

The Phyllosphere of Nigerian Medicinal Plants *Euphorbia lateriflora* and *Ficus thonningii* is inhabited by a specific Microbiota

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Abstract

Background

Medicinal plant microbiota is highly specific and can contribute to medicinal activity. However, the majority of plant species have not yet been studied. Here, we investigated the phyllosphere composition of two common Nigerian medicinal plants, *Euphorbia lateriflora* and *Ficus thonningii*, by a polyphasic approach combining analyses of metagenomic DNA and isolates.

Results

Microbial abundance estimated via qPCR using specific marker gene primers showed that all leaf samples were densely colonized with up to 10^8 per gram of leaf with higher bacterial and fungal abundance than Archaea. While no statistically significant differences between both plant species were found for abundance, amplicon sequencing of 16S rRNA and ITS genes revealed a distinct microbiota composition, and only seven of the 27 genera isolated were represented on both plants. We observed dominance of *Sphingomonas* spp. on both plants, and members of *Xanthomonadaceae* and *Enterobacteriaceae* were also found in high numbers. The most dominant fungal families on both plants were *Cladosporiaceae*, *Mycosphaerellaceae* and *Trichosphaeriaceae*. In addition, 225 plant-specific isolates were identified, with *Pseudomonadota* and *Enterobacteriaceae* being dominant. Interestingly, 29 isolates are likely species previously unknown, and 14 of these belong to *Burkholderiales*. However, a high proportion, 56% and 40% of the isolates from *E. lateriflora* and *F. thonningii*, respectively, were characterized as various *Escherichia coli*. The growth of most of the bacterial isolates was not influenced by extractable secondary metabolites of plants.

Conclusions

Our results suggest that a specific and diverse microbial community inhabits the leaves of both plants, including potentially new species and producers of antimicrobials.

Background

The phyllosphere refers to the shoots of plants, including leaves, stems, fruits and flowers [1, 2]. As with almost every other environmental niche of plants, the phyllosphere is colonized by microorganisms, with bacteria being the most dominant [1–4]. Microorganisms coexisting with multicellular organisms confer a wide range of benefits [1], including protection from pathogens and stress, nutrient modulation, synthesis of secondary metabolites and growth promotion. For many host organisms, the microbiome in each niche is shaped by antimicrobial principles produced by the host. While this was shown for the rhizosphere [5, 6], less is known about the phyllosphere.

A great proportion of research on medicinal plants has focused on their antimicrobial activity, leading to the discovery of compounds with biological activity, including antibacterial activity. Medicinal plants should be seen as meta-organisms consisting of the plants themselves and the microbes they harbour. Efforts are thus being made to investigate the microbiology of medicinal plants' phyllospheres. This interest is aroused partly because some bacteria coexisting with medicinal plants contribute to the production of secondary metabolites and active compounds [5, 7, 8] and play roles in the metabolism and ecology of the plant. Indeed, some studies have confirmed that some plant-associated microbes themselves directly produce antimicrobial compounds, while others are involved in the production of secondary metabolites by such plants [9–14]. It is also possible that the microbiomes themselves are shaped by bioactive agents produced by the plants. Phyllosphere studies are increasingly featured in the literature, but the few from Africa focus predominantly on plant pathogens of agricultural crops, and few studies worldwide examine the culturable phyllosphere.

Euphorbia lateriflora and *Ficus thonningii* are medicinal plants of the Euphorbiaceae and Moraceae families, respectively. *E. lateriflora* is a shrub growing to a height of approximately 1.7 metres with nearly vertical waxy branches [15]. Abundant whitish latex is produced when the leaves shed or when the plant is otherwise injured. The latex is toxic to the eyes and may result in blindness in some cases. Scientific investigation into the toxicity of *E. lateriflora* discovered varying levels of dose-dependent toxicity in experimental animals [16, 17]. *E. lateriflora* is used in ethnomedicine for treating prostatic diseases, skin and soft tissue infections, sexually transmitted infections and a variety of other syndromes [15, 18], and various solvent extracts produce *in vitro* antimicrobial activity. *F. thonningii* is a large, multistemmed, flowering tree with a dense spreading crown. The tree bark on old branches is grey and smooth, while younger branches have hairy bark. The leaves are green, simple, entire, glossy and puberulous [19]. Its flowers range from red to yellow and may be globose or ellipsoid, hairy or smooth and glabrous or pubescent [20]. The fruits of *F. thonningii* are small, round, yellow or pink and usually occur singly or in pairs. *F. thonningii* is generally considered safe, with minimal or no toxicity recorded [21–23]. Several parts of *F. thonningii* have been reportedly used in ethnomedicine, but the leaves are more commonly used in treating diarrhea, gonorrhea, wound infections, urinary tract infections and skin and soft tissue infections [24–28]. In addition to surveys, various extracts of *F. thonningii* have been shown to exhibit *in vitro* antimicrobial activity against microbial pathogens [29–36].

