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Microbes and metabolites of a plant-parasite interaction: Deciphering the ecology of *Tetrastigma* host choice in the world's largest parasitic flower, *Rafflesia*

Jeanmaire Molina [a,*], Roche C. de Guzman [b], Rinat Abzalimov [c], Wenkai Huang [a], Anusha Guruprasad [a], Ronniel Pedales [d], Adhityo Wicaksono [e], Destiny Davis [f], John Rey Callado [g], Hans Bänziger [h], Piyakaset Suksathan [i], William Eaton [a], Pride Yin [a], Marco Bürger [j,k], Mick Erickson [f], Stephen Jones [f], James Adams [f], Susan Pell [f]

[a] *Biology Department, Pace University, New York, NY, USA*
[b] *Bioengineering Program, Department of Engineering, Hofstra University, Hempstead, NY, USA*
[c] *Biomolecular Mass Spectrometry Facility, Advanced Science Research Center, City University of New York, New York, NY, USA*
[d] *Institute of Biology, University of the Philippines, Diliman, Quezon 1101, Philippines*
[e] *Genomik Solidaritas Indonesia (GSI) Lab, Jl. Sultan Agung No.29, Setiabudi, Jakarta 12980, Indonesia*
[f] *US Botanic Garden, Washington, DC, USA*
[g] *Department of Science and Technology-Forest Products Research and Development Institute, Laguna, Philippines*
[h] *Department of Entomology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand*
[i] *Herbarium (QBG), Queen Sirikit Botanic Garden, P.O. Box 7, Mae Rim, Chiang Mai 50180, Thailand*
[j] *Plant Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA, USA*
[k] *Howard Hughes Medical Institute, Salk Institute for Biological Studies, La Jolla, CA, USA*

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ABSTRACT

Rafflesia, known for producing the world's largest flowers, is a holoparasite found only in Southeast Asia's rapidly diminishing tropical forests. Completely dependent on its *Tetrastigma* host plants, *Rafflesia* grows covertly within its host until flowering, but the ecological factors driving host susceptibility are unknown. With most *Rafflesia* species on the brink of extinction due to habitat loss, understanding the complex ecological interactions between *Rafflesia* and its host is crucial for conservation. In this study, we integrated metagenomic data with metabolomic profiles to identify potential functional relationships between microbial communities and specific metabolites, shedding light on their ecological roles in *Rafflesia's* life cycle. Key findings reveal that microbial taxa such as Microbacteriaceae and Nocardioidaceae correlate with elevated levels of polyphenols, particularly gallic acid derivatives, which may shape the chemical environment conducive to *Rafflesia* development. Complex-carbon-degrading bacteria thrive in the chemically distinct environment of *Rafflesia* buds, while an unknown group of Saccharimonadales was enriched in *Tetrastigma* host species. Docosenamide production in *Rafflesia* buds and their hosts may facilitate parasitic infection, while coumarin compounds in non-host *Tetrastigma* species may exert allelopathic effects. The enrichment of gallic acid derivatives, the phytohormone adenine, and gall-associated bacteria suggests that *Rafflesia* buds may function similarly to plant galls, manipulating host tissues to support their reproductive development. This study highlights the dynamic microbial shifts during *Rafflesia*'s development, emphasizing its symbiotic relationship with microbial communities and hosts. In identifying essential microbial and chemical conditions that could improve propagation techniques, this research has practical applications in ex situ conservation efforts, aiding in the rescue of the world's largest flowers from the brink of extinction.

* Corresponding author.
E-mail address: jmolina2@pace.edu (J. Molina).

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1. Introduction

Producing the largest flowers in the world, up to a meter in diameter, *Rafflesia* is considered the "greatest prodigy of the vegetable world [12]", yet it is a holoparasite lacking stems, roots, leaves. All 40 + of *Rafflesia* spp., even their closest relatives, *Rhizanthes* and *Sapria,* within Rafflesiaceae are only found in the rapidly deteriorating tropical forests of Southeast Asia. Entirely dependent on their sole host, the genus *Tetrastigma* (Vitaceae), species of Rafflesiaceae grow inside the host until ready to flower [53]. The evolution of this intimate relationship may have created the conditions leading to the loss of *Rafflesia's* chloroplast genome [47], prompting the question, "When is a plant no longer a plant?" [61]. This finding, initially controversial, was confirmed in confamilial *Sapria,* with no detectable plastome [13]. Extensive horizontal gene transfer (HGT) has been observed between parasite and host with transferred host genes remarkably functional in the parasite [13, 85,86]. However not all *Tetrastigma* spp. appear to serve as hosts. There are only 11 of the c. 95 species of *Tetrastigma* that have shown evidence of Rafflesiaceae infection [16], though this number may have been underestimated because of cryptic *Tetrastigma* spp. [5]. However, the features that drive host susceptibility in *Rafflesia* remain elusive. There is also no phylogenetic evidence for co-speciation in that *Tetrastigma* species that host multiple *Rafflesia* species are parasitized by more distantly related *Rafflesia* species [57].

Growing surreptitiously as minute strands of undifferentiated cells within the host plant's vascular cambium and developing into a network of clonal clusters, the vegetative stage of *Rafflesia* can last for years [8]. As it transitions to reproduction, each cluster may form a protocorm with rudimentary vascular tissues. The protocorm triggers host defenses but continues to grow, eventually developing into a floral bud [53]. Bud mortality is high (>90 %), but surviving buds bloom into large, distinct flowers. Most *Rafflesia* species produce unisexual flowers, with a higher ratio of males to females, although some species produce bisexual flowers [3]. The flowers emit odors that attract carrion flies for pollination. Successful pollination results in a fruit that sheds millions of seeds with seed dispersal possibly facilitated by small mammals and ants [52,59].

Population genetic studies have shown *Tetrastigma* vines infected by numerous closely related *Rafflesia* individuals [58,60], and some single vines hosting 25 buds from the same *Rafflesia* individual [7]. Pollination seems to ensure some outcrossing in certain *Rafflesia* species [7]. These studies highlight the detrimental consequences of the destruction of individual host plants and the importance of maintaining connectivity through biological corridors between host plants [78]. Thought to be extinct until it was rediscovered in 2006, the largest flower in the Philippines reaching 80 cm wide, *R. schadenbergiana* is only known from less than 5 populations ([6]; M. Tabamo and J. V. Cruz, pers. comm). One population residing within a single host plant further reinforces the importance of conservation efforts.

All *Rafflesia* species are endangered, with most on the brink of extinction due to habitat loss from deforestation [44]. In Indonesia, *Rafflesia* was previously harvested for ethnobotanical uses [51]. Anthropogenic causes, compounded by high bud mortality, disproportionate number of male flowers, unknown process of seed germination all contribute to *Rafflesia's* endangered status. The ex situ propagation of *Rafflesia* has largely been unsuccessful, despite numerous attempts. The most promising method to date involves grafting infected *Tetrastigma* plants onto uninfected rootstocks. The only documented success has been at Indonesia's Bogor Botanic Garden (BBG), where grafted *Tetrastigma* cuttings have produced multiple blooms of *Rafflesia patma* which have been made available for public viewing [51,82]. More recently, however, a blooming *Rafflesia arnoldii* flower was reported in 2022 at BBG, presumably the result of a seed inoculation experiment conducted several years prior (S. Mursidawati and D. Latifah, personal communication, January 5, 2023). This marks the first confirmed success of ex situ propagation from *Rafflesia* seed, building upon early work by Teijsmann [74], whose 19th-century experiments demonstrated propagation of *Rafflesia arnoldii* by inserting seeds into incisions made in the bark of host roots, resulting in *Rafflesia* developing at varying distances from the inoculation site. These advancements provide valuable insights into the potential for *Rafflesia* cultivation.

Rafflesia and its relatives have yet to be successfully cultivated in a Western botanical garden, resulting in missed opportunities for public education and conservation awareness. Since 2015, Molina et al. [48] have been transporting viable *Rafflesia*-infected *Tetrastigma* cuttings from the Philippines to the US Botanic Garden (USBG) in Washington D. C. for propagation. However, efforts to propagate these cuttings at the USBG have been largely unsuccessful, as the cuttings failed to produce shoots and eventually died. Uninfected *Tetrastigma* host vines have been inoculated with *Rafflesia* seeds since 2017, but no buds have emerged. Germination experiments using various phytohormones for induction have also proven futile [48]. To better understand *Rafflesia's* seed biology, its seed transcriptome was sequenced for clues to its germination. The *Rafflesia* seed transcriptome revealed genes responsive to germination-related compounds like laccase, karrikin, and ethylene, which could be prioritized in stimulating germination. Unlike some species with similar "dust seeds" lacking endosperm, the *Rafflesia* seed transcriptome showed no expressed genes involved in mycorrhizal association [50]. Given the uniqueness of *Rafflesia's* life cycle, a better understanding of its biology and symbiotic ecology is crucial for successful cultivation.

A recent study by Molina et al. [46] began to identify the complex microbiome interactions between *Rafflesia* and its host, providing information on the role of endophytic microorganisms living within the plant tissues in influencing plant health and development. Holoparasites harbor a diverse microbiome, yet the functions of these microbial communities have largely remained unexplored. In other holoparasites, such as *Langsdorffia hypogaea* and *Phelipanche* spp., specific endophytes have been found to produce hormones, inhibit pathogens, and enhance host resistance to parasitism [23,32]. Characterization of the bacterial microbiome in *Rafflesia speciosa* seeds and *Tetrastigma* cuttings revealed that *R. speciosa* seeds have bacteria in common with their infected host, suggesting that the seeds may sequester certain host bacteria while also acquiring unique bacterial taxa from biotic associates of the fruit [46].

