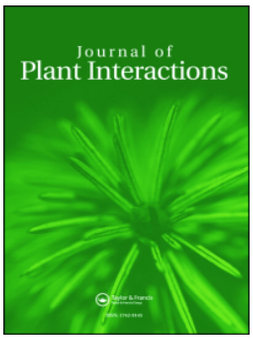

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The endophyte's endophytes: the microbial partners of the endangered plant parasite *Rafflesia speciosa* (Rafflesiaceae) reveal clues about its cryptic biology and cues for cultivation

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ABSTRACT

Rafflesia is an endangered endophytic holoparasitic plant that lives the majority of its life inside the tissues of its sole plant host, *Tetrastigma*. *Rafflesia* floral buds emerge to produce the world's largest single flower. Like other plants, holoparasites harbor a diverse microbiome, the role(s) of which has remained largely unstudied. We characterized the bacterial microbiome of seeds of *Rafflesia speciosa* and cuttings of its host. We found evidence that *R. speciosa* seed has similar bacterial profiles to its infected host, which suggests that seeds sequester certain host bacteria, as well as acquire unique bacterial taxa from biotic associates of the fruit. We did not find evidence of mycorrhizal taxa in the microbiome. This is the first study of the microbial endophytes associated with any *Rafflesia* species and its host, a tripartite holobiont, and provides insights on its cryptic microbial partners. We discuss how this may aid horticultural propagation of *Rafflesia*.

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Holobiont; holoparasite; *Orobanche*; phhytobiome; *Tetrastigma*

Introduction

Rafflesia (Rafflesiaceae, Malpighiales) is a holoparasitic plant genus that is completely dependent on its sole host plant, *Tetrastigma* (Vitaceae) for nutrition and produces the largest flowers in the plant kingdom (Davis 2008). The genus is also dependent on its host for genetic architecture, pilfering and appropriating host genes as its own (Xi et al. 2012; Xi et al. 2013). *Rafflesia* plants exist mostly as inconspicuous strands interwoven into host tissue (Nikolov et al. 2014) and/or clusters (Mursidawati et al. 2019) of cells inside its host. Its tissues only emerge outside of the host to flower, with its full bloom emitting sulfurous compounds reminiscent of decaying meat and attracting carrion flies in deceptive pollination (Wee et al. 2018). *Rafflesia* and the confamilial genus *Sapria*, are the only known plants to have completely lost their chloroplast genome (Molina et al. 2014; Cai et al. 2021), though *Rafflesia* seems to retain the plastid compartments for synthesis of amino acids and lipids while missing all other photosynthesis components (Ng et al. 2018).

There are over 40 *Rafflesia* species endemic to the tropical forests of Southeast Asia, including 15 endemic to the Philippines (Tobias et al. 2023). All but one of these are endemic to a single island (Pelser et al. 2019). Habitat destruction and harvest for medicinal uses have made all *Rafflesia* spp. vulnerable to extinction (Malabrigo Jr et al. 2023). Hailed as the 'panda of the plant world' for its endearing but endangered status, *Rafflesia* has proved incredibly challenging to propagate, severely limiting conservation efforts. The only successful propagation efforts recorded have been grafting of *Rafflesia*-infected host cuttings at Bogor Botanic Garden in Indonesia (Wicaksono et al. 2016). However, *Rafflesia* has never been propagated outside its native range, which severely limits *in* and *ex situ* conservation opportunities. Molina et al. (2017) have attempted to propagate *Rafflesia* species from the Philippines in the US Botanic Garden in Washington DC, USA by importing live *Rafflesia*-infected host cuttings from the Philippines. However, inevitable prolonged transit stressed the plants, and many perished along the way, and the few that rooted in the garden, despite utmost care, eventually succumbed after a few months. Non-parasitized *Tetrastigma* seedlings brought to the garden, however, were successfully grown, and these have been inoculated with *Rafflesia* seeds since 2017, but *Rafflesia* buds have yet to be observed. *Rafflesia* seeds were also incubated in various plant growth regulators known to induce germination in other plants, including other holoparasites, though none have resulted in observable germination (Molina et al. 2017).

Rafflesia seeds become transcriptionally active after imbibition, which may indicate readiness for germination

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This article has been corrected with minor changes. These changes do not impact the academic content of the article.

pending host stimulation (Molina et al. 2023). Genes responsive to strigolactone, which induces germination in plant parasites of Orobanchaceae, were not detected in the *Rafflesia* seeds. However, genes for wood-degrading laccase enzymes (involved in haustorium formation in Orobanchaceae) were identified in the *Rafflesia speciosa* seed transcriptome. This may suggest that *Rafflesia* seed inoculation in *Tetrastigma* may benefit from application of laccase, which is a lignolytic enzyme also derived from diverse fungi and bacteria (Janusz et al. 2017). In *Cytinus hypocistis* (Cytinaceae), another rosid endophytic holoparasite, mycorrhizae were found embedded in its tissues and that of its host, suggesting that mycorrhizae may play an important physiological role in the *Cytinus* life cycle. Mycorrhizae are a group of fungi that exist in a mutualistic relationship with plants enhancing plant growth by improving nutrient uptake (Giovannini et al. 2020). However, Molina et al. (2023) were unable to find homologs of genes involved in mycorrhizal symbiosis in *R. speciosa* seeds, casting doubt on the hypothesis that mycorrhizae may be required for *Rafflesia* germination or infection.

Though mycorrhizal partners may not be involved in *Rafflesia* seed germination, it is possible that *Rafflesia* enlists other microbial partners to facilitate its germination or other aspects of its life cycle (Truyens et al. 2015; Mahmood et al. 2016; Vujanovic and Germida 2017; Wicaksono et al. 2021). Only 11 of the c. 95 spp. of *Tetrastigma* are known to support *Rafflesia* infection (Chen et al. 2011), but the lack of phylogenetic evidence for cospeciation between *Rafflesia* species and its *Tetrastigma* host species (Pelser et al. 2016) suggests that ecological factors, such as microbial symbionts, may be involved in *Rafflesia*'s host choice of *Tetrastigma* species. Moreover, only certain individuals within these species are parasitized, perhaps due to infraspecific genetic variation resulting in decreased production of defensive compounds such as benzylisoquinoline alkaloids (Fondevilla et al. 2010; Rowntree et al. 2011; Molina et al. 2022).

Microbial symbionts, together with the plant host, form the holobiont, a partnership involving the host and the associated microbial community coevolving with one another (Vandenkoornhuyse et al. 2015; Rosenberg and Zilber-Rosenberg 2018). But in the case of *Rafflesia,* a ternary connection exists, involving the holoparasite, its host, and the microbial communities enmeshed within both their respective tissues. Microbial symbionts include endophytes residing within plant tissues that can improve plant growth, development and health by increasing nutrient acquisition, producing phytohormones, performing nitrogen fixation, and/or priming host defenses against pathogens (Felestrino et al. 2017; Shahzad et al. 2018; Afzal et al. 2019). Endophytic bacteria in seeds have also been found to promote seed germination and to confer stress tolerance, with host plants recruiting a beneficial microbial community that can be vertically transmitted (Shahzad et al. 2018).

