*GriHB, *w*GriZJ, *w*GriFJ, and *w*GriYN were identified as the same strain, designated as *w*Gri. Thus, *w*Gri was classified as a potentially CI-inducing *Wolbachia* strain in supergroup B.

Effects of *Wolbachia* on *E. grisescens* Reproduction

To explore the effects of *Wolbachia* strain *w*Gri on *E. grisescens* reproduction, we treated *E. grisescens* with antibiotics to eliminate *w*Gri and carried out reciprocal cross analyses. To

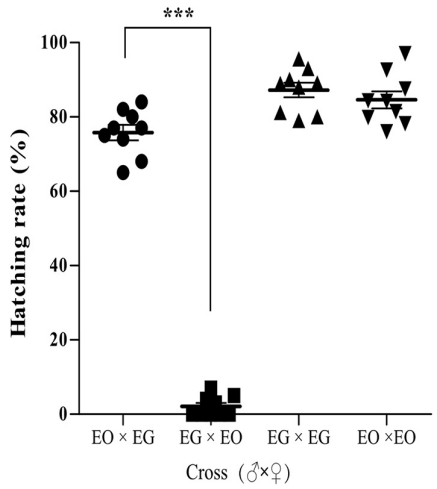

FIGURE 9 | Crossing experiments between *E. grisescens* (EG) and *E. obliqua* (EO). Significant differences were detected by unpaired two-tailed *t*-test; ***$p < 0.0001$.

eliminate the effects of antibiotic interference on the cross results, *E. obliqua* and *S. subpunctaria* were treated with the same antibiotics.

The survival rates of *E. grisescens* infected with *Wolbachia* (EG-CK) or treated with antibiotics (EG-T) showed no significant difference (unpaired and two-tailed *t*-tests; $p > 0.5$) (**Figure 8A**). Egg production (fecundity) was significantly greater in infected females (♂ × ♀; EG-CK × EG-CK: 320.56 ± 35.13 eggs per female and EG-T × EG-CK: 322.56 ± 77.48 eggs per female) than in uninfected females (♂ × ♀; EG-T × EG-CK: 225.67 ± 54.99 eggs per female and EG-T × EG-T: 189.12 ± 26.64 eggs per female) in both tests (**Figure 8D**). This indicated that the presence of *w*Gri increased the number of eggs produced.

The fertility (total number of eggs hatched) rates of *E. grisescens* infected with *Wolbachia* (EG-CK) or treated with antibiotics (EG-T) were not significantly different (unpaired and two-tailed *t*-tests; $p > 0.5$). However, the uninfected females mated with infected males showed 0% eggs hatched per female (100% embryo mortality). Infected females mated with uninfected males had high fertility rates (**Figure 8G**). These results indicated that *Wolbachia* strain *w*Gri induces strong unidirectional CI in *E. grisescens*.

The survival (**Figures 8B,C**), fertility (**Figures 8E,F**), and fecundity rates (**Figures 8H,I**) of *E. obliqua* and *S. subpunctaria* showed no significant difference between the control and the antibiotic treatments, confirming that antibiotics did not affect the results of the crossing analyses.

These data indicated that *w*Gri resulted in a high CI level in *E. grisescens* and enhanced the fecundity of the female hosts.

Reciprocal Cross Analysis

Crossing experiments were conducted using *E. grisescens* and *E. obliqua*. The hatching rate of the eggs produced by *E. grisescens* females mated with *E. obliqua* males was 75%, while the eggs produced by *E. obliqua* females mated with *E. grisescens* males did not hatch, or very few hatched (**Figure 9**).

These findings indicated that *Wolbachia* strain *w*Gri influences reproductive communication between *E. grisescens* and *E. obliqua* through CI.

DISCUSSION

The microbiomes of insects play important roles in mediating their health and fitness, and hence, microbiomes are attracting the attention of entomologists (Oliver et al., 2010; Engel and Moran, 2013; Blow and Douglas, 2019). In this study, a culture-independent approach was used to compare the composition and diversity of gut bacteria among three tea Geometridae moths, *E. grisescens*, *E. obliqua*, and *S. subpunctaria*. The results revealed that *Enterococcus* was the dominant bacterial genus in the gut of the three Geometridae larvae. Many studies have reported that *Enterococcus* is active in the gut throughout the larval life cycle of most Lepidoptera and probably plays a key role in insect defense against potentially harmful microorganisms (Dillon and Dillon, 2004; Chen B. S. et al., 2020). The results of the metagenomic analyses demonstrated that *E. mundtii* was the main *Enterococcus* species in the gut of *E. grisescens*.

Although the alpha- and beta-diversity indexes of gut bacteria were lowest in *E. grisescens* larvae, the composition of core gut bacteria (except for *Wolbachia*) was similar among the three Geometridae species. *Wolbachia* are the most widespread endosymbiotic bacteria among arthropods. In these hosts, they are notorious for their reproductive parasitism, which ensures their spread, even though it may lower host fitness (Vavre et al., 2002). For instance, CI provides the infected females with a reproductive advantage in the population, allowing *Wolbachia* to rapidly expand in the host population (Werren et al., 2008). In this study, we detected *Wolbachia* in all the tested *E. grisescens* samples collected from five different representative tea plantations. Molecular typing analyses demonstrated that these *Wolbachia* were the same strain, designed as *w*Gri. Our analyses showed that *w*Gri has a strong capability to induce CI. Besides, in a previous study, *Wolbachia* were detected in all the tested *E. grisescens* samples that had been collected from eight different geographical tea-producing areas in China (Wang et al., 2020). Consequently, we speculate that the 100% *w*Gri infection rate of *E. grisescens* in nature has resulted in the spread of *w*Gri in *E. grisescens via* induced unidirectional CI.

Wolbachia also have the potential to engage in mutualistic relationships with their hosts (Zug and Hammerstein, 2015). In insects, there are a few cases whereby *Wolbachia* act as an obligate mutualist. For example, in the bed bug *Cimex lectularius*, elimination of *Wolbachia* (supergroup F) through antibiotic treatment renders abnormal development of eggs, which can be restored by dietary supplementation of synthesizes biotin and riboflavin (Hosokawa et al., 2010; Nikoh et al., 2014). *Wolbachia* also act as facultative mutualists whereby hosts benefit from infection; they do not depend on *Wolbachia* for survival or fecundity (Kaur et al., 2021). The results of the mating experiment showed that *w*Gri could enhance the fecundity of its host, indicative of a

mutually beneficial symbiosis between *w*Gri and *E. grisescens*. Meanwhile, elimination of *w*Gri through antibiotic treatment did not affect its host survival rate. Based on this, we hypothesize that *Wolbachia* strain *w*Gri as the facultative symbiont of its host *E. grisescens* has contributed to its rapid spread in tea gardens by enhancing its fecundity.

The tea Geometrid moths *E. grisescens* and *E. obliqua* are closely related species that feed on tea leaves. In China, *E. grisescens* and *E. obliqua* are treated as the same species in tea garden management because their morphology is so similar that it is difficult to tell them apart (Wang et al., 2019). According to the records, *E. obliqua* has always been one of the main tea pests in China. Previous data showed that it was distributed in the main tea-producing areas, while *E. grisescens* was mainly distributed in southern tea-producing areas (Luo et al., 2017). However, recent surveys have found that the distribution of *E. obliqua*, which was once distributed in all tea-producing areas, has greatly reduced. In contrast, *E. grisescens*, once a relatively minor pest species, is now distributed in almost all tea-producing provinces in China (Jiang et al., 2014; Li et al., 2019). These sibling species can mate. Our results showed that mating between *E. grisescens* and *E. obliqua* conformed to the phenomenon of unidirectional CI. In other words, the eggs produced by female *E. grisescens* mated with male *E. obliqua* are able to hatch. The characteristics of *Wolbachia* maternal inheritance have promoted rapid evolution of its host. We suggest that the gradual decrease in the distribution of *E. obliqua* and the gradual increase in the distribution of *E. grisescens* is related to the presence of *Wolbachia* strain *w*Gri.

The results of this study show that *Wolbachia* strain *w*Gri results in a high level of CI in *E. grisescens* and enhances the fecundity of its female hosts. We propose that the presence of *w*Gri in its host *E. grisescens* has contributed to host rapid spread among tea gardens by improving the host's fertility and interfering with the reproduction of its competitors (*E. obliqua*).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MW630082-MW630111 and https://www.ncbi.nlm.nih.gov/, PRJNA720281.

AUTHOR CONTRIBUTIONS

YY, YZ, and YlZ designed the research. YL supervised the study. YZ, SL, RJ, CZ, and TG performed the experiments. YZ analyzed and wrote the manuscript. YZ, RJ, and CL revised the manuscript. All authors approved the final version for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.694466/full#supplementary-material

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Characterization and Comparison of Intestinal Bacterial Microbiomes of *Euschistus heros* and *Piezodorus guildinii* Collected in Brazil and the United States

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Background: Herbivorous insects are one of the main biological threats to crops. One such group of insects, stink bugs, do not eat large amounts of tissue when feeding on soybean, but are damaging to the quality of the seed yield as they feed on green developing seeds leading to poorly marketable harvests. In addition to causing physical damage during sucking-feeding activities, the insects can also transmit microbial pathogens, leading to even greater yield loss. Conducting surveys of the insect intestinal microbiome can help identify possible pathogens, as well as detail what healthy stink bug digestive systems have in common.

Methods: We used the conserved V4 region of the 16S rRNA gene to characterize the bacterial microbiome of the red-banded stink bug *Piezodorus guildinii* collected in Brazil and the United States, as well as the neotropical brown stink bug *Euschistus heros* collected in Brazil.

Results: After quality filtering of the data, 192 samples were kept for analyses: 117 samples from *P. guildinii* covering three sites in Brazil and four sites in the United States, and 75 samples for *E. heros* covering 10 sites in Brazil. The most interesting observations were that the diversity and abundance of some bacterial families were different in the different ecoregions of Brazil and the United States.

Conclusion: Some families, such as Acetobacteraceae, Bacillaceae, Moraxellaceae, Enterobacteriaceae, and Rhodocyclaceae, may be related to the better adaptation in some localities in providing nutrients, break down cellulose, detoxify phytochemicals, and degrade organic compounds, which makes it difficult to control these species.

Keywords: distribution, glycine max, insect pests, microflora, stink bugs

INTRODUCTION

One of the largest threats to crops is insects, either directly through their feeding or indirectly *via* pathogen transmission (Gullan and Cranston, 2014; Hirakuri and Lazzarotto, 2014; EMBRAPA, 2018). An example of important crop-damaging insects is the stink bugs (Hemiptera) and feed by piercing their stylets into the plant host tissue (Esquivel, 2019), which can cause direct injuries, inject toxic substances, or transmit pathogens, reducing host tissue health (Venzon et al., 1999; Gallo et al., 2002; Gullan and Cranston, 2014; Fujihara et al., 2016; Hosokawa et al., 2016). The attack on crops can be very damaging as the insects prefer feeding on the nutrient-rich developing seed. Stink bugs like to feed on legumes such as soybean (*Glycine max* L.), common bean (*Phaseolus vulgaris* L.), peas (*Pisum sativum* L.), and alfalfa (*Medicago sativa* L.), in addition to multiple alternative host plants that might be found within or near an agricultural field (Panizzi et al., 2012).

Throughout their lives, stink bugs can explore several environments and diverse food sources, which can modulate de gut microbiome. As seen in many insects, the bacterial community varies in population size, composition, location, and function within the gut, which can affect the health of the insects, as well as how likely they transmit pathogens to the plant host. Most of the microorganisms that enter into the gut lumen transiently pass through or are eliminated by the host immune system, but some of them remain longer and proliferate in the gut lumen. The wide range of insect associations with intestinal microorganisms is illustrated within Hemiptera (Engel and Moran, 2013; Yun et al., 2014; Lee et al., 2017).

The microbiota of an insect can be strictly hereditary, allowing nutrient supply to the host who lives in a restricted environment (Engel and Moran, 2013). In stink bugs, gut microbiome would be vertically transmitted, these gut symbionts are deposited on eggs every generation and co-evolve with the host and are important for basic host functions such as developmental programs, or essential adaptations. The intestinal microbiome also can be acquired through horizontally transmitted, which are better adapted for host colonization than environmental strains, and can facilitate host adaptation to new niches or environmental conditions. Beyond vertical and horizontal transmission, the environmental microbes can be acquired by feeding, which is advantageous in facilitating host adaptation to new conditions but are not adapted to the host (Kikuchi et al., 2007; Hosokawa et al., 2016; Shapira, 2016).

The absence of endosymbionts in the stink bug gut microbiome slows their growth and increases mortality and sterility since the gut microbiome plays an important role in insect health (Zchori-Fein and Bourtzis, 2011). Furthermore, prolonged interaction with a specific member of the gut microbiota can have an impact on a wide range of host physiologies (Engel and Moran, 2013; Lee et al., 2017).

Several cases of insects resistance by endosymbionts bacteria are reported in the literature, as the stink bug *Riptortus pedestris* that takes part in a mutualistic symbiosis with members of the *Burkholderia* genus, which are acquired from the soil during the second instar of its development stage. The insect-bacteria association was found in treated fields which showed enrichment for insecticide-degrading *Burkholderia* (Kikuchi et al., 2012). Similar to *R. pedestris,* the intestinal symbiotic bacteria *Citrobacter freundii* enhances the fruit flies' resistance to trichlorfon insecticide (Cheng et al., 2017).

In addition to containing beneficial microbes, the stink bug intestinal track may also contain pathogens that could be passed to their host plant (Mitchell, 2004). Conducting surveys of the insect intestinal microbiome can help identify possible pathogens, as well as detail what healthy stink bug digestive systems have in common. A study on bacteria associated with *Piezodorus guildinii* included isolating those that can or cannot be transmitted by feeding, *via* the sequencing of PCR products amplified from the V4 region of the 16S rRNA gene (Husseneder et al., 2017). The sequencing of the V4 region of the 16S rRNA gene from *P. guildinii* gut DNA identified 51 putative bacteria species, including several potential plant pathogens. Somewhat similar results were reported in bacteria transmitted by *N. viridula* (Ragsdale et al., 1979).

The unprecedented work presented here focuses on the gut microbiome of two stink bugs that are major pests of soybean, the red-banded stink bug (*Piezodorus guildinii*) collected in Brazil and the United States, and the neotropical brown stink bug (*Euschistus heros*) collected in Brazil. These pests normally colonize pre-flowering soybean and peak infestations are seen at the end of grain filling. They feed by inserting their stylets into the pods, directly reaching the seed (Foster and Daugherty, 1969; Turnipseed and Kogan, 1976; Corrêa-Ferreira et al., 1999; Kimura, 2007). According to Guedes et al. (2012), one stink bug per square meter can cause losses of 125 kg ha^{-1}, and losses can reach United States $3.06 billion (CEPEA – USP, 2020; USDA, 2020). Here, we hypothesize that (1) the intestinal microbiome of stink bugs in the United States and Brazil are different, (2) microbiome of stink bugs differ based on ecoregion, and (3) the two stink bug species differ in microbiome composition. To elucidate this hypothesis, we characterized and compared the intestinal microbiome of these two species collected from different locations in Brazil and the United States (Marchesi and Ravel, 2015), and investigated possible microbial families associated with the adaptation of these stink bugs to environments modified by agricultural management.

MATERIALS AND METHODS

Insect Collection and Dissection

In the field, insects were collected in soybean crops in Brazil and the United States and stored in falcon tubes with 70% ethanol at −20°C (**Supplementary Table 1**). Insects were dissected by removing heads and digestive tracts (including fore-, mid-, and hind guts with attached Malpighian tubes) using sterile forceps and storing them in 1.5 mL microfuge tubes at −20°C until used for DNA extraction.