A comparison of metabolites in the shoots of *Rafflesia*-infected and non-infected *Tetrastigma loheri* provided further insights into their symbiotic chemistry. LC–MS-based untargeted metabolomics analysis showed that benzylisoquinoline alkaloids were more abundant in uninfected shoots, marking the first report of these metabolites in *Tetrastigma* and the grape family Vitaceae [49]. These alkaloids are implicated in plant defense mechanisms and may prevent *Rafflesia* infection. In contrast, *Rafflesia*-infected shoots exhibited elevated levels of oxygenated fatty acids (oxylipins) and a flavonoid associated with plant immune response [48]. These findings suggest that *Rafflesia* infection triggers specific metabolomic changes in *Tetrastigma.*

The conservation of *Rafflesia* species requires a multifaceted approach that integrates habitat preservation, innovative propagation techniques, and a better understanding of ecological interactions between *Rafflesia* and *Tetrastigma* host spp. It is unknown what host metabolites could facilitate a *Rafflesia* infection [49]. It is suspected that *Tetrastigma* host spp., possess (or lack) certain metabolites that make them vulnerable to *Rafflesia* infection, compared to *Tetrastigma* non-host spp. It is also hypothesized that there are specific microbiota in host and parasite that may facilitate symbiosis. By enhancing our understanding of these interactions, we can develop more effective strategies to ensure the survival of these remarkable plants.

2. Materials & Methods

2.1. Sampling

Two species systems of *Rafflesia-Tetrastigma: R. lagascae* and

R. speciosa and their associated host spp. as well as sympatric non-host species were collected from the Philippine localities of San Lorenzo Ruiz, Camarines Norte (CAM), and Miagao, Iloilo (ILO), and exported to the US (with all necessary permits from the Philippine Department of Natural Resources). *Rafflesia speciosa* is endemic to the Negros and Panay islands of the Philippines and is known to infect only two host species, *T. harmandii* and *T. cf. magnum* (T), even when sympatric with *T. loheri* and *T. spA* [57]. Additionally, *T. loheri* and *T. spA* (T) are the host species of *R. lagascae,* another Philippine endemic but restricted to Luzon island. *Tetrastigma scariosum*, *T. papillosum,* and a reddish morphospecies of *T. aff. loheri* are *Tetrastigma* spp. that have never been observed to support a *Rafflesia* infection and are considered "non-hosts" (Tn). These *Tetrastigma* species described are widespread throughout the Philippines. Analogous samples of *Sapria himalayana* buds (two) and their associated *Tetrastigma* spp. (three infected and one uninfected but none for non-host) from Queen Sirikit Botanic Garden (QSBG), Chiang Mai, Thailand (THA) were also collected with permission from the National Research Council of Thailand (NRCT) [Fig. 1]. Fifty-three (53) plant tissue samples collected from eight *Rafflesia speciosa* seeds (R), 14 *Rafflesia/Sapria* flower buds (RT), 14 cuttings of Rafflesiaceae-infected host *Tetrastigma* spp. (TR within five cm of flower bud), 12 uninfected host spp. (T), and five cuttings of non-host *Tetrastigma* spp. (Tn) [Fig. 2] were outsourced for microbiome sequencing (ZymoBiomics, Irvine, CA) and metabolite profiling (Advanced Science Research Center, CUNY).

Samples were processed for microbiome metagenomics sequencing following methods in Molina et al. [46] and for metabolic profiling (except for THA samples given limited sampling), following methods in Molina et al. [49]. Samples for microbiome metagenomics sequencing were surface-sterilized with 2 % sodium hypochlorite, and without rinsing, sent immersed in 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). Samples for metabolic profiling were standardized to a concentration of 0.05 mg per microliter (mg/μL) during methanol extraction. THA samples were excluded because of limited material. Samples were prepared for injection by reconstituting in 0.3 mL (v/v) of 1:1 (v/v) MeOH/water. Samples were analyzed using a Bruker Daltonics maXis-II UHR-E-SI-QqTOF mass spectrometer coupled to a Thermo Scientific Ultimate-3000 UHPLC system following methods in Molina et al. [49].

2.2. Metagenomics analysis

FASTQ sequences were processed in QIIME2 [9] using WSL2, VS Code, and Excel (Microsoft), Galaxy (usegalaxy.org), and MATLAB (Mathworks), similarly based on our earlier paper [46]. Briefly, after demultiplexing and DADA2 denoising to acquire the feature table and

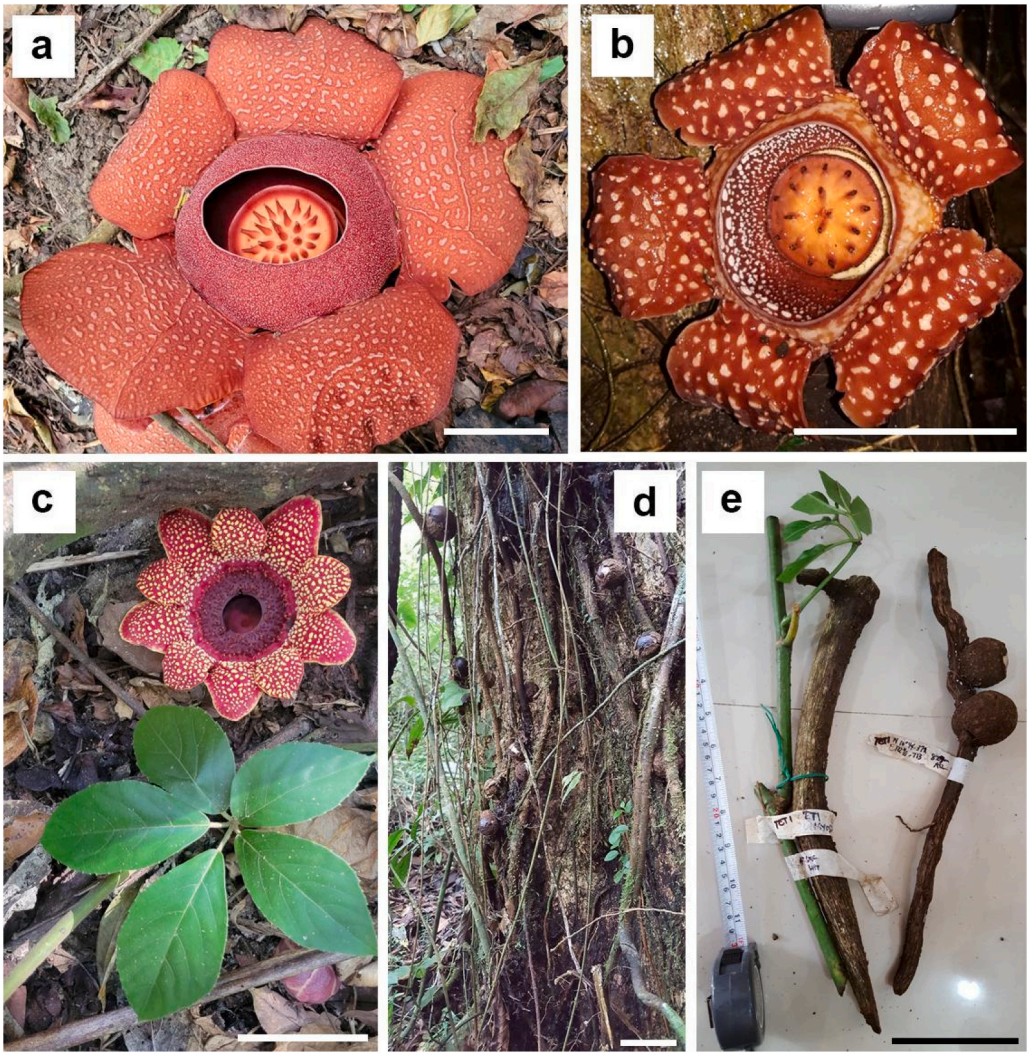

Fig. 1. Species sampled in this study. *Rafflesia speciosa* (a), *R. lagascae* (b), *Sapria himalayana* and its host *Tetratigma obovatum* (c), infected *T. loheri* aerial stems with *R. lagascae* in its natural habitat (d), uninfected shoot and infected *T. magnum* root with *R. speciosa* (e). Scale bars = 10 cm.

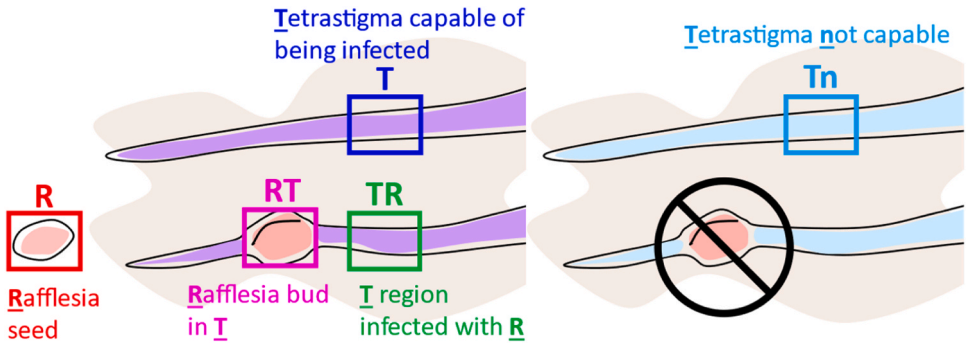

Fig. 2. Sampling represented as 5 colored groups, namely, *Rafflesia*: seed (**R**) and bud (**RT**) in its host, and *Tetrastigma*: infected with *Rafflesia* (**TR**), capable of being infected (**T**), and non-host or found incapable of supporting *Rafflesia* (**Tn**).

representative sequences, taxonomic classification was performed using study amplicon-refined reference sequences and Silva 13.8 sklearn classifier, and samples merged together. Taxa levels were collapsed and exported to acquire OTUs with corresponding bacterial counts, then processed for relative frequencies (abundance). Analysis of composition with bias correction (ANCOM-BC) [42] was employed for differential abundance, comparing T vs. Tn groups (and other pairs) with q value (false discovery rate (FDR)-corrected or adjusted p value) < 0.05 deemed statistically significant. Phylogeny reconstruction used a pipeline (MAFFT, masking, FastTree, and midpoint-rooted). Diversity analysis was conducted with a sampling depth of 5000 counts: alpha and beta reported Shannon's and Faith's, and Bray-Curtis and weighted UniFrac tests (without and with phylogenetic data). Kruskal-Wallis and permutational multivariate analysis of variance (PERMANOVA) pairwise tests were performed, with q value < 0.05 assigned for statistical significance. For visualization of central tendencies: bar graphs represent mean abundances, while boxplots (bar and whisker) show median-inclusive quartiles (Q) 1–3 (box) with Q2 = median (horizontal line), Q0 and Q4 as minimum and maximum, respectively (whiskers), mean as ×, and outliers as circles or separate points that fall outside the 150 % of the interquartile range (IQR = Q3 - Q1). The emperor plot was generated using QIIME2 view (view.qiime2.org).