In the holoparasite *Langsdorffia* (Balanophoraceae), which grow in the Brazilian Iron Quadrangle, associated microbiota were found to be in mutualism by producing iron-chelating siderophores, indole-3-acetic acid (IAA), and fixing nitrogen, in addition to inhibiting pathogens (Felestrino et al. 2017). Iasur Kruh et al. (2017) have also observed that the microbiome community of the broomrape holoparasite *Phelipanche aegyptiaca* (syn. *Orobanche aegyptiaca,* Orobanchaceae) was significantly different from the non-parasitized tomato host but similar to that of the infected host suggesting horizontal bacterial exchange between host and parasite. A strain of *Pseudomonas.* spp. in tomato was also found to suppress c. 80% of *Phelipanche* seed germination demonstrating that certain bacterial taxa can be used to protect host plants from parasitism. In another study, growth-promoting rhizobacteria were isolated from the seeds of *Phelipanche ramosa* (syn. *O. ramosa*), the most aggressive broomrape weed (Durlik et al. 2021). Fitzpatrick and Schneider (2020) also analyzed the microbiomes of *Orobanche hederae* and its ivy host and found that the parasitic plant microbiome is also derived but still distinct from host plant microbiota, with the microbiome of the infected host showing modest changes compared to the uninfected individual. In a recent study that characterized the microbiome of the seeds of the holoparasite *Cistanche armenia* (Orobanchaceae), a desert plant, most of the bacterial isolates were a spore-forming, halotolerant, alkaliphile *Bacillus* spp. and produced metabolites that seem to benefit the parasite's survival in its arid saline environment (Petrosyan et al. 2022). In the confamilial hemiparasite *Striga hermonthica*, the soil microbiome was found to suppress *Striga* infection in its host depleting the parasite's haustorium inducing factors as one mechanism (Kawa et al. 2022). These studies suggest that microbial symbionts are physiologically important in plant growth and development, even in parasitic plants.

The available literature motivates a metagenomic study of microbial taxa associated with *Rafflesia* and its host in an effort to identify microbial partners that can potentially promote *Rafflesia* growth and development to aid in conservation efforts. In this study, we characterized the microbiota of *Rafflesia speciosa* seeds to understand if there are bacteria acquired from its host and/or if there are bacteria possibly transferred from parasite to host that may be altering the host microbiome in comparison to uninfected host roots. *Rafflesia speciosa* primarily grows on host roots (an important distinction as many *Rafflesia* spp. colonize host stems). We also characterized the endophytic fungal taxa within two accessions of *Rafflesia speciosa* seeds to ascertain if mycorrhizal taxa are present. Lastly, we compared the *Rafflesia*-associated microbiome with that of *Orobanche*, another holoparasite distantly related to *Rafflesia,* to determine if there are bacterial endophytes common to holoparasites that may be relevant for plant parasitism across plant families. To our knowledge, this is the first study of microbial endophytes associated with a *Rafflesia* species and its host and provides insights on the microbial partnerships that may promote *Rafflesia* development and could be leveraged for *ex situ* conservation.

Materials & methods

Samples (ranging from 50 mg to 500 mg) of the following were collected: seeds of *Rafflesia speciosa* ('R', with some pulp, from 4 accessions), root cuttings of *Rafflesia*-infected host *Tetrastigma* species (4x *T. magnum* and 4x *T. harmandii*, each from different plants, sampled immediately next to the *Rafflesia* bud or at the bud junction; designated 'TR' hereafter) and cuttings of their uninfected counterparts (2x *T. magnum*, 2x *T. harmandii,* each from different plants, designated 'T' hereafter), were surface-sterilized with 2% sodium hypochlorite (prepared from reagent

Table 1. Microbial richness (alpha) diversity within groups.

Kingdom	Groups	Alpha Metrics with *p*-values			
Bacteria		*Shannon's Entropy* $p = 0.065$	*Observed Features* $p = 0.071$	*Faith's Phylogenetic Diversity* $p = 0.059$	*Pielou's Evenness* $p = 0.076$
	T	9.37 ± 0.13	793.25 ± 61.68	69.14 ± 3.37	0.97 ± 0
	TR	7.51 ± 1.64	374.5 ± 354.88	30.61 ± 27.22	0.95 ± 0.02
	R	6.43 ± 1.04	140.75 ± 84.11	15.78 ± 8.47	0.93 ± 0.03
	O	4.29	40	5.52	0.81
Fungi		*Shannon*	*Observed Species*	*Simpson's Reciprocal*	*Simpson*
	R	3.7 ± 2	75.85 ± 58.05	11.96 ± 12.51	0.82 ± 0.19

Note: Kruskal-Wallis one-way ANOVA of the group's bacterial species number indicates similarity (at $p > 0.05$). However, post-hoc multiple comparison suggests that T > R for all metrics (*$p = 0.021$).

grade solution with available chlorine 10-15%, Sigma-Aldrich Cat. 425044), agitated for 15 min, and without rinsing sent immersed in 1 mL DNA/RNA shield (Zymo cat# R1100) for DNA extraction and 16S rRNA (V3-V4 region) microbiome sequencing (Zymo cat# Q2001) to Zymo Research, Irvine CA. Zymobiomics sequencing service included a positive control (mock microbial community of defined composition) and negative control (blank). Non-parasitized status of *Tetrastigma* samples were confirmed by mitochondrial 16s rRNA sequencing with *Rafflesia*-parasitized samples possessing *Rafflesia* amplicons, as expected. *Tetrastigma* samples were DNA-barcoded to confirm species identity following methods in Molina et al. (2018).

Rafflesia speciosa seeds were obtained from 1 dehisced fruit and from 3 intact fruits. All samples were collected from Miag-ao Iloilo (Aug 2018, Aug 2019, Jan 2020, July 2022, and Jan 2023), with appropriate permits from the Philippine Biodiversity Management Bureau (gratuitous permit 275, 295, and 315) and imported into the US with USDA import permit P526P-18-02136. Seeds from one accession of *Orobanche hederae* (purchased from Plant World Seeds, Devon, UK) were also sent to Zymo. Plastid PCR blockers were applied to reduce chloroplast contamination (Zymo cat#Q2032). ITS fungal metagenomic sequencing (Zymo cat#Q2003) was also performed on two *Rafflesia speciosa* seed samples.

Raw sequence data in FASTQ were processed and analyzed in QIIME2 (Bolyen et al. 2019) using WSL2-VS Code (Microsoft). Visualization was done in QIIME2 View, Excel, and NCBI Tree Viewer. Briefly, after denoising into amplicon sequence variants (ASVs) with DADA2 to get the feature table and representative sequences, taxonomic classification was performed (with a confidence range of 70%−100%) using a weighted Silva 13.8 (at 99% full-length sequences OTUs, or operational taxonomic units) pre-trained scikit-learn classifier. Taxa levels were collapsed and filtered based on groups and abundances to look for trends. Phylogenetic trees were constructed using a pipeline containing alignment with MAFFT, masking, FastTree, then rooted at its midpoint. Diversity core-metrics-phylogenetic pipeline was employed to output alpha and beta diversity data and principal coordinates analysis (PCoA) emperor plots. Alpha and beta groups statistical significance tests were conducted using Kruskal–Wallis and permutational multivariate analysis of variance (PERMANOVA) with pairwise multiple comparison, respectively. Finally, differential abundance was utilized using Gneiss balances with Ward hierarchical bifurcating tree and analysis of composition with bias correction (ANCOM-BC) (Lin and Peddada 2020) for finding statistically enhanced taxa in R and TR groups relative to the T group.

16S rDNA sequences for bacterial genera with at least 0.1% abundance in R (*Rafflesia* seed) were aligned and phylogenetically analyzed in Geneious Prime (Biomatters, Ltd). Bacterial abundance information was also mapped on the resulting phylogeny using ItoL (Interactive Tree of Life; Letunic and Bork 2021).

Results

A total of 1,990,005 bacterial 16S rDNA sequences from 375,305 high quality reads (total frequency), and 263,212 ITS fungal sequences from 123,781 reads were obtained resulting in 13,568 (features) OTUs of bacteria and 140 OTUs of fungi respectively. Raw data were submitted to NCBI SRA under BioProject PRJNA996588.

Collectively, the 4 samples of uninfected *Tetrastigma* (T) had the highest richness indices among the samples, and *Orobanche* (O) had the least (Table 1). When evaluated as a group (uninfected *Tetrastigma*: T, infected *Tetrastigma*: TR, *Rafflesia* seed: R, and *Orobanche* seed: O), the non-parametric Kruskal–Wallis ANOVA demonstrated statistical similarity within the group since *p* values are >0.05 (the lowest value being 0.059). However, post-hoc pairwise tests showed $p = 0.021$ for all alpha diversity metrics for T vs. R groups, indicating that T has statistically higher bacterial diversity than R.