DNA Extraction

For bacterial DNA extraction, a modified CTAB method was used as follows. The digestive tracts samples were placed in 1.5 mL microtubes containing 350 μL 2% CTAB extraction buffer (20 mM EDTA, 0.1 M Tris–HCl pH 8.0, 1.4 M NaCl, 2% CTAB, plus 0.4% b-mercaptoethanol added just before use), 10 μL proteinase k, and five stainless steel 2.8 mm beads. An additional 350 μL of 2% CTAB was added per tube and the solution incubated at 65°C for 60 min, and mixed by inversion every 15 min to macerate. Next, 600 μL chloroform-isoamylalcohol (24:1) was added and the tubes were gently mixed for 1 min, followed by centrifugation for 15 min at 10,000 rpm. Immediately after centrifuging, 600 μL of the supernatant of each tube was transferred to a fresh tube with 350 μL cold isopropanol (−20°C). Samples were mixed by inversion and held at −20°C for 60 min, followed by centrifugation at 14,000 rpm for 10 min. After centrifugation, it was possible to visualize the DNA at the bottom of each tube. The supernatants were removed, and the DNA pellets were washed with 1,000 μL of 70% ethanol, centrifuged at 14,000 rpm for 5 min. The ethanol was discarded and 500 μL 100% ethanol was added, centrifuged at 14,000 rpm for 10 min, the ethanol discarded, and the tubes were set to dry for at least 3 min in a sterile cabinet with the tubes inverted over a filter paper at room temperature. The DNA pellets were suspended in 50 μL TE buffer (10 mM Tris–HCl pH 7.6, 1 mM EDTA pH 7.6) plus 2 μL ribonuclease (RNAse 20 mg/mL), incubated at 37°C for 1 h, and stored at −20°C (Doyle and Doyle, 1987).

PCR Amplification and Sequencing

To fully characterize the microbiome at the *Bacteria* Domain, the PCR primer pair 515F (5′-GTGYCAGCMGCCGCGGTAA) and 806R (5′-GGACTACNVGGGTWTCTAAT) was used for amplifying the V4 region of the bacterial 16S rRNA gene, resulting in an amplicon length of 292 bp. PCR reactions were performed on a high-throughput Fluidigm PCR platform (Biomark HD) at the Roy J. Carver Biotechnology Center, the University of Illinois following the procedure outlined by Muturi et al. (2017). DNA samples were diluted to 2 ng/μL prior to amplification and processed with the Roche High Fidelity Fast Start Kit and 20X Access Array loading reagent according to Fluidigm protocols. To generate final PCR amplicons prepared for subsequent Illumina sequencing, two sets of primers were utilized simultaneously in one reaction. The first primer set had the Fluidigm-specific primers CS1 (5′-ACACTGACGACATGGTTCTACA) and CS2 (5′-TACGGTAGCAGAGACTTGGTCT) added to the 5′ end of all the ribosomal-specific primers mentioned above. The second primer set contained the same Fluidigm-specific primers attached to the Illumina i5 primer (5′-AATGATACGGCGACCACCGAGATCT) and barcoded i7 primer (5′-CAAGCAGAAGACGGCATACGAGAT-XXXXXXXXXX). All primers were synthesized by IDT Corp., (Coralville, IA, United States). The mastermix was aliquoted into 48 wells of a PCR plate. To each well, 1 μL DNA sample and 1 μL Fluidigm Illumina linkers with unique barcodes were added. On a separate plate, primer pairs were prepared and aliquoted. 20X primer solutions were prepared by adding 2 μL of each forward and reverse primer (50 μM), 5 μL of 20X Access Array Loading Reagent, and water to a final volume of 100 μl. The final primer concentration in the reactions was 50 nM each. Samples (4 μL each) were loaded in the sample inlets and 4 μL of primer loaded in primer inlets of a previously primed Fluidigm 48.48 Access Array IFC. The IFC was placed in the Juno microfluidic machine (Fluidigm Corp.) for the loading of all primer/sample combinations, amplification, and harvest. All samples were run on a Fragment Analyzer (Advanced Analytics, Ames, IA, United States), and amplicon regions and expected sizes were confirmed. Samples were then pooled in equal amounts according to product concentrations. The pooled products were size selected on a 2% agarose E-gel (Life Technologies) and extracted from the isolated gel slices with a Qiagen Gel Extraction kit (Qiagen) using a Qiacube robot. Cleaned, size-selected products were run on an Agilent Bioanalyzer to confirm appropriate profiles and determination of average sizes.

PCR amplicons for the 288 samples (286 insects and two water negative control) were sequenced on two MiSeq flow cells of 301 cycles from each end of the fragments using a MiSeq 600-cycle sequencing kit version 3 at the Roy J. Carver Biotechnology Center, University of Illinois. Read length was 300 nt. The resulting fastq files were demultiplexed with the bcl2fastq v2.17.1.14 Conversion Software (Illumina).

Sequence Data Processing

For 288 sequence libraries of the bacterial 16S rRNA gene V4 region, the software Cutadapt v1.12 was used to trim specific V4 primer sequences on both 5′ and 3′ ends of the reads (Martin, 2011). Since the primers for amplifying this region contained ambiguous positions, the option -match-read-wildcards was set to "on." Adapter-trimmed paired-end reads were imported into software Pear v0.9.5 (Zhang et al., 2014) that merged sequences into single fragments using overlapping regions to correct sequencing errors and yield higher quality. Reverse complement sequences of merged fragments were created with an in-house script.

The software IM-TORNADO v2.0.3.2 was used to do quality filtering and taxonomy assignment for processed sequences generated by the V4 regions above (Jeraldo et al., 2014). The Trimmomatic program was implemented by IM-TORNADO to do quality trimming, with a hard cutoff of a PHRED score of Q30 at ends of reads (LEADING: 3 and TRAILING: 3), a 4-base average score cutoff of Q15 (SLIDING WINDOW: 4:15) and a minimum read length cutoff of 75% of the original read length. For the taxonomy assignment, reads from the bacterial V4 region were aligned against the Ribosomal Database Project 10 (RDP10) (Cole et al., 2014) and the OTUs with 97% of similarity were clustered. Three BIOM files were generated separately by forward-reads, reverse-reads, and paired-reads for the region, containing the OTUs and counts corresponding to each OTU in each sample; forward-read BIOM files were used for downstream analyses due to the best result. Quality filtering was performed to remove low-quality OTUs and samples using QIIME-v1.9.1 (Caporaso et al., 2010).

The sufficiency of sequencing coverage was evaluated by alpha diversity and rarefaction analyses. Three metrics were used to estimate the alpha diversity of the microbiome among all the stink bug samples, i.e., the number of observed OTUs, Chao1, and Shannon.

The datasets generated for this study can be found in the NCBI Sequence Read Archive under the identification PRJNA764175 (*Euschistus heros*) and PRJNA764176 (*Piezodorus guildinii*).

Data Analysis

To compare the bacterial community structure and composition among the samples the data was rarefied and we used the Non-metric Multidimensional Scaling (NMDS) using Bray-Curtis as a distance metric, and the Phyloseq (McMurdie and Holmes, 2013) package in R. To test whether the samples harbored significantly different bacterial community compositions, permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was performed.

Diversity measurements were calculated using the Phyloseq package in R and included alpha diversity (Shannon index) and richness (number of observed OTUs, and Chao1 richness estimator). The Kruskal–Wallis non-parametric test was used to compare multiple groups and the pairwise Wilcoxon non-parametric test was used to compare samples grouped in ecoregions. Further, to determine the differential abundance of the top ten bacterial families between the ecoregions, we performed the Wilcoxon non-parametric parameter test for each bacterial family used.

Recent discoveries indicated that *E. heros* from the north and the south of Brazil were composed of two distinguished lineages. With the expansion of soybean in the Brazilian Cerrado, these two lineages could meet and produce hybrids more adapted to the climates and even to new hosts, such as cotton (Soares et al., 2018; Zucchi et al., 2019b). Thus, we performed analyses to investigate the relationships between the stink bug intestinal microbiomes and the proposed lineages of *E. heros*: north (Teresina/PI, Palmeirante/TO), south (Piracicaba/SP, Anhembi/SP, and Ponta Grossa/PR), and hybrids (the other localities).

RESULTS

Sequences and Samples Quality Control

For the 286 stink bug PCR samples of the bacterial 16S rRNA V4 sequence, the Illumina MiSeq sequencing produced 7.5 million reads in one lane, and the mean number of raw reads ranges from about 7,000–53,000 per sample for different locations. Reads filtering procedures including merging reads and trimming low-quality bases removed large percentages of reads from most locations, with 33–73% reads remaining, but the stringency of filtering left high-quality sequence data for subsequent analyses (**Supplementary Table 2**). After OTU identification, low-quality samples were filtered out for downstream analysis, leaving a total of 192 samples out of 286, with 75 samples from *E. heros* collected from 10 sites in Brazil and 117 samples from *P. guildinii* collected from three sites in Brazil and four sites in the United States (**Supplementary Table 2**).

The depth of sequencing was sufficient as shown by *P. guildinii* rarefaction plot that began to plateau by 1,000–1,500 reads per sample (**Supplementary Figure 1B**). Similarly, the *E. heros* rarefaction plot also began to plateau by 1,000–1,500 reads per sample for most locations, although the Teresina and Uberlândia locations might have benefited from additional sequencing as those plotlines still had a slightly positive slope at 1,500 (**Supplementary Figure 1A**). To accomplish this study we used a total of 2,141 OTUs.

Microbiome Characterization of *Euschistus heros*

Comparing the different collection sites of *E. heros* using the non-metric multidimensional scaling (NMDS) analysis, the bacterial communities present in the gut of these stink bugs clustered according to the following ecoregions of Brazil: tropical dry forests (TDF), tropical moist forests (TMF), and tropical savanna (TS) (**Figures 1A,B**, PERMANOVA $P = 0.0001$). Therefore, subsequent analyses were based on these ecoregions.

The species richness, based on the number of observed OTUs, is different among the ecoregions ($p = 0.015$). *E. heros* collected in tropical dry forests ($p = 0.0059$) and tropical savanna ($p = 0.034$) had significantly higher observed OTUs than those collected in tropical moist forests. This result was confirmed by the richness estimator Chao1 ($p = 0.0004$). *E. heros* collected in sites from tropical dry forests ($p = 3.8e\text{-}05$) and Tropical Savanna ($p = 0.012$) had more richness of species than *E. heros* collected in tropical moist forests. However, Shannon's diversity, an index that takes into account both abundance and evenness of the species present, did not show significant differences among ecoregions. A noticeable observation would be that the *E. heros* microbiome from tropical dry forest showed the highest richness of species (OTUs and Chao1) but the lowest Shannon diversity index, indicative of low evenness/equitability of species within this bacterial community (**Figure 1C**).

The most abundant bacterial families in the intestinal microbiome of *E. heros* were Acetobacteraceae, Bacillaceae, Bradyrhizobiaceae, Enterobacteriaceae, Enterococcaceae, Hyphomicrobiaceae, Methylobacteriaceae, Moraxellaceae, Polyangiaceae, and Sphingomonadaceae. The Acetobacteraceae and Bacillaceae were unique to insects sampled from the tropical moist forests (**Figure 1D**).

Comparing the bacterial families across ecoregions, stink bugs from the tropical moist forests had a significantly higher mean proportion of the Bradyrhizobiaceae than dry forests ($p = 0.012$) and savanna ($p = 0.016$) (**Supplementary Figure 2**). Although Enterobacteriaceae was the most abundant family in stink bugs from all three ecoregions (**Figure 1D**), it was more abundant in the tropical dry forests, followed by the tropical savanna and moist forests (**Supplementary Figure 2**). The Moraxellaceae only existed abundantly in stink bugs from tropical savanna (∼20%) whereas in the other two ecoregions they were not detected, or present at a very low level.

Comparing the different lineages, the NMDS analysis showed that the bacterial species present in our samples clustered significantly by stink bug lineages (**Figures 2A,B**, PERMANOVA

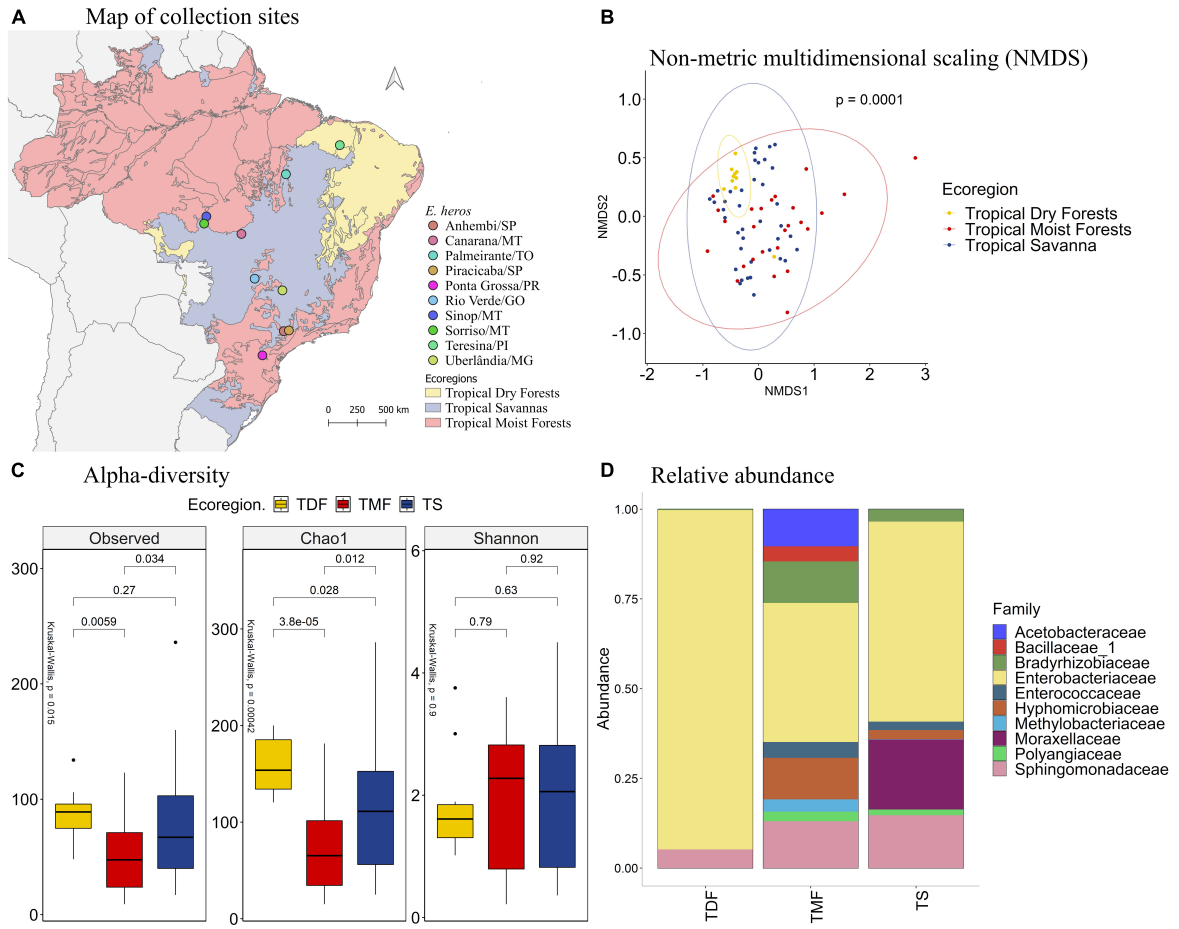

FIGURE 1 | (A) Map of collection sites of *E. heros*, classified by ecoregion in Brazil. **(B)** NMDS of Bray-Curtis similarity matrix among 75 samples from the intestinal microbiome of *E. heros*, for the different ecoregions from Brazil. The ellipses are showing clustering between ecoregions. Permutational multivariate analysis of variance (PERMANOVA) is indicated in the upper right. **(C)** Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different ecoregions. The non-parametric test (Kruskal–Wallis) is indicated in the upper left of each graph. Pairwise comparison (Wilcoxon) is indicated between Ecoregions. **(D)** Analysis of the ten most abundant bacteria families in the intestinal microbiome of *E. heros* collected in Brazil, classified by ecoregion.

$p = 0.0001$). The species richness, based on the observed OTU and Chao1 index, is higher in samples from the north lineage than the south lineage, and the hybrids have an intermediate species richness. The species diversity, based on the Shannon index, is bigger on the north and hybrid lineages than the south lineage of *E. heros* (**Figure 2C**).