2.3. Metabolite Analysis

All spectra were processed using Metaboscape-2023b software (Bruker Inc, USA). The software supports workflows for comprehensive analysis of LC-MS based un-targeted metabolomics data from identification of the observed ions to advanced statistics. The raw data files (.d) were converted into.CSV files, which included details on retention times, peak intensities, and m/z (mass-to-charge) ratios. In MetaboScape, "intensity" refers to the signal strength or abundance of a detected compound as measured by the mass spectrometer, represented by the peak height or area in the data. This metric helps quantify the relative concentration of metabolites across samples, allowing for the comparison of specific metabolite levels under varying experimental conditions. Higher intensity indicates a greater abundance of an ion, while lower values reflect a lower concentration. Several mass-spectral databases including Bruker's MetaboBASE Personal library-3.0, open-community mass spectra repository MassBank of North America, HMDB metabolite library, and in-silico fragmentation algorithms available to Bruker's MetaboScape and Sirius software [19] were used to identify metabolites.

3. RESULTS

3.1. Metagenomics analysis

After denoising and valid data selection in QIIME2, the number of samples and average bacterial counts obtained are summarized in Table 1. A representative taxa bar graph displays all the 53 samples of the 5 groups (R, RT, TR, T, and Tn, Fig. 3). The diversity analyses (Table 2) indicate that *Rafflesia* seeds (R) and buds (RT) have distinct microbial community compositions compared to *Tetrastigma* plants (T, TR, Tn). There was no significant difference between TR and T communities implying that the core microbiome of *Tetrastigma* remains relatively stable, even when infected. The lack of significant differences between Tn (non-host) and RT might suggest that *Rafflesia* buds, despite being embedded within a host, share similarities with non-host *Tetrastigma* species, though this finding could also be a statistical artifact due to the small sample size of Tn. The clustering pattern observed (Fig. 4**)** supports the representative taxa results (Table 2**)** showing R microbial community composition as distinctly separate from the other groups, while RT communities appear as a transitional state. The variability observed in the communities from the RT samples may reflect differences in how *Rafflesia* buds integrate or interact with the host microbiome. Meanwhile, the close clustering of T and TR sample communities across localities suggests that *Rafflesia* infection does not significantly disrupt the core microbiome of *Tetrastigma.*

The mean phyla abundance frequencies of the *Rafflesia* seed (R) illustrated a pattern that is qualitatively different from the *Tetrastigma* groups (Ts): TR, T, and Tn; while the abundance frequencies of *Rafflesia* bud in *Tetrastigma* (RT) group seemed to be intermediate between R and Ts for select bacteria (Fig. 5**a**). More specifically, this trend is clearly displayed when comparing the phyla Acidobacteriota and Planctomycetota (Fig. 5**b**), which were greatly reduced in R, at ~5 % in Ts, and at ~2 % in RT. At the genus level, the pattern is also apparent for the 14 most abundant genera (Fig. 5**c**), particularly at the following family/genus: Paenibacillaceae/*Paenibacillus*, Micromonosporaceae/, Xanthobacteraceae/, Streptomycetaceae/ *Streptomyces*, Mycobacteriaceae/ *Mycobacterium*, and Gaiellales (order) trending with relatively higher values for Ts, low for R, and intermediate amounts for the RT group (Fig. 5**d**). Moreover, RT was found to have the highest amount of outliers or with high variance/variability (Fig. 6**a-b**).

Some genera were statistically greater in T and TR compared to small proportion/absence in Tn. Abundances data in TR are generally comparable to T (Fig. 7). An unidentified Saccharimonadales is substantial in R, more abundant in RT, decreasing in TR and T, and very limited in Tn. Pyrinomonadaceae/RB41, *Opitutus,* and *Vicinamibacter* are both significantly greater in host spp. (TR/T), whether *Rafflesia*-infected or not, and absent in Tn. There were also differences in the mean relative abundance of certain bacterial genera (or families) across Rafflesiaceae species systems and their associated *Tetrastigma* species (Fig. 8). The graph displays bacterial taxa present at ≥ 0.1 % mean abundance in each group (RT, TR, T, Tn), except for R, where a threshold of 1 % was used. Notable patterns include a higher prevalence of certain bacteria (pink tones e.g. *Marmoricola, Nocardioides*) in RT. Certain bacteria are also enriched in host spp. *Rafflesia*-infected or not (TR/T, green tones, e.g. Saccharimonadales) compared to non-host *Tetrastigma* spp. (blue tones, e.g. Polyangiaceae).

Table 1
Sample sizes and average bacterial counts of groups and localities. The ILO locality has all representative groups and with the highest average count (– not sampled).

Locality	Sample Size						Average Bacterial Count					
	R	RT	TR	T	Tn	All	R	RT	TR	T	Tn	All
Iloilo, Philippines (ILO)	8	8	8	8	3	35	10256	13094	22386	19356	14178	16093
Camarines Norte, Philippines (CAM)	–	4	3	3	2	12	–	3504	17817	16373	22081	13396
Chiang Mai, Thailand (THA)	–	2	3	1	–	6	–	496	17364	8031	–	10186
All	8	14	14	12	5	53	10256	8554	20331	17666	17339	14814

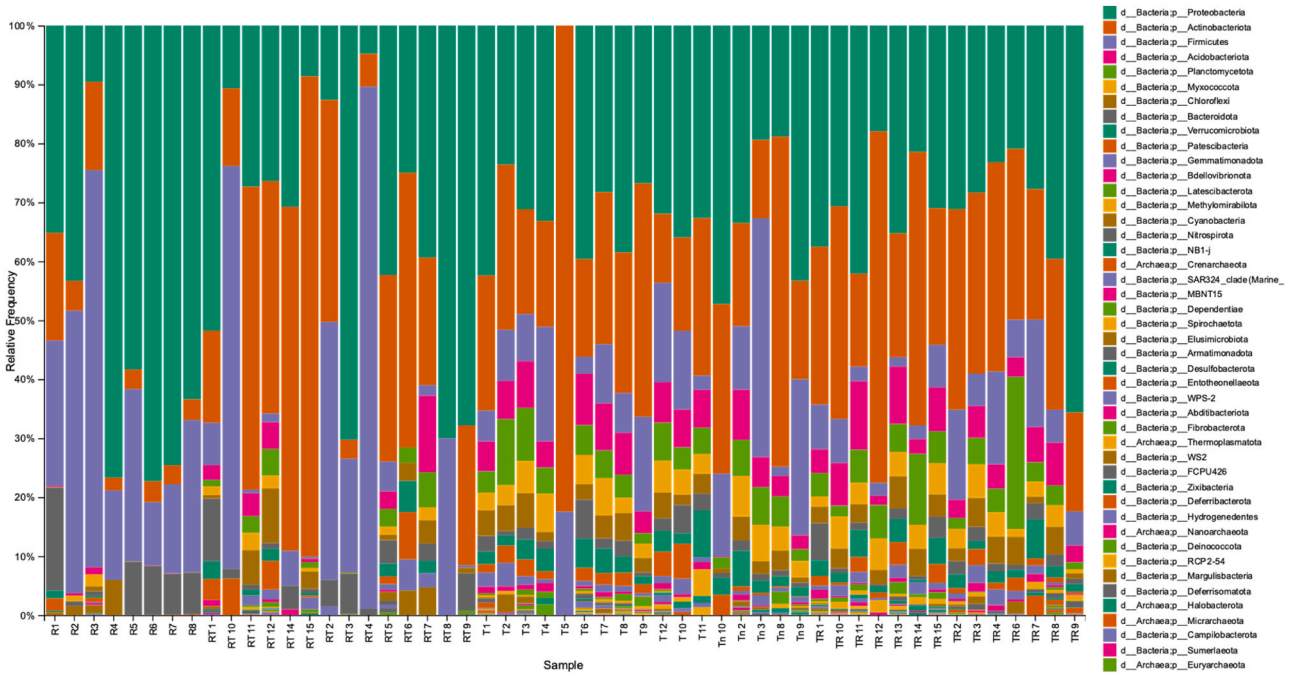

Fig. 3. Phylum bar graph showing all 53 samples in 5 groups. A few RT have similar profiles to R, while some are more similar to Ts.

Table 2
Diversity of bacterial microbiota among groups. Quantitative tests: Shannon and Bray-Curtis and tests incorporating the rooted tree phylogeny: Faith and Weighted UniFrac, employing pairwise comparison. Significance is represented as q values: * ** < 0.001, * * < 0.01, and * < 0.05, while non-significance is left blank.

Group Pairs	Alpha				Beta			
	Shannon		Faith		Bray-Curtis		Weighted UniFrac	
	q value	significance	q value	significance	q value	significance	q value	significance
R vs. RT	0.548		0.487		0.003	* *	0.149	
R vs. TR	0.005	* *	0.005	* *	0.003	* *	0.005	* *
R vs. T	0.029	*	0.022	*	0.003	* *	0.005	* *
R vs. Tn	0.029	*	0.026	*	0.025	*	0.042	*
RT vs. TR	0.029	*	0.013	*	0.047	*	0.010	*
RT vs. T	0.197		0.033	*	0.034	*	0.015	*
RT vs. Tn	0.391		0.103		0.173		0.491	
TR vs. T	0.702		0.547		0.218		0.660	
TR vs. Tn	0.548		0.239		0.139		0.077	
T vs. Tn	0.668		0.240		0.309		0.509	

3.2. Metabolite analysis

The differential presence of metabolites across samples based on peak intensities, which correlate with their concentrations were assessed (Fig. 9**),** excluding THA samples because of limited material. This showed that *Rafflesia* buds (RT, *R. lagascae* and *R. speciosa*) and their host species (TR/T) possess docosenamide, which was not evident in non-host spp (Tn). The *Rafflesia* buds possessed substantial amount of gallotannins (e.g. gallic acid derivatives, GAD) along with flavonoids, and phytohormones. However, these compounds were either absent or present at lower levels in host and non-host species. The metabolites detected in *Rafflesia* buds included adenine and ethylene precursors, which were specific to buds and not observed in significant levels in other sample types. However, the two *Rafflesia* spp. systems also differed—CAM samples, except for the non-host, were enriched in isoquinoline alkaloids (IA e.g. such as magnoflorine, methylococlaurine). However, in ILO, the non-host spp. contains the IA muricinine, which is lacking in *R. speciosa* and sympatric host spp. Both CAM and ILO non-host species (Tn) exhibited elevated levels of coumarins, including umbelliferone, which were found in lower concentrations in host species and buds. Table 3 lists the retention time, *m/z* values, and molecular formula of these compounds.

