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Analysis of *Ficus hirta* Fig Endosymbionts Diversity and Species Composition

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Abstract: Endosymbionts living in plants and insects are pervasive. *Ficus* (Moraceae) has very special inflorescences (which we also call figs) enclosed like an urn, and such inflorescence is usually parasitized by fig wasps. *Ficus* breeds fig wasp larvae in its figs and adult fig wasps pollinate for *Ficus*, *Ficus* and its obligated pollinator formed fig-fig wasp mutualism. Previous studies have found that this confined environment in figs may have provided protection for fig wasps and that this has left some imprints on the genome of fig wasps during the coevolution history of figs and fig wasps. Research on the diversity of both bacteria and fungi in figs are fewer. Our study explored the diversity of endosymbionts in *Ficus hirta* figs. We utilized high-throughput sequencing and biological database to identify the specific microorganism in figs, then conducted microorganism communities' diversity analysis and function annotation analysis. As a result, we identified the dominant endosymbionts in figs, mainly some insect internal parasitic bacteria and fungi, plant pathogen, endophytes, and saprotroph. Then we also found bacteria in *Ficus hirta* figs were more diversified than fungi, and bacteria communities in female figs and functional male figs were different. These findings may give us more insight into the coevolution and interaction among endosymbiont, fig, and fig wasp.

Keywords: endosymbiont; microbial diversity; fig and fig wasp; *Ficus hirta*; interaction; alpha and beta diversity

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1. Introduction

Endosymbiotic microorganisms living in plants and insects are very common in nature, such as plant rhizospheric bacteria, insect gut microbes, and intracellular symbiotic bacteria. According to the previous research, almost all higher plants that have been studied can be found with endosymbionts, so plant endosymbionts prevail in nature [1,2]. For plants, endosymbionts can be found in leaves, petioles, fruits, thorns, seeds, bark branches, and roots, and has rich biodiversity [3,4]. Then it is also very common for insects to live with endosymbionts in their bodies [5]. Intracellular symbiotic bacteria can reside in many different organs and tissues of insects and even live inside the cell [6]. Endosymbionts usually have interactions with their hosts, they can not only cause diseases to the host, but also have many other effects on the host. For instance, plant endophyte can promote vegetative growth of host, increase biomass (yield), and increase the stress resistance of plants [7–9], while insect endosymbionts can improve nutrient metabolism of host insects, enhance host stress resistance and immunity [10–12], manipulate the reproduction of hosts, and influence insect growth, development, longevity, and evolution [13,14].

Ficus(Moraceae) is one of the largest genera of higher plants which has about 800 species worldwide [15]. *Ficus* spp. produce enclosed inflorescences (syconia) commonly called figs. About one-half of all *Ficus* spp. are dioecious, whereas the rest, including the Neotropical species, are monoecious, with both male and female florets occurring in an individual syconium. In dioecious figs, figs of functional male trees grow male and short-styled female

florets which can be parasitized by fig wasps, whereas figs of female trees produce long-styled female florets that can only be pollinated to be seeds and cannot be parasitized by fig wasp due to their short ovipositors. Fig wasp larvae parasitized in figs, and figs provide nutrition to breed up the larvae, after the larvae grow into adults, fig wasps come out of the figs and pollinate new adaptive figs. By such interaction, fig and fig wasp have formed a strong obligate mutualism system which is a coevolutionary mode system with a long history and close relationship among animals and plants [16,17]. This mutualism system is a defining model for plant-insect coevolution and interaction researches and contributes greatly to ecosystem functioning, biodiversity, and agriculture [18,19]. The development of next-generation sequencing has led to a surge in effort to characterize the microbiomes of various vertebrate hosts, a necessary first step to determine the functional role these communities play in host evolution or ecology [20]. However, utilizing the next-generation sequencing to explore the endosymbionts in figs is rare in the past. Thus, to find out the influence fig internal endosymbionts having on fig and fig wasp coevolution or ecology, it is necessary to explore the fig inner microorganisms by next-generation sequencing as well. Fig has an enclosed inner environment, the past research of fig wasp genome assembly and annotation indicated that antibacterial and immune gene families and their metabolic pathways were contracting, this might demonstrate that airtight environment had a certain protective effect on fig wasp [21]. However, some researches have shown that although the fig was closed, there were still many microbes inside [4,22]. So, whether or not these microorganisms in figs have an influence on fig wasps, and if they do, what are the effects? To answer this question, first we should explore which microorganisms exist inside figs.

So, we chose *Ficus hirta*, a southeast Asia widely distributed dioecious fig, to record the fig endosymbionts using high throughput sequencing and various biological databases. The following questions were addressed: How about the diversity and functions of microbial communities including bacteria and fungi in figs of *F. hirta*? Is there any difference of them between female and functional male figs?

2. Materials and Methods

2.1. Microorganism Sampling

Our studies were carried out at the South China Botanical Garden (SCBG), in Guangdong Province (112°57′ E to 114°3′ E, 22°26′ N to 23°56′ N) where there is a subtropical maritime climate.

In total, we collected two samples of female figs and functional male figs, and each sample had three replicates: Fhf_1, Fhf_2, Fhf_3 for female figs, and Fhm_1, Fhm_2, Fhm_3 for functional male figs (Figure 1). All figs were picked at inter-floral phase (A period after fig wasp pollinating for figs, then female florets in female fig bear seeds while functional male florets in male fig grow into galls and fig wasp larvae develop into adults in the galls), and the picking time was at 12:00 to 3:00 p.m. Afterward, these figs were asepticized and stored in liquid nitrogen at −80 °C.