The relative abundance of the bacterial phylum Firmicutes (38.2%) in R was much higher compared to those in T (3.5%)

Table 2. Bacterial genus frequency across all samples sorted according to the most abundant in R.

Genus	O	R	TR	T
Acetobacter	0%	8.54%	0.06%	0%
Bacillus	0%	8.53%	1.80%	1.00%
Enterobacter	0%	5.40%	4.47%	0.20%
Gluconobacter	0%	5.35%	0.40%	0%
Clostridium_sensu_stricto_1	0%	3.84%	0.68%	0.01%
Paenibacillus	0.30%	2.73%	0.77%	0.93%
Cohnella	0%	2.17%	0.01%	0.20%
Dysgonomonas	0%	1.77%	0%	0%
Streptomyces	0%	1.62%	2.85%	1.17%
Staphylococcus	0%	1.57%	0.23%	0%
Lactobacillus	0%	1.43%	0.86%	0%
Leuconostoc	0%	1.20%	0.79%	0%
Frateuria	0%	1.19%	0%	0%
Robbsia	0%	1.03%	0%	0%
Ralstonia	0.21%	1.02%	0%	0%
Others (unidentified and <1%)	99.49%	52.62%	87.06%	96.50%

Table 3. Fungal genus frequency in the R group totaling to 100%.

Genus	Relative Abundance
Fusarium	8.6%
Penicillium	6.5%
Aspergillus	5.0%
Pichia	4.9%
Lipomyces	4.0%
Wickerhamomyces	4.0%
Sporopachydermia	3.8%
Clavispora	3.6%
Gliocladiopsis	2.6%
Ogataea	2.4%
Talaromyces	1.2%
Hanseniaspora	1.1%
Others (unidentified and <1%)	52.3%

and TR (9.4%), while Planctomycetota, Chloroflexi and Acidobacteriota generally showed the reverse trend, lower in R than in T and TR (Figure 2; Table 2). O was primarily Proteobacteria (91.9%). To note, the taxonomic names follow the latest SILVA database, which may differ from the taxonomy of Genome Taxonomy Database (GTDB; Oren and Garrity 2021). For example, phylum Firmicutes is synonymous with Bacillota, Proteobacteria is syn. Pseudomonadota, Bacteroidetes is syn. Bacteroidota, Actinobacteriota syn. is Actinomycetota, etc. As for bacterial families, Acetobacteraceae and Bacillaceae were higher in R than in T and TR. Xanthobacteraceae and Gemmataceae were higher in T than in TR and R. Microbacteriaceae was the most abundant bacterial family in O compared to the other groups.

Only R was sampled for fungal endophytes (Figure 2), which revealed Ascomycota (56.3%), Basidiomycota (0.1%), with the rest unclassified even at the phylum level (43.6%). The fungal families Nectriaceae (*Fusarium* spp., *Gliocladiopsis* spp.), Aspergillaceae (*Penicillium* spp., *Aspergillus* spp.) and Pichiaceae (*Pichia* spp., *Ogatea* spp.) were the top 3 most abundant collectively making up 34.5% of R fungal diversity (Table 3).

Beta diversity metrics' (Jaccard, Bray–Curtis, and UniFrac: unweighted and weighted) PERMANOVA (Table 4) indicated that bacterial populations among R, T, and TR groups were statistically different (all p-values < 0.05) or clustering separately. Multiple comparisons also generally indicated that each group has unique bacterial populations as shown in pairwise differences (9 out of 12, $p < 0.05$). However, looking at the weighted UniFrac outcome (Table 4, last column), T vs. TR groups showed similarity (at $p = 0.093$). The PCoA emperor scatter plot (Figure 3(A), at different views) revealed that 3 individual samples belonging to the TR group clustered tightly with the T groups (circled), which likely influenced the statistical similarity outcome. Samples from groups O, R, and 5 samples from TR (pointed by the arrow) demonstrated diffusion across the 3 major principal coordinates. Expanding the coordinates or features to 5 majors using parallel plots also displayed the tightness of the T group cluster (with some TR samples) versus the other samples (Figure 3(B)). The emperor scatter and parallel plots represented about 69% and 81%, respectively, of total features or coordinates (Figure 3(C)).

Gneiss balance differential abundance of the group's bacterial phyla showed that Myxococcota, Verrucomicrobiota, Acidobacteriota, Chloroflexi, and Planctomycetota were generally enriched in T samples compared to both R and TR. However, Firmicutes, Bacteriodota, Actinobacteriota, and Proteobacteria were mostly more abundant in R and TR samples than in T (Figure 4).

Seven bacterial phyla were identified in R (genera having at least 0.1% abundance (Figure 5)), with members of Bacteriodetes, Proteobacteria, Firmicutes, and Actinobacteriota well-represented. The taxonomic groups Bacteroidales, Acetobacteraceae, Lactobacillales were enriched in R and/or TR, but depleted in T. *Ralstonia* and *Xanthomonas* were only present in both R and O.

Using ANCOM-BC with T as the reference group, Proteobacteria (T vs. R, at $^{***}p = 0.00019$ and T vs. TR, at $^{***}p = 0.00002$), Firmicutes (T vs. R, at $^{***}p = 7e\text{-}60$ and T vs. TR, at $^{*}p = 0.03$), and Actinobacteriota (T vs. R, at $^{***}p = 5e\text{-}17$ and T vs. TR, at $^{***}p = 1e\text{-}6$) phyla were statistically enhanced in both R and TR groups (Figure 6(A)). The genera that were at least 0.1% abundant in R that were significantly more abundant in both R and TR versus T are: *Enterobacter, Clostridium* sensu stricto 1, *Staphylococcus, Lactobacillus, Leuconostoc, Gluconacetobacter, Cellulomonas, Burkholderia-Caballeronia-Paraburkholderia, Lachnospiraceae* NK4A136 group, *Galbitalea, Enterococcus, Clostridium* sensu stricto 19, and *Kaistia* (Figure 6(B)). Of interest are: *Enterobacter* (a Proteobacteria at 5.4% in R and 4.5% in TR, but only at 0.2% in T, for a log fold change (LFC) of 3.15 and 3.23 in R and TR, respectively, compared to T), *Lactobacillus* (a Firmicutes at 1.4% in R and 0.9% in TR, but only at 0.0% (near zero) in T, for a log fold change (LFC) of 5.68 and 2.22 in R and TR, respectively, compared to T), *Leuconostoc* (a Firmicutes at 1.2% in R and 0.8% in TR, but only at 0.0% in T, for a log fold change (LFC) of 5.07 and 2.71 in R and TR, respectively, compared to T), *Cellulomonas* (an Actinomycetota at 0.7% in R and 0.6% in TR, but only at 0.0% in T, for a log fold change (LFC) of 4.21 and 3.34 in R and TR, respectively, compared to T) and *Burkholderia-Caballeronia- Paraburkholderia* (a Proteobacteria at 0.5% in R and 0.7% in TR, but only at 0.0% in T, for a log fold change (LFC) of 4.85 and 3.24 in R and TR, respectively, compared to T).

Discussion

Synopsis. In this study, we analyzed the microbiome of *Rafflesia speciosa* seeds and root cuttings from *Rafflesia*-parasitized *Tetrastigma* and compared them to non-parasitized host root samples. We hypothesized that the seeds and the parasitized host cuttings would share a microbiome that

Table 4. Microbial population (beta) diversity across groups.

	Beta Diversity p-values			
Pairwise Groups	*Jaccard* **0.001*	*Bray-Curtis* **0.002*	*Unweighted UniFrac* **0.003*	*Weighted UniFrac* **0.001*
R vs. **T**	*0.032	*0.032	*0.03	*0.032
R vs. **TR**	*0.004	*0.012	0.103	*0.028
T vs. **TR**	*0.049	0.054	*0.041	0.093

*Statistically-significant based on PERMANOVA and pairwise multiple comparison.