This study investigated the phyllosphere microbiome of *E. lateriflora* and *F. thonningii*. We hypothesized that *E. lateriflora* and *F. thonningii* are colonized by diverse bacteria that are nonsusceptible to the antimicrobial principles of the plants.

Methods

Plant collection and authentication

Euphorbia lateriflora for this study was sampled from the botanical garden of the University of Ibadan, Ibadan, Nigeria (GPS coordinates 7.377536, 3.94704), while *Ficus thonningii* was sampled from a mature

tree near Ojoo, Ibadan, Nigeria (GPS coordinates 7.49529, 3.93415). Up to 10 leaves of *E. lateriflora* were sampled in contrast to 3 leaves of *F. thonningii* due to differences in leaf surface area. Healthy, fresh leaves of the plants were aseptically plucked from the petiole with the aid of presterilized forceps, placed directly into sterile laboratory collection bags and transported to the laboratory for culture and DNA extraction within one hour. For solvent extraction, the plants were collected and dried to constant weight at ambient temperatures, away from direct sunlight, before further processing. *E. lateriflora* and *F. thonningii* were authenticated and deposited at the Forestry Research Institute of Nigeria (FRIN) with the respective voucher numbers FRIN 110801 and FRIN 1106898.

DNA Extraction, qPCR and Amplicon Sequencing

DNA was extracted from two biological replicate leaf samples of both plants with the PowerSoil DNA Isolation Kit according to the manufacturer's instructions.

qPCR

Quantitative polymerase chain reaction (qPCR) was performed with the aim of estimating the abundance of archaea, bacteria and fungi in the extracted community DNA. Archaeal and bacterial qPCRs targeted the 16S rRNA gene with the respective domain-specific primer pairs 344aF and 517uR [37] and Unibac-II-515f and Unibac-II-806r [38], while fungal qPCR targeted the internal transcribed spacer (ITS) gene with ITS1f and ITS2r primers [39]. All reactions consisted of the following components: sample DNA (1 µl) KAPA SYBR Green (5 µl) (Bio-Rad, Hercules, CA, USA), 0.5 µl each of forward and reverse primers (10 µM) and PCR-grade water (3 µl) making up a total volume of 10 µl. Tenfold dilutions of appropriate archaeal, bacterial and fungal standards that contained known abundance estimates were prepared according to previously described protocols [38] and were used to calculate gene copy numbers. All reactions were performed in duplicate and run on a Rotor-Gene™ 6000 series thermal cycler (Corbett Research, Sydney, Australia). The qPCR protocol for archaea was as follows: initial denaturation (95°C/5 minutes) followed by 39 cycles of denaturation (95°C/15 seconds), annealing (60°C/30 seconds) and extension (72°C/30 seconds) with a final step from 72°C to 96°C. The protocol for bacteria and fungi was as follows: initial denaturation (95°C/5 minutes) followed by 35 cycles of denaturation (95°C/10 seconds), annealing (54°C/15 seconds) and extension (72°C/10 seconds) with a final step from 72°C to 96°C. Statistical analyses of gene abundance estimates were computed with the Kruskal–Wallis test.