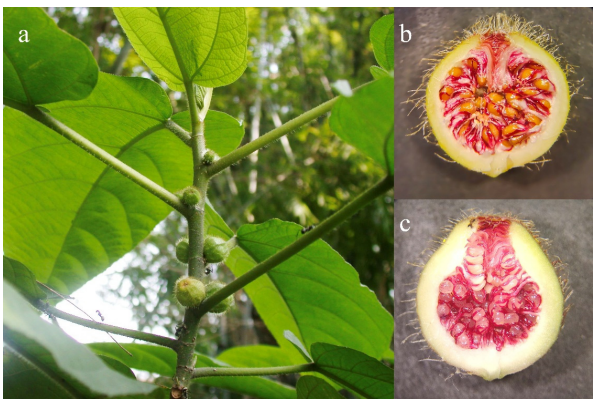

Figure 1. (**a**). *Ficus hirta* tree with its figs grow on the branch. (**b**). Female fig of *Ficus hirta*. (**c**). Functional male fig of *Ficus hirta*.

2.2. Microorganism DNA Extraction and High-Throughput Sequencing

Microbial community genomic DNA was extracted from six samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, NC, USA). The extracted DNA of bacterial and fungal were amplified with primer pairs 799F (5′-AACMGGATTAGATACCCKG-3′)/1193R (5′-ACGTCATCCCCACCTTCC-3′) and ITS1F(5′-CTTGGTCATTTAGAGGAAGTAA-3′)/ITS2R(5′-GCTGCGTTCTTCATCGATGC-3′) respectively by an ABI GeneAmp® 9700 PCR thermocycler (ABI, Vernon, CA, USA). The PCR amplification of microbial gene was performed as follows: initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s, and single extension at 72 °C for 10 min, and end at 4 °C. The PCR mixtures contain 5 × TransStart FastPfu buffer 4 µL, 2.5 mM dNTPs 2 µL, forward primer (5 µM) 0.8 µL, reverse primer (5 µM) 0.8 µL, TransStart FastPfu DNA Polymerase 0.4 µL, template DNA 10 ng, and finally ddH2O up to 20 µL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, Madison, WI, USA). Finally, purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, CA, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.3. OTU Clustering and Species Classification

The raw bacterial and fungal gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 [23] and merged by FLASH version 1.2.7 [24] with the following criteria: (i) The 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region is 0.2. Reads that could not be assembled were discarded; (iii) samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching, 2 nucleotide mismatch in primer matching.

Operational taxonomic units (OTUs) with 97% similarity cutoff [25,26] were clustered using UPARSE version 7.1 [25], and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed, the corresponding sequence was respectively allocated to each taxonomic level: Domain, Kingdom, Phylum, Class, Order, Family, Genus, Species. The database used for comparison was as follows. Bacteria and archaea 16 s rRNA database: Silva (http://www.arb-silva.de, accessed on 10 March 2021) and Greengene (http://greengenes.secondgenome.com/, accessed on 10 March 2021). Fungi Unite ITS database: Release 8.0 http://unite.ut.ee/index.php (accessed on 10 March 2021); The function of the GeneBank gene database: http://fungene.cme.msu.edu/ (accessed on 10 March 2021). Here the matching software we used was Qiime platform (http://qiime.org/scripts/assign_taxonomy.html, accessed on 10 March 2021), RDP Classifier [27] (version 2.11 http://sourceforge.net/projects/rdp-classifier/, accessed on 10 March 2021), the default confidence threshold was 0.7. Second, we also used the Nucleotide Sequence Database of NCBI (ftp://ftp.ncbi.nih.gov/blast/db/, accessed on 10 March 2021), the comparison method here used was blast, and e-value was default 1×10^{-5}.

2.4. Analysis of Microbial Community Diversity

α-diversity refers to the diversity within a particular region or ecosystem and β-diversity is used to compare diversity between different ecosystems. So, to further explore the diversity and constitution of microbes in figs and the differences of microbial diversity

between different gender figs, both α-diversity and β-diversity analyses were implemented. α-diversity of each sample was qualified with Ace and Shannon's diversity index.

Ace index is one of the commonly used indexes for estimating the total number of species in ecology, the index is bigger and the community abundance is higher. Here we use the following algorithm:

$$\mathrm{SAce} = \begin{cases} \mathrm{Sabund} + \frac{\mathrm{Srare}}{\mathrm{CAce}} + \frac{n1}{\mathrm{CAce}}\hat{\gamma}^2_{\mathrm{Ace}}, & for\ \hat{\gamma}_{\mathrm{Ace}} < 0.80 \\ \mathrm{Sabund} + \frac{\mathrm{Srare}}{\mathrm{CAce}} + \frac{n1}{\mathrm{CAce}}\tilde{\gamma}^2_{\mathrm{Ace}}, & for\ \hat{\gamma}_{\mathrm{Ace}} \geq 0.80 \end{cases}, \quad (1)$$

and

$$\mathrm{Nrare} = \sum_{i=1}^{\mathrm{abund}} ini,\ \mathrm{CAce} = 1 - \frac{n1}{\mathrm{Nrare}}, \quad (2)$$

$$\hat{\gamma}^2_{\mathrm{Ace}} = \max\left[\frac{\mathrm{Srare}\sum_{i-1}^{\mathrm{abund}} i(i-1)ni}{\mathrm{CAce}\ \mathrm{Nrare}(\mathrm{Nrare}-1)} - 1, 0\right], \quad (3)$$

$$\tilde{\gamma}^2_{\mathrm{Ace}} = \max\left[\hat{\gamma}^2_{\mathrm{Ace}}\left\{1 + \frac{\mathrm{Nrare}(1-\mathrm{CAce})\sum_{i-1}^{\mathrm{abund}} i(i-1)ni}{\mathrm{Nrare}(\mathrm{Nrare}-\mathrm{CAce})}\right\}, 0\right]. \quad (4)$$

ni = The number of OTUs containing *i* sequences; Srare = The number of OTUs containing "abund" sequence or less than "abund"; Sabund = The number of OTUs more than "abund" sequence; abund = Advantage OTU threshold. The default value is 10.