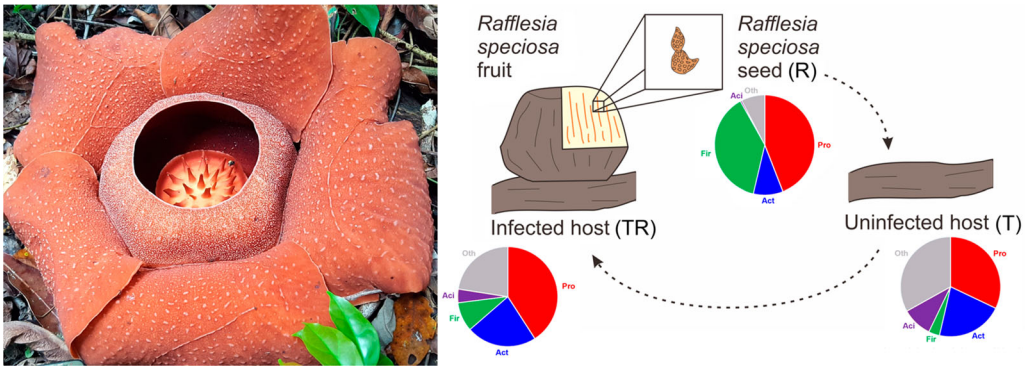

Figure 1. Hypothetical microbiome changes upon *Rafflesia* infection of the *Tetrastigma* host. During development, *Rafflesia speciosa* seed sequesters certain bacteria (e.g. Firmicutes) from its infected host and transfers this to a new *Tetrastigma* host altering its microbiome compared to uninfected roots. Whether this is a strategy to facilitate and sustain a *Rafflesia* infection needs to be tested. A blooming flower of *Rafflesia speciosa* (c. 45 cm wide) is seen on the left.

may be different from that of the uninfected host, suggesting that *Rafflesia* acquires certain bacteria from its host (Figure 1). Despite sampling only 4 accessions, the uninfected *Tetrastigma* roots (T) had higher diversity indices than 8 accessions of *Rafflesia*-infected *Tetrastigma* roots (TR) (Table 1), which may suggest that a *Rafflesia* infection could diminish host microbial diversity, possibly due to an alteration of host metabolism by the holoparasite.

We found evidence that *Rafflesia* seeds (R) and the *Rafflesia*-parasitized host cuttings shared bacteria not present in the uninfected host (Figure 2–3; Table 2), which suggests that during reproductive development, *Rafflesia* seeds acquire certain host bacteria. We hypothesize that these newly acquired bacteria become available for transfer to a new host, potentially altering the parasitized host's microbiome. In all pairwise comparisons (Table 4) R was significantly different from T, but as expected, TR was intermediate, sharing some taxa with R, and having some taxa in common with T (Figure 2). There were two TR groups, one that associated with R, and another that formed a tight cluster with T (Figure 3). The two TR accessions that associated with R were the actual host plants for 3 R samples, which supports the idea that R seeds do sequester some of their host's microbes. However, for the other TR group that clustered with T, we were not able to sample R seeds from those, since they were collected in January 2023, when *Rafflesia* fruits/seeds were not available. It is possible that this second TR cluster are hosts that have been relatively recently infected (within a few years), which is why its microbiome is still more similar to T, compared to the hosts from which R fruit/seeds have been collected and imply prolonged infection given the long life cycle of *Rafflesia*. Bacterial taxa that contribute to these patterns are indicated in Figure 4, which shows that different accessions of TR demonstrate varying levels of abundance for certain bacterial phyla, either mirroring R's or T's. Notably, Firmicutes appear highly enriched in all accessions of R, yet depauperate in all samples of T, while different accessions of TR are either enriched or diminished for this phylum. However, we cannot discount the potential influence of tissue type differences (roots vs. seeds) on the makeup of the sampled R compared to the T/TR microbiomes.

The phylogeny of the most abundant bacterial genera in R is shown in Figure 5. Certain taxa of Firmicutes, in particular lactic-acid genera of the order Lactobacillales such as *Enterococcus, Streptococcus, Leuconostoc,* and *Lactobacillus,* were found to be enriched in both R and TR but lacking in T (Figure 6). In addition, another group of fermenting bacteria, acetic-acid producing *Gluconacetobacter* spp. of Acetobacteraceae (Proteobacteria), were also enriched in both R and TR but absent in T (Figures 5 and 6). The prevalence of these acidophilic fermenting bacteria could indicate an acidified environment surrounding R and TR, or at least a portion of the latter infected with R, reducing bacterial diversity compared to T, which explains the lower bacterial diversity of R and TR (Table 1). Whether this acidified environment is a strategy to facilitate infection by *Rafflesia* waits to be seen. There were also unique bacterial taxa in R not found in other groups that R may have acquired from the fruit pulp and/or other biotic associates of the fruit.

Planctomycetes, Chloroflexi and Acidobacteriota showed the reverse trend–lacking in R but increased in T (Figure 2). The ecological significance of these bacterial phyla in T (vs R/TR) is unknown. At the family level, Xanthobacteraceae, a group of N-fixing rhizobia, was higher in T than in TR and R. It is possible that the metabolic alteration in the *Rafflesia*-infected host affects abundance of rhizobia that typically thrive in neutral pH. It was interesting that cyanobacteria were not well represented in any of the microbiomes. The plastid PCR blockers used by Zymo seemed to have depleted cyanobacterial reads since a closer inspection revealed the plastid PCR blocker to be identical to the 16S rRNA cyanobacterial gene (Molina pers. obs). The full ecological picture may not be appreciated until this missing taxonomic group is also characterized. Nonetheless, for the microbes that were identified, they revealed clues about *Rafflesia's* cryptic biology.

The endophyte's endophytes – *Rafflesia's* microbial partners: There were 4 phyla well represented in R's endophytic composition: Bacteriodetes, Proteobacteria, Firmicutes, Actinobacteriota. Members of Bacteroidetes were unique in R, especially in seeds obtained from the dehisced fruit. For example, *Dysgomonas* and *Bacteroides* were present in R but not in TR and T. *Dysgomonas* sp. is widely distributed in xylophagous insects such as termites and wood-eating cockroaches (Bridges and Gage 2021). The termite gut bacterial species *Bacteroides reticulotermitis* (Sakamoto and Ohkuma 2013) was identified in R. An unidentified species of *Bergeyella* (0.11%) was also found in R. The species *Bergeyella zoohelcum* is associated with mammalian animal bites (Chen et al. 2017). Interestingly, an unidentified member of the family Muribaculaceae, reported to be abundant in