Amplicon Sequencing

Amplicon libraries of the bacterial 16S rRNA and fungal ITS genes were prepared as described below. PCR targeting the 16S rRNA gene was conducted with the 515f and 806r primer pair [40]. Both forward and reverse primers contained barcodes at the 5 prime ends, and these were assigned to samples to yield unique combinations for sample multiplexing and sequencing. To minimize the amplification of plant mitochondrial 16S rRNA and plastid genes, peptide nucleic acid (PNA) clamps were incorporated in the PCRs [41, 42]. Each PCR contained 6 µl of 5XTaq & GO premix for PCR (MP Biomedicals), 0.6 µl each of forward and reverse primers, 0.45 µl each of mPNA and pPNA, 1 µl of template DNA and 20.9 µl of molecular biology grade water to yield a final volume of 30 µl. The PCR conditions consisted of an initial

denaturation at 60°C for 5 minutes and subsequent 30 cycles of 94°C for 60 seconds, 78°C for 5 seconds, 54°C for 1 minute and 72°C for 60 seconds with a final extension at 72°C for 10 minutes. PCRs were run in triplicate. The respective replicates were pooled and stored at -20°C for onwards processing.

The ITS primer pair ITS1f and ITS2r was used to amplify the ITS 1 region of the fungal ITS gene [39, 43]. The PCR consisted of 1 µl of template, 6 µl of 5XTaq &Go pre-PCR mix (MP Biomedicals), 0.6 µl each of forward and reverse primers and 21.8 µl of molecular biology grade water to a final volume of 30 µl. The PCR conditions included an initial denaturation at 98°C for 5 minutes and then 30 cycles of denaturation at 98°C for 60 seconds, primer annealing at 58°C for 60 seconds, extension at 74°C for 60 seconds with a final extension at 72°C for 10 minutes. PCRs were run in three replicates, and the replicates were combined and kept at -20°C prior to further processing.

Pooled amplicons were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Ref: A9282), after which DNA concentrations were estimated with a Qubit dsDNA Broad Range Kit. Samples were normalized to equimolar concentrations and pooled before being sent for Illumina sequencing by the commercial company Novogene Corporation Incorporated. Sequences were deposited in the NCBI SRA database with accession number PRJNA1036807.

Sequence Analyses

Raw sequence reads obtained were demultiplexed. Reads with identical barcodes were combined, and primers/barcodes were removed with cutadapt [44]. The processed reads were then imported into QIIME2 [45], where the input was summarized, quality filtered, denoised (with the DADA algorithm) [46] and classified. Operational taxonomic units were apportioned with the VSEARCH algorithm [47] implemented within QIIME2 using curated reference databases, SILVA132 for bacterial 16S rRNA [48, 49] and UNITE for fungal ITS [50].

Statistical analyses and data visualization were performed in Rstudio in addition to the online microbiome analyst platform [51]. The generated OTU table and taxonomic classification were analysed with phyloseq [52] and vegan packages within RStudio. The Shannon index was used to estimate alpha diversity after rarefaction of data to the minimum library size. Upon realization, after initial analyses that the bacterial sequences were overdominated by OTUs mapping to the *Sphingomonas* genus, the genus was omitted for subanalyses, creating two sets of 16S rRNA data. The relative abundance of taxa was computed across three levels of taxonomy: class, order and family.

Isolation of bacteria

Fresh leaves (2–4) were aseptically collected into sterile bags, transported to the laboratory within an hour and processed following previously established procedures [53]. In summary, the leaves were washed off in sterile water containing Tween 20 with 5 minutes of sonication at ambient temperature. The sonicate was serially diluted, and aliquots were inoculated into 0.1X tryptone soya agar (TSA) containing 100 µg/mL cycloheximide. Plates were then incubated at room temperature for 5–7 days. Plate counts were computed after incubation, after which approximately 150 colonies were selected for

each plant. The colonies were selected from all plates with distinctive colonies (counts between 30 and 300). All the different colony morphologies were picked in relation to their abundance as much as possible. The selected colonies were then subcultured to purity, Gram-stained and cryo-preserved at -80°C.