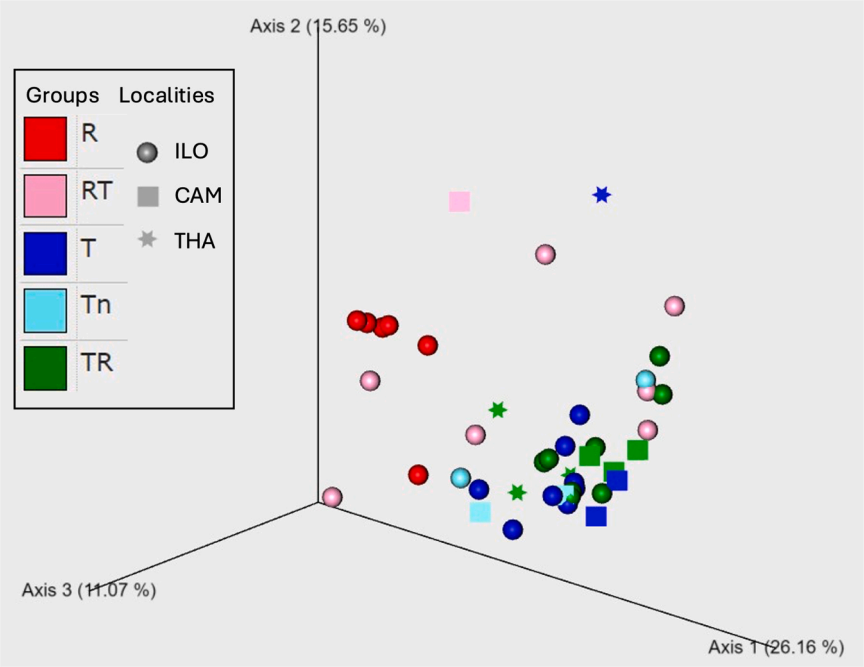

Fig. 4. Emperor plot of beta diversity among groups in different localities: ILO, CAM, and THA. T and TR generally cluster together regardless of localities. The RT group samples are the most diffuse.

3.3. Correlation Analysis Between Metagenomic and Metabolomic Data

A Spearman correlation analysis was performed (Fig. 10) to explore the relationships between microbial community composition and metabolite groups across the samples. This showed that *Rafflesia* buds exhibited elevated levels of flavonoids, gallic acid derivatives, other compounds (e.g. docosenamide), phytohormones, and terpenoids (i.e. inotodiol) compared to other sample groups. These metabolites showed positive correlations with the enrichment of specific bacterial taxa, including Microbacteriaceae (correlation coefficients: 0.2–0.61), Comamonadaceae (0.33–0.88), Nocardioidaceae (e.g., *Nocardioides*, *Marmoricola*; 0.29–0.76), and *Sphingomonas* (0.43–0.86). Interestingly, the bacterial taxa Saccharimonadales, Pyrinomonadaceae/RB41, *Opitutus,* and *Vicinamibacter*, that were abundant in host species (TR/T) but sparse or absent in *Tetrastigma* non-host species (Tn), exhibited strong positive correlations (0.59–0.81) with terpenoid compounds (e.g. inotodiol/uvaol). Conversely, bacteria from Polyangiaceae and Burkholderiaceae, which were more abundant in non-host species, were associated with the presence of aromatic acids and coumarins, showing correlation coefficients ranging from 0.48 to 0.76.

4. DISCUSSION

In this study we characterized the microbial communities and chemical compounds associated with Rafflesiaceae spp. and their *Tetrastigma* host plants to better understand the ecological factors driving host susceptibility.

5. Microbial symbionts of the world's largest flowers

When all samples were combined, regardless of locality, the bacterial taxonomic frequencies in *Rafflesia* seeds (R) showed a distinct pattern compared to all *Tetrastigma* groups (TR, T, Tn), while *Rafflesia* buds (RT) exhibited a microbiome that was intermediate between *Rafflesia* seeds and *Tetrastigma* hosts. This suggests that as *Rafflesia* seeds infect *Tetrastigma,* their original microbiome does not persist, likely due to interactions with the host microbiome as the endophyte grows inside and emerges as buds. The variability seen in RT samples might reflect differences in how the buds interact with or adapt to the host microbiome at various developmental stages. However, given that seeds and floral buds represent distinct developmental stages, the observed microbiome differences may also be influenced by physiological changes. It is likely that the shifts result from a combination of both developmental and host-related factors. The close clustering of T and TR samples across different localities indicates that *Rafflesia* infection does not significantly disrupt the core microbiome of *Tetrastigma,* or that the host maintains microbiome stability even when infected.

The high variability in RT could be attributed to some type of feedback between *Rafflesia*-associated and host-associated bacteria, leading to fluctuating community compositions that range from R-like to more T-like, depending on the degree of influence from the host. Such interactions may involve competition or even synergistic associations between microbes in host and holoparasite, causing shifts that result in high variability within the RT microbiome. This variability may also explain the observed significant differences observed between RT and TR. Meanwhile, the lack of significant difference in the bacterial communities between RT and Tn could suggest the shared presence of bacterial subsets that either discourage parasitism or the absence of parasitism-encouraging bacteria, as *Rafflesia* buds and non-host *Tetrastigma* were the only tissues in this study seemingly unable to host parasitism, leading to a convergence of their microbiomes.

5.1. Tetrastigma host spp. vs. non-host species

Pyrinimonadaceae RB41 and *Vicinamibacter* were enriched in host species, whether infected or not, across both CAM and ILO localities, compared to non-host species. Both bacterial taxa belong to the phylum Acidobacteriota, which is generally acidophilic and physiologically well adapted in fluctuating soil environments [20], though it remains unclear if these traits play a role in influencing susceptibility to *Rafflesia* infection. *Rafflesia*-infected *Tetrastigma* species were also greatly enriched in an unidentified group of Saccharimonadales, a rare microbial group known to enhance soil phosphorus cycling [81]. Although not statistically significant. CAM/ILO non-host species were relatively enriched in Burkholderiaceae, which are known for allelopathic properties [28,63,76], as well as an unidentified group of Polyangiaceae, which includes

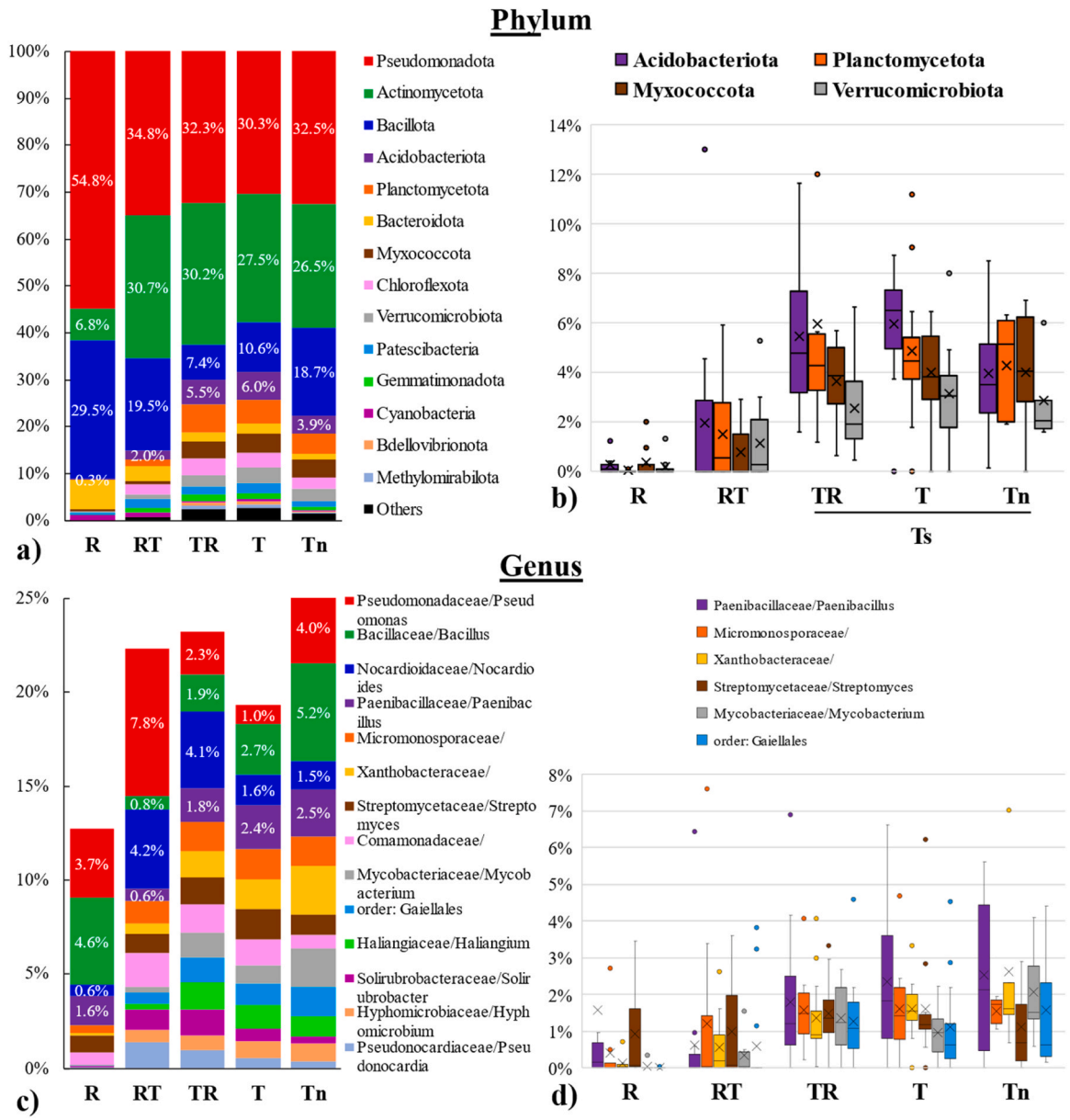

Fig. 5. Phylum (A-B) and genus (C-D)-level abundance. Phylum (**A**) and genus (**C**) bar graphs of the mean relative frequency distribution of the top 14 most abundant taxa among groups. Phylum (**B**) and genus boxplots (with outliers as separate points) of the median of selected taxa highlighting the frequencies associated with RT belonging to the intermediate between R and Ts: TR, T, and Tn **(D)**.

predatory myxobacteria [62]. It remains uncertain whether these microbial features actively deter *Rafflesia* infection or are simply reflective of chemotaxonomic differences between host and non-host species.