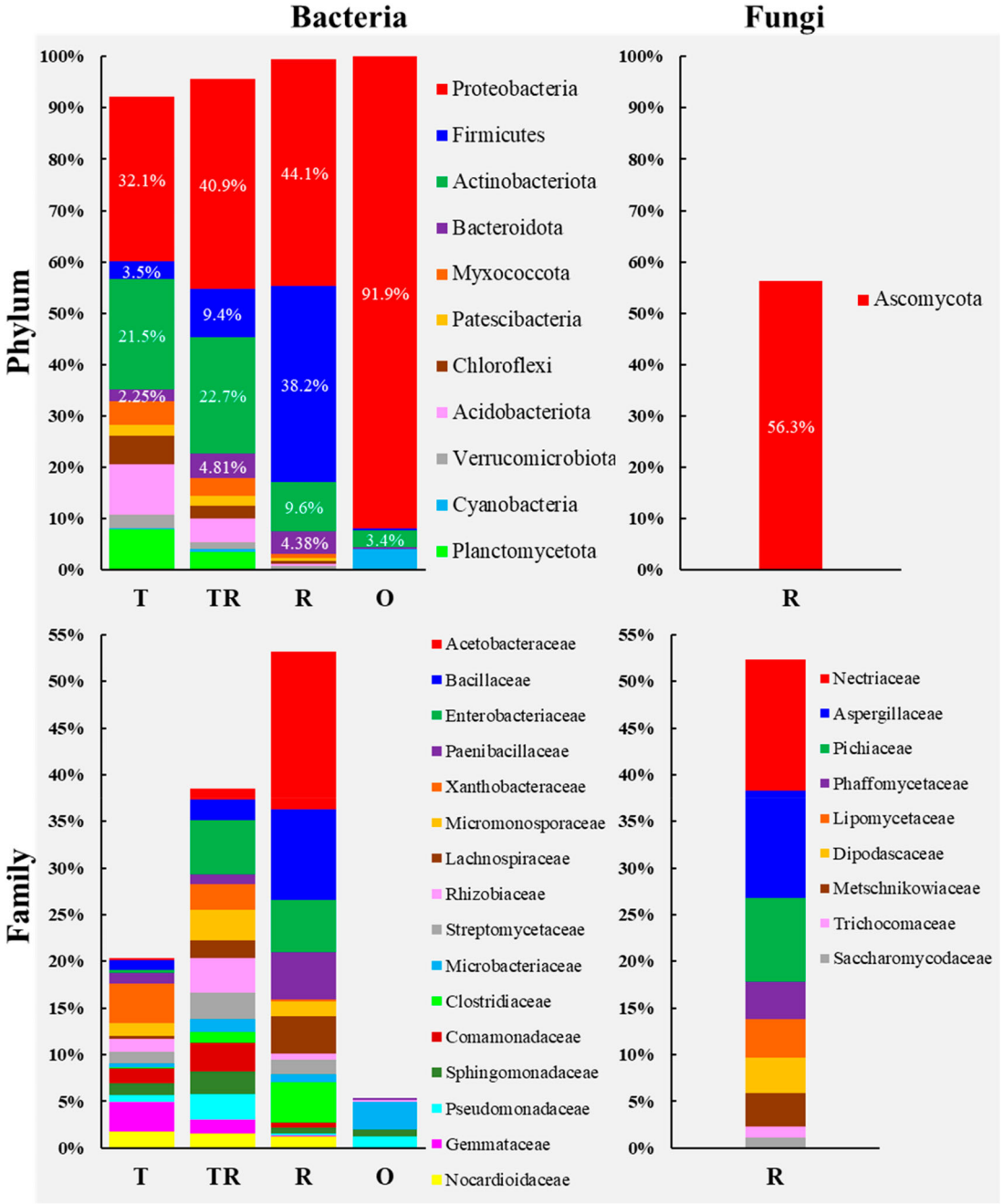

Figure 2. Relative abundance (>1%) of bacterial and fungal microbiota in sample averages based on phylum and family-level taxonomic classification. Data values for the top 4 most abundant phyla, relative to the R group are also displayed.

mouse gut intestines (Lagkouvardos et al. 2019), was also found in R. Both *Bergeyella* and Muribaculaceae were only detected in the seeds collected from the dehisced fruit, not from those in intact fruits. These findings could suggest animals, especially rodents, could have been feeding on the *Rafflesia* fruit. This finding bolsters the report by Bouman and Meijer (1994) that squirrels and tree shrews feed on *Rafflesia keithii* fruits and are seed dispersers, and that they possibly injure *Tetrastigma* vines with their claws as they also forage for worms and termites allowing *Rafflesia* seeds to penetrate. Seed endophytes may also be acquired through pollen (Ambika Manirajan et al. 2016) with *Rafflesia*'s carrion fly pollinators likely transporting microbes from a carcass, hence the recovery of microbes typically associated with mammals from *Rafflesia* seeds.

Within Actinobacteriota, *Micrococcus* was unique in R. A species of *Micrococcus, M. luteus,* was found to promote root branching in *Arabidopsis* and produced growth-promoting auxins in *Helianthus* (García-Cárdenas et al. 2022), but it is not clear what the ecological implication of this species in R. This genus is a typical seed endophyte (Truyens et al. 2015). One member, *Cellulomonas*, which is known for producing cell-wall degrading enzymes (Rajoka and Malik 1999) were enriched in both R and TR, but lacking in T, and may facilitate dissolution of *Tetrastigma* root surface to allow R to penetrate and infect. *Galbitalea* was also similarly present in R and TR but given this genus is a fairly recent discovery (Kim et al. 2014), its ecology is not well-understood.

Within Phylum Proteobacteria (Pseudomonadota), there were some taxa unique in R, while others were shared between R and TR. Interestingly, various phytopathogenic genera such as *Robbsia*, *Pectobacterium, Tatumella, Ralstonia,* and *Xanthomonas* were detected in R but not found in TR nor T. They infect plants by secreting cell-wall degrading

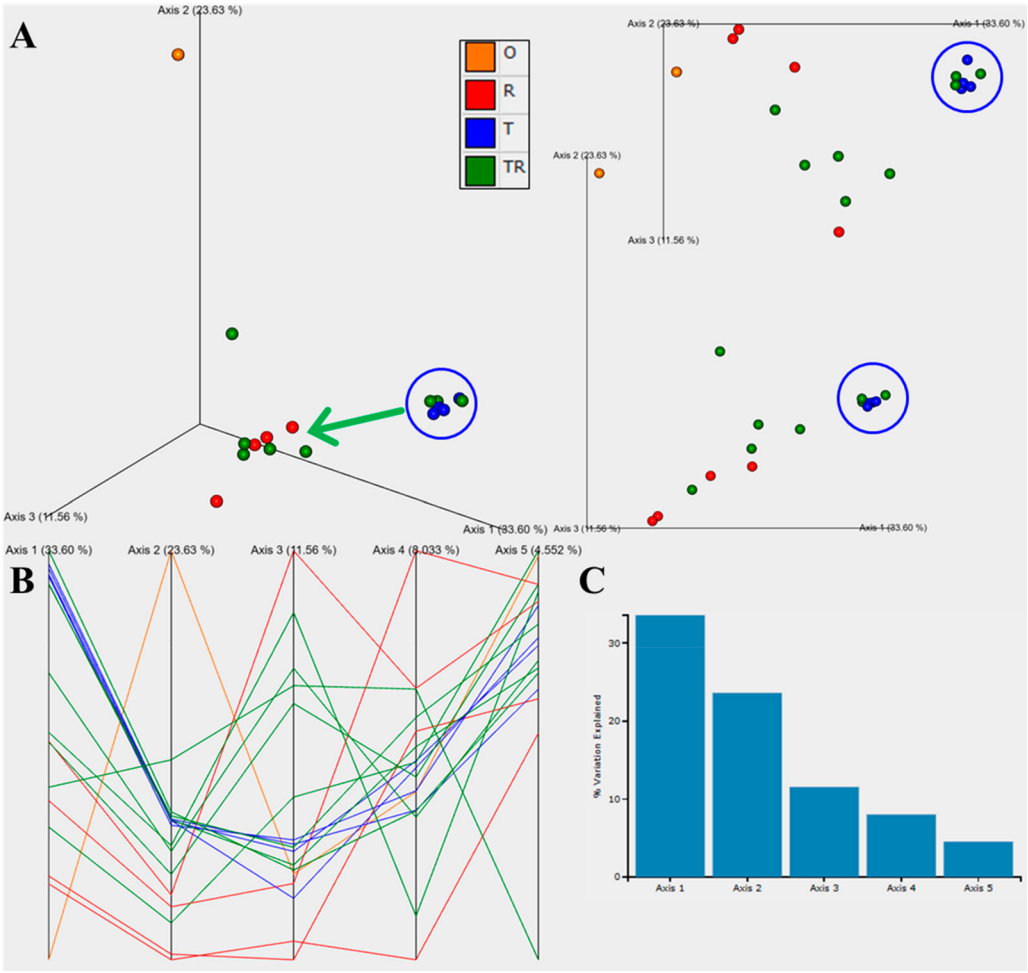

Figure 3. (A) Weighted UniFrac principal coordinates analysis (PCoA) emperor scatter plot at different views using the 3 major axes or coordinates (corresponding to 68.79% of features) shows clustering of the T group (blue circle) with 3 TR samples, while the green arrow indicates that 5 TR samples diverged mostly toward the R group. (B) Parallel plot using (C) the top 5 axes (81.37% of features) also displays the T clustering up to the 4th and 5th axes of some TR samples.

enzymes (cellulases, xylanases, pectinases) (Aguilar-Marcelino et al. 2020) which conceivably may be exploited by R allowing it to penetrate *Tetrastigma* host tissue. Interestingly, both *Ralstonia* and *Xanthomonas* were also found in O, which hints at the facilitatory ecological role of such phytopathogenic bacteria in host infection by holoparasites.