The Shannon index is one of the indicators used to estimate microbial diversity in a sample, the bigger the index is the higher the diversity is. The calculation formula is as follows:

$$H\mathrm{shannon} = -\sum_{i=1}^{sobs} \frac{ni}{N} ln \frac{ni}{N}. \quad (5)$$

Thereinto, *sobs* = The number of OTUs actually observed; *ni* = The number of sequences contained in the *i*-th OTU; *N* = All sequence numbers.

Then inter-group difference test of index was conducted by the Student's *t*-test and Wilcoxon rank-sum test.

To identify whether the microbe constituents varied between different gender figs, β-diversity was applied for comparison. In the β-diversity analysis (Including PCoA statistical analysis and PERMANOVA analysis), the distance between two pairs of samples needs to be calculated by the statistical algorithm to obtain the distance matrix, which can be used in the subsequent β-diversity analysis and visual statistical analysis. In PCoA statistical analysis we calculated and graphed based on the weighted unifrac-distance matrix [28,29]. In the subsequent PERMANOVA analysis, we used the Bray-Curtis distance matrix to decompose the total variance, analyzed the degree of explanation of sample differences by different grouping factors, and used the permutation test to analyze the statistical significance of the groupings [30,31]. The vegan bag in R (Version 3.3.1) was used for calculation. These operations were performed on the cloud platform (http://cloud.majorbio.com/, accessed on 10 July 2021), which provided a variety of statistical analysis-related software programs and scripts, we just needed to follow the default steps provided by the cloud platform to operate.

2.5. Functional Prediction Analysis

To find out the major physiological types and functions of microbes in figs, we carried out Bugbase phenotype prediction and FaproTax function prediction analysis for bacteria OTUs and FUNGuild function prediction analysis for fungi OTUs [32,33]. After the functional prediction, the corresponding phenotype and functional prediction table were obtained. Then, the functional differences of the microbes from different genders of figs were tested by the Wilcoxon rank-sum test. These operations are also implemented on the cloud platform (http://cloud.majorbio.com/, accessed on 14 July 2021).

3. Results

3.1. Result of OTUs Analysis

Total 685,210 clean reads were obtained in six samples of *F. hirta*, of which 315,536 and 369,674 were amplified by primers of 799F_1193R and ITS1F_ITS2R respectively. These

reads were then aggregated at 97% sequence similarity to generate 867 OTUs, of which 718 and 149 OTUs belonged to bacterium and fungi respectively.

3.2. Results of OTUs Taxonomic and Community Species Composition Analyses

We tried to match all obtained OTUs of six samples with the specific taxonomic levels. In total, we identified 25 phyla, 65 classes, 155 orders, 240 families, 389 genera, and nearly 547 species for bacteria; 5 phyla, 16 classes, 37 orders, 76 families, 93 genera, and 109 species for fungi. However, about 78.97% OTUs of bacteria and 46.98% OTUs of fungal could not be annotated to species level, most of OTUs we could only classify into genus, family, order, or phylum level. Hence, for these unclassified microbes OTUs which we could not specify into species level, we designated these OTUs by their annotated genus, family, order, or phylum names.

From Figure 2a and Table S1, we can see that *Wolbachia* almost only exists in the functional male figs sequence library with 64.32% ± 26.90% abundance, while in the female figs library the abundance is 0.17% ± 0.16%. *Ralstonia solanacearum* and *Burkholderia gladioli*'s sequences mainly exist in female figs with 37.37% ± 27.20% and 28.39% ± 48.35% abundance, but for functional male figs, abundances are 1.83% ± 1.24% and 6.29% ± 10.84%. Then, *Rhodococcus erythropolis* and Enterobacteriaceae exist in all six samples' sequence libraries, with 6.95% ± 7.26% and 6.15% ± 14.62% abundance in all libraries. But Enterobacteriaceae has a prominent quantity merely in Fhm_3, which is 35.99%, and in the rest of the groups, the abundance is 0.18% ± 0.22%. Moreover, *Burkholderia-Caballeronia-Paraburkholderia* and *Rickettsia bellii* also exhibit distribution bias between functional male and female figs, of which *Burkholderia-Caballeronia-Paraburkholderia* has 5.11% ± 0.76% abundance in female figs libraries and 0.62% ± 0.50% abundance in functional male figs. *Rickettsia bellii* only exists in Fhm_1 and Fhm_2 with 4.48% and 3.10% abundance.