Analyzing the relative abundance of the 10 most abundant bacterial families in each lineage, we can see that the hybrid lineages had more bacterial families than the north and south lineages and the proportion of these families present in the gut of the hybrids lineages are more evenly distributed than in pure lineages (**Figure 2D**). Furthermore, the family Bacillaceae is unique in the hybrids lineages and the Acetobacteraceae family is unique in the south lineage.

Comparing the bacteria families among lineages, the family Bradyrhizobiaceae was the most abundant in *E. heros* from hybrids lineages than either of the pure lineages. The family Hyphomicrobiacea was the most abundant in *E. heros* from hybrid lineages than north lineages. The families Moraxellaceae and Sphingomonadaceas were the most abundant in hybrid lineages than south lineages and the Enterobacteriaceae family was the most abundant in both pure lineages than hybrids (**Supplementary Figure 3**).

Microbiome Characterization of *Piezodorus guildinii*

The comparison of the different collection sites of *P. guildinii* by NMDS analysis (**Figure 3B**) revealed that the bacterial species present in the gut of these stink bugs also clustered (p of 0.0001) according to the ecoregions of Brazil and the United States: temperate broadleaf forests (TBF), temperate conifer forests (TCF), temperate grasslands (TG), and tropical savanna (TS) (**Figure 3A**). Therefore, subsequent analyses were based on these ecoregions.

The bacterial species diversity across ecoregions was significantly different based on the alpha diversity index, the number of OTUs, and Chao1 index ($p = 0.0072$ and $p = 0.0035$,

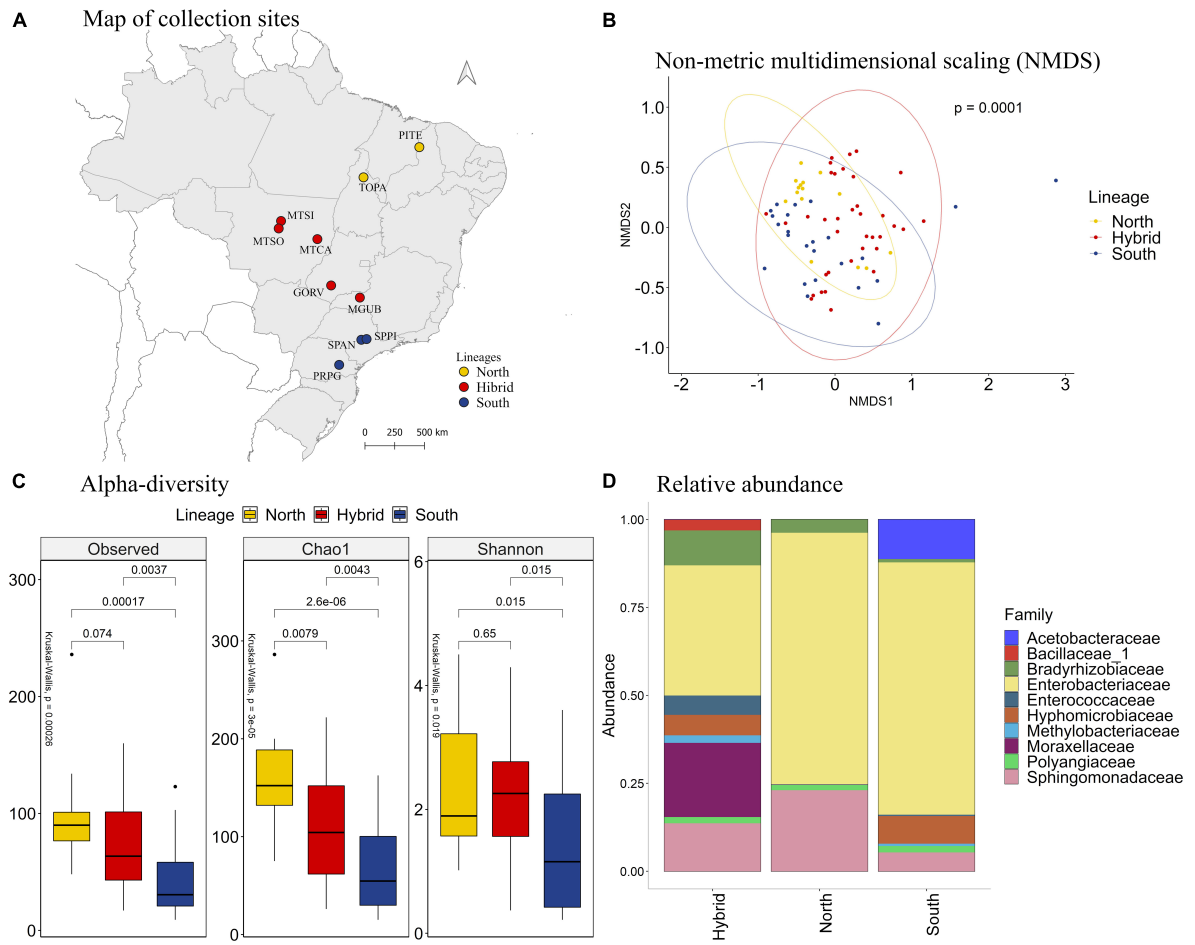

FIGURE 2 | (A) Map of collection sites of *E. heros*, classified by lineages in Brazil. **(B)** NMDS of Bray-Curtis similarity matrix among 75 samples from the intestinal microbiome of *E. heros*, for the different lineages. The ellipses are showing clustering between lineages. Permutational multivariate analysis of variance (PERMANOVA) is indicated in the upper right. **(C)** Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different lineages. The non-parametric test (Kruskal–Wallis) is indicated in the upper left of each graph. Pairwise comparison (Wilcoxon) is indicated between lineages. **(D)** Analysis of the ten most abundant bacteria families in the intestinal microbiome of *E. heros* collected in Brazil, classified by lineages.

respectively) (**Figure 3C**). On average, *P. guildinii* collected in temperate grasslands had more bacterial species than *P. guildinii* collected in the other ecoregions. Considering the richness estimator Chao1, the values were also significantly different (p = 0.0035). *P. guildinii* from tropical savanna were richer in species than insects from temperate broadleaf forests (p = 0.0025). Shannon's diversity showed a significant difference among ecoregions (p = 0.018). *P. guildinii* from temperate conifer forests (p = 0.015) and tropical savanna (p = 0.044) had higher equitability than temperate broadleaf forests, confirming the lowest species diversity from insects of temperate broadleaf forests at Shannon index (**Figure 3C**).

When focusing on the microbial abundance of *P. guildinni* at the family level, the most abundant bacterial families were Acetobacteraceae, Aurantimonadaceae, Bradyrhizobiaceae, Deinococcaceae, Enterobacteriaceae, Enterococcaceae, Moraxellaceae, Rhodocyclaceae, Sinobacteraceae, and Sphingomonadaceae. Among these families, the Sinobacteraceae family was found only in insects from temperate broadleaf forests and it existed at a low proportion. The Rhodocyclaceae family was found only in insects from this ecoregion and temperate broadleaf forests in low proportion (**Figure 3D**).

Comparing the bacterial families present in the intestine of *P. guildinii* insects shared by more than one ecoregions, the Acetobacteraceae family was significantly more abundant in insect guts from temperate broadleaf forests than insect guts from tropical savanna (p = 0.044). The Aurantimonadaceae was more abundant in *P. guildinii* from temperate conifer forests than temperate broadleaf forest and tropical savanna (p = 0.015 and 0.029). The Bradyrhidobiaceae family was more abundant in *P. guildinii* from temperate broadleaf forests than tropical savanna. Lastly, the Enterobacteriaceae family, as a major component in all four ecoregions, exhibited a lower proportion in temperate conifer forests than the other three ecoregions, but the difference did not reach statistical significance either, due to large variation (**Supplementary Figure 4**).

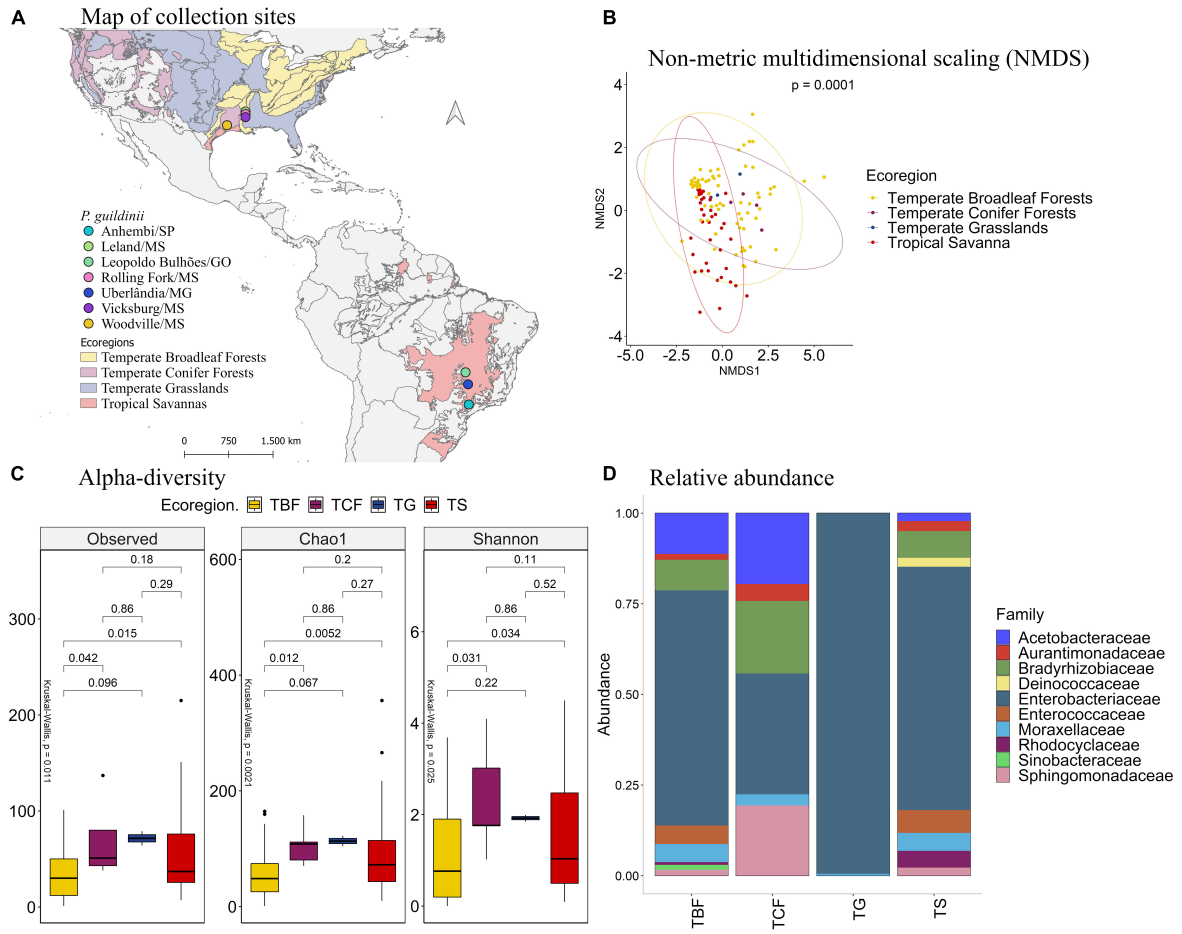

FIGURE 3 | (A) Mapping of collection sites of *P. guildinii*, classified by ecoregion in Brazil and the United States. **(B)** NMDS of Bray-Curtis similarity matrix among 117 samples from the intestinal microbiome of *P. guildinii*, for the different ecoregions from Brazil and United States. The ellipses are showing clustering between ecoregions. Permutational multivariate analysis of variance (PERMANOVA) is indicated in the upper right. **(C)** Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different ecoregion. The non-parametric test (Kruskal–Wallis) is indicated in the upper left of each graph. Pairwise comparison (Wilcoxon) is indicated between Ecoregions. **(D)** Analysis of the ten most abundant bacteria families in the intestinal microbiome of *P. guildinii* collected in Brazil and the United States, classified by ecoregion.

Since the species *P. guildinii* co-occurred in both Brazil and the United States, we further explored the association between its intestinal microbiome and the country of origin (**Figure 4A**). The NMDS analysis (**Figure 4B**) showed distinct groups of bacterial species in insects from Brazil and the United States ($p = 0.0107$). According to the number of observed OTU ($p = 0.015$) and the richness estimator Chao1 ($p = 0.0087$), *P. guildinii* sampled from Brazil were richer in intestinal bacterial species than insects from the United States (**Figure 4C**).

The Deinococcaceae family occurred only in stink bugs collected from Brazil, and the Sinobacteraceae occurred only in insects collected from the United States (**Figure 4D**). The families Acetobacteraceae, Bradyrhizobiaceae, and Moraxellaceae were significantly more abundant in *P. guildinii* from the United States than Brazil, and the family Rhodocyclaceae was more abundant from Brazilian insects (**Supplementary Figure 5**). Enterobacteriaceae, the most abundant bacterial family in both countries, constituted about 80% of the gut microbiome of both countries and showed no significant difference (**Figure 4D** and **Supplementary Figure 5**).

Analysis of *Euschistus heros* and *Piezodorus guildinii* Combined

We further combined the microbiome data of *E. heros* and *P. guildinii* and clustered samples by ecoregions (**Figure 5A**). With PERMANOVA, the bacterial species present in stink bugs displayed distinct compositions by different ecoregions with a significant p at 0.0001 (**Figure 5B**). As seen in the NMDS analysis (**Figure 5B**), samples from tropical dry forests grouped and were separated from samples from tropical moist forests and tropical savanna. Therefore, subsequent analyzes were based on ecoregions.

According to the number of observed OTUs, we found that stink bugs from tropical dry forests contained more abundant bacterial species than stink bugs from the tropical moist forests

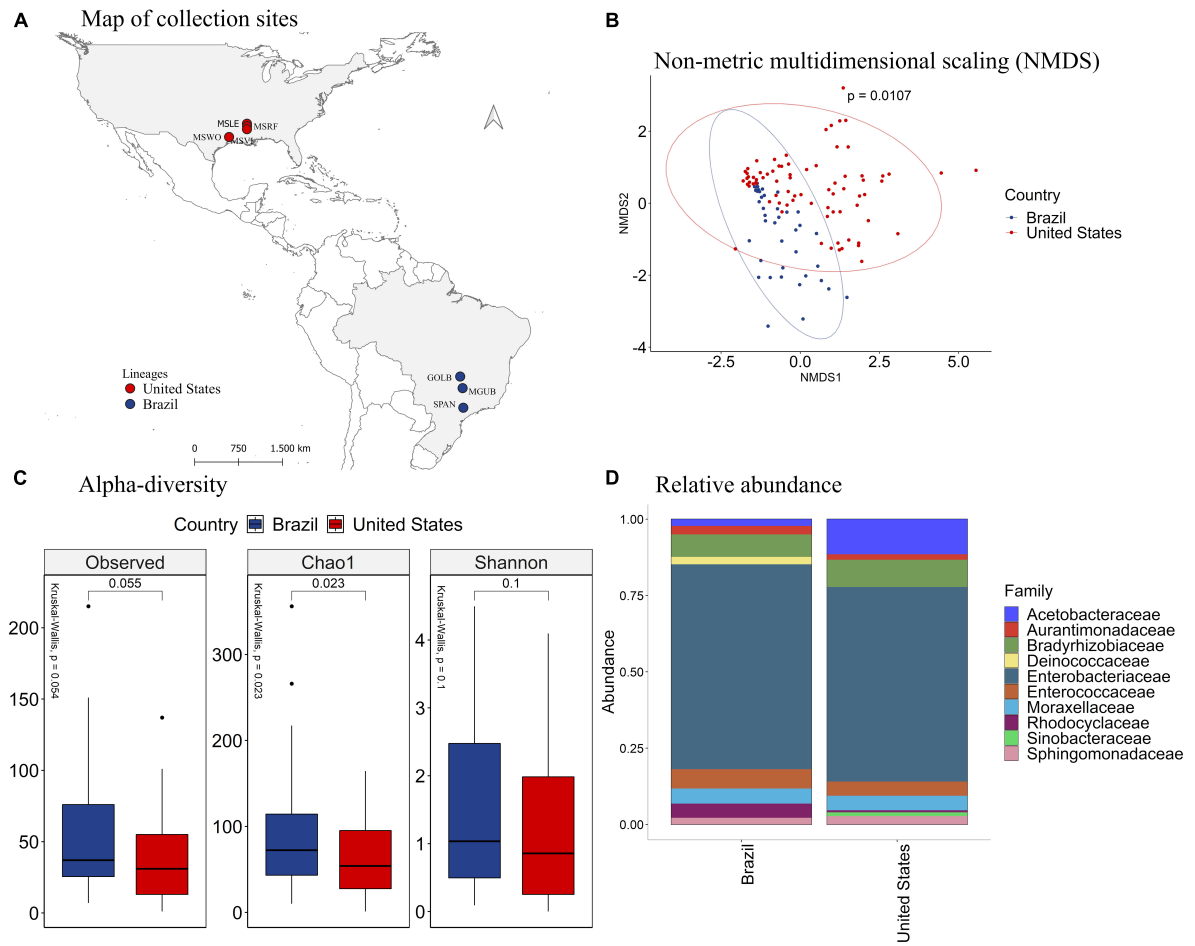

FIGURE 4 | (A) Mapping of collection sites of *P. guildinii*, classified by Country in Brazil and the United States **(B)** NMDS of Bray-Curtis similarity matrix among 117 samples from the intestinal microbiome of *P. guildinii* for the different countries, Brazil and United States. The ellipses are showing clustering between countries. Multivariate analysis of variance (PERMANOVA) is indicated in the upper right. **(C)** Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different countries. The non-parametric test (Kruskal–Wallis) is indicated in the upper left of each graph. Pairwise comparison (Wilcoxon) is indicated between countries. **(D)** Analysis of the ten most abundant bacteria families in the intestinal microbiome of *P. guildinii* collected in Brazil and the United States, classified by country.