In addition to phytopathogens, there were also Proteobacteria unique in R that have plant-growth promoting properties (PGPP) such as *Frateuria* which increases plant potassium uptake (Subhashini 2015), and *Brevundimonas,* which is diazotropic and increases plant nitrogen uptake (Naqqash et al. 2020). Noteworthy is the abundance of acetic acid bacteria of Acetobacteraceae (8.5%) in R, low in TR (0.67%) and interestingly absent in T. *Acetobacter, Gluconobacter,* and *Gluconacetobacter* are acetic acid-producing, oxidative-fermenters, and nitrogen-fixing genera that have been reported to enhance plant growth (Sevilla et al. 1998; Tapia-Hernández et al. 2000; Reis and Teixeira 2015; Kandel et al. 2017; He et al. 2022). Enrichment of these bacteria in R also suggests that the seed environment is sugar-rich and acidic since the optimum pH range for these bacteria is 5.0-6.0, which could inhibit the growth of acid-intolerant bacteria, and conceivably facilitate a microbial consortium that thrives at acidic pH. Other Proteobacteria such as *Enterobacter, Burkholderia-Caballeronia-Paraburkholderia*, and *Kaistia* were significantly enriched in both R and TR but diminished/absent in T. Multiple *Enterobacter* and *Burkholderia* spp. have known PGPP (Angus et al. 2014), while not so much is known about *Kaistia's* ecology other than it may be parasitic and antagonistic to other bacterial cells (Duda et al. 2009). Their potential ecophysiological functions in *Rafflesia*'s development, if any, need to be demonstrated.

The most abundant phylum in R was Firmicutes (Bacillota), which was also the phylum that dominated in cucurbit seeds (Khalaf and Raizada 2016), primarily of class Clostridia. These are obligately anaerobic spore-formers and will thrive within the oxygen-deficient tissues of seeds (Thomas and Sahu 2021). Within Phylum Firmicutes, *Clostridium* spp. represented 4.2% of R bacterial composition. *Clostridium* has shown agroecological benefits including nitrogen fixation and phosphate solubilization making these nutrients more available to plants (Figueiredo et al. 2020). Another clostridial member enriched in R/TR was Lachnospiraceae NK4A136 which are gut/rumen microbes and can ferment plant polysaccharides to fatty acids and ethanol (Boutard et al. 2014; Wu et al. 2020). Clostridial species are butanediol producers, as are lactobacilli (O. Lactobacillales) (Sabra et al. 2011), another member of Firmicutes enriched in R/TR. Though butanediol is a bacterial volatile compound with PGPP (Sharifi and Ryu 2018), it is also secreted by phytopathogenic bacteria of phylum Proteobacteria (e.g. *Pectobacterium)* activating enzymes that break down plant host tissue (Effantin et al. 2011).

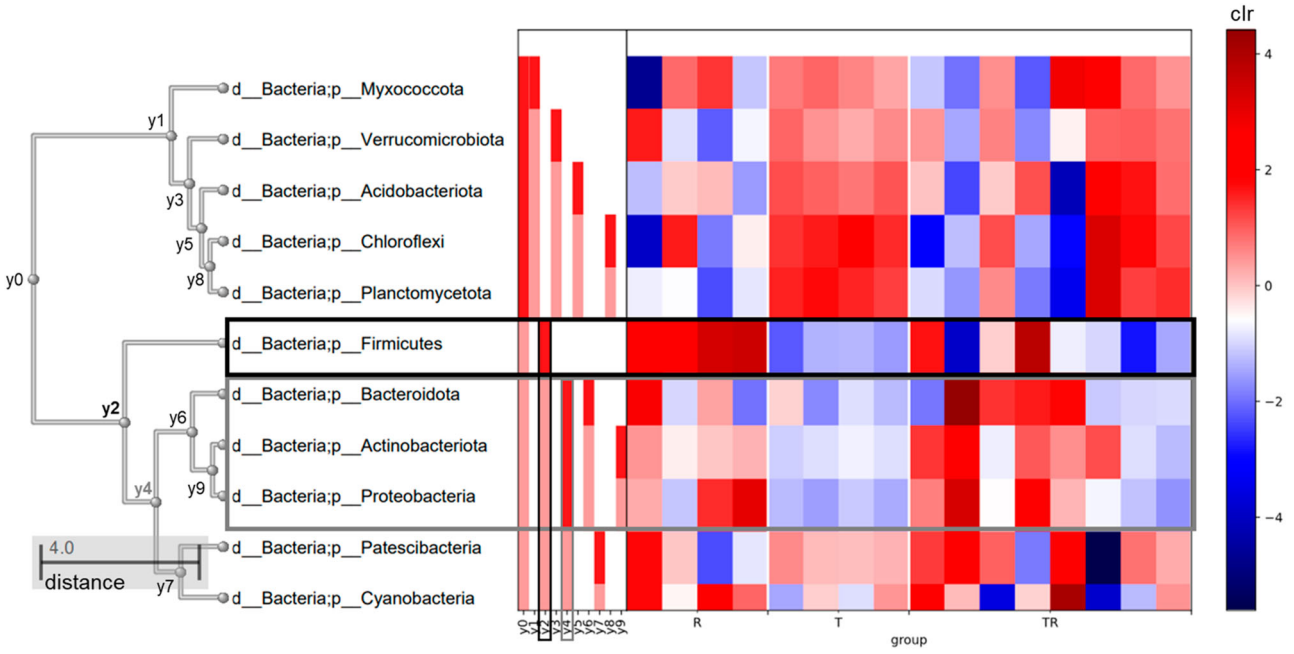

Figure 4. Gneiss balance (y-denominator, dark red bars from y0-y9) differential abundance dendrogram-heat map of top-identified phyla with distance values of individual samples in group's (R, T, and TR) relative frequency center log ratio (clr) transform. The 2nd (y2) and 4th (y4) hierarchy branches showed increased proportion of Firmicutes (black box) and 3 phyla (Bacteriodota, Actinobacteriota, and Proteobacteria; gray box), respectively, in groups R and TR compared to T.

The abundant presence of a diverse consortium of acidophiles (Proteobacteria: Acetobacteraceae, Firmicutes: Clostridia) provides the chemical milieu that perhaps allowed the co-existence of members of Lactobacilli, which are typically rare as plant endophytes. However, they can increase in number when carbohydrates are released as plant tissues are damaged (Duar et al. 2017), Thus, it is conceivable that the R seeds and/or the surrounding pulp were undergoing fermentation when collected perhaps resulting from activities of the abundant Acetobacteraceae (He et al. 2022), as well as from the various clostridia species, consequently providing the sugars encouraging lactobacilli growth. *Lactobacillus* spp. have been reported to have PGPP (Jaffar et al. 2023) enhancing plant tolerance to pH changes (Msimbira and Smith 2020), protecting plants from pathogenic microorganisms (Liu et al. 2019), and solubilizing phosphates (Khalaf and Raizada 2016). Other unique Firmicutes bacteria in R include *Monoglobus*, mesophilic bacteria in Therminoactinomycetaceae, as well as *Geobacillus* and *Anoxybacillus*. They were of low abundance (0.1-0.4%) and their ecological roles are unclear.

***Rafflesia* seed mycobiome: fungal friends, foes, or just opportunists?** We also characterized the fungal taxa within two accessions of *Rafflesia speciosa* seeds to determine if there are mycorrhizal taxa. The majority of fungal taxa classified were Ascomycota, but incredibly, 43.6% were unclassified even at the phylum level. The most abundant fungi were yeasts of Saccharomycetales (27.6%), followed by Hypocreales (14.9%), then Eurotiales (12.7%), which were also found as endophytes in the mandarin orange, *Citrus reticulata* (Sadeghi et al. 2019), as well as in various crops (Xia et al. 2019) suggesting that these fungal orders are typical endophytes.