Isolate identification by 16S rRNA gene sequencing

Isolates were identified by analysis of the DNA sequences of their 16S rRNA genes. Five to six colonies of each isolate were harvested into sterile, nuclease-free microcentrifuge tubes containing 100 µl of sterile molecular biology grade water. This was then boiled at 95°C for 10 minutes [53] and used as a template for PCR. PCR amplification of 16S rRNA genes was conducted for all isolates using (illustra TM) puRe Taq Ready-To-Go PCR beads in a 25 µl reaction volume. The amplification protocol for 10F and 1507R began with initial denaturation at 94°C for 2 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 1 minute and 30 seconds, with a final step of 72°C for 5 minutes. The 27F and 1492R primer set was as follows: initial denaturing temperature 94°C for 2 minutes, denaturing temperature 94°C for 30 seconds, annealing temperature 53°C for 30 seconds, extension temperature 72°C for 1 minute and 30 seconds, and final step 72°C for 5 min for a total of 36 cycles. The primers employed are listed in Table 1.

Table 1
Oligonucleotide primers employed to amplify 16S rRNA gene

Gene name	Primer	Primer sequence	Product size	Reference
16S rRNA (10-1507)	10F	AGTTTGATCATGGCTCAGATTG	1494 bp	[54]
	1507R	TACCTTGTTACGACTTCACCCAG		
16S rRNA (27-1492)	27F	GAGAGTTTGATCCTGGCTCAG	1466 bp	[54]
	1492R	GGTTACCTTGTTACGACTT		

Following amplification, 5 µl of each PCR product was electrophoresed on a 1.5% (w/v) agarose gel containing gel red at 110 volts for 45 minutes. The gels were thereafter visualized on a transilluminator, after which PCR amplicons were Sanger sequenced by Inqaba Biotec (Ibadan). There was no amplification for some isolates after trying both universal primers, and as a result, their 16S rRNA genes were not sequenced. Raw reads with acceptable peak quality on traces were analysed using the Basic Local Alignment Search Tool (BLAST) [55] version 2.6.0 + tool of the NCBI (National Center for Biotechnology Information) 16S rRNA database for bacteria and archaea. Strains with sequences 97% identical to the database match were presumed to belong to the same species as the matching organism in the database, and a 95% cut-off was used to define genera [56]. Sequence data with low quality were exempted from downstream analyses. The sequences were deposited in GenBank with accession numbers OQ396403-OQ396569.

Multiple sequence alignments and phylogenetic tree construction were performed using the MEGA tool (<http://www.megasoftware.net/>) following directions and recommendations by Hall (2013). All

sequences were trimmed to approximately 840 bp, equivalent to positions 20 to 855 of the *E. coli* 16S rRNA gene. The alignment was then executed, and the data were exported as a mega file, after which they were used to build a phylogenetic tree (Construct/Test Maximum Likelihood Tree). Phylogenetic trees were visualized and annotated with the interactive Tree of Life tool (iTOL) [58].

Isolate characterization by phenotypic coliform test

All identified *E. coli* and *E. fergusonii* isolates were tested for lactose fermentation at 44°C to determine if they are type 1 *E. coli* typically associated with the mammalian gastrointestinal tract [59, 60]. Briefly, isolates were subcultured on 0.1X tryptone soy agar for 24 hours and subsequently incubated in 5 mL of MacConkey broth at 44°C for 24 hours. A colour change from purple to yellow indicated a positive test and was used to differentiate type 1 from other *E. coli*.

Isolate characterization by antimicrobial screening using plant extracts

Ethyl-acetate extracts of dried pulverized leaves of *E. lateriflora* and *F. thonningii* were obtained with the Soxhlet extraction method [61]. The extracts were then concentrated to constant weight with the aid of a rotary evaporator before storage at -20°C for future use.

To determine whether the plants had antimicrobial activity against phyllosphere isolates, the agar diffusion method was employed. Briefly, the ethyl acetate extracts of both plants were dissolved in methanol to a concentration of 100 mg/mL. Phyllosphere isolates and standard control strains were grown on 0.1X TSA, after which they were suspended in 0.85% physiological saline to a turbidity level equivalent to 0.5% McFarland's standard. Sterile cotton swabs were used to evenly spread the inoculum on pre-prepared sterile plates of 0.1X TSA. Equidistant wells were thereafter bored with the aid of an 8 mm cork-borer. One hundred microliters of prepared extracts was then dispensed in the respective wells and allowed sufficient time to diffuse, after which the plates were incubated at room temperature with daily observations. At the end of 5 days of incubation, zone diameters were recorded.