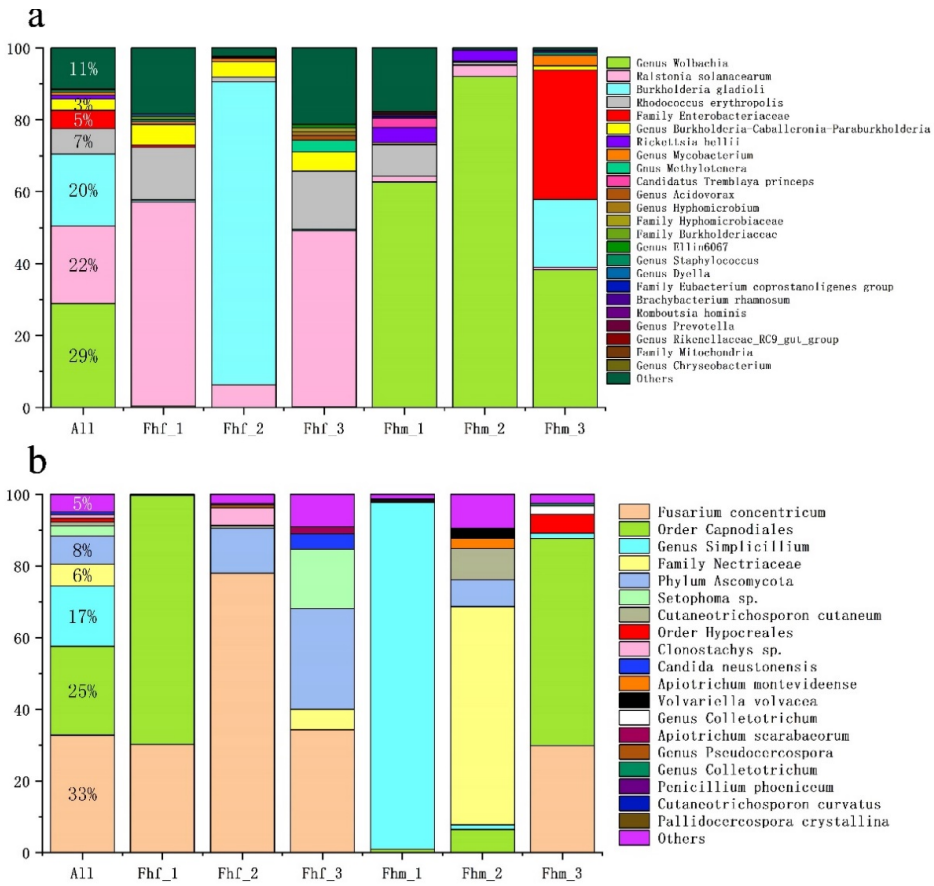

Figure 2. Abundance proportions of main microorganisms in total and single samples. (**a**) Percentage stack bar chart of bacteria; (**b**) percentage stack bar chart of fungi.

In terms of fungus, *Fusarium concentricum*, species of Capnodiales, *Simplicillium*, Nectriaceae, Ascomycota, and *Sctophoma* are major fungi in all samples sequence libraries, with 28.72% ± 28.67%, 22.33% ± 32.36%, 16.61% ± 39.32%, 8.11% ± 11.07%, 11.29% ± 24.81%, and 2.77% ± 6.78% abundance in order. Of the above-listed fungi, only Ascomycota have detected sequences in all six samples, while others are absent at some samples. *Fusarium concentricum* has 47.47% ± 26.52% abundance in female figs sequence library, and 9.96% ± 17.21% abundance in functional male figs, and Capnodiales has 23.23% ± 40.14% and 21.43% ± 31.68% abundance in female and male figs respectively, while *Simplicillium* has 33.21% ± 55.12% in functional male figs and only one sequence is detected in female figs. (Figure 2b and Table S2).

3.3. Results of Microorganism α-Diversity and β-Diversity Analyses

For the α-diversity in each fig, Shannon's diversity index of bacteria can be 1.689 (0.78–2.52), while that of fungi can be 1.033 (0.225–1.75), but no significant difference between them showing the similar species diversity between fungi and bacteria (Table 1). The Ace index of bacteria can be 190.410 (88.651–261.193), while that of fungi can be 35.004 (24.694–59.507) with significantly higher than that of fungi ($p = 0.0002$) showing species abundance in bacteria is higher (Figure 3; Table 1). For the diversity indexes between female and functional male figs, there is no significant difference for both Shannon's diversity index and Ace index (Table 2). The index of Shannon's diversity and Ace for the bacteria is 1.82 ± 0.91 and 219.1 ± 25.58 in female figs, and 1.557 ± 0.79 and 161.72 ± 89.25 in functional male figs; while for fungi is 1.118 ± 0.64 and 38.41 ± 17.49 in female figs, and 0.997 ± 0.69 and 48.94 ± 25.52 in functional male figs (Table 2).

Table 1. *t*-test for two types of indexes between bacteria and fungi.

Index Type	Shannon Diversity Index		Ace Index	
Microbial type	Bacterium	Fungus	Bacterium	Fungus
Fhf_1	0.783	0.634	88.651	14.869
Fhf_2	0.793	0.870	192.565	41.354
Fhf_3	1.515	1.750	135.317	33.600
Fhm_1	2.516	0.225	243.601	36.000
Fhm_2	2.153	1.549	221.131	24.694
Fhm_3	2.372	1.172	261.193	59.507
Average	1.689	1.033	190.410	35.004
p-value	0.1273		0.0002 **	

p-value < 0.01 means difference is extremely significant, and we marked the extremely significant *p*-value of the differences with **.

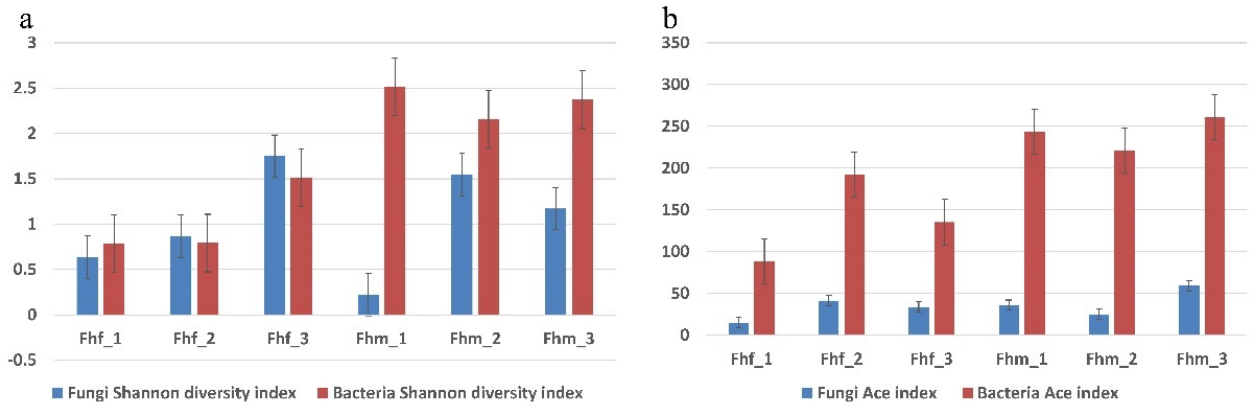

Figure 3. (**a**) Shannon diversity index clustered column chart of fungi and bacteria; (**b**) Ace index clustered column chart of fungi and bacteria.