At the generic level (Figure 2), the most abundant fungi (at least 5% each) in R were *Penicillium, Aspergillus*, and *Fusarium* which collectively represented c. 20% of the fungi composition of the seeds. These genera have been reported to be either saprotrophs, phytopathogens and/or plant mutualists in the literature (Zakaria and Ning 2013; Toghueo and Boyom 2020; Jing et al. 2022). It has been hypothesized that some fungal endophytes may be latent saprotrophs that switch to this new ecological role upon host senescence (Promputtha et al. 2007).

Fusarium, which is a common plant endophyte, was also reported in the pistil stigmas of the holoparasite *Orobanche* (Ruraż et al. 2023). In a study of the endophytic fungal diversity of the flower of *Rafflesia cantleyi*, *Gliocladiopsis* was isolated (Refaei et al. 2011), which was also detected in R in our study, though at lower abundance (<5%). Though *Gliocladiopsis* spp. has been recovered from diseased plants, they are considered as secondary pathogens or saprobes, and may even have benefits to their host plant reported in a study of inoculated avocado roots (Lombard and Crous 2012). Typical arbuscular mycorrhizal taxa (of c. Glomeromycota) as well as basidiomycete symbiotic genera of mycoheterotrophic orchids were not detected in the *Rafflesia* seed, which lends support to a recent genomic study that did not find mycorrhizal symbiotic genes in *Rafflesia* seeds (Molina et al. 2023). While it may be possible that there are ectomycorrhizal taxa among the unclassified fungi given the expansive taxonomic breadth of ectomycorrhizal fungi (Tedersoo and Smith 2013), we now have multiple lines of evidence that mycorrhizal fungi do not play a role in *Rafflesia* infection. Other less abundant genera recovered in R included *Talaromyces, Wickerhamomyces, Hanseniaspora,* which have been reported to have mutualistic associations with plants (Rabosto et al. 2006; Sahu et al. 2019; Poitevin et al. 2020), including production of hydrolytic enzymes to control plant pathogens, auxin and siderophore synthesis, as well as phosphate solubilization.

There were some genera in R that were mycoparasitic (i.e. parasitize other fungi) such as *Clavispora* (Pereyra et al. 2020) and *Pichia* (Agrios 2005), as well as entomopathogenic (i.e. parasitize insects) such as *Arthrobotrys* (Barron 2004)

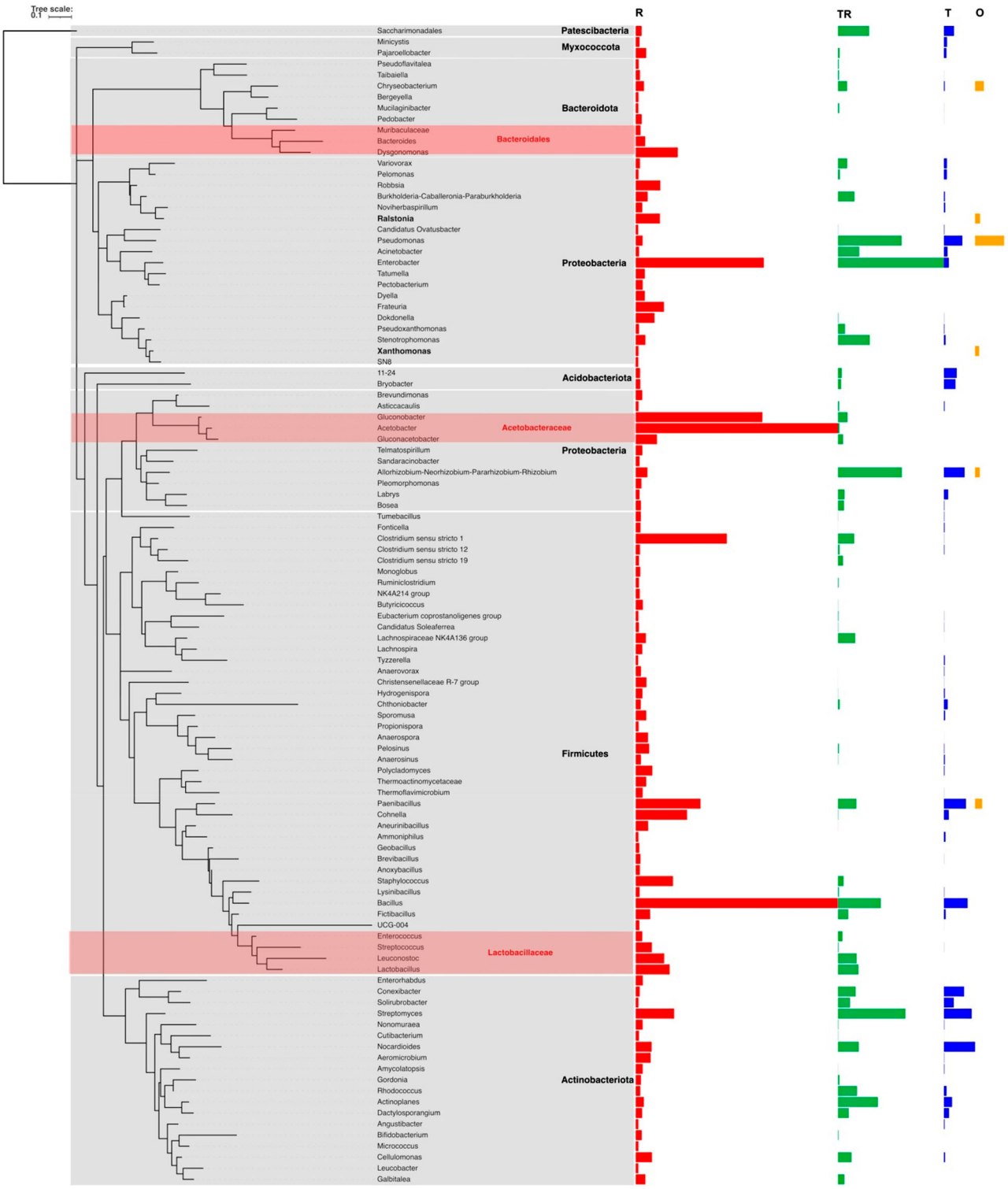

Figure 5. Phylogeny of the bacterial endophytes in R (>0.1%), with bacterial proportions per genus as colored bars with length proportional to abundance. Note that Proteobacteria is not monophyletic based on the 16S rRNA (V3-V4 region). R (red): proportion in *Rafflesia* seed; TR (green): infected host; T (blue): uninfected host; O (orange): *Orobanche*. Seven unknown genera were not included. The taxonomic groups Bacteroidales, Acetobacteraceae, Lactobacillales were enriched in R and/or TR, but depleted in T. The genera *Ralstonia* and *Xanthomonas* were only present in both R and O.

and *Purpureocillium* (Castillo Lopez et al. 2014). Upon maturity, the *Rafflesia* fruit dehisces, revealing a coconut-scented white pulp holding the millions of *Rafflesia* seeds (Molina et al. 2017). The mesocarp is very attractive to various insects (beetles, flies, ants, etc.) that feed on the pulp and/or lay eggs in it (Molina, pers. obs), which would explain the presence of opportunistic entomopathogenic fungi in R.