5.2. Flower buds

The microbiomes of *Rafflesia* buds (RT) were qualitatively similar to their sympatric *Tetrastigma* species; however, they showed an enrichment of certain bacterial taxa. Unknown genera from families like Microbacteriaceae, Enterobacteriaceae, and Comamonadaceae, as well as multiple genera from Nocardioidaceae (*Nocardioides, Marmoricola*) and Lachnospiraceae (*Blautia, Sellimonas*), were notably more abundant in the buds of *R. lagascae* and *R. speciosa* compared to their infected hosts. *Leuconostoc, Staphylococcus, Sphingomonas,* and *Pseudomonas* were also enriched in the buds. These findings suggest that specific bacterial species may accumulate due to a chemically distinct microenvironment (e.g., polyphenol-rich) in the *Rafflesia* buds, which promotes the growth of particular bacteria. Families like Microbacteriaceae and Nocardioidaceae are known to degrade polyphenols [80,83] and produce auxins, which have plant-growth-promoting properties [10,77]. Similarly, Lachnospiraceae (e.g., *Blautia and Sellimonas)* have been reported as plant endophytes associated with polyphenols [11].

The high abundance of complex carbon-degrading bacteria (e.g., Microbacteriaceae, Nocardioidaceae, Lachnospiraceae) in *Rafflesia* buds suggests a role in breaking down lignin, hemicellulose, cellulose, and other complex carbon compounds, which could facilitate *Rafflesia's* parasitism of woody *Tetrastigma* hosts. Microbacteriaceae and Nocardioidaceae are actinomycetes which have been a source of lignocellulolytic enzymes [64]. The absence of simple carbon-fixing bacteria (CO_2/C1 fixers) in both *Rafflesia* species' buds may be attributed to their parasitic nature, as Rafflesiaceae depend entirely on *Tetrastigma* for nutrients and water, eliminating the need for carbon fixation. The significant differences in bacterial composition between RT and TR could also imply antagonistic feedback between their microbiomes,

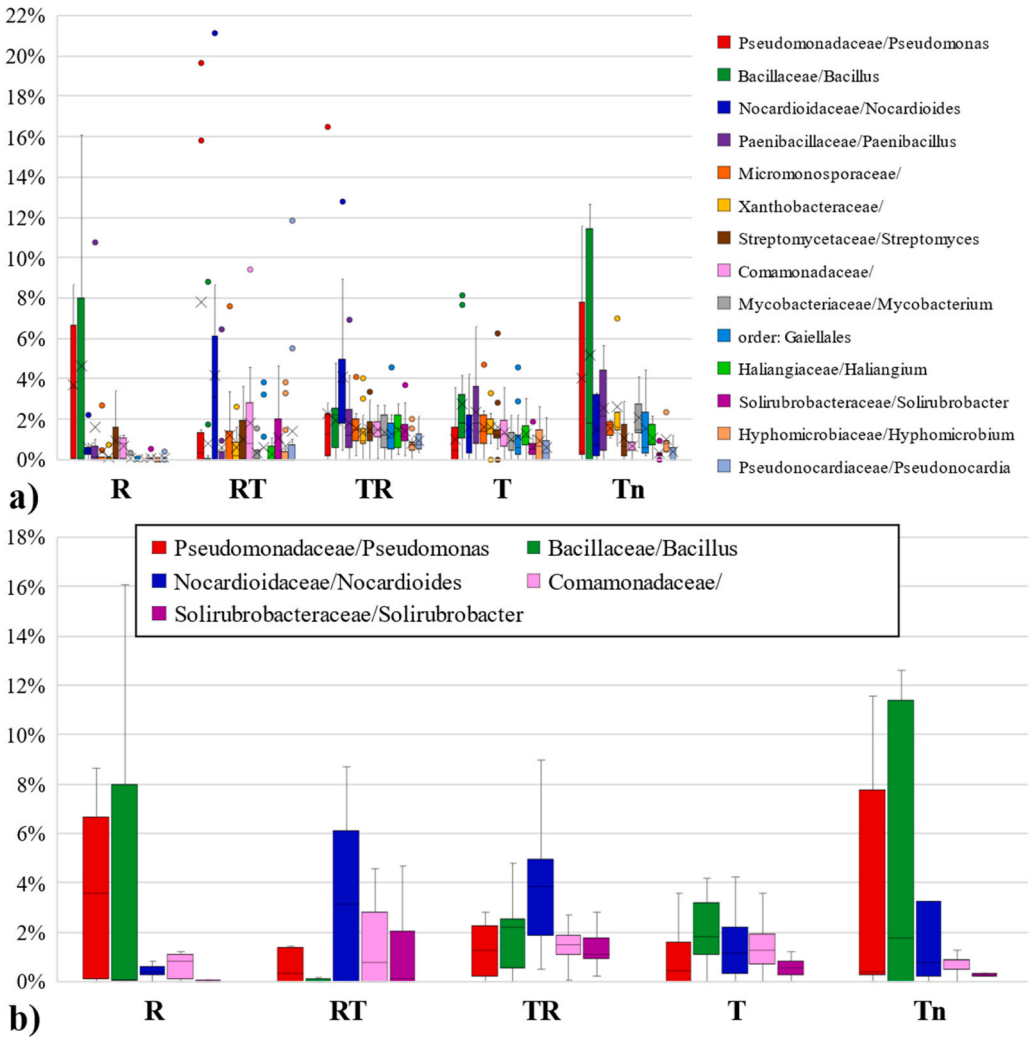

Fig. 6. Boxplots of the 14 most-abundant genera with outliers as separate points. **A.** R different from the rest while RT as the most variable (with most outliers that are widely spread) **B.** Five genera, excluding the outliers and mean for clarity. T looks more similar to TR than Tn while Tn seemed to exhibit more similarities to R compared to T.

contributing to the observed variability and enrichment of specific taxa in the buds.

5.3. *Rafflesia speciosa seeds*

The expanded sampling of *R. speciosa* seeds confirmed findings from previous studies [46], showing an enrichment in acidophilic Acetobacteraceae (>5.9 %) and Lactobacillales (>3.9 %), though *Clostridium,* previously reported at 4 % [46], was less abundant. A notable difference was the presence of an unidentified Enterobacteriaceae group, comprising 25 % of the seed microbiome (previously 1.7 % in [46]), potentially linked to plant-growth-promoting properties [30,35,84]. *Sapria* bud samples also showed an abundance of an unknown Enterobacteriaceae.

Additional R-dominant genera included *Pseudomonas* (>5 %) and *Bacillus* (>2 %), which were similarly enriched in non-hosts**.** Whether this reflects ecological significance is unclear, though intrageneric competition [14,55] may play a role in preventing *Rafflesia* infection, making these species unsuitable hosts. Certain phytopathogenic bacteria (e.g. *Xanthomonas*) have been hypothesized to aid in *Rafflesia's* infection [46] through cell-wall degrading enzymes [2,73], while Chitinophagaceae and Rhodobacteriaceae were also detected (>1 %). Chitinophagaceae, being chitin-degraders [24], may be opportunistic due to the high fungal content of *Rafflesia* seeds [46], while the ecological role of Rhodobacteraceae remains unclear given their diverse adaptations [67].

The shift from *Rafflesia* seeds to buds marked a change in microbial composition, with taxa like Acetobacteraceae and Lactobacilli becoming sparse in buds. This suggests that *Rafflesia* undergoes distinct microbiome shifts throughout its life stages, likely due to physiological changes that alter bacterial interactions. However, certain bacteria, such as Enterobacteriaceae, persisted from seed to bud, potentially indicating a core component of the *Rafflesia* microbiome, possibly transmitted vertically. The transition from seed to bud may involve shifts in nutrient needs, influencing which microbes are maintained or recruited, a pattern observed in other plants where microbiomes evolve with development [1,15,87]. During the endophytic stage, the seed microbiota may partly be replaced by host-associated bacteria, some of which remain as the bud matures.

6. Metabolites of Holoparasite and Hosts

The relationship between *Rafflesia* and its *Tetrastigma* host involves complex metabolic exchanges that remain poorly understood. To address this gap, we compared the metabolomic profiles of two *Rafflesia* spp. systems including their sympatric *Tetrastigma* host spp., to determine key compounds that may influence their interactions and elucidate the chemical basis of their symbiosis.

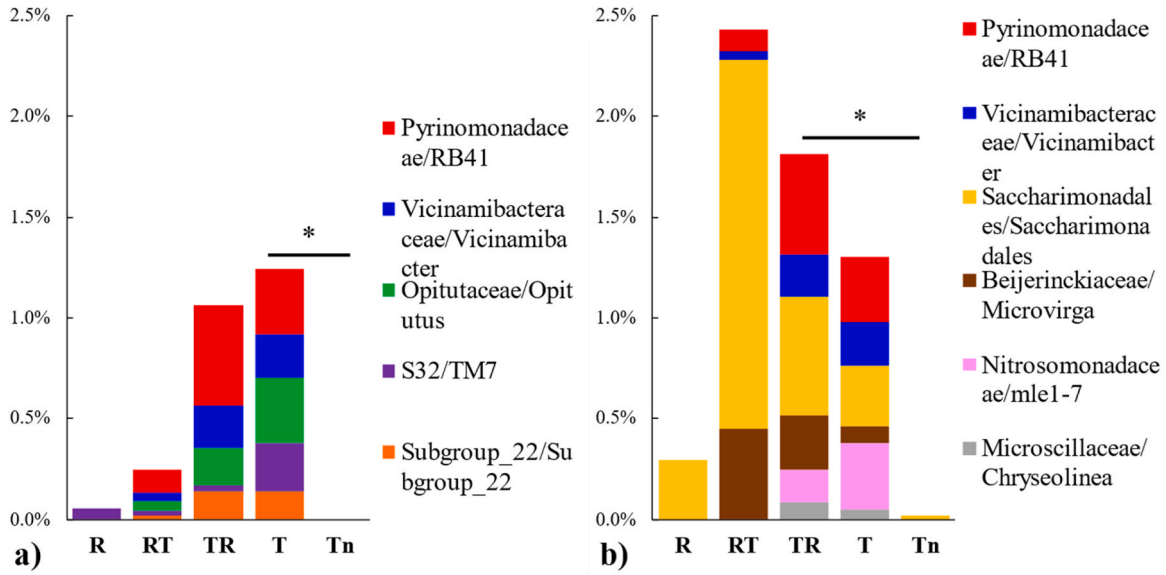

Fig. 7. ANCOM-BC of statistically (*at least q < 0.05) differentially-abundant genera between. **A.** T versus Tn, and **B.** TR versus Tn (including the mean frequency values for the other groups) **A.** The 5 genera are statistically enriched in T but absent in Tn, which are also almost as abundant in TR, and absent in R except for TM7. **B.** RB41 and *Vicinamibacter,* which were also elevated in T, are further enriched in TR, alongside four other genera. In contrast, these genera are absent in Tn, except for Saccharimonadales, which is present in trace amounts.