Table 2. Wilcoxon rank-sum test for Shannon diversity index and Ace index of bacteria and fungi between functional male and female figs.

Microbial Type	Bacterium		Fungus	
Index type	Shannon	Ace	Shannon	Ace
female-Mean	1.821	219.1	1.118	38.41
female-Sd	0.91	25.58	0.64	17.49
male-Mean	1.557	161.72	0.997	48.94
male-Sd	0.79	89.25	0.69	25.52
p-value	0.66	0.66	0.83	0.59
Q-value	0.83	0.83	0.99	0.95

Mean is mean, Sd is standard deviation, *p*-value is the false positive probability value, and Q-value is the FDR value.

The similarity of species between female and functional male figs was further calculated by PCoA analysis (Figure 4a,b). Bacteria are disparate in two types of figs; however, fungi are overlapped. The results of PERMANOVA analysis between different groups are similar to that of PCoA (Table S3). For bacteria, the R2 value between female and functional male figs is the highest (R2 = 0.5908), showing bacteria community divided by gender is more reliable than any other grouping modes, while for fungi it seems that communities do not vary with the fig's gender.

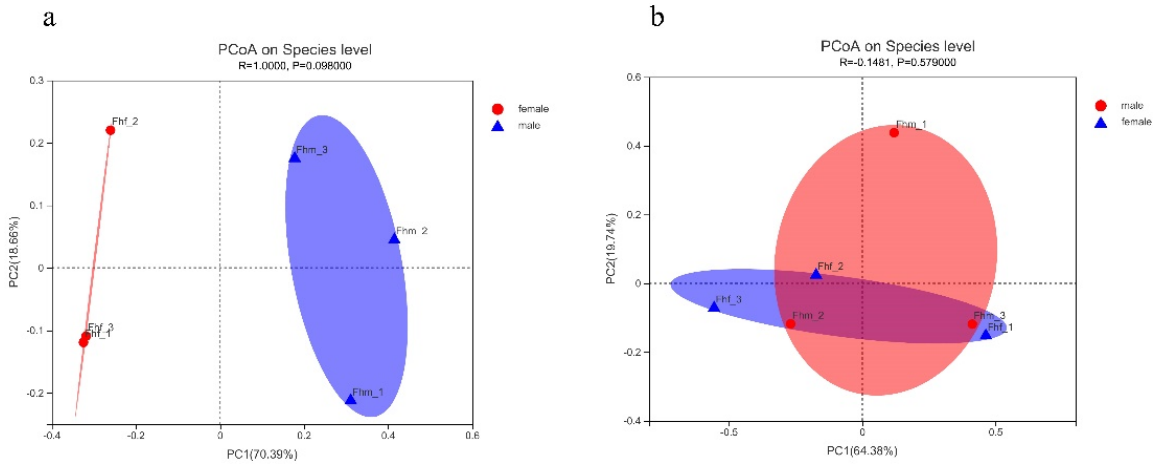

Figure 4. (**a**,**b**) are PCoA statistical analysis results of bacteria and fungi respectively.

3.4. Result of Functional Prediction Analysis

The comparison of phenotype for bacteria between female and functional male figs is shown in Figure 5. There are five phenotypes with a slightly significant larger proportion in female figs: Gram-positive, facultatively-anaerobic, stress-tolerant, potentially pathogenic, and contain-mobile-elements, with the first phenotype's prominent group being *Rhodococcus* and the last four phenotypes being *Ralstonia* and an unclassified genus of Enterobacteriaceae; while two phenotypes with slightly significant larger proportion in functional male figs are aerobic and Gram-negative, and Wolbachia is the dominant group in both of them. According to the phenotype, the functions of functional male and female figs microbial communities are also different with slight significance with more proportion of plant-pathogen in female figs and more proportion of intracellular-parasites in functional male figs (Figure 5a,b).