(p = 0.0059) and tropical savanna (p = 0.041). The same occurs when the richness estimator Chao1 is compared (p = 3.8e-05 and p = 0.0042, respectively). Lastly, Shannon's index showed that the species diversity of all three ecoregions did not differ significantly from each other (**Figure 5C**).

The most abundant bacterial families present in the digestive tracts of *E. heros* and *P. guildinii* were Acetobacteraceae, Bacillaceae, Bradyrhizobiaceae, Deinococcaceae, Enterobacteriaceae, Enterococcaceae, Hyphomicrobiaceae, Moraxellaceae, Rhodocyclaceae, and Sphingomonadaceae, with Enterobacteriaceae comprising more than 80% of the bacterial community. The families Deinococcaceae and Moraxellaceae only existed in the tropical savanna group (**Figure 5D**). Comparing the bacterial families present in two or more ecoregions, the Bradyrhizobiaceae family was significantly more abundant in insects from tropical moist forests than tropical dry forests and tropical savanna. The Enterobacteriaceae family was most abundant in insects from tropical dry forests, followed by insects from tropical savanna (**Supplementary Figure 6**).

Comparing the species of stink bugs between *E. heros* and *P. guildinii* (**Figure 6A**), the NMDS analysis showed that these pentatomids had different bacterial species in their intestines, which can be separated into two groups with a p = 0.0001 (**Figure 6B**). Analyzing the diversity indexes, the number of observed OTUs showed that *E. heros* had more bacterial species than *P. guildinii*. Based on Shannon's index, the bacterial community in the gut of *E. heros* had significantly higher diversity than *P. guildinii*, which also suggests a relatively even distribution of species in the *E. heros* intestines (**Figure 6C**).

The composition of bacterial communities in the two stink bugs was similar at the family level (**Figure 6D**). Enterobacteriaceae was the major family and constitute more than 50% of the bacterial species in both stink bugs. The two

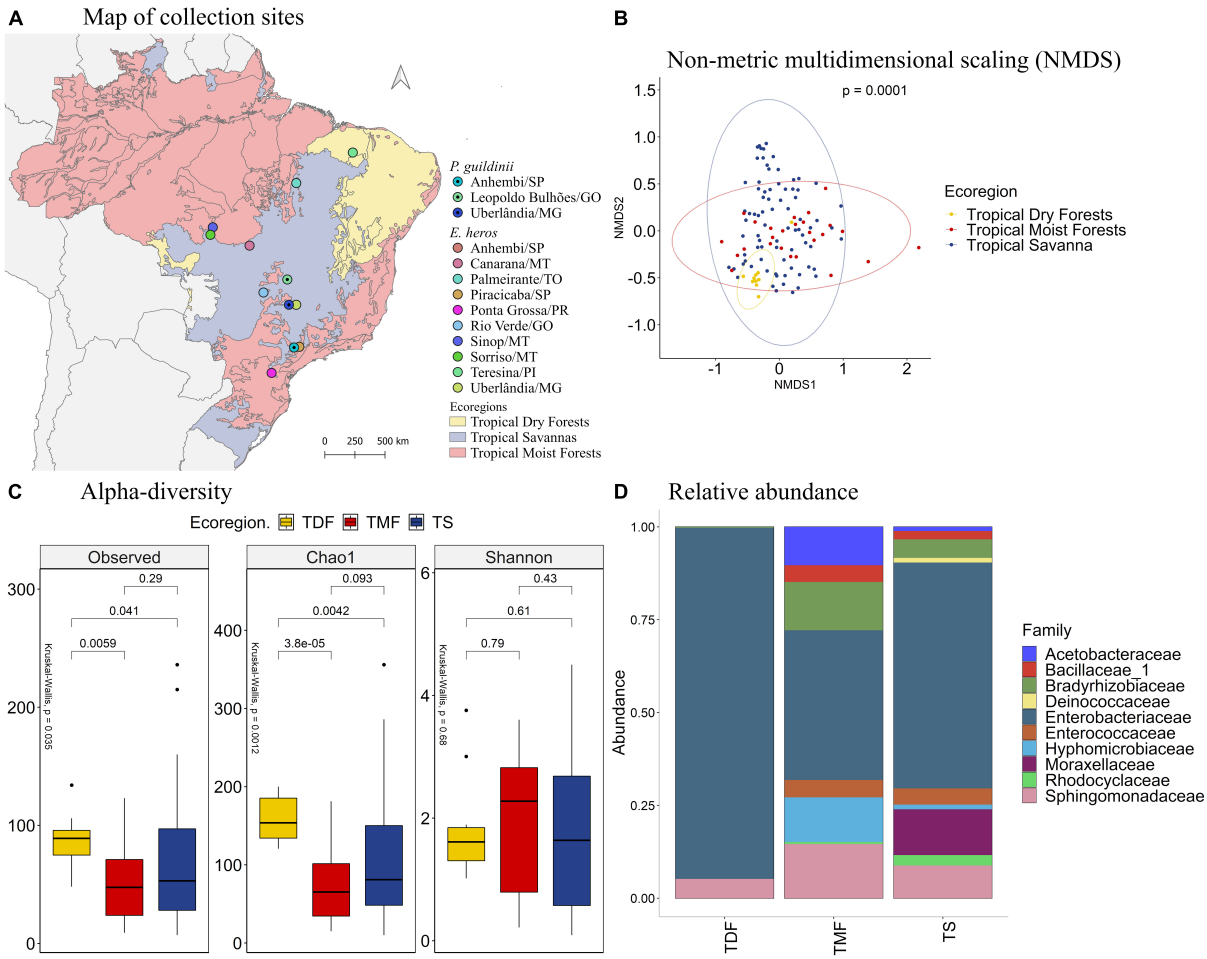

FIGURE 5 | (A) Mapping of collection sites of *E. heros* and *P. guildinii*, classified by ecoregion in Brazil. **(B)** NMDS of Bray-Curtis similarity matrix among 115 samples from the intestinal microbiome of *E. heros* and *P. guildinii*, for the different ecoregion from Brazil. The ellipses are showing clustering between ecoregion. Multivariate analysis of variance (PERMANOVA) is indicated in the upper right. **(C)** Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different ecoregion. The non-parametric test (Kruskal–Wallis) is indicated in the upper left of each graph. Pairwise comparison (Wilcoxon) is indicated between Ecoregions. **(D)** Analysis of the ten most abundant bacteria families in the intestinal microbiome of *E. heros* and *P. guildinii* collected in Brazil, classified by ecoregion.

other major bacterial families shared by the two stink bug species were Bradyrhizobiaceae and Moraxellaceae. However, differences can be observed as well. Hyphomicrobiaceae occurred only in *E. heros* and Deinococcaceae occurred only in *P. guildinii* (**Supplementary Figure 7**).

The phylogenetic relationship of the OTUs from the top 10 most abundant bacterial families was identified to understand their distribution (**Figure 7**). Analyzing the OTUs among *P. guildinii* from Brazil and the United States, 13 unique OTUs belonged only to Brazil and 19 unique OTUs belonged only to the United States. Most OTUs were not related among countries. The families Enterococcaceae (2 OTUs), Acetobacteraceae (1 OTU), Enterobacteriaceae (15 OTUs), Aurantimonadaceae (1 OTU), and Bradyrhizobiaceae (1 OTU) belonged to *P. guildinii* from both countries. This difference may be related to bacterial diversity among environments. On the other hand, comparing the OTU from the ten most abundant bacterial families among *E. heros* and *P. guildinii* from Brazil, 16 unique OTUs belong only to *E. heros* and seven unique OTUs belong only to *P. guildinii*. The families Enterococcaceae (2 OTUs), Bacillaceae (2 OTUs), Acetobacteraceae (1 OTU), Rhodocyclaceae (2 OTUs), Moraxellaceae (3 OTUs), Enterobacteriaceae (12 OTUs), Sphingomonadaceae (7 OTUs), and Bradyrhizobiaceae (3 OTUs) belonged to both stink bug species. This difference is most interesting, *E. heros* had twice the number of unique bacteria than *P. guildinii*, which may be favoring the adaptation of this species over the other since it is the most difficult to control today (**Figure 7**).

DISCUSSION

This study evaluated the insect intestinal microbiome, seeking to describe the structure and composition among different regions. Our results revealed a higher bacterial species richness in the stink bug intestinal tract from dry regions in *E. heros* and

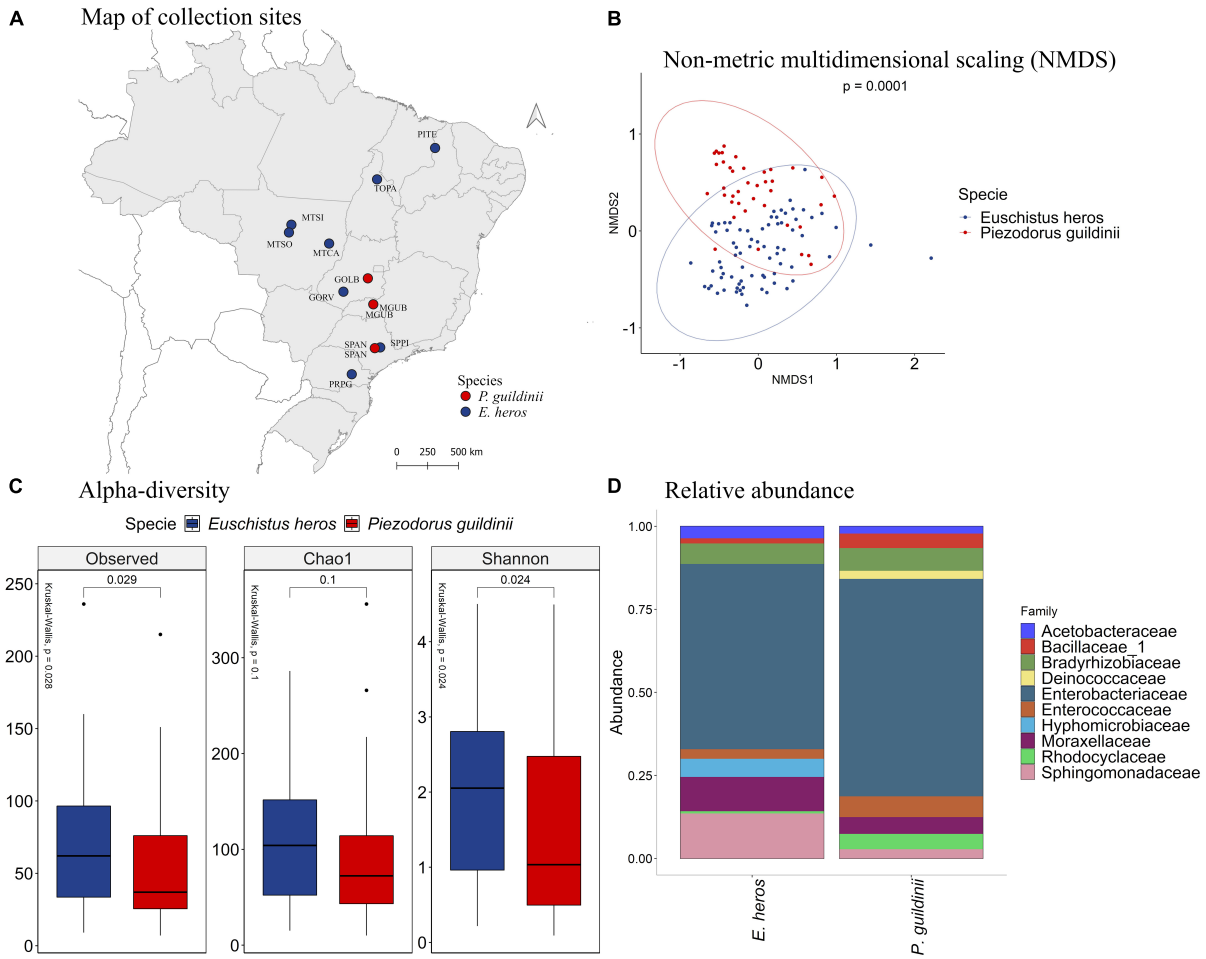

FIGURE 6 | (A) Mapping of collection sites of *E. heros* and *P. guildinii*, classified by species in Brazil. **(B)** NMDS of Bray-Curtis similarity matrix among 115 samples from the intestinal microbiome of *E. heros* and *P. guildinii* collected in Brazil. The ellipses are showing clustering between species. Multivariate analysis of variance (PERMANOVA) is indicated in the upper right. **(C)** Alpha diversity levels based on Observed OTU, Chao1 index and Shannon index for the different species. The non-parametric test (Kruskal–Wallis) is indicated in the upper left of each graph. Pairwise comparison (Wilcoxon) is indicated between Ecoregions. **(D)** Analysis of the ten most abundant bacteria families in the intestinal microbiome of *E. heros* and *P. guildinii* collected in Brazil, classified by species.

temperate grasslands in *P. guildinii*. Comparing the *P. guildinii* intestinal microbiome in both countries, we observed a higher bacterial species richness in stink bugs collected in Brazil than in the United States. On the other hand, comparing both species in Brazil, *E. heros* was richer in bacterial species than *P. guildinii*.

Microbiome Structure and Diversity

Differences in bacterial species richness found in *E. heros* and *P. guildinii* from different ecoregions, lineages, and species can be mainly explained by species behavioral, environmental, and genetic factors. *E. heros* and *P. guildinii* can feed on different food sources, including native and weed plants, this pattern allows individuals to frequently transfer to various areas throughout their development (Panizzi et al., 2012). According to Engel and Moran (2013), the microorganisms present in the insect gut can be acquired in each generation from the outside environment, such as soil. Studies that evaluated the diversity and biogeography of soil bacterial communities found that bacterial diversity varied across the ecosystem types, e.g., dry forest and dry grassland soils were more diverse than humid temperate forest and tropical forests (Fierer and Jackson, 2006). Our results showed that *E. heros* had higher species richness in tropical dry forests than other ecoregions, which might support the previous finding that the intestinal bacterial diversity could be affected by environmental factors.

Once acquired from the environment, the microorganism goes through a series of physicochemical conditions of gut compartments, such as pH, redox potential, and availability of particular substrates, that can be selective for particular species (Engel and Moran, 2013). In many insects, gut bacterial communities vary among individuals within a species and appear to consist largely of bacteria not specifically adapted to living in the guts of their host species (Engel and Moran, 2013). Corroborating our study, Priya et al. (2012) found highly variable gut communities in field-collected *Helicoverpa armigera* from different locations with the influence of the host plant.