6.1. *Rafflesia flower buds and host species*

Though we were unable to chemically characterize *Sapria* samples, the 2 *Rafflesia* spp. (*R. lagascae* and *R. speciosa*) and their sympatric *Tetrastigma* species revealed differences in the abundance of compounds. One particularly interesting finding was the abundance of docosenamide, a fatty acid amide, in both *Rafflesia* buds and their host species, which was notably absent in non-host species. While the ecological role of docosenamide is not well understood, it is possibly produced by endophytic microorganisms [70] Intriguingly, docosenamide has been identified in various symbiotic species systems, highlighting its potential ecological roles. It is produced by the endophytic bacterium *Streptomyces sp.*, isolated from the plant *Sonchus oleraceus* [71], and by the cotton-endophyte *Nocardiopsis alba*, where it has demonstrated antifungal properties [45]. Additionally, endophytic fungi such as *Serendipita indica* [66] and *Penicillium setosum* [27] have been shown to synthesize docosenamide. Moreover, it is also produced by *Teredinibacter turnerae*, a cellulolytic bacterium symbiotic with shipworms [79], and by symbiotic dinoflagellates such as *Symbiodinium* [56]. Docosenamide is also present in the root exudates of duckweed, where it plays a role in stimulating denitrification in rhizospheric bacteria [68]. These diverse instances of docosenamide production suggest its widespread ecological function in various symbiotic relationships. Interestingly, docosenamide has also been detected as an abundant compound in oak galls [4]. In addition, infected *Tetrastigma* spp. in both CAM and ILO localities also possessed uvaol/inotodiol, a triterpenoid which in plants have diverse functions including defense, symbiotic signaling, and even regulation of seed germination [43].

In addition to docosenamide, buds from both *Rafflesia* species were found to contain an abundance of polyphenols, particularly gallic acid derivatives (GAD, e.g. epicatechin gallate), which have been previously detected in *Rafflesia* flowers [39] and *Sapria* [33]. These metabolites also characterize plant galls [4,65] and function as herbivore-deterrents. Several antioxidant compounds such as ellagic acid, flavonoids pinocembrin, genistein, naringenin, phloretin, and stilbenoids such as gaylussacin were also profiled.The anthraquinone glycoside emodin was also detected [34]. Other plant parasites have been reported to produce similar antioxidant compounds. Ellagitannins were detected in the holoparasite *Balanophora japonica* [36]. Branches of the mistletoe *Phoradendron perrottettii* contained significantly higher levels of flavonoids compared to its host, *Tapirira guianensis*, and Furlan et al. [25] suggested that this increase is linked to the mistletoe's antioxidant activity, which is likely a response to the host plant's defense against the infiltrating parasite.

Interestingly, phytohormonal compounds were also detected in *Rafflesia* buds, including adenine (a cytokinin) and 1-(malonylamino) cyclopropane-1-carboxylic acid, a precursor to ethylene. Cytokinins such as adenine are critical regulators of cell division and development [40], and their presence in *Rafflesia* suggests an active role in the control of parasitic growth and development. Cytokinins have also been associated with plant gall formation and nutrient mobilization [29]. Ethylene is a hormone known to mediate plant stress responses, and its precursor, 1-(malonylamino)cyclopropane-1-carboxylic acid [37], was elevated in *Rafflesia* buds compared to *Tetrastigma* plants. This finding aligns with previous studies showing that ethylene plays a critical role in facilitating the host invasion process [18].

6.2. *Metabolic differences between the 2 Rafflesia spp. systems*

Isoquinoline alkaloids (IA) were also differentially enriched across CAM samples. In the CAM system, isoquinoline alkaloids such as magnoflorine and methylococlaurine were detected in all host samples, except for the non-host species, and were similarly reported in a previous study by Molina et al. [49], in which IA were previously thought to deter *Rafflesia* infection in uninfected CAM host sp. *Tetrastigma loheri*. However, increased sampling in the present study suggests that IA may be associated with CAM species more generally, including those hosting *Rafflesia*. Rather than deterring infection, IA may serve a broader ecological function in host plants. These alkaloids may even be associated with development of plant galls [17]. In contrast, in the ILO system, non-host species *T. scariosum* and red *T. aff. loheri* were found to contain the IA muricinine, which was absent in both *R. speciosa* and its sympatric host species. The presence of muricinine exclusively in ILO non-host species could suggest a potential role in deterring *Rafflesia speciosa* infection, though this hypothesis requires further investigation.

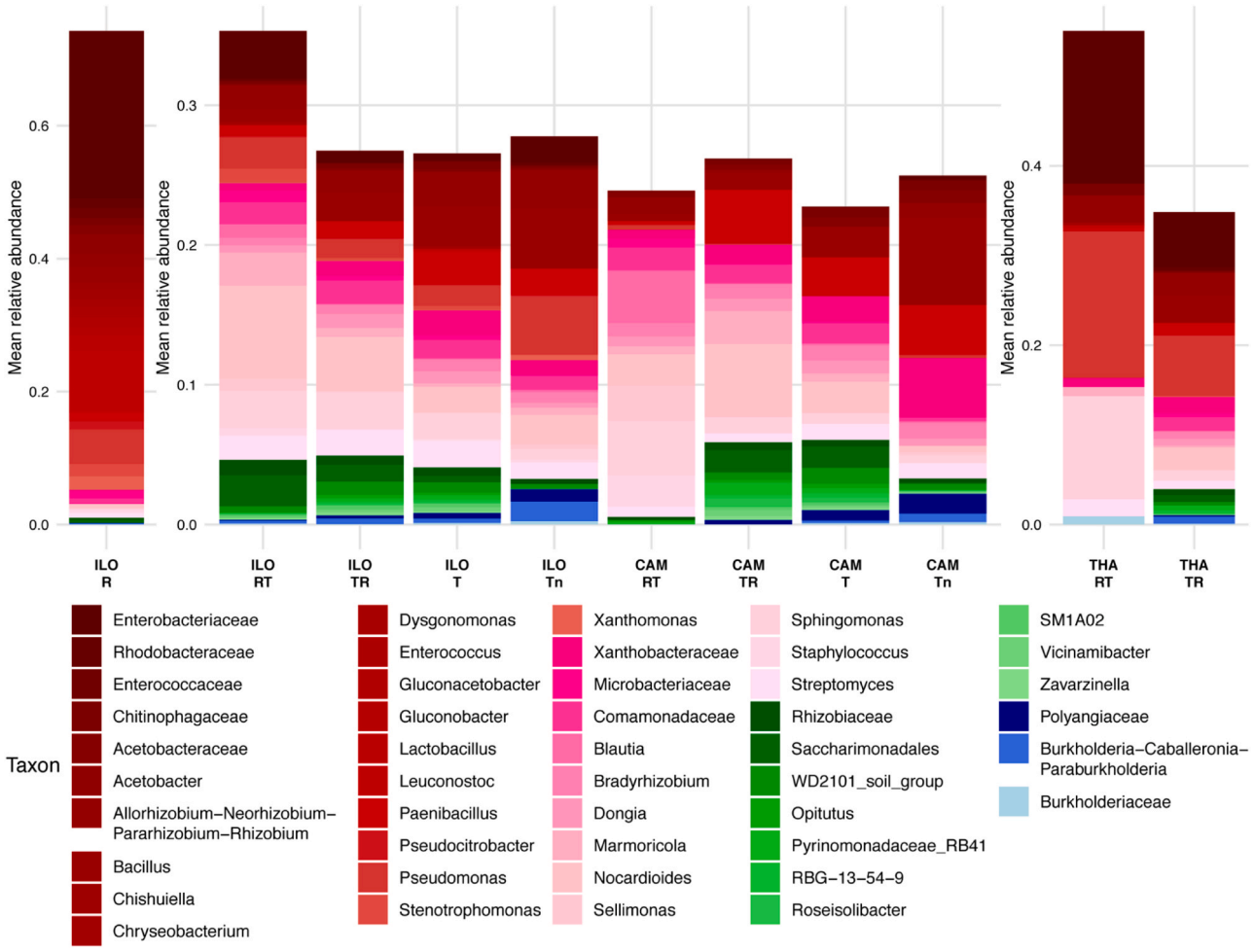

Fig. 8. Differentially abundant bacterial genera (or families, if genus identification was not possible) across Rafflesiaceae species systems and their associated *Tetrastigma* spp. Bacteria are shown with at least 0.1 % mean abundance in each group (RT, TR, T, Tn), except for R, where a threshold of 1 % was used. Common bacteria found in buds of both Rafflesia spp. (CAM and ILO) were selected for. Additionally, bacterial taxa shared between CAM and ILO host species (with at least 0.1 % abundance) were selected for, if their abundance was below this threshold in both CAM and ILO non-hosts. Conversely, bacteria with at least 0.1 % presence in non-hosts but below this in host species were also excluded. Red tones correspond to bacteria proportionally abundant in R; pink tones: RT; green tones: host spp., infected or not; blue: non-host spp.