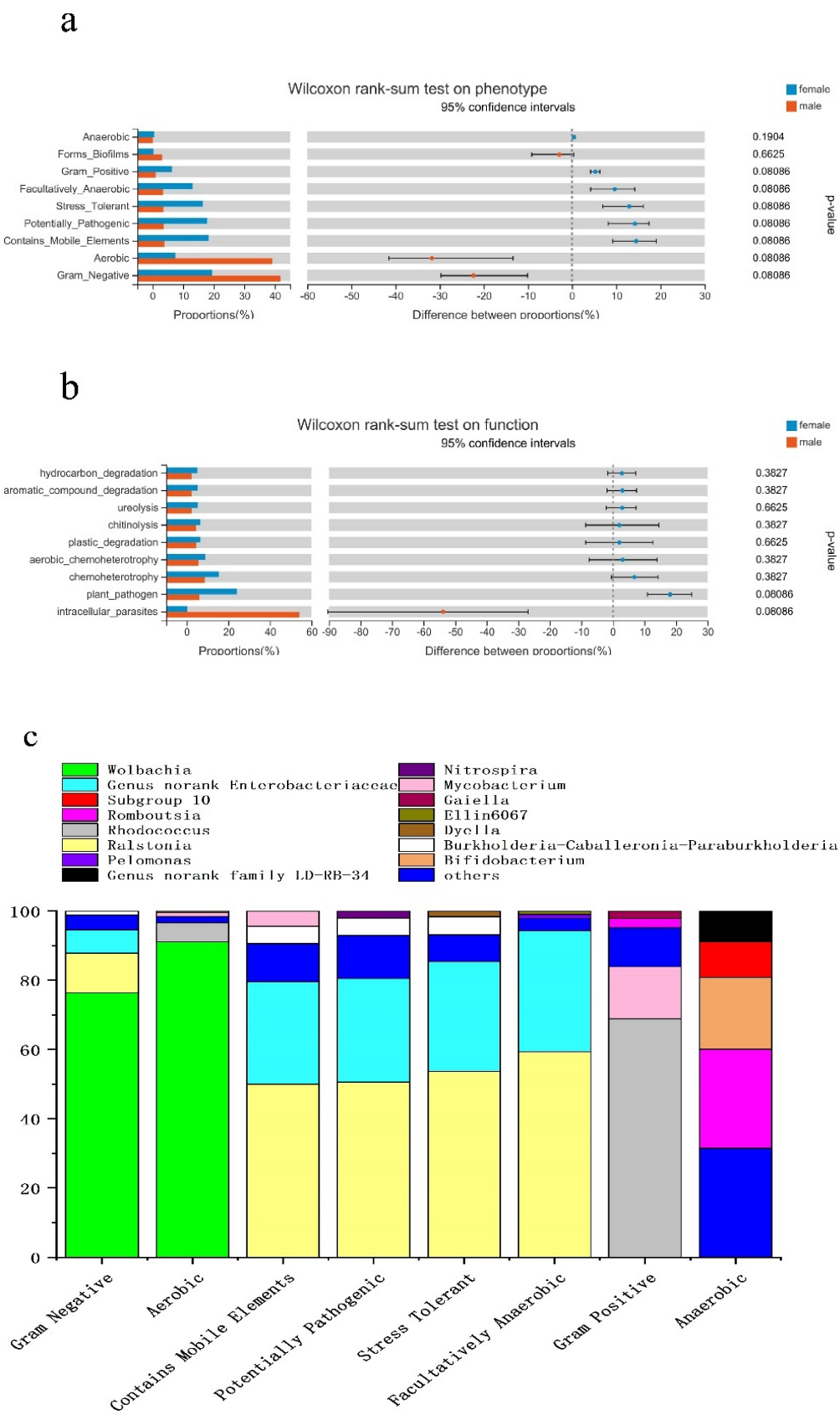

Figure 5. (**a**,**b**) are clustered column charts of difference test of phenotype and function between male and female figs microbial communities; (**c**) is a percentage stack bar chart of phenotype contribution of several main bacteria in total samples for a phenotype.

Fungi in figs are mainly animal or plant pathogens and soil, wood, and undefined Saprotroph, but about 34.81% of fungi's physiological types were unknown (Figure 6). As preceding results showed that there was no difference of fungi communities in functional male and female figs, and the functional annotation results of fungi are not that specific, so we did not conduct the Wilcoxon rank-sum test for fungi function difference between different gender.

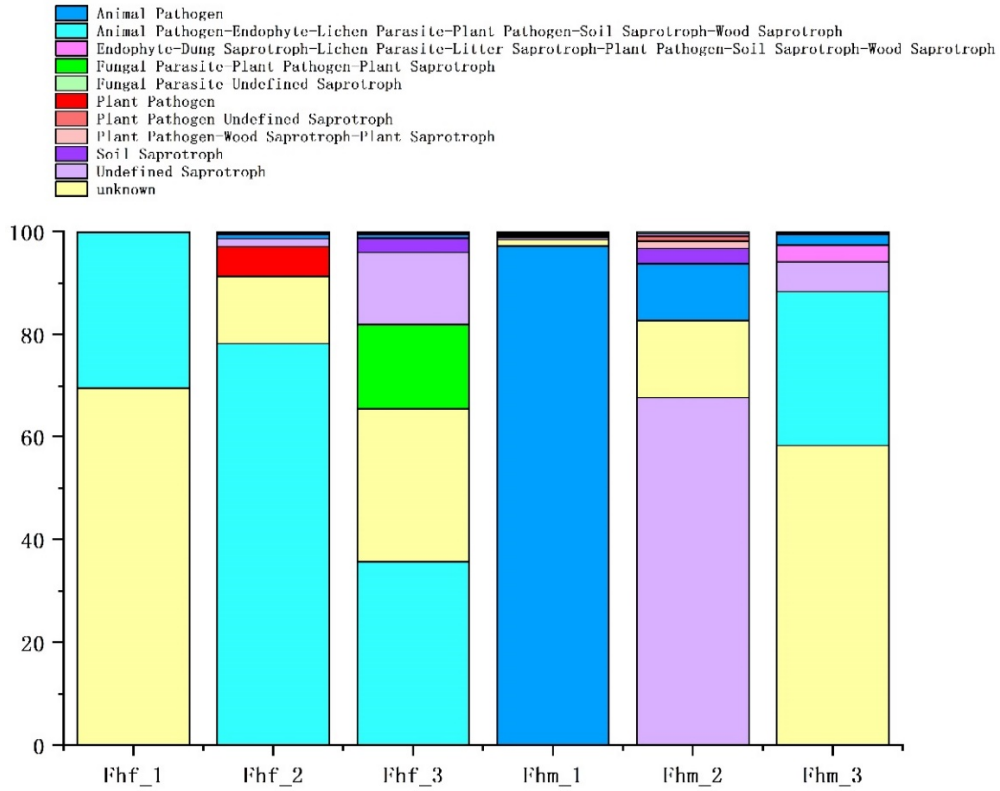

Figure 6. Percentage stack bar chart of several main fungal phenotypes in each sample.