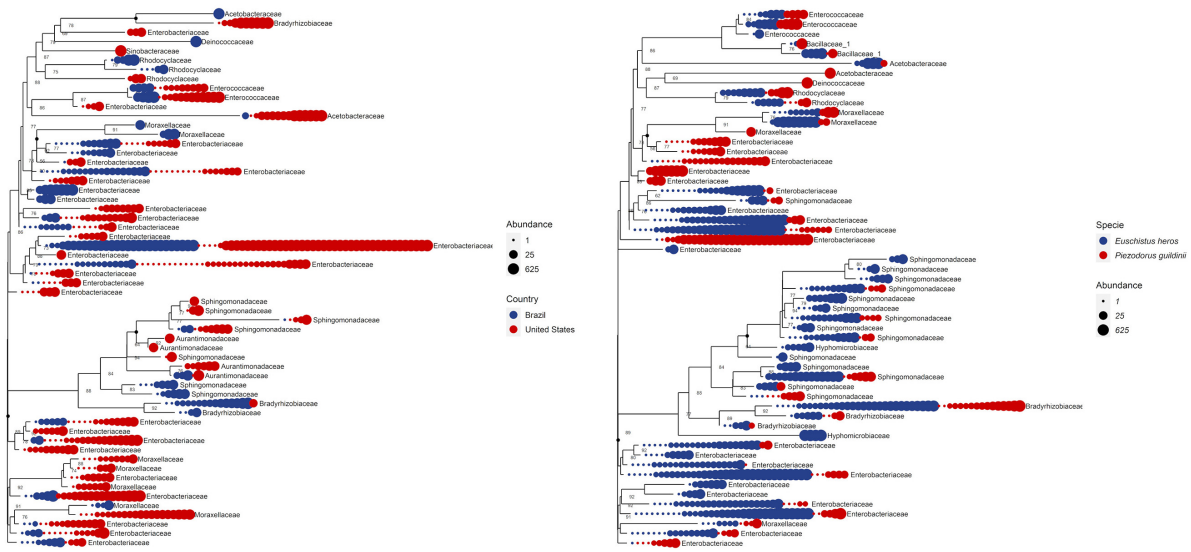

FIGURE 7 | (A) Phylogenetic tree comparing top 10 bacteria abundance at family level among countries. **(B)** Phylogenetic tree comparing top 10 bacteria abundance at family level among species.

The indiscriminate and large-scale use of phytosanitary products in soy production regions in Brazil may affect the soil microbiome and even insect's gut microbiota, which may be the main explanation for the grater diversity of bacteria in dry tropical forests.

(Spehar, 1995). A previous study on the gut microbiota composition of honey bees indicated that the neonicotinoids pesticides exposition may influence dominant honey bee gut bacteria (Jones et al., 2018). As stated earlier, stink bugs can feed on native plants and Tropical dry forests, due to the less intensive cultivation of soybeans than other ecoregions, may have a greater diversity of native plants to feeding.

Analyzing the species *E. heros* separated by northern and southern lineages, northern lineage individuals present higher species richness, followed by individuals of the hybrid and southern lineage. An explanation would be that individuals of the northern lineage (specialized in cotton) have genetic characteristics that when expressed allow the establishment of a greater number of bacterial species when compared to the southern lineage (specialized in soy) (Soares et al., 2018; Zucchi et al., 2019b). Thus, the hybrids of these two lineages present intermediate species richness.

It is important to note that the insect's lineage and the ecoregion to which it belongs can be equally important to the difference in the microbial community, as evidenced by the PERMANOVA.

We found that *P. guildinii* displayed a higher intestinal bacterial diversity from Brazil than the United States, which might be mainly explained by genetic and environmental factors. For genetic factors, Zucchi et al. (2019a) evidenced distinct *P. guildinii* lineages from Brazil and the United States using Genotype-By-Sequencing. These genetic differences in stink bug lineages might account for the differences in the intestinal microbiome changing the physicochemical conditions of the intestine and, consequently, the microbial composition of the different lineages. For environmental factors, the discrepancy of climatic conditions of each country could be relevant because the performance of seed-sucking heteropterans is affected by a variety of abiotic factors. The high temperature could increase food ingestion and, consequently, the ingestion of bacteria (Slansky-Júnior and Panizzi, 1987).

Tropical areas feature broad offerings of resources to bacteria species due to a higher diversity of environment and microclimates. Other pentatomids such as *E. heros*, enter in reproductive diapause under a photoperiod of 12 h or less, reducing feeding activity (Mourão and Panizzi, 2002). Collections in Brazil were made at latitudes below 25°, while in the United States they were made at latitudes above 30°. This difference means that in winter Brazil has more hours of light than the United States, possibly resulting in greater food activity of stink bugs in Brazil and thus affecting the diversity of the intestinal microbiota. The bacterial diversity found in the gut of insects in different ecoregions of the country and both countries is possibly related to the microbial diversity of the environment in which this insect is inserted.

Microbiome Composition

Bacterial abundance may be related to the stink bug environment and eating habits. Previous studies of insect gut symbionts have shown that diet change shifts relative abundances of bacteria in the insect gut rather quickly (Husseneder et al., 2009). Besides that, within the insect, symbiotic microorganisms have to face the host's innate immune system (Gross et al., 2009).

A vast majority of microbes constituting the gut microbiota are referred to as commensal microbes. By definition, commensals gain benefits from the host but are neither beneficial nor harmful to the host. However, commensals can be viewed as a pool of gut-interacting microbes with potential regulatory functions in host physiology (Lee et al., 2017).

The Acetobacteraceae family was found in both species of stink bugs. However, they were the majority in insect guts collected in the tropical moist forests and temperate broadleaf and mixed forests. Members of this family are symbionts of a wide variety of insects, providing nutrition to insects on limited sugar-rich diets (Crotti et al., 2010) and are commonly found in the guts of honey bee (Corby-Harris et al., 2014). These locations have less intensive soybean cultivation than other collection sites, allowing these insects to feed on less nutrient-rich crops. Possibly these bacteria are providing nutrients necessary for the survival of these insects in this environment.

The family Bacillaceae was found only in *E. heros* individuals collected in tropical moist forests. As discussed earlier, this ecoregion has less intensive cultivation of soybean, forcing this species to remove nutrients from the cultural remains between two cycles (Panizzi et al., 2012). So, the Bacillaceae family has a fundamental role in the survival of this stink bug, as many *Bacillus* isolates have the ability to break down cellulose, hemicellulose, and pectin. They are saprophytes that participate in the carbon, nitrogen, sulfur, and phosphorus cycles in natural habitats (Mandic-Mulec et al., 2015), enabling the acquisition of nutrients from these previously inaccessible cells. According to Husseneder et al. (2017), the genus *Bacillus* is the second major genus transmitted to the plant by *P. guildinii* feeding. Species belonging to this genus is known as a promoter of plant growth, biocontrol agent, and crop protective inhibit fungal and bacterial pathogens on seedlings. Bacteria belonging to the γ-Proteobacteria class, as Enterobacteriaceae and Moraxellaceae, supplement the nutritionally poor diet of phloem sap through the provision of essential amino acids (Douglas, 2006).

The Moraxellaceae family was found in both stink bug species. *E. heros* had this family only in individuals collected in the tropical savanna, known as the Cerrado. However, this family was found in *P. guildinii* in all ecoregions. This family can have an important function for these insects to acquire new hosts such as cotton. According to Zucchi et al. (2019b), hybrids lineages of *E. heros* are colonizing the cotton crop in Cerrado due to its improvement of the detoxification capability acquired of existing adaptations from recombination of genes from northern populations (specialized in cotton) with southern populations (specialized in soybean). It is interesting because this family was found in all sites where the use of agrochemicals is high. Moreover, some Acinetobacter species are supposedly aiding herbivores in overcoming plant defenses through detoxification of phytochemicals (Mason et al., 2014). Furthermore, some bacteria of the Acinetobacter genus can be transmitted by the red-banded stink bug feeding and have potentially beneficial effects on insects (Husseneder et al., 2017).

The Enterobacteriaceae was the most abundant family in both stink bugs. Corroborating our study, Enterobacteriaceae was found in *Spodoptera frugiperda* as the most abundant family (Rolim, 2014). Many species can exist as free-living in diverse ecological niches, both terrestrial and aquatic environments, and some are associated with animals, plants, or insects only (Teixeira and Merquior, 2014). Besides that, the genus *Erwinia* of the family Enterobacteriaceae contains mostly plant pathogens. *Erwinia persicina*, one of the major bacteria species found in the guts of red-banded stink bug, is a pathogen of legume plants (Husseneder et al., 2017). Enterobacteriaceae was found mostly in insect guts from the tropical dry forest and tropical savanna. Endosymbionts from this family may be performing some functions assisted in adapting these insects to new hosts.

The Rhodocyclaceae family was found mostly in stink bugs from Brazilian tropical savanna. Representatives of this family have been isolated from diverse environments have considerable potential for biodegradation of organic waste material and bioremediation of polluted environments (Rosenberg et al., 2014). So, members of this family may be associated with insecticides degradation and consequently better adaptations of these stink bugs.

CONCLUDING REMARKS

In this work, we assessed the microbiome of the red-banded stink bug *Piezodorus guildinii* collected in Brazil and the United States, as well as the neotropical brown stink bug *Euschistus heros* collected in Brazil. Our analysis showed that the diversity and abundance of intestinal bacteria vary among the two species, ecoregions, and countries within the same species. This variability allows individuals to perform different functions to better adapt to the environments in which they live. The capacity of endosymbionts bacteria for nutrient supply, cellulose breakdown, phytochemical detoxification, and degradation of organic compounds suggested by the analysis is directly related to adaptability, survival, and control difficulty in the field of these stink bugs. As Brazil and the United States are countries with great environmental diversity, we cannot generalize the control method. To find a more sustainable way to control stink bugs, we need to understand the relationship and dependence on intestinal bacteria, and this relationship must be better explored in future studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA764175 and https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA764176.

AUTHOR CONTRIBUTIONS

MM: conceptualization, data analysis, original draft writing, and review and editing. XW: investigation methodology, data analysis, and review and editing. WW and LM: data analysis and

review and editing. KA and JP: insect collection and review and editing. SC: funding, conceptualization, and review and editing. MZ: funding, conceptualization, insect collection, and review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.769965/full#supplementary-material

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Suppression of Midgut Microbiota Impact Pyrethroid Susceptibility in *Aedes aegypti*

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Aedes *aegypti* is a mosquito that transmits viral diseases such as dengue, chikungunya, Zika, and yellow fever. The insect's microbiota is recognized for regulating several biological processes, including digestion, metabolism, egg production, development, and immune response. However, the role of the bacteria involved in insecticide susceptibility has not been established. Therefore, the objective of this study was to characterize the resident microbiota in a field population of *A. aegypti* to evaluate its role associated with susceptibility to the insecticides permethrin and deltamethrin. Mosquitoes were fed 10% sucrose mixed with antibiotics and then exposed to insecticides using a diagnostic dose. DNA was extracted, and sequencing of bacterial 16S rRNA was carried out on Illumina® MiSeq™. Proteobacteria (92.4%) and Bacteroidetes (7.6%) were the phyla, which are most abundant in mosquitoes fed with sucrose 10%. After exposure to permethrin, the most abundant bacterial species were *Pantoea agglomerans* (38.4%) and *Pseudomonas azotoformans-fluorescens-synxantha* (14.2%). *Elizabethkingia meningoseptica* (38.4%) and *Ps. azotoformans-fluorescens-synxantha* (26.1%) were the most abundant after exposure to deltamethrin. Our results showed a decrease in mosquitoes' survival when exposed to permethrin, while no difference in survival when exposed to deltamethrin when the microbiota was modified. We found that the change in microbiota modifies the response of mosquitoes to permethrin. These results are essential for a better understanding of mosquito physiology in response to insecticides.

Keywords: microbiome, *Aedes aegypti*, insecticide susceptibility, deltamethrin, permethrin

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INTRODUCTION

According to the World Health Organization (WHO), vector diseases represent 17% of the estimated emerging infectious diseases in the world (WHO, 2020). This results from vector's geographical expansion, world transport, unplanned urbanization, and climate change (Jupatanakul et al., 2014). Viruses like dengue, zika, and chikungunya are the most important arboviruses, which are the leading cause of the emerging infectious diseases worldwide (Patterson et al., 2016). The main vectors of these diseases are mosquitoes belonging to the genus *Aedes* (Diptera: Culicidae) (Mayer et al., 2017). In Mexico, the control of these mosquitoes depends on integrated management with insecticides (CENAPRECE, 2018); however, as in many other countries, the effectiveness of vector control programs is compromised by the development of resistance to insecticides (Musso and Gubler, 2015). Moreover, there is concern about the environmental impact of this approach (Nkya et al., 2013; Smith et al., 2016). Recent studies have shown that insecticide resistance is a diverse and global process related to metabolic mechanisms (increased enzymatic activity) and resistance to the target site (Smith et al., 2016; Dada et al., 2019). These problems have created the need to develop alternative methods for controlling mosquito populations (Ramirez et al., 2012).

Previous research indicates that mosquitoes can host different bacterial communities that vary depending on sex, stage of development, and environmental factors (Cirimotich et al., 2010; Ramirez et al., 2012; Terenius et al., 2012; Coon et al., 2016). The functions of these microorganisms in the insects are correlated with nutrition, immune response, protection against pathogens, digestion, and development, among others (Gaio et al., 2011; Douglas, 2014). Multiple studies have addressed the bacterial community in *A. aegypti*, where species of the genera *Bacillus, Elizabethkingia, Enterococcus, Klebsiella, Pantoea, Serratia,* and *Sphingomonas* have been found (Terenius et al., 2012). Other studies have demonstrated the role of microbiota as a modulator of vector competence in *Anopheles* during infection with *Plasmodium* (Abdul-Ghani et al., 2012; Dennison et al., 2014). In *A. atropalpus,* the intestinal microbiota has been found to be associated with variations in survival, size, and egg production (Coon et al., 2016). In addition, new control strategies and environmentally friendly measures have focused on endosymbiotic bacteria such as *Wolbachia* to reduce or block the transmission of pathogens such as dengue, chikungunya, and Zika viruses (Bian et al., 2010; Bennett et al., 2019; Scolari et al., 2019). Recently, the microbiota of insects has been found to be involved in the detoxification processes (Aislabie and Lloyd-Jones, 1995; Itoh et al., 2018). Studies have shown that bacteria such as *Bacillus cereus, Enterobacter asburiae,* and *Pantoea agglomerans* can degrade acephate, an organophosphate in the diamondback moth *Plutella xylostella* (Kikuchi et al., 2012, van den Bosch and Welte, 2017). Knowing that the detoxification mechanism is a complex process that is regulated by metabolic (increase in enzyme activity) or genetic (mutations) systems, our study aimed to characterize the resident microbiota in *A. aegypti* mosquitoes and their potential role in their susceptibility to the insecticides: permethrin and deltamethrin.

MATERIALS AND METHODS

Biological Material

The *A. aegypti* mosquitoes were obtained locally from San Lorenzo, which is located in the municipality of Umán in the state of Yucatán, Mexico. Larvae were reared under controlled and aseptic conditions in the rearing room: 28 ± 1°C, 80 ± 5% RH, and a 12:12 h light: dark photoperiod. Larvae were fed with a mixture of tilapia feed meal and yeast (90:10 ratio, respectively). The adult colonies were incubated at 27 ± 2°C, 75 ± 5% RH, and under a 12:12 h light: dark regime in the insectarium of the Collaborative Unit for Entomologic Bioassays (UCBE) at the Universidad Autónoma de Yucatán (UADY).