There were also many fungi in the *Rafflesia* seed that were classified as phytopathogenic and presumably destructive to *Rafflesia's Tetrastigma* host. Phytopathogens have convergently evolved to produce cell-wall degrading enzymes like pectinase, cellulase, and proteases to dissolve plant host tissue (Uchiyama et al. 2020). Interestingly, these are also enzymes produced by parasitic plants of Orobanchaceae (Yang et al. 2015). Such enzymes have also been reported in many plant-associated fungi (Lebeda et al. 2001) in addition to amylases, lipases and laccases (Hawar 2022; Raghav et al. 2022), the genes of which were also identified in the seed transcriptome of *Rafflesia speciosa* (Molina et al. 2023). The prevalence of a diverse

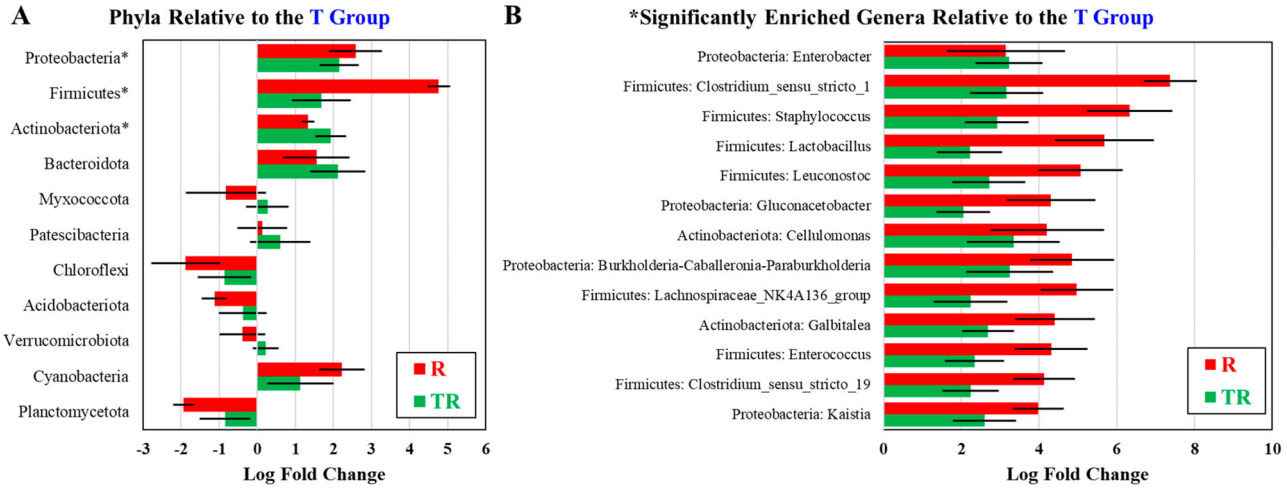

Figure 6. ANCOM-BC of differential abundances log fold change (LFC) of bacterial (A) top phyla (with *statistically higher for both R and TR groups), and (B) statistically-elevated genera of R and TR groups compared to the T group, sorted based on relative abundance to the R group.

community of phytopathogens in *Rafflesia* seeds is notable, and understanding their ecophysiological roles in the context of facilitating *Rafflesia* infection and reproductive success is needed.

Holoparasite microbiomes – do phytopathogens play a role? We also compared the *Rafflesia*-associated microbiome with that of *Orobanche*, another holoparasite but unrelated to *Rafflesia,* to determine if there are bacterial endophytes common to holoparasites that may be relevant for plant parasitism. Though we were only able to include one sample of *Orobanche* seeds, it was apparent this had a very different microbial composition compared to *Rafflesia* (Figure 2), being enriched in Microbacteriaceae, in particular, *Curtobacterium*, which was absent in R. *Curtobacterium* was one of the dominant endophyte in the seeds of *Cistanche armena*, another Orobanchaceae holoparasite, though the ecology of this bacterial genus in these parasites is unknown (Petrosyan et al. 2022). Though most species of *Curtobacterium* are not considered pathogenic, there is one species that can infect a variety of legumes and ornamental plants facilitated by plasmids carrying genes for cellulases, pectate lyase, serine proteases – again hydrolytic enzymes. Interestingly, some of these genes are homologous to those found in *Xanthomonas* spp., exemplifying convergent evolution among phytopathogens (Evseev et al. 2022). Though *Curtobacterium* was not detected in R, *Xanthomonas*, as well as the related phytopathogen *Ralstonia,* were found in common in *Orobanche* and *Rafflesia* seeds sampled here, but not in any of the *Tetrastigma* samples. It is thus tempting to speculate that holoparasites may be exploiting the cell-wall degrading enzymes secreted by these phytopathogens (Agrios 2005) to infiltrate host tissue.

Members of bacterial genera, including *Pseudomonas, Chryseobacterium, Paenibacillus* and *Allorhizobium* were also found in both R and O, as well as in *Tetrastigma* samples. These genera were also recovered from the seeds of *Cistanche* (Petrosyan et al. 2022). Some of these bacterial genera were also found in the microbiome of roots of other holoparasitic plants including *Orobanche* spp. (Fitzpatrick and Schneider 2020), *Cistanche* spp. (Miao et al. 2023) and *Langsdorffia* spp. (Felestrino et al. 2017). Their ubiquitous presence in many plants, regardless of taxonomy or habitat, suggests their possible involvement in synergistic interactions that may ultimately promote plant growth (Eid et al. 2021; Vandana et al. 2021; Yin et al. 2021; Gómez-Godínez et al. 2023). In all these microbiome studies of holoparasitic plants and their hosts, researchers observed that host and parasite tend to share similar microbial composition, compared to unparasitized host plants, with the parasite's microbiome derived from that of the host. Fitzpatrick and Schneider (2020) noted that the relative abundance of members of Burkholderiales was strongly correlated between *Orobanche* and its host. This group was also significantly enriched in R/TR but not in T, represented by the *Burkholderia-Caballeronia-Paraburkholderia* complex, which includes plant pathogens as well as environmental/plant-growth promoting species (Sawana et al. 2014).

Conclusion: clues and cues from *Rafflesia's* microbiome: In this study we found evidence that during reproductive development, the *Rafflesia speciosa* seed acquires certain host bacteria such as fermenting acidophilic butanediol-producing bacteria (e.g. Clostridia, Lactobacilli) from its infected host, but at the same time acquires unique bacterial taxa (e.g. Acetobacteraceae, Bacteroidales, phytopathogens) from biotic associates of the fruit. This may have ecological consequences when the seed infects a host and may potentially alter the parasitized host's microbiome compared to uninfected roots. Whether this is a strategy to facilitate a *Rafflesia* infection remains to be seen.

The full ecological picture is still fragmentary but seems to support a scenario of *Rafflesia* seeds packaged with butanediol-producing bacteria and phytopathogenic hitchhikers and dispersed by rodents. Though typical mycorrhizal fungi were not detected in R, at least 40% of the R seed mycobiome have yet to be characterized, and it is conceivable that they modulate bacterial constituents and vice versa. When the *Rafflesia* seed lands on a suitable *Tetrastigma* host, hydrolytic enzymes secreted by its microbiome purportedly dissolve host tissue facilitating *Rafflesia* germination. The *Rafflesia* seed, after imbibition, is transcriptionally and metabolically active, seemingly primed for germination upon stimulation – not just from the *Tetrastigma* host (Molina et al. 2023), but from its holobiont as well. Hypothetically, the susceptible host harbors microbes that interact with those of *Rafflesia* promoting and sustaining an infection.

Future seed germination experiments should explore the application of butanediol and inoculation of Clostridia, lactobacilli, Acetobacteraceae as well as hydrolytic enzymes in the presence of *Tetrastigma* roots. It is also worthwhile to understand how the microbiome changes in the *Rafflesia* bud, which is intimately connected to the host, unlike the seed, to determine whether any of the *Rafflesia* seed microbiome persists, as well as identify new microbial colonists that may promote *Rafflesia* flower development. Our study reinforces the notion that the holoparasite and its host plant are a conjoined microcosm of microbes whose ecophysiological functions in maintaining the holobiont may be important but largely understudied. To succeed in propagating the 'panda of the plant world', cultivating the *Rafflesia* holobiont – the endophyte and its endophytes – may be key.

Author contributions

JM conceived the project. RP, DT, JC, SJ, ME collected samples with JM. RCG, AW, TM, D. Diaz, CL, HM, FK, AE, PA, MB, and JM analyzed the data. JM drafted the manuscript with all authors editing and approving the final manuscript.

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