4. Discussion

4.1. Ficus Hirta Figs Microbial Composition

According to microbial OTUs clustering results, we obtained 718 OTUs for bacteria and 149 OTUs for fungi. Although more sequences of fungi were produced than bacteria, bacteria sequences aggregated much more OTUs than fungi. This may show that bacteria in figs are more diversified than fungi.

For *Ficus hirta*, in functional male figs, *Fusarium concentricum*, fungi of Capnodiales and Ascomycota, and bacteria of order Rickettsiales (Including *Wolbachia* and *Rickettsia bellii*) are dominant communities; while in female figs, family Burkholderiaceae bacteria (Including *Ralstonia solanacearum*, *Burkholderia gladioli* and Burkholderia-Caballeronia-Paraburkholderia) and fungi of *Simplicillium*, Capnodiales, and Nectriaceae are dominant communities (Figure 2).

Wolbachia (Anaplasmataceae) is a maternally inherited endosymbiont that mainly exists in arthropods and filarial nematodes [34,35]. Past research has manifested that *Wolbachia* can infect about 40% of arthropod species, *Wolbachia* is likely to be the most abundant endosymbiont among arthropods [36,37]. In addition, previous surveys have also shown that the incidence of *Wolbachia* in fig wasps is up to 59–67%, which is remarkably higher than that in other insects [38]. *F. hirta* functional male inflorescence grows a lot of gall florets which are parasitized by fig wasp larvae, so in functional male figs abundance of *Wolbachia* is distinctly higher than any other bacteria and there is low abundance in female figs as female figs without galled flowers inside. Another bacteria *Rickettsia bellii* has a similar distribution pattern to *Wolbachia*, *Rickettsia bellii* only exists in functional male fig specimens

Fhm_1 and Fhm_2 (Table S1). *Rickettsia* is a facultative eukaryotic intracellular symbiotic bacterium belonging to the subgroup Rickettsiaceae of Proteobacteria, similar to insect endosymbiont *Wolbachia*, *Rickettsia* is associated with reproductive manipulation in host insects and the 16S rDNA of *Rickettsia* was 86% similar to that of *Wolbachia* as well [39,40]. Both of them can affect the reproduction of the host by parthenogenesis induction and killing of male progeny from infected females. Recent research show that *Rickettsia* has an effect on the environmental fitness of host insects and it can also enhance the host insect's ability to resist environmental adversity [40–42]. Bacteria of family Enterobacteriaceae have a large amount in Fhm_3, second only to *Wolbachia* (Figure 2a). Enterobacteriaceae are widely distributed and its host range is large including people, animals, plants, where they can establish themselves as parasites, symbionts, epiphytes, or saprophytes. Moreover, they can also live in soil or water. The previous study has also found Enterobacteriaceae bacterium is the dominant bacteria flora in *Ficus hispida* fig wasp [43].

Both *Burkholderia gladioli* and *Ralstonia solanacearum* belong to the family Burkholderiaceae, including some other bacteria of the same family, and they are prominent and dominant bacterial groups in female figs, while their numbers are relatively low in functional male figs. Family Burkholderiaceae is characterized by the presence of ecologically extremely diverse organisms and contains truly environmental saprophytic organisms, phytopathogens, opportunistic pathogens, as well as primary pathogens for humans and animals [44]. *B. gladioli* was initially known as a plant pathogen, but currently, *B. gladioli* is isolated not only from plants [45], but also found in diverse habitats, including soil, environmental water [46,47], and even the respiratory tract of immunosuppressed humans [48,49]. Studies have shown that *B. gladioli* often symbioses with fungi and plants as well [50]. *B. gladioli* is widely regarded as a germ that exhibits significant ecological niches divergence even within species [51], so it exists in all fig specimens with remarkable divergence in species abundance. *Ralstonia solanacearum* is a Gram-negative soil-borne pathogen that can cause bacterial wilt disease and lead to destructive losses of some economic crops, such as potato, eggplant, tomato, peanut, and tobacco [52]; it is a common plant pathogen. Perhaps because female figs without fig wasp larvae parasitize and florets mainly develop into seeds, so *R. solanacearum* existed in much more quantity in female fig than that in functional male fig.

Then let us turn to the fungi of figs, *Fusarium concentricum* (Family Nectriaceae), which was first described by Nirenberg and O'Donnell et al. from bananas in Central America and aphids in Korea [53]. *F. concentricum* has a wide host range, associating with multiple diseases on different hosts such as stem rot of *Paris polyphylla* var. *chinensis* and fruit rot of pepper and banana [54]. *Fusarium* fungi is a kind of important plant pathogenic fungi, widely distributed in nature [55]. Maybe just because of this, *F. concentricum* prefers existing in female figs and relatively exists less in functional male figs which are full of galled flowers (Figure 2b). The Capnodiales incorporate plant and human pathogens, endophytes, saprobes, and epiphytes, with a wide range of nutritional modes; several species are lichenized or occur as parasites in fungi or animals [56]. These biological characteristics may contribute to the distribution pattern of Capnodiales in *Ficus hirta* figs, both female and functional male figs have Capnodiales fungi without regularity. Moreover, the *Simplicillium* species are commonly found in soil, seawater, rock surface, decayed wood, air, and as symbiotic, endophytic, entomopathogenic, and mycoparasitic fungi, while a few insect-associated species have also been reported [57]. We can find that *Simplicillium* almost only existed in functional male figs (Table S1), this may indicate that the *Simplicillium* fungi we found in *Ficus hirta* figs are associated with fig wasps.