6.3. Non-host species

An intriguing finding was the elevated presence of coumarin compounds in both CAM and ILO non-host species (Tn). Coumarins (e.g. umbelliferone) are known for their strong allelopathic properties, inhibiting the growth of neighboring plants [21]. The detection of more abundant coumarins in both CAM and ILO non-host species (on average >36 fold higher in Tn than other samples RT, TR, T) raises the possibility that it plays a role in preventing *Rafflesia* infection through allelopathic interactions. Such allelopathic effects have been demonstrated in various systems where coumarins act as plant defense compounds [54]. It remains an open question whether coumarins directly inhibit *Rafflesia* or interact with other compounds to exert this effect. To address this, future research should explore the bioactivity of coumarins against *Rafflesia's* endophytic stages. Such studies could help validate the hypothesis that coumarins may serve as potential biochemical markers for host resistance, providing insights into their role in mediating plant-parasite interactions.

6.4. Microbe-metabolite connection

The integrated analysis of metagenomic and metabolomic data elucidates the functional implications of microbial communities in *Rafflesia's* life cycle. The enrichment of Microbacteriaceae and Nocardioidaceae in *Rafflesia* buds highlights their potential role in degrading complex polyphenols like gallic acid derivatives. These compounds, characteristic of the bud's chemically distinct environment, likely act as selective pressures shaping the microbial community. Additionally, restricted presence of docosenamide in *Rafflesia* buds and host species aligns with its proposed role in symbiotic signaling. This fatty acid amide, identified in other symbiotic systems, may facilitate microbial colonization and host-parasite interactions by modulating the chemical environment to favor parasitism. Conversely, non-host species showed enrichment of Polyangiaceae and Burkholderiaceae, correlating with aromatic acids and coumarins, which are known to exhibit allelopathic effects, potentially deterring *Rafflesia* parasitism.

6.5. A speculative synthesis–microbial and chemical ecology of the life cycle of Rafflesia speciosa

Given our expanded sample collection across various stages of the *Rafflesia speciosa* life cycle, including seeds, we can begin to decipher the microbial and chemical ecology that supports this species. Previous research [46] has shown that *Rafflesia* seeds inherit some bacteria from their host but also develop a unique microbial profile, being enriched in Enterobacteriaceae, Microbacteriaceae, and *Xanthomonas*. This unique

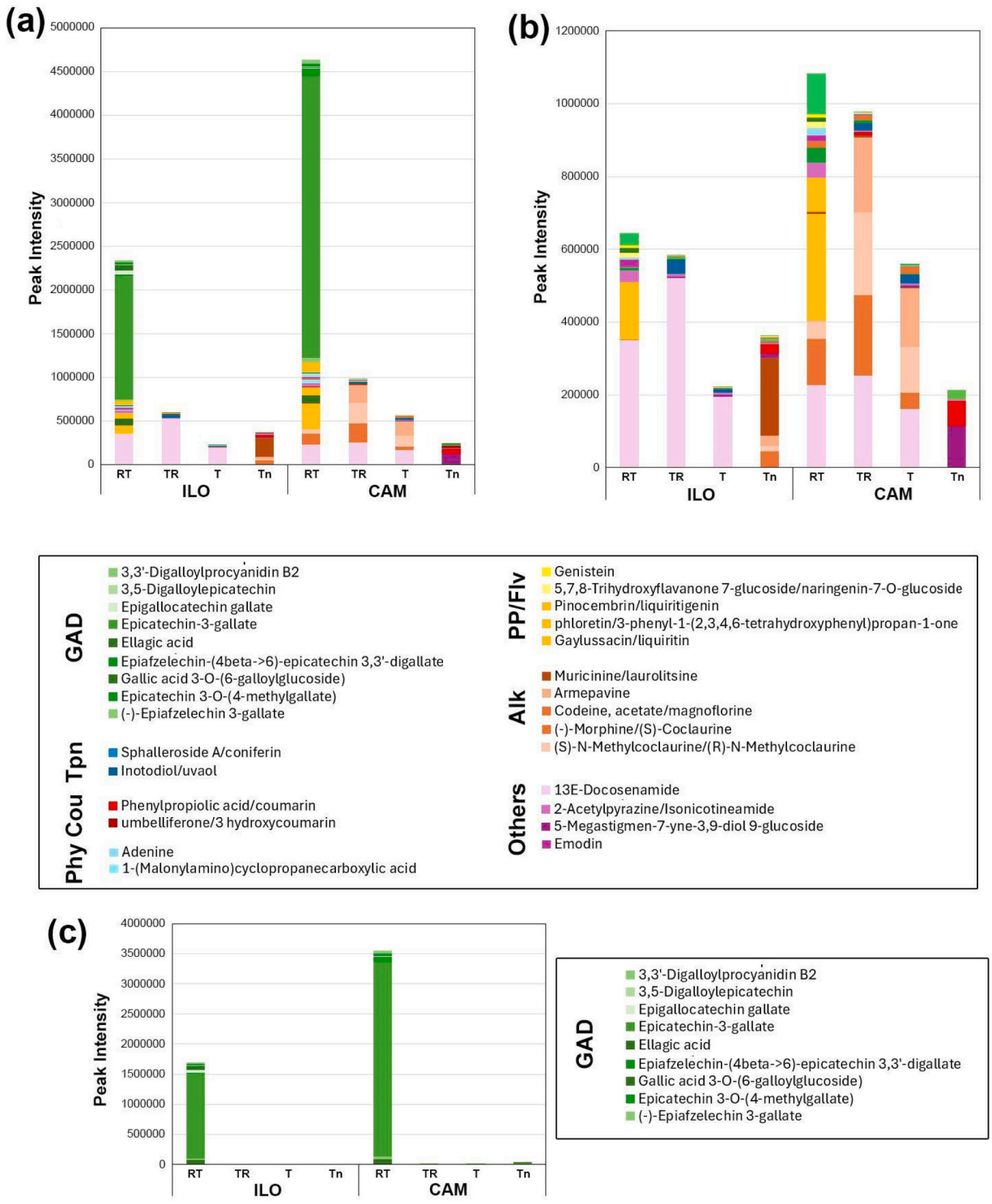

Fig. 9. Metabolites differentially present among samples based on peak intensities (which are proportional to concentration). **A.** All metabolites shown including gallic acid derivatives (GAD). **B.** Without GAD. **C.** Only GAD intensities shown. Tpn, terpenoids; Cou, coumarins; Phy, phytohormones; PP/Flv, phenylpropanoids/flavonoids; Alk, alkaloids.

microbiota likely arises from both the seed's chemical composition and interactions with the fruit's biotic associates. When the *Rafflesia* seed locates a suitable host, microbial enzymes from its endophytic microbes may facilitate germination within the host. During this phase, *Rafflesia* remains covert, living as an endophyte until a physiological trigger induces the proliferation of the parasite, causing it to emerge from the host's epidermis as a bud. The *Rafflesia* bud initially mirrors its host's microbiota, reflecting its origin as a host outgrowth. However, the bud accumulates specific bacteria that thrive in its chemically distinct environment, notably rich in gallotannins [69], which fosters high levels of Microbacteriaceae and Nocardioidaceae.

The abundance of gallic acid derivatives, commonly associated with plant galls, suggests that *Rafflesia* buds may act similarly to these abnormal growths produced by a foreign "invader", typically bacteria or insects [31] to benefit the intruder nutritionally. However, in this case, endophytic *Rafflesia* cells reorganize the development of its *Tetrastigma*

Table 3
Metabolites differentially present among samples. Retention time (R/t) in minutes, *m/z* values, molecular formulas, potential metabolite identification and peak intensities (which are proportional to concentration) are provided.