Minimum inhibitory concentrations (MICs) were determined using the agar dilution method. TSA (0.1X) plates containing doubling dilutions of extract in concentrations ranging between 0.39 mg/mL and 50 mg/mL were prepared in distilled methanol. Methanol was also used as a solvent control for the assay. Plates containing twofold dilutions of chlorocresol from 7.8 µg/mL to 1000 mg/mL were similarly prepared. An overnight broth culture of each isolate on 0.1X tryptone soy broth (TSB) was diluted, and 200 µl of dilutions equivalent to a 0.5 McFarland standard was transferred into a sterile 96-well microtiter plate, which was used to surface-inoculate duplicate plates of each extract or chlorocresol concentration using a sterile 48-point multipoint inoculator. Sterile plates of 0.1X TSA and MacConkey Agar with neither extract nor antibacterial were similarly inoculated. Plates were incubated at ambient temperatures for up to 5 days, with daily inspection. The lowest concentration of antimicrobials that inhibited the growth of each isolate over the entire incubation period was recorded as its MIC.

Results

Marker gene copy estimation for archaea, bacteria and fungi by qPCR revealed higher copy numbers for fungi and bacteria than for archaea for both *E. lateriflora* and *F. thonningii* (Fig. 1). Kruskal–Wallis statistics indicated a significant difference in gene abundance between archaea, bacteria and fungi ($p = 0.02$) but no significant difference comparing the estimated abundance of these domains across the two plants ($p = 0.52$).

Leaves of *E. lateriflora* and *F. thonningii* were abundantly colonized: the colony forming units per gram of leaf were 1.72×10^7 and 7.63×10^6 , respectively, with significantly more colonies from *E. lateriflora* in comparison to *F. thonningii*. Overall, 127 and 98 isolates were identified from *Euphorbia lateriflora* and *Ficus thonningii*, respectively. Bacterial species of Actinomycetota, Bacillota and Pseudomonadota were identified from both plants (Table 2). A total of 11 and 16 different genera were identified on *E. lateriflora* and *F. thonningii*, respectively. Only seven genera, *Bacillus*, *Lysinibacillus*, *Staphylococcus*, *Methylobacterium*, *Sphingomonas*, *Escherichia* and *Pseudomonas*, were shared across both plants. Four genera were unique to *E. lateriflora*, while nine were exclusive to *F. thonningii*. Some isolates could not be assigned to the genus level and were thus classified as ‘unassigned’ and placed in the family of the closest BLAST hit. There were 15 such isolates from *E. lateriflora* and 14 from *F. thonningii*.

Table 2
Taxonomic summary of identified isolates from *E. lateriflora* and *F. thonningii*

Taxonomic summary of identified isolates from <i>E. lateriflora</i> and <i>F. thonningii</i>				
	<i>E. lateriflora</i>		<i>F. thonningii</i>	
	Isolate number	Number of species	Isolate number	Number of species
Actinomycetota				
Actinomycetales				
<i>Brevibacterium</i>	5	3	0	0
Micrococcales				
<i>Brachybacterium</i>	0	0	1	1
<i>Curtobacterium</i>	0	0	1	1
<i>Microbacterium</i>	0	0	7	2
<i>Micrococcus</i>	0	0	1	1
Bacillota				
Bacillales				
<i>Bacillus</i>	4	1	10	2
<i>Lysinibacillus</i>	2	1	2	1
<i>Staphylococcus</i>	2	2	8	5
Unassigned	0		3	
Alpha-Proteobacteria				
Hyphomicrobiales				
<i>Methylobacterium</i>	1	1	1	1
Sphingomonadales				
<i>Sphingomonas</i>	2	2	1	1
Beta-Proteobacteria				
Burkholderiales				
<i>Aquabacterium</i>	2	2	0	0
<i>Methylibium</i>	1	1	0	0
<i>Pelomonas</i>	0	0	1	1
<i>Rivibacter</i>	1	1	0	0