4.2. Microbial Diversity of Ficus Hirta Figs

The above discussion presents that in different genders of figs the dominant microbes are different but the α-diversity analysis reveals that there is no significant difference between female and functional male fig microbial communities in diversity and species abundance (Table 2). Student's *t*-test conducted between fungi and bacteria for Shannon's

diversity index and Ace index shows that bacteria Ace index is remarkably higher than fungi while the difference of Shannon's diversity index between bacteria and fungi is not conspicuous (Table 1). Relatively speaking, *F. hirta* figs bacteria community has high species abundance and higher uniformity of species distribution than fungi, but this kind of discrepancy does not exist between different gender fig endosymbiont communities.

Results of the β-diversity analysis show that bacteria communities differ with figs' gender whereas fungi communities in different gender figs are similar (Figure 4). Meanwhile, PERMANOVA analysis also confirms that the differences between the two bacteria groups are best explained by gender as a grouping factor, however, this trend is not distinct for fungi communities (Table S3). So far, with the addition of previous microbial species composition analysis, we can firmly conclude that in *Ficus hirta* figs bacteria are more abundant and diversified than fungi, and bacteria communities are different between female and functional male figs.

Differences of bacteria communities between different gender figs may be due to the large amounts of endosymbionts like *Wolbachia*, *Rickettsia bellii or* Enterobacteriaceae coexisted in fig wasp larvae. First, a large quantity of fig wasp symbiotic bacteria almost merely exist in functional male figs have caused the difference in bacteria communities between female and functional male figs; then, insect symbiotic bacteria can induce the host to produce antimicrobial peptides and defensins or symbiotic bacteria can secrete antibiotics to resist the infection of pathogenic microorganisms [58,59], and this may result in relatively less plant bacterium pathogen in functional male figs compared to that in female figs (Figure 5a,b). Thus, the different functions of female fig and functional male fig would influence the microbial communities inside indeed.

4.3. Functional Prediction Analysis

We analyzed figs endosymbionts from facets including species constitution and abundance, community structure, and diversity; perhaps we can get new points of view by analyzing the microbial function and physiological phenotype. In functional male figs, there are mainly some Gram-negative, aerobic, and intracellular parasite bacteria, this reflects the prevalence of *Wolbachia*, *Rickettsia*, Enterobacteriaceae such as Gram-negative bacteria in functional male figs (Figure 5). We can also find that aerobic such as phenotype is mainly contributed by *Wolbachia*, and *Wolbachia* is a kind of intracellular parasite bacteria of fig wasp, so this may indicate that fig wasp's larva exists in oxygenated condition. However, fig syconium is relatively enclosed and fig wasp's larva parasitizes in a completely closed gall, therefore figs may have some mechanisms to let outside air come into the gall or can provide oxygen for the fig wasp larva. However, there are fewer research on such mechanisms so far and we have no idea about this phenomenon as well, hence further study is required on this. Then in female figs, there are more plant pathogen, Gram-positive, containing mobile elements, and stress-tolerant bacteria (Figure 5a,b), and these phenotypes are mainly contributed by *Ralstonia solanacearum* according to BugBase phenotype prediction results (Figure 5c), as Enterobacteriaceae only appears in functional male figs (Figure 2a).

Fungi in figs are mainly animal pathogen, endophyte, lichen parasite, plant pathogen, soil saprotroph, and wood saprotroph; but many are still functionally unknown (Figure 6). Then it is also worth noting that animal pathogen is prominently rich in Fhm_1, and species annotation results show that the *Simplicillium* fungi make up 96.86% proportion in Fhm_1 (Table S2). Accordingly, we can speculate *Simplicillium* may be a type of endophyte in the fig wasp.

Fig interior microorganisms do have influences on figs or fig–fig wasp interaction system. For example, plant-pathogen can infect figs and cause figs rot [60–62]. Some intracellular parasite bacteria can enhance fig wasp stress resistance, manipulate reproduction, and influence the growth of fig wasp, as we have discussed previously. The endosymbionts of fig wasps also have an impact on the evolution or speciation of fig wasps; the interaction between fig wasps and *Wolbachia* alters the genome of fig wasps, and *Wolbachia* may also

promote speciation of fig wasps [63]. The contraction of the gene family associated with antibacterial defense and immune response in fig wasps mentioned earlier was explained by the fact that fig provided a closed and relatively safe environment for the development of fig wasp larvae, so that fig wasps were less exposed to the harsh external environment and numerous antagonists [21,64,65]. But we guess that interaction between fig wasp and endosymbionts such as *Wolbachia* and *Rickettsia* can increase host resistance to diseases or adverse factors [41,42,66–69], may also have a role in such gene family contraction.

5. Conclusions

Ficus hirta is a dioecious plant in which female trees only grow female inflorescence that can bear seeds and male trees grow functional male inflorescence with galled flowers that are parasitized by fig wasps. Such special structure and function of different gender figs would result in different endosymbionts contained in figs. Our research demonstrated that bacteria involved in different gender figs were different but the bacteria community diversity was similar. In gross samples of *F. hirta* figs, bacterial diversity was higher than fungi. Although we did not identify the significant difference of fungi communities between female and functional male figs, we found the distribution of a special fungi *Simplicillium* showed bias in different gender figs. If we increase the number of samples, we can probably find that fungi communities vary with the figs' gender.

Our research found out the main endosymbionts existed in *F. hirta* figs, but more samples and deeper and broader sequencing are required to discover new specific species. Further research with new techniques and advanced methods are needed to explore how these endosymbionts interact with figs and affect fig and fig wasp interaction. Our preliminary study of fig endosymbionts diversity is the base for subsequent studies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13120636/s1, Table S1: Number of sequences of bacteria in each sample, Table S2: Number of sequences of fungi in each sample, Table S3: Chart of PERMANOVA analysis result.

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