				ILO				CAM			
R/t (min)	*m/z*	Molecular formula	Potential metabolite ID	RT	TR	T	Tn	RT	TR	T	Tn
4.3	123.055	C6H6N2O	2-Acetylpyrazine/Isonicotineamide	30975.75	6884.44	5311.50	5275.50	39861.00	4162.33	5131.33	3112.00
12.5–12.53	136.061	C5H5N5	Adenine	6037.25	783.11	1054.75	1144.75	22368.67	2810.00	1512.00	0.00
16.52–17.55	147.044	C9H6O2	Phenylpropiolic acid/coumarin	388.75	393.11	167.25	25984.00	1072.67	8627.00	219.00	68807.00
13.14–14.8	163.039	C9H6O3	umbelliferone/3 hydroxycoumarin	732.50	7395.33	1606.75	7271.13	130.17	1269.00	1333.83	25354.00
4.97–5.74	188.055	C7H9NO5	1-(Malonylamino)cyclopropane-carboxylic acid	8922.25	89.56	1203.00	1140.75	40965.67	8075.00	2133.33	0.00
20.93–20.95	208.133	C12H17NO2	Synephrine acetonide	355.50	10360.22	3399.25	551.50	292.33	8834.67	4339.67	0.00
24.51–25.18	257.080	C15H12O4	Pinocembrin/liquiritigenin	62459.00	39.11	19.25	107.25	93844.33	166.67	101.67	128.00
16.92–16.98	271.059	C15H10O5	Emodin	21299.25	273.56	550.75	551.00	14466.33	1130.33	966.33	0.00
16.42–16.46	271.060	C15H10O5	Genistein	8267.00	1885.78	444.75	1212.75	9169.00	1509.67	499.33	225.00
21.47–21.6	275.091	C15H14O5	phloretin/3-phenyl−1-(2,3,4,6-tetrahydroxyphenyl)propan−1-one	34343.00	70.44	10.50	1600.75	111348.67	432.33	707.00	217.00
33.82–33.83	279.232	C18H30O2	Calendic acid/pinolenic acid	6352.75	21445.11	16073.25	5639.00	1988.67	6860.00	10983.67	5257.00
14.81–15.07	286.140	C17H19NO3	(-)-Morphine/(S)-Coclaurine	0.00	0.00	0.00	4403.00	19007.00	13996.00	20721.67	0.00
15.03–15.14	287.149	C14H22O6	methyl 2-ethyl−4-[(3 R,4 R,5S)−5-hydroxy−4,5-dimethyl−2-oxooxolan−3-yl]−2-methyl−3-oxobutanoate	311.00	1081.11	1732.00	6814.25	3586.33	3038.67	3792.67	25529.00
14.64–15.75	289.072	C15H12O6	okanin/3,5,7-trihydroxy−2-(3-hydroxyphenyl)−3,4-dihydro-2H−1-benzopyran−4-one	17837.75	13054.67	24583.25	1219.50	6841.00	11101.67	10414.67	4299.00
14.83–15.17	300.159	C18H21NO3	(S)-N-Methylcoclaurine/(R)-N-Methylcoclaurine	0.00	0.00	0.00	15434.42	45765.00	227833.67	125116.00	0.00
17.99–18.62	303.015	C14H6O8	Ellagic acid	79135.00	29.33	47.25	0.00	90836.33	381.67	123.00	40.00
14.67–14.92	314.140	C18H19NO4	Muricinine/laurolitsine	0.00	0.00	0.00	214826.25	5568.33	4440.00	1429.00	0.00
14.24	314.173	C19H23NO3	Armepavine	402.50	0.00	115.75	26915.00	4130.00	206993.00	161889.33	984.00
12.84–12.87	330.169	C19H23NO4	Sinomenine/(S)-Reticuline	0.00	17.78	86.25	1515.00	36.67	5196.67	8243.33	82.00
37.53–38.61	338.342	C22H43NO	13E-Docosenamide	351049.50	521214.67	194875.75	0.00	226849.33	252552.00	161277.33	0.00
16.46	342.168	C20H23NO4	Isocorydine	0.00	8.89	17.25	2094.00	269.00	7112.67	3355.33	0.00
15.29–15.69	342.170	C20H23NO4	Codeine, acetate/magnoflorine	357.00	133.89	74.25	44758.75	127550.33	220396.00	44753.67	0.00
13.36–13.59	365.120	C16H22O8	Sphalleroside A/coniferin	13363.25	175.11	31.50	0.00	11221.67	116.33	55.33	0.00
19.12–19.8	371.206	C19H30O7	5-Megastigmen−7-yne−3,9-diol 9-glucoside	476.00	3491.33	4877.25	10163.42	0.00	1149.00	4992.33	113091.00
25.19–25.38	419.132	C21H22O9	Gaylussacin/liquiritin	94627.25	0.00	0.00	0.00	292538.67	77.33	0.00	0.00
20.5	427.101	C22H18O9	(-)-Epiafzelechin 3-gallate	21874.00	0.00	0.00	0.00	44076.00	0.00	0.00	799.00
18.44–18.48	435.128	C21H22O10	5,7,8-Trihydroxyflavanone 7-glucoside/naringenin−7-O-glucoside	11663.75	631.33	208.00	1427.25	16560.00	627.67	385.00	194.00
18.43–18.65	443.098	C22H18O10	Epicatechin−3-gallate	1411540.00	0.00	0.00	0.00	3226890.67	1825.33	233.00	25083.00
33.18–34.54	443.390	C30H50O2	Inotodiol/uvaol	0.00	40168.00	9538.00	46.25	0.00	20073.00	25315.33	0.00
20.54–20.78	457.110	C23H20O10	Epicatechin 3-O-(4-methylgallate)	11511.00	444.22	217.75	213.50	87696.00	126.00	183.33	413.00
14.31–14.39	459.090	C22H18O11	Epigallocatechin gallate	49275.75	17.56	0.00	0.00	6151.33	0.00	130.67	77.00
13.32–13.38	485.090	C20H20O14	Gallic acid 3-O-(6-galloylglucoside)	62375.25	10.67	0.00	0.00	9549.67	0.00	0.00	72.00
20.43–25.71	595.110	C29H22O14	3,5-Digalloylepicatechin	6662.00	0.00	0.00	0.00	9236.00	0.00	0.00	0.00
18.62–21.76	867.180	C44H34O19	Epiafzelechin-(4beta->6)-epicatechin 3,3′-digallate	26774.00	0.00	0.00	9.50	30501.33	36.00	25.00	0.00
20.5–20.6	883.170	C44H34O20	3,3′-Digalloylprocyanidin B2	26760.00	0.00	0.00	0.00	43974.33	15.67	41.33	0.00

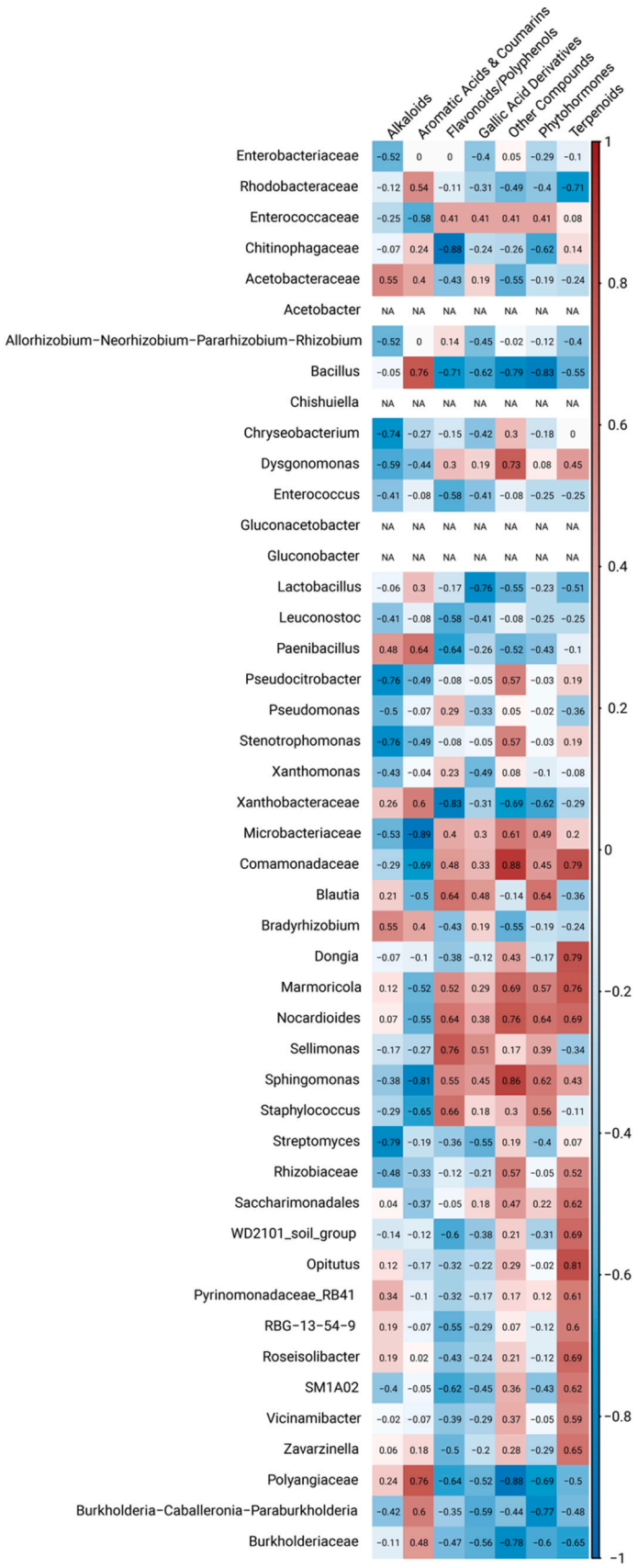

Fig. 10. Spearman correlation analysis between bacterial taxa and metabolite groups across samples. The heatmap shows correlation coefficients ranging from −1 (strong negative correlation, blue) to 1 (strong positive correlation, red). White indicates no correlation (values close to 0). Numbers in each cell represent the correlation coefficient between the corresponding taxon and compound group. 'NA' indicates cases where correlation could not be calculated due to lack of variation in the data or unavailability of data. Taxa are shown on the y-axis and metabolite groups on the x-axis.

host tissues to support the enlarging holoparasite. Enterobacteriaceae sp, *Pseudomonas* sp., and *Allorhizobium s.l.,* which are the most dominant bacteria in crown gall disease in grape, *Vitis vinifera* [22], may be involved, given the abundance of these bacteria in *Rafflesia* seed and/or buds. The enrichment of gall-associated bacteria [22,26,41,72] in floral buds of both *Rafflesia* species, alongside elevated levels of adenine/cytokinin—a key phytohormone involved in gall formation—supports the 'gall hypothesis' for *Rafflesia.* While Teixeira-Costa et al. [75] highlighted mistletoes as unique among plant parasites for their ability to induce woody galls in hosts, our current findings suggest that members of the Rafflesiaceae may also be capable of forming gall-like structures. Notably, transcriptomic studies on *Rafflesia* and *Sapria* have revealed gene expression patterns akin to those observed in oak galls [38], suggesting that these parasitic plants exhibit transcriptomic features reminiscent of gall structures (M. Burger and J. Molina, unpubl.).

7. Conclusion

This study highlights the intricate microbial and chemical interactions that underpin the life cycle of *Rafflesia*, shedding light on the microbial shifts occurring throughout its developmental stages, from seed to bud. These findings emphasize the specialized symbiosis between *Rafflesia*, its microbial partners, and its *Tetrastigma* hosts. The presence of specific bacterial communities in *Rafflesia* buds suggests that these microbes are adapted to the chemically distinct environment of the bud, thriving in conditions enriched with gallotannins and other polyphenols. Key evidence supports the hypothesis that *Rafflesia* buds function similarly to plant galls, manipulating host tissues to promote their reproductive development. The abundance of gall-associated bacterial families, along with the detection of adenine—a cytokinin involved in gall formation—indicates that these bacteria may play a role in modulating host tissue responses to support parasitism. Conversely, the enrichment of coumarins and potentially allelopathic bacteria in non-host species appears to deter *Rafflesia* infection, further emphasizing the role of microbial and chemical factors in shaping host susceptibility. These findings have practical applications for ex situ conservation. Incorporating beneficial microbes, such as those involved in polyphenol degradation or parasitic signaling, into host propagation systems could improve host compatibility and parasitic success in controlled environments. Additionally, screening *Tetrastigma* hosts for favorable chemical profiles and reducing the influence of allelopathic compounds could further enhance propagation efforts. By leveraging these microbial and chemical pathways, this research provides actionable strategies to optimize ex situ conservation techniques, ultimately aiding in the rescue of the world's largest flowers from the brink of extinction.

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CRediT authorship contribution statement

JM conceived the project. RP, JRC, AW, HB, PS, ME, SJ collected samples with JM. JM, RCG, RA, KH, AG, RP analyzed data. JM drafted the manuscript with all authors editing and approving the final manuscript.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT in order to improve readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Bacterial metagenomic data were submitted to Genbank SRA under submission number SUB14821153.

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