Microbiota Suppression

Once the larval development cycle was completed, the pupae were transferred to beakers for the emergence of adults in rearing cages 25 cm × 25 cm × 25 cm (Bug Dorm-1) previously disinfected with 70% ethanol to maintain the aseptic conditions. At 24 h post-emergence, groups of 100–125 females were selected for each treatment. In the first treatment, the female mosquitoes were fed with sterilized 10% sucrose solution (denoted as group A). For the modification of the microbiome, female mosquitoes (100-125) were placed and fed with sterilized 10% sucrose solution with 10 U/ml for penicillin/streptomycin (Gibco Life Technologies) (denoted as group H: treated with penicillin/streptomycin). In other treatments, female mosquitoes (100-125) were placed and fed with sterilized 10% sucrose solution with 15 μg/ml gentamicin (Gibco Life Technologies) (denoted as I: treated with gentamicin). The antibiotic concentrations used in this study were previously reported by Dong et al. (2009). The treatments were done for 3 consecutive days and no mortality of mosquitoes was recorded during the assay. Microbiota of mosquitoes treated with antibiotics was analyzed to ensure changes in the microbiota.

Mosquito Susceptibility to Pyrethroid Insecticides

After modification to antibiotics, groups of mosquitoes were used for susceptibility bioassays. These assays were performed according to the CDC bottle method bioassay (Brogdon and McAllister, 1998), with 20 to 25 females with microbiota modification and without modification. Survival against each insecticide was evaluated in six groups of mosquitoes as follows: B: exposed to permethrin (control group), C: treated with penicillin/streptomycin permethrin exposed, D: treated with gentamicin permethrin exposed, E: exposed to deltamethrin (control group), F: treated with penicillin/streptomycin deltamethrin exposed and G: treated with gentamicin deltamethrin exposed). Groups were exposed to the diagnostic dose of permethrin of 15 μg/bottle and deltamethrin of 10 μg/bottle (>95% purity; Chemservice,

West Chester, PA, United States). Each bioassay consisted of four replicates per treatment and one control bottle without insecticide. The bioassays were carried out as described above (average temperature of 27 ± 2°C and relative humidity of 75 ± 5%, under aseptic conditions). The specimens that survived the diagnostic time (TD, 30 min for both pyrethroids) were transferred to recovery cups. The gut was then extracted according to the method described by Liu et al. (2018) with modifications. Briefly, before dissection, adult insects were sterilized with 96% ethanol for 3 min, then rinsed 3 times with sterile deionized water. The guts of 20–25 adult females were dissected on a plate containing 2 ml of sterile phosphate-buffered solution (10 mmol/L, pH 7.4; Ambion, Thermo Fisher Scientific, Madison, WI, United States) using a pair of flame-sterilized entomological forceps and with the aid of a stereomicroscope (Leica MZ16, 1.6X). The extracted guts were placed in 1.5-ml plastic Eppendorf® tubes containing a DNA shield and stored at –70°C for DNA extraction and sequencing.

Microbiome Analysis

Extraction of DNA

The samples used in this study were analyzed using the ZymoBIOMICS® service performed by Zymo Research (Irvine, CA, United States). DNA was extracted from samples collected and processed with the ZymoBIOMICS™ Service - Targeted Metagenomic Sequencing (Zymo Research, Irvine, CA, United States). The ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research, Irvine, CA, United States) was used to extract DNA.

Targeted Library Preparation

Bacterial 16S rRNA gene-targeted sequencing was performed using the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA, United States). Bacterial 16S primers amplified the V1-V2 or V3-V4 region of the 16S rRNA gene. These primers have been custom designed by Zymo Research to provide the best coverage of the 16S gene while maintaining high sensitivity. The sequencing library was prepared using a library preparation process in which PCR was performed in real-time PCR instruments to control cycles and prevent/limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned up with the Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA, United States) and then quantified with TapeStation® and Qubit®. The final library was sequenced on Illumina® MiSeq™ with a v3 reagent kit (600 cycles). The sequencing was performed with > 10% PhiX spike-in.

Bioinformatics Analysis

Raw reads were quality-filtered to remove low-quality data and chimeric sequences using Dada2 pipeline (Callahan et al., 2016). The resulting data were analyzed using the Quantitative Insights Into Microbial Ecology (Qiime v.1.9.1) pipeline. Reads were clustered into operational taxonomic units (OTUs) with representative sequences and calculated read counts (abundances) into OTUs at 97% (species-level) sequence identity to compare OTUs abundance between treatments. If an OTU contained fewer than 5 reads, they were omitted from downstream analyzes. Taxonomy assignment was performed using Uclust from Qiime v.1.9. with Greengenes database as reference with a 0.80 confidence threshold.[1] If applicable, a taxonomy that showed significant abundance between different groups was identified by LEfSe (Segata et al., 2011) using default settings. Alpha-diversity (Shannon diversity) and beta-diversity (Chao1) analyzes were performed with Qiime v.1.9.1 pipeline (Caporaso et al., 2010). We performed a principal coordinates analysis (PCoA) on Bray–Curtis distances using Qiime v.1.9 to compare the microbial community differences between different treatments. Permutational multivariate analysis of variance (PERMANOVA) was applied to Bray-Curtis similarity matrices to compute similarities between groups using PAST statistical software version 2.17. Other analyzes such as heatmaps and abundance plots were performed with internal scripts (see Supplementary 3 and 4).

Statistical Analysis

Kaplan–Meier survival and Mantel–Haenszel analysis tests were conducted using IBM SPSS Statistical Software version 20.0 to determine the statistically significant differences in the survival of mosquitoes treated with antibiotics and control after treatment with the insecticide. Differences were significant when $p \leq 0.05$.

RESULTS

Mosquito Susceptibility to Pyrethroid Insecticides After Treatment With Antibiotics

Here, we examined the impact on the mosquito microbiota after treatment with one concentration of two antibiotics including penicillin/streptomycin and gentamicin as described above. As expected, a decrease in the survival rate in mosquitoes exposed to permethrin (30.8%) and deltamethrin (55.8%) was observed compared to mosquitoes that were fed with 10% sucrose and no exposed to insecticides. In turn, a significant difference was observed ($p \leq 0.05$) between the different groups exposed to permethrin after treatment with penicillin/streptomycin (16%) or gentamicin (28%) compared to control group fed with 10% sucrose exposed to permethrin. Further, no difference was seen in the group of mosquitoes exposed to deltamethrin that were treated with penicillin/streptomycin (2%). Likewise, the group treated with gentamicin showed a decrease in survival (4%) (**Figure. 1**).

Aedes aegypti Microbial Communities

A total of 1,48,300 rawseqs were generated from adult *A. aegypti*. Proteobacteria (92.4%) and Bacteroidetes (7.6%) were the most abundant bacterial phyla found in adult mosquito populations fed with 10% sucrose. Briefly, the bacterial diversity was predominantly composed of the classes Gammaproteobacteria

[1] https://greengenes.secondgenome.com/

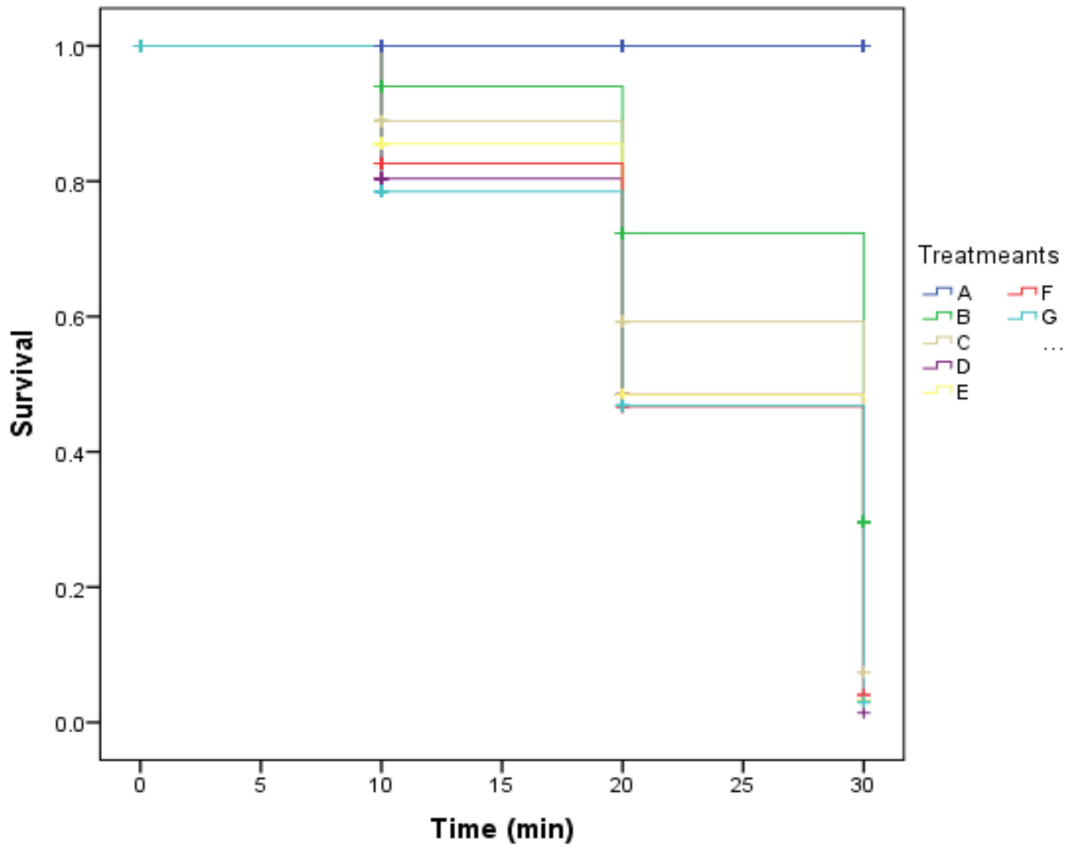

FIGURE 1 | Kaplan-Meier survival curve after exposure to the pyrethroid insecticides permethrin and deltamethrin in an adult population of *Aedes aegypti* using the CDC bottle bioassay ($p \leq 0.05$). A: fed with sucrose 10%, B: exposed to permethrin. (control group), C: treated with penicillin/streptomycin permethrin exposed, D: treated with gentamicin permethrin exposed, E: exposed to deltamethrin (control group), F: treated with penicillin/streptomycin deltamethrin exposed, and G: treated with gentamicin deltamethrin exposed.

(91.6%), Flavobacteria (7.6%), Betaproteobacteria (0.5%), and Alphaproteobacteria (0.3%). Additionally, Enterobacteriales (77.7%), Pseudomonales (13.6%), Flavobacteriales (7.6%), Burkholderiales (0.5%), and Aeromonadales (0.2%) were found as the main bacterial orders. Overall, Enterobacteriaceae (74.7%), Pseudomonadaceae (13.1%), and Flavobacteriaceae (7.6%) were the most abundant families. The genus *Pantoea* (43.0%) was the most abundant, whereas *P. agglomerans* was the most predominant species. Other species found were: *Serratia marcescens* (17.2%), *Serratia marcescens-nemathodiphila* (17.1%), *Pseudomonas azotoformans-fluorescens-synxantha* (12.9%), *Elizabethkingia meningoseptica* (5.6%) and *Chryseobacterium zeae* (2%) (**Figure 2**).

Microbiota *Aedes aegypti* Population After Treatment With Antibiotic

Here, the microbiota of *A. aegypti,* when the groups were treated with penicillin/streptomycin, was composed only of the phylum Proteobacteria (99.99%), while in the groups treated with gentamicin was represented by Proteobacteria (96.23%) and Firmicutes (3.77%). Also, the family that was found in gentamicin treatments was *Alcaligenacea* (95.2%), and the main genera were *Bordetella* (95.2%), *Staphylococcus* (3.7%), and *Serratia* (1.1%). In the group treated with penicillin/streptomycin, the main families were *Comammonodaceae* (86%) and *Pseudomonace*ae (13.2%). The genera *Delfia* (86.0%) and *Pseudomonas* (13.36%) were the most abundant in this group (**Supplementary Table 1**).

Changes in the Gut Microbiota Composition of *Aedes aegypti* Exposed to Insecticides

In PERMANOVA, pairwise comparisons indicated that the microbiota profiles of mosquitoes treated with antibiotics and later exposed to permethrin were statistically different from those exposed to deltamethrin ($p \leq 0.05$).

Exposure to Permethrin

The most abundant phyla in mosquitoes without modification of microbiota that were exposed to permethrin were Proteobacteria (74.1%) and Bacteroidetes (26%). In contrast, Proteobacteria (99.9%) was the dominant phylum in the mosquitoes treated with gentamicin that were exposed to permethrin. In the phylum Proteobacteria, the most representative class in all treatments was Gammaproteobacteria. Families found in mosquitoes that were

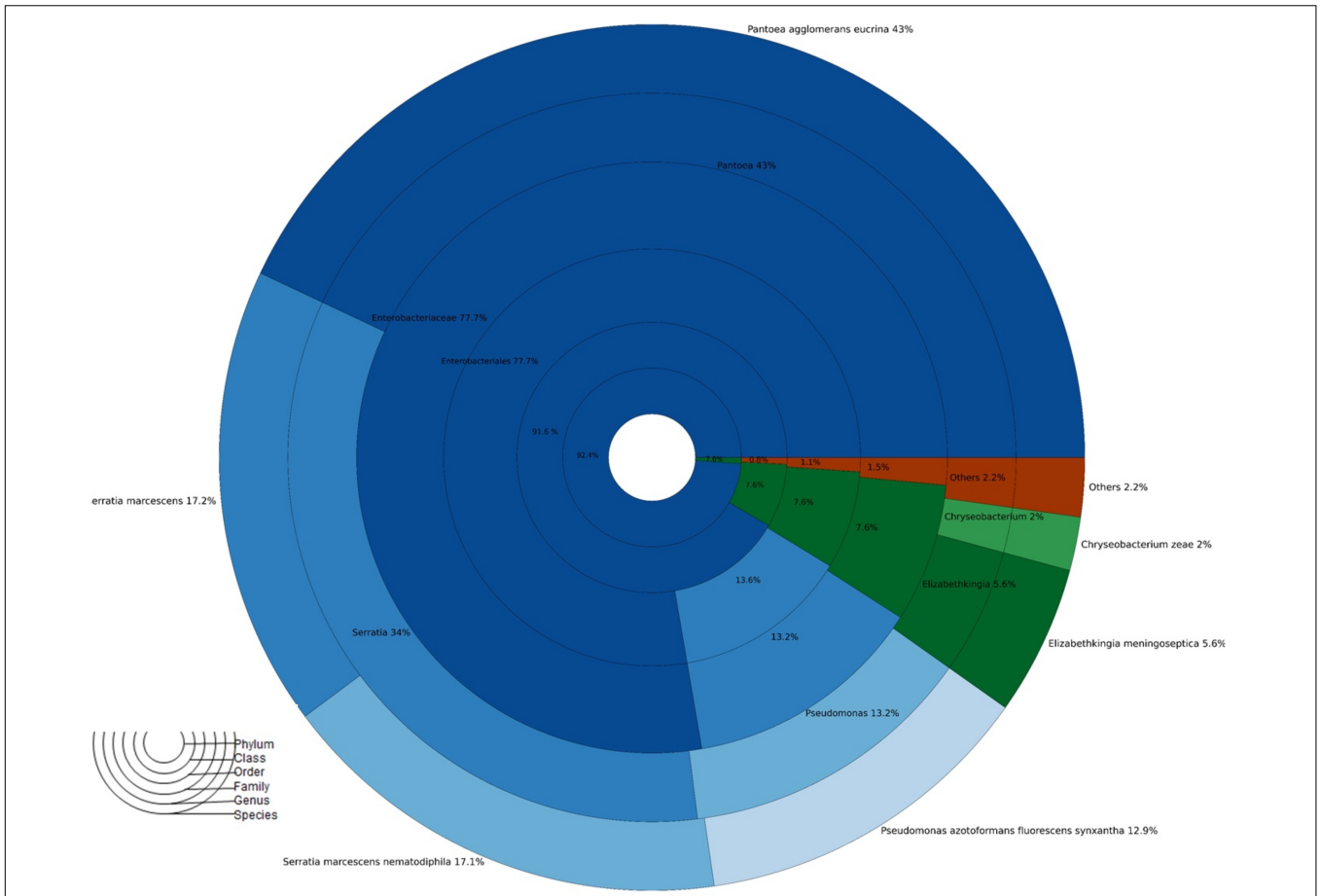

FIGURE 2 | Taxonomic abundance of *A. aegypti* mosquitoes fed 10% sucrose no exposed to insecticides.

only exposed to permethrin were Enterobacteriaceae (51.6%), Flavobacteriaceae (25.8%), Pseudomonadaceae (14.20%), and Moraxellaceae (7.6%). For the mosquitoes treated with penicillin/streptomycin and exposed to this insecticide, an increase in the family Pseudomonadaceae (93.9%) and decrease in the family Moraxellaceae (2.4%) was observed. Meanwhile, in mosquitoes treated with gentamicin and exposed to this insecticide, two families predominated Enterobacteriaceae (57.9%) and Aeromonadaceae (41.7%) (**Figure 3**).