	<i>E. lateriflora</i>		<i>F. thonningii</i>	
Unassigned	14		3	
Gamma-Proteobacteria				
Enterobacterales				
<i>Pantoea</i>	0	0	1	1
<i>Enterobacter</i>	0	0	2	2
<i>Escherichia/Shigella</i>	70	2	35	2
Unassigned	1		7	
Pseudomonadales				
<i>Pseudomonas</i>	21	2	7	3
Vibrionales				
Unassigned	0		1	
Xanthomonadales				
<i>Xanthomonas</i>	0	0	2	2

The microbiome of both plants was dominated by Pseudomonadota (Alpha- and Gamma-Proteobacteria), with over 65% of OTUs assigned to the *Sphingomonas* genus (Fig. 2). Excluding *Sphingomonas*, Gammaproteobacteria, Bacilli, Alphaproteobacteria and Actinomycetia were the most commonly detected taxonomic classes with visible but statistically insignificant variation between replicates. Bacillales, Burkholderiales, Enterobacterales, Rhizobiales and Xanthomonadales were the dominant orders across replicates of both plants. A high proportion of Pseudomonadales was observed for *F. thonningii* compared to *E. lateriflora*. At the family level, Bacillaceae, Beijerinckiaceae, Enterobacteriaceae and Xanthomonadaceae were abundant on both plants. While Beijerinckiaceae and Vagococcaceae were relatively more abundant in *E. lateriflora*, Moraxellaceae was more abundant in *F. thonningii*, and Oxalobacteriaceae was exclusively detected in *F. thonningii*. Overall, both plants were similar in their bacterial composition at higher taxonomic levels, but the similarity was reduced at lower taxonomic levels.

The two most frequently detected fungal classes were Dothideomycetes and Sordariomycetes (Fig. 2). The orders Capnodiales, Mycosphaerellales and Pleosporales were commonly identified from both plants. Higher, almost exclusive abundances of Glomerallales and Hypocreales were observed in *Ficus*, while Trichosphaeriales and Xylariales were observed in Euphorbia. At the family level, Cladosporiaceae, Corynesporascaceae and Mycosphaerellaceae were identified at high frequencies on both plants. Didymellaceae, Massarinaceae, Nectriaceae and Neodevriesiaceae were almost exclusive to *F. thonningii*, and Dissoconiaceae, Schizothyriaceae and Amphispheariaceae were more commonly identified in *E.*

lateriflora. Similar to the observation for 16S rRNA sequences, both plants were composed of similar taxa, but the proportions decreased at lower taxonomic levels.

Figure 3 shows 16S rRNA sequence-based phylogeny of the identified isolates with associated metadata-source plant and bacterial classification at the phyla, order and family levels. Isolates clustered according to established phylogeny.

A total of nine families were identified in *E. lateriflora* (Fig. 4A) and 13 in *F. thonningii* (Fig. 4B). While Enterobacteriaceae, Pseudomonadaceae, Bacillaceae, Staphylococcaceae, Methylobacteriaceae, Sphingomonadaceae, Comamonadaceae, and Burkholderales were common to both plants, Brevibacteriaceae (5 isolates) was exclusive to *E. lateriflora*, while Microbacteriaceae (8 isolates), Micrococcaceae (1 isolate), Xanthomonadaceae (2 isolates), Dermabacteriaceae (1 isolate) and Vibronaceae (1 isolate) were unique to *F. thonningii*.

Figure 5 shows the phylogeny of representative strains from both plants and four control strains that were subjected to antimicrobial susceptibility testing of ethyl acetate extracts of the plants and chlorocresol. Zones of inhibition from the agar diffusion method ranged from 13 mm to 34 mm, depending on the plant and isolates. Overall, the extract of *E. lateriflora* produced wider zones of inhibition when compared to *F. thonningii*. Both extracts consistently produced wider zones of inhibition than ciprofloxacin (10 µg/mL) and chlorocresol (500 µg/mL). The MICs of the plant extracts ranged from 3.125 mg/mL to 12.5 mg/mL, while those of chlorocresol ranged from 62 µg/mL to > 1000 µg/mL. Overall, the MICs produced by *E. lateriflora* extracts were lower than those produced by *F. thonningii* extracts. However, *Xanthomonas perforans*, *Micrococcus luteus* and two strains of *Microbacterium testaceum* from *F. thonningii* had relatively higher MICs to the *Euphorbia lateriflora* extract.