Differences were found at the species level within the groups evaluated. In mosquitoes without modification of microbiota and exposed to permethrin, the most abundant species were *P. agglomerans* (38.4%), followed by *E. meningoseptica* (22.6%) (**Figure 4**). Diversity profiles showed that only bacterial communities exposed to permethrin were significant ($\alpha \leq 1$), according to their non-parametric estimator Chao1 value (21.52; 95% CI = 8.97–23.1), compared to the control group, which showed an estimator Chao1 value (18.70; 95% CI = 6.4–20). This indicated that exposure to the insecticide depended directly on the abundance of the bacterial species. PCoA plot with Bray-Curtis distance comparison showed a difference between the bacterial communities of the groups ($F = 6.38$, $p = 0.009$) with microbiota modification exposed to insecticide including groups treated with penicillin/streptomycin without exposition and groups only exposed to the insecticide (**Figure 5**). Even though the treatments with penicillin/streptomycin exposed to this insecticide were dominated by only one species, i.e., *P. azotoformans-fluorescens-synxantha* (93.7%), the estimator Chao1 was greater (30.1 95% CI = 1.4–34.1) when compared to the other treatments, so that the species found in greater proportion were *E. meningoseptica* (2.4%) and *A. baumannii-calcoaceticus* (6.1%), and about 16 species showed an abundance of < 1%. On the other hand, there was less diversity ($\alpha > 1$) in the mosquitoes exposed to insecticide-treated with gentamicin because of the predominance of three species: *Aeromonas dhakensis-hydrophila-taiwanensis* (41.7%), *Serratia marcescens* (28.8%) and *S. marcescens-nematodiphila* (29.1%).

Exposure to Deltamethrin

The bacterial communities in the mosquitoes exposed only to deltamethrin were dominated by the phyla Proteobacteria (54.8%) and Bacteroidetes (38.9%). In the same way, the mosquitoes treated with penicillin–streptomycin that were exposed to deltamethrin were represented by Proteobacteria (46.2%) and Bacteroidetes (53.8%). In groups treated with

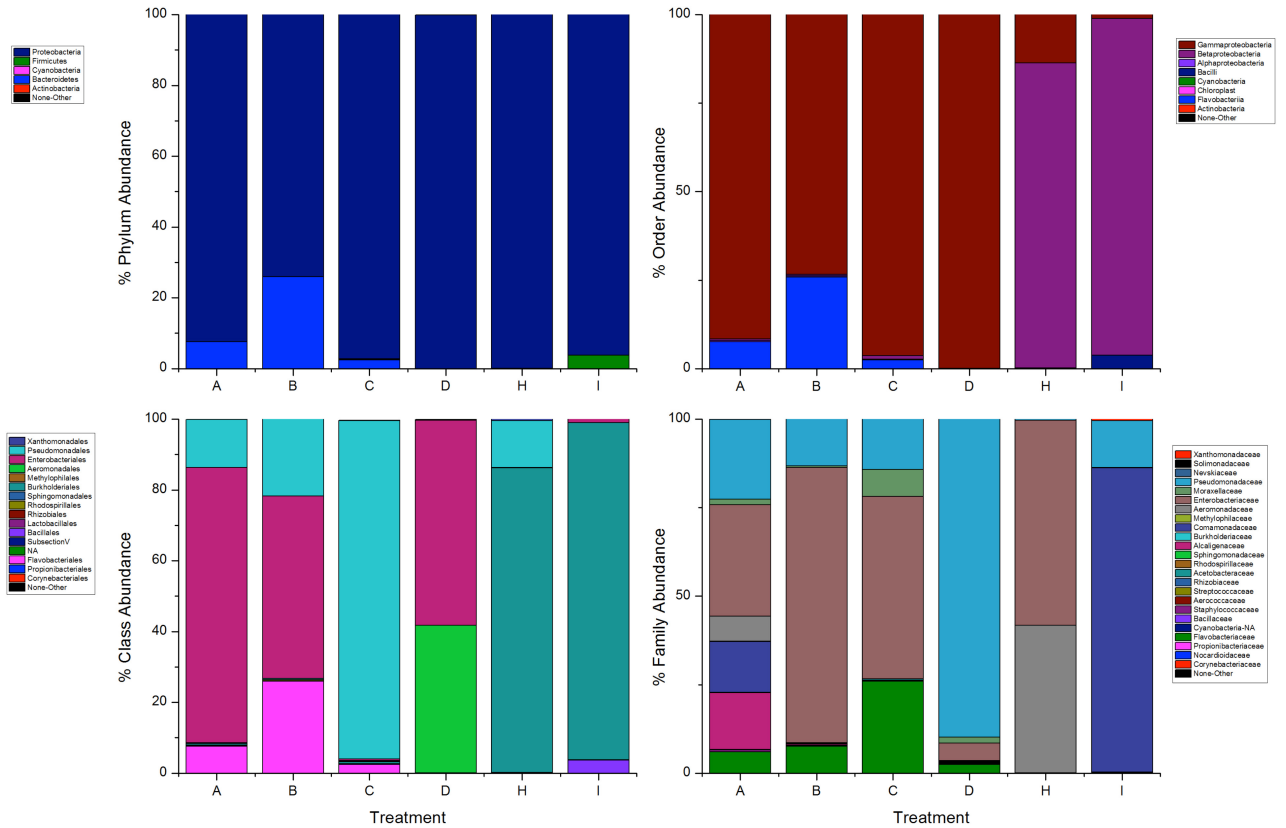

FIGURE 3 | Taxonomic variation between groups of *A. aegypti* exposed to permethrin. A: fed with sucrose 10%, B: exposed to permethrin (control group), C: treated with penicillin/streptomycin permethrin exposed, D: treated with gentamicin permethrin exposed, H: treated with penicillin/streptomycin (not exposed), I: treated with gentamicin (not exposed).

gentamicin and exposed to deltamethrin, the main phyla were Bacteroidetes (5.7%) and Firmicutes (25.3%) (**Figure 6**). *E. meningoseptica* was found in mosquitoes exposed to deltamethrin and the groups treated with penicillin-streptomycin exposed to this insecticide. The family Staphylococcaceae was only found in the mosquitoes with microbiota modified with penicillin-streptomycin, showing *Staphylococcus arlettae* (25.3%) as the most representative species. Species such as *P. agglomerans-eucrina, S. marcescens,* and *S. marcescens-nematodiphila* complex found in the control group as predominant in this group were found in a lower proportion (**Figure 7**). In the family Pseudomonadaceae, there was an increase in concentration after treatment with deltamethrin and exposure to penicillin-streptomycin. There was a significant difference in the diversity profiles ($\alpha \leq 1$) according to the non-parametric estimator Chao1, which was 74.10 (95% CI = 7.3–82.45), compared to the control group [Chao1 estimator: 18.70 (95% CI = 6.4–20)]. This indicated a substantial increase in the abundance of mosquitoes exposed to deltamethrin. While groups whose microbiota were modified showed less diversity ($\alpha > 1$) compared to the control group according to Chao1 estimator of 12.63 (95% CI = 1.4–34.1, penicillin-streptomycin) and 10.36 (95% CI = 4.5–11, gentamicin). PCoA plot with Bray–Curtis distance comparison showed differences between bacterial communities of the control group and group of mosquitoes fed with sucrose 10%.

DISCUSSION

Insects are a broad group of organisms with a large variety of lifestyles, which depends directly on microorganism associations (Douglas, 2014; Guégan et al., 2018). Adaptations of mosquitoes to selective pressure have made it necessary to determine new ways to approach their control. There are only a few studies regarding the effect of insecticides on mosquito microbiota, and thus in this study, we determined the microbiota of a field population of *A. aegypti* in southern Mexico with sequencing techniques and the characterization of key bacteria in response to two pyrethroid insecticides. Previous studies have been conducted to determine the microbiota of mosquitoes in different geographical regions, which have allowed the determination of the microbial diversity of these species of mosquitoes (Ramirez et al., 2012). In our study, the natural microbiota of the *A. aegypti* population (control) was mainly dominated by the phyla Proteobacteria and Bacteriodetes; these results are like those previously reported by Wang et al. (2011). Bacterial populations differ because of the habitat in which they interact as

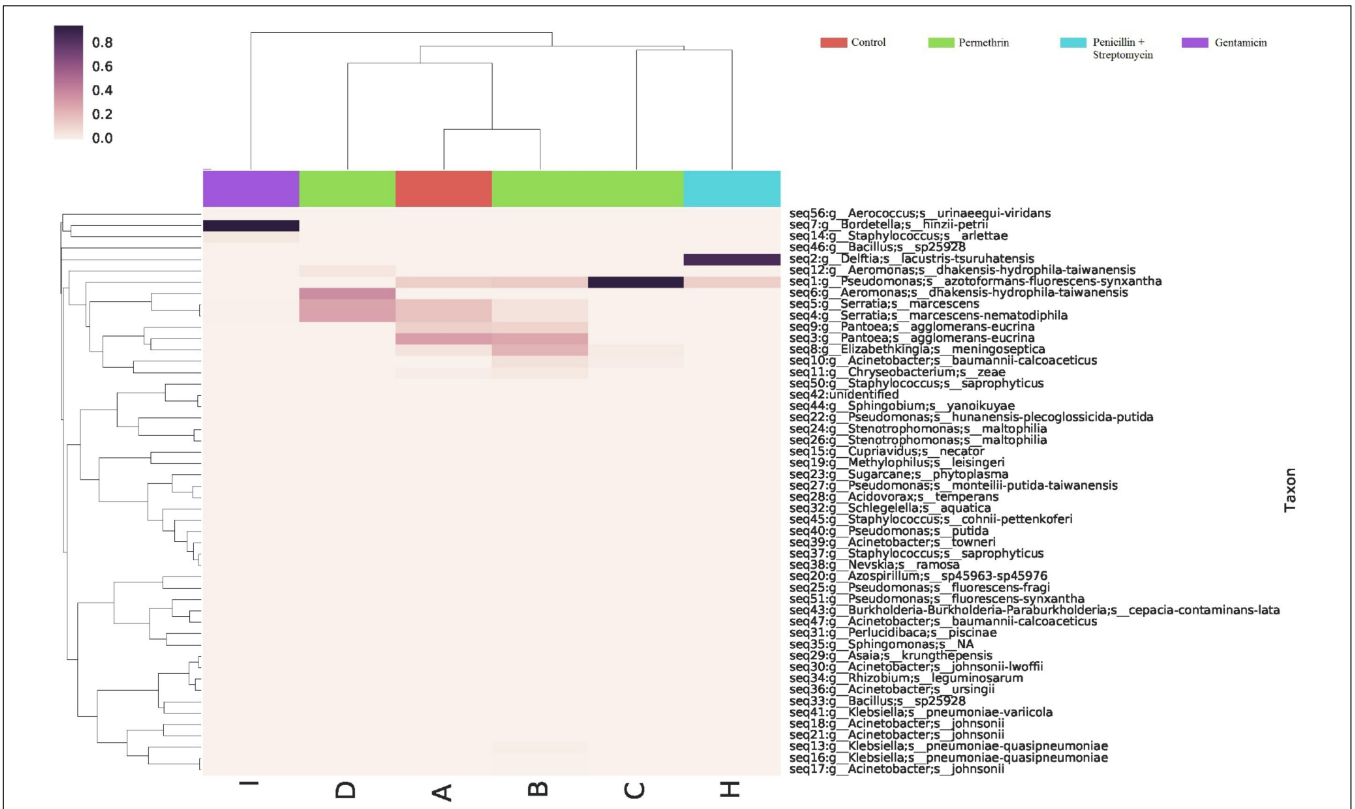

FIGURE 4 | Abundance heatmap of taxa with simple clustering (species) of bacterial communities of *A. aegypti* mosquitoes with permethrin exposure after microbiome suppression. A: fed with sucrose 10%, B: exposed to permethrin (control group), C: treated with penicillin/streptomycin permethrin exposed, D: treated with gentamicin permethrin exposed, H: treated with penicillin/streptomycin (not exposed), I: treated with gentamicin (not exposed).

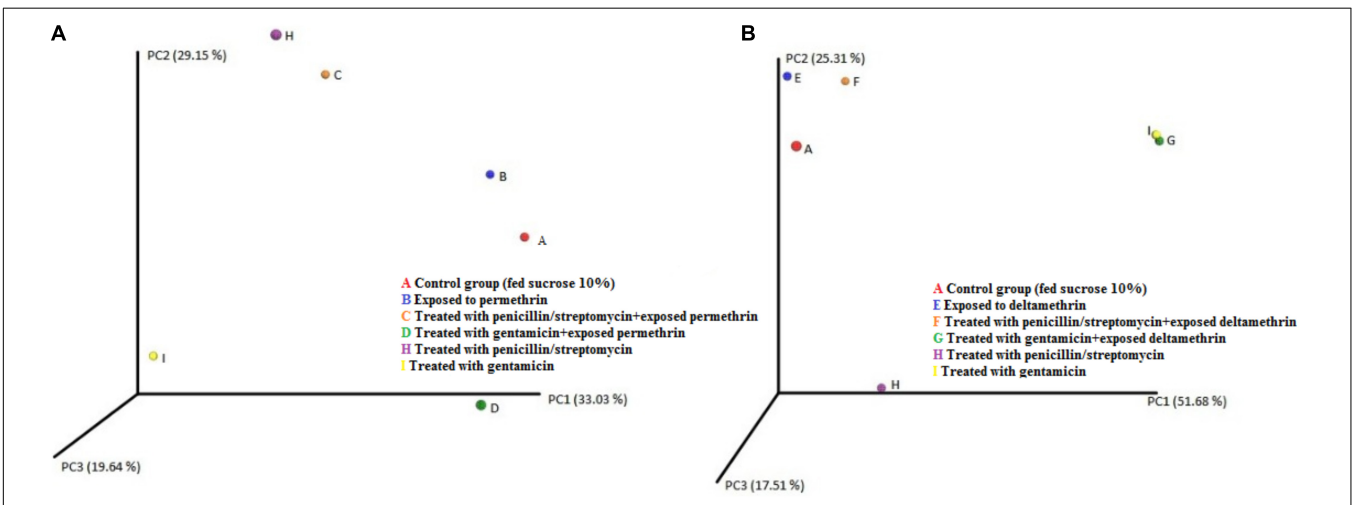

FIGURE 5 | PCoA plot using Bray–Curtis distance showing the distribution of bacterial community composition in *A. aegypti.* **(A)** permethrin exposure **(B)** deltamethrin exposure.

it depends on food, through plants for sugar sources, or through blood in female mosquitoes (Guégan et al., 2018). There are several studies about the microbiota in *A. aegypti* populations of endemic areas of Panama where the predominant phyla are Proteobacteria and Firmicutes (Ramirez et al., 2012).