Sixty-five and thirty-three isolates from *E. lateriflora* and *F. thonningii*, respectively, were identified as *Escherichia coli*, and sequencing found nucleotide differences within their 16S rRNA genes, suggesting the possibility of multiple clones. Figure 6 shows the result of the coliform test on *Escherichia* isolates from both plants. Thirty-eight out of forty-one isolates of *E. lateriflora* fermented lactose at 44°C, while only one of eleven isolates of *F. thonningii* was positive. Hence, a greater proportion of *Escherichia* isolates of *E. lateriflora* are type 1 *E. coli* associated with mammalian intestinal colonization.

Discussion

Our research on two common Nigerian medicinal plants, *Euphorbia lateriflora* and *Ficus thonningii*, showed that they are densely colonized by different phyllosphere bacteria.

Fungal and bacterial marker genes were abundantly detected through qPCR in relation to archaea. Bacteria and fungi are the most abundant groups inhabiting the phyllosphere, and several reports have confirmed this with high marker gene abundance using quantitative PCR, amplicon and metagenome sequencing approaches [1, 62, 63]. It has been argued that the abundance of archaea is frequently underestimated, and the full potential of the archaeaome is poorly understood due to limitations in

current assays [64–66]. However, archaea have been reported in high abundances in the rhizosphere [63, 66, 67]. The phyllosphere is probably less suited for archaeal proliferation.

The high abundance determined by estimation of colony forming units of bacteria per gram (CFU/g) of leaves suggests that the leaves of *E. lateriflora* and *F. thonningii* in Ibadan are heavily colonized. Phyllosphere bacterial populations typically range from 10^1 to 10^8 CFU/g depending on the plant species and on its environmental, climatic and geographical habitat [5, 53, 68–72]. The numbers recorded in this study are in the upper range, similar to those obtained from sampling of roots and rhizosphere environment [5, 73]. This may be attributable to the plants' intrinsic properties and differences in environmental conditions from other phyllosphere studies, as the vast majority of studies originated outside Nigeria and West Africa. There are relatively less fluctuations in temperature and humidity in tropical Nigeria, which may favour the proliferation of bacteria in the phyllosphere, resulting in large populations.

Overall, Pseudomonadota was the most abundant phylum in all replicates of the two plants. Pseudomonadota is frequently the most dominant phylum in several phyllosphere studies [1, 74]. We observed that the dominance of Pseudomonadota was highly influenced by Alphaproteobacteria (75 to 90% of total ASVs depending on the sample), which was due to an overabundance of the *Sphingomonas* genus. *Sphingomonas* is a major genus in the phyllosphere of many plants and colonizes a wider variety of plants when compared to the more commonly detected *Pseudomonas* genus [75–79]. Sphingomonads are well adapted to withstand harsh conditions and competitive life in the phyllosphere [75, 80, 81], where they are associated with antagonism, plant protection from disease [76, 82, 83] and geochemical cycling.

To visualize the composition of other taxa in the microbiome more clearly, we removed all ASVs mapping to *Sphingomonas* spp. and repeated the analyses. The most abundant class shifted to Gammaproteobacteria, followed by Bacilli, Alphaproteobacteria and Actinomycetota. There were visible differences in the abundance between the two plants. For instance, Actinomycetia, Alphaproteobacteria and Bacilli were more abundant in *E. lateriflora*, while Gammaproteobacteria were more abundant in *F. thonningii*. However, the observed differences were not statistically significant. The differences in composition and abundance between the plants were more evident at lower taxonomic levels, where Oxalobacteraceae was exclusively detected in *F. thonningii*. Overall, the compositions of the microbiome of both plants were similar, which may be partly ascribed to similar environmental and climatic conditions, as the plants were collected in close locations. It has been established that environmental conditions and geographical location have a profound influence on phyllosphere microbiome composition [84]. The host plant is also considered a significant influential factor of microbiome composition and abundance and is argued to be the most significant single factor from studies that have observed similar composition from identical plants in different locations [85, 86]. However, several studies have also reported similarities in different plants within the same location and differences between identical plants in different locations [84, 87]. It is evident that an interplay of factors influences the composition of the phyllosphere microbiome, and none