The predominant species in our control group were *P. agglomerans-eucrina, S. marcescens-nematodiphila, P. azotoformans-fluorescens-synxantha,* and *E. meningoseptica.* Previously, these bacterial genera have been isolated from other species of mosquitoes such as *Anopheles gambiae* and

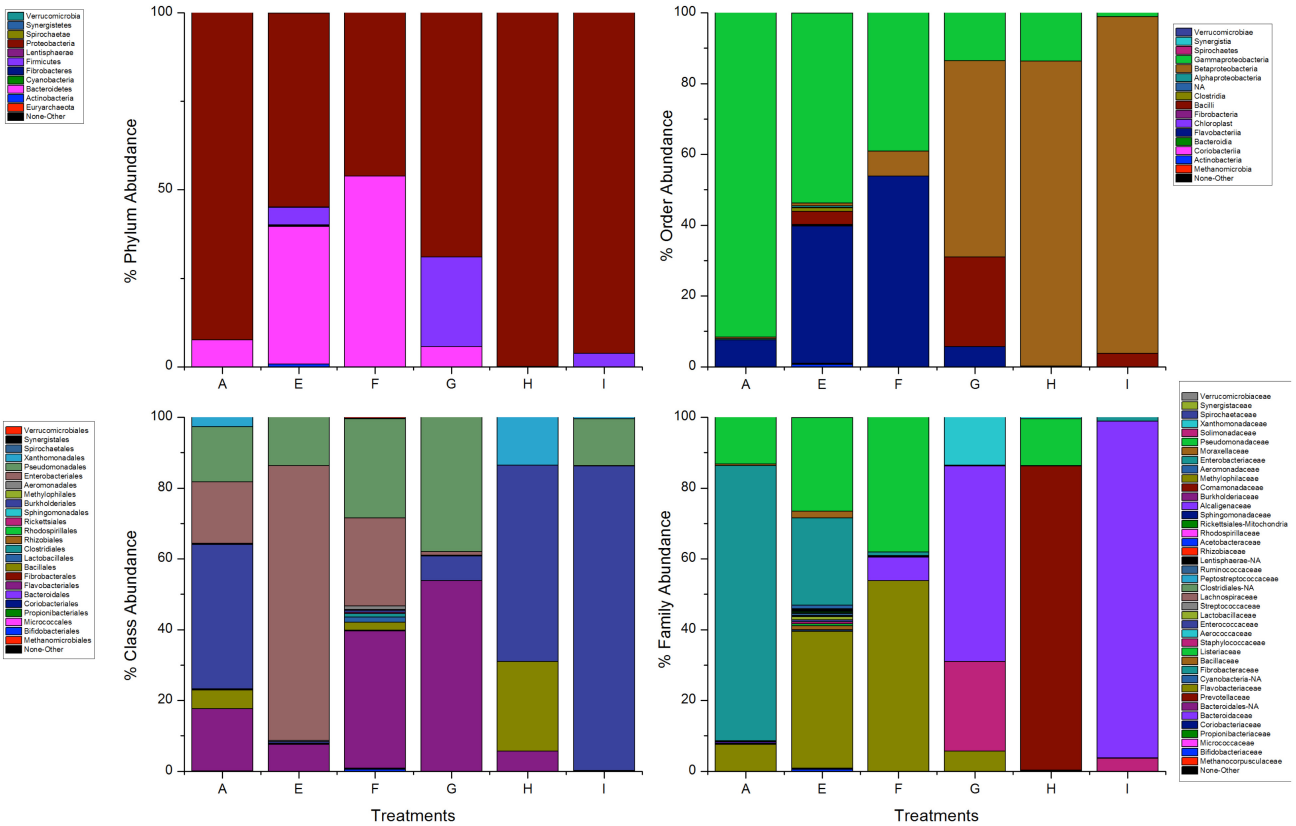

FIGURE 6 | Taxonomic variation between groups of *A. aegypti* exposed to deltamethrin. A: fed with sucrose 10% E: exposed to deltamethrin (control group), F: treated with penicillin/streptomycin deltamethrin exposed, G: treated with gentamicin deltamethrin exposed, H: treated with penicillin/streptomycin (not exposed), I: treated with gentamicin (not exposed).

A. funestus from Kenia and Mali (Straif et al., 1998). Similarly, in *A. aegypti* (Rockefeller), the genera *Bacillus, Elizabethkingia, Enterococcus, Klebsiella, Pantoea, Serratia,* and *Sphingomonas* (Gusmão et al., 2010; Terenius et al., 2012) have been isolated. In Brazil, the most abundant genera detected in *A. aegypti* populations have been *Pseudomonas, Acinetobacter,* and *Aeromonas* (David et al., 2016). In addition, in *Anopheles* species from Vietnam, *Acinetobacter spp.* has been found to be the main component of the gut microbiota (Ngo et al., 2016). In the same way, microbiota in *Anopheles* has included *Pseudomonas, Comamonas, Acinetobacter, Rhizobium, Burkholderia,* and members of the family Enterobacteriaceae (Tchioffo et al., 2016). The midgut microbiota of *Culex quinquefasciatus* has been shown to harbor bacterial species such as *Acinetobacter junii, Ac. calcoaceticus, Aeromonas culicicola, Bacillus thuringiensis, Microbacterium oxydans, P. agglomerans, P. aeruginosa, Staphylococcus epidermidis*, and *Stenotrophomonas maltophila* (Pidiyar et al., 2004).

Adult mosquitoes may contain a bacterial diversity concentrated mainly in aerobic and anaerobic gram-negative bacteria (Coon et al., 2014, 2016). Many of the related associations between bacterial communities of an insect are known to be mutualists where the host provides bacteria with nutrients and habitat, having selectivity due to the physicochemical conditions present in the host intestine (e.g., alkaline pH, redox potential, oxygen level below 5%, etc.) (Dada et al., 2019). It has been reported that many gram-negative bacteria are frequently found in the middle intestine of vector insects, influencing differential growth by contributing to the modulation of vector competition (Azambuja et al., 2005). In mosquitoes, the bacteria of the phylum Proteobacteria, especially the family Enterobacteriaceae, which are the main components of the middle gut microbiome, can tolerate the redox stress of blood digestion (Jupatanakul et al., 2014). In our study, the most representative species in *A. aegypti* population (control group) was *P. agglomerans*. It is common to find this bacterium as a symbiont in mosquitoes since it is a natural inhabitant of the environment (Segado Arenas et al., 2012; Mayer et al., 2017). In mosquitoes, this bacterium participates in nitrogen fixation, creating a nitrogen-rich environment ideal for the development of eggs and larvae (MacCollom et al., 2009).

The microbiota of our population under study was modified by reducing important enterobacteria species to observe their impact on insecticide response. The populations of *A. aegypti* treated with penicillin/streptomycin and then exposed to permethrin showed a decrease in survival of 16% compared to the mosquitoes not treated. The populations modified with gentamicin decreased by 28% compared to

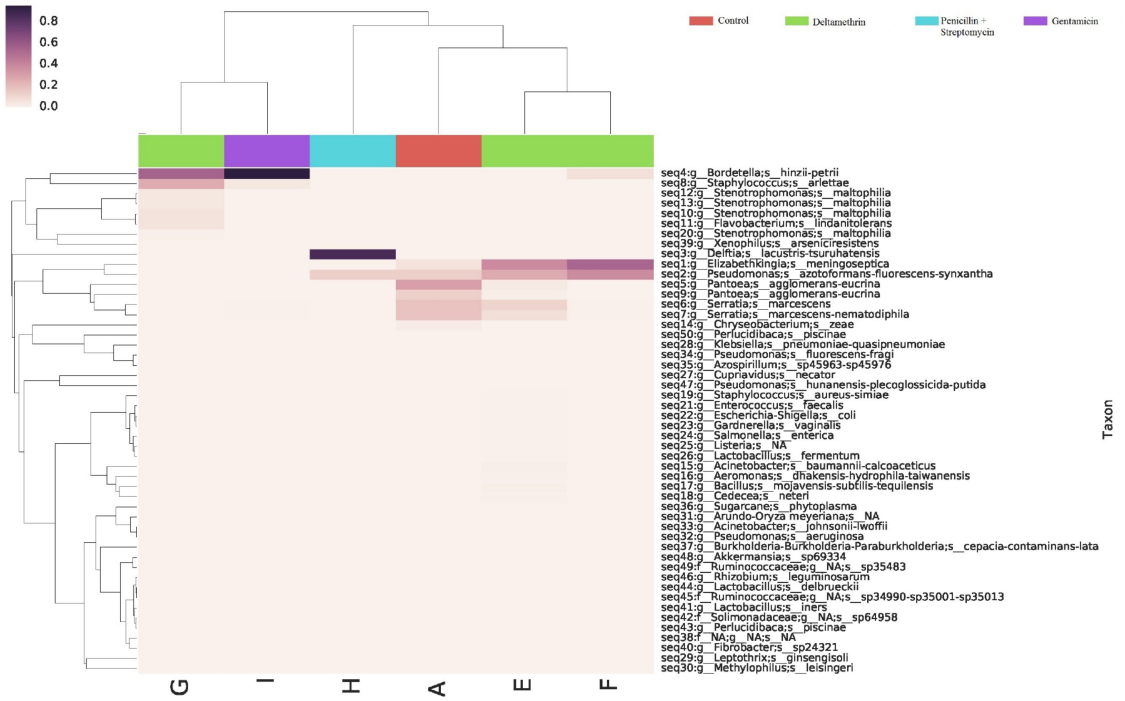

FIGURE 7 | Abundance heatmap of taxa with simple clustering (species) of bacterial communities of *A. aegypti* mosquitoes in deltamethrin exposure after microbiome suppression. A: fed with sucrose 10%, E: exposed to deltamethrin (control group), F: treated with penicillin/streptomycin deltamethrin exposed, G: treated with gentamicin deltamethrin exposed, H: treated with penicillin/streptomycin (not exposed), I: treated with gentamicin (not exposed).

those mosquitoes not treated but exposed to permethrin. The results showed that >90% of bacteria in these treatments were represented by the species *P. azotoformans-fluorescens-synxantha* with penicillin/streptomycin treatment, while in treatments with gentamicin, *A. dhakensis-hydrophila-taiwanensis* and *S. marcescens-nematodiphila* accounted for 29.10%. The strains of *Pseudomonas* are gram-negative, ubiquitous bacteria, characterized by primary nutritional needs and presence in various environments (soil, decaying organic material, atmospheric dust, vegetation, and water), with a wide range of plants and animals (Andreani et al., 2015). This genus has been found as part of the bacterial community in insects. Some research points to the toxic effectiveness of *P. aeruginosa* in the larvae of different mosquito species of the genera *Aedes, Anopheles, Culex,* and *Culiseta. In vivo* experiments with *P. fluoresences* cultures have found effectiveness against pupae of three mosquito species: *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* (Prabakaran et al., 2009). Similarly, Nabar and Lokegaonkar (2015) reported the larvicidal power of metabolites extracted from *Pseudomonas* spp. The species *P. fluorescens* has active metabolites against the larvae and pupae of *Cx quinquefasciatus* mosquitoes (Sadanandane et al., 2003; Brammacharry and Paily, 2012).

On the other hand, with the modification of the microbiota with gentamicin, we found the complex *A. dhakensis-hydrophila-taiwanensis.* This genus of gram-negative bacteria inhabits many environments (aquatic, fish, food, domesticated pets, invertebrate species, birds, ticks and insects, and natural soils) (Figueras et al., 2005; Janda and Abbott, 2010), and has been isolated from the intestines of *Cx. quinquefasciatus* (Pidiyar et al., 2002, 2004). Similarly, the species *A. hydrophila* has been reported to possess chitinolytic enzymes with activity against *Cx. quinquefasciatus* under laboratory conditions (Halder et al., 2012). As we observed in our results, when modifying the microbiota, enterobacteria were eliminated, indicating that these bacteria may play an important role in maintaining the response to insecticides. Certain bacterial communities such as *Pseudomonas* spp. and *Aeromonas spp*. were increased; it has been reported that some of these bacteria may produce metabolites that can compromise the physiological functioning of the mosquitoes when they are exposed to insecticides. Therefore, we could associate this type of response to this event resulting in decreased survival in our results.

The response with deltamethrin was different from what we observed in the permethrin group. There was a non-significant 2% decrease in mosquito survival with exposure to deltamethrin and penicillin/streptomycin treatment and a reduction of 4% with gentamicin compared to those whose microbiota was not modified. However, we could observe a change in the microbiota populations exposed to deltamethrin. The mosquitoes treated with penicillin/streptomycin exposed to this insecticide showed as predominant species *E. meningoseptica* and *P. azotoformans-fluorescens-synxantha*, while *B. hinzii-petrii* and *S. arletta* in those exposed to gentamicin. *E. meningoseptica* is a non-motile gram-negative bacillus, ubiquitous in nature (Kim et al., 2005). The members of the genus *Elizabethkingia* are found in wet

habitats, particularly in water supplies (Kämpfer et al., 2011). In mosquitoes, it has been reported that this bacterium can modulate anti-*Plasmodium* effects, thus prolonging the lifespan of the infected mosquito vector (Akhouayri et al., 2013). In addition, it has been consistently found in laboratory conditions to be an important part of the functionality of mosquitoes (Wang et al., 2011). *E. anophelisis* is known to be a dominant bacterium in the gut microbiota in the mosquito *A. gambiae* (Rani et al., 2009; Gusmão et al., 2010; Kämpfer et al., 2011; Boissière et al., 2012; Akhouayri et al., 2013), and *E. meningoseptica* has been found in *Cx. quinquefasciatus* (Terenius et al., 2012). Similarly, the genus *Elizabethkingia* was detected in 68% of mosquito populations collected in Cameroon (Chen et al., 2015). The species *Bordetella sp.,* which was the most abundant bacterium in the modification with gentamicin, is found in various aquatic environments and terrestrial environmental sources, also associated with plants (Shamim et al., 2019). The genus *Bordetella* has been isolated from adult mosquitoes of *An. stephensi* (Chavshin et al., 2012). Although these species are not common among microbes in mosquitoes, they can be found in small amounts in the intestinal ecosystem or tissues of mosquitoes, having an ecological connection to behavior and protection against adverse conditions (Kukutla et al., 2013).

Recent studies have demonstrated the role of symbiotic bacteria in detoxification processes (Itoh et al., 2018). This was observed in arthropods with phenothion applied in agricultural fields which were shown to be degraded by *Pseudomonas, Flavobacterium,* and *Burkholderia* (Kikuchi et al., 2012). *Bacillus cereus,* isolated from the guts of the *P. xylostella* moth, has been found to have a high degradation capability for the pesticide indoxacarb (Ramya et al., 2016a). Similarly, *Enterobacter asburiae* and *P. agglomerans* were found to degrade acephalate (Ramya et al., 2016b). Also, *Citrobacter* sp. was isolated from the intestine of *B. dorsalis*, capable of hydrolyzing trichlorphon (McFall-Ngai et al., 2013). Recently, a study determined the effects of pyrethroid insecticides in *An. albimanus* microbiota, finding differences with respect to mosquitoes not exposed to pyrethroids, determining *Klebsiella, Pantoea,* and *Asia* as key species in resistance to pyrethroids (Dada et al., 2019). In our study, we did not find species with evidence of metabolism of deltamethrin; however, we observed that with exposure to deltamethrin, there were different patterns of bacterial profiles compared to those seen with exposure to permethrin, which could influence the detoxification process. Our results clearly showed a different response to two insecticides from the same family, previously noted in Dada et al. (2019)'s study, where they obtained different microbiota patterns against pyrethroid insecticides. It is very important in future investigations to study the participation of key bacteria in the mechanisms of detoxification in mosquitoes since there is limited information.

CONCLUSION

The results of this study describe the microbiota of *A. aegypti*, which were dominated by species of *Proteobacteria* and *Bacteroidetes.* In nature, insects frequently face unfavorable environmental conditions that can alter symbiotic bacteria. In this study, we observed that changes in the microbiota affect the mosquito's response to exposure to pyrethroid insecticides. Symbiont microorganisms associated with mosquitoes have been shown to play a key role in response to insecticide exposure. The search for key symbionts related to response to insecticides will allow us to understand mosquito physiology and then create tools and new targets for controlling mosquito populations. The development of this research will generate basic knowledge of key bacteria of each population analyzed.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Center for Biotechnology Information (NCBI) BioProject database under accession numbers OK646404-OK646460 entitled "Uncultured Prokaryotic 16S rRNA/Microbiome Aedes aegypti Group 1" and OK648490-OK648570 entitled "Uncultured Prokaryotic 16SrRNA/Group 2".

ETHICS STATEMENT

Ethical review and approval were not required for the animal study because the manuscript presents research results on invertebrate animals (*Aedes aegypti*).

AUTHOR CONTRIBUTIONS

All authors contributed to the Replication of experiments, data analysis, and manuscript review.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.761459/full#supplementary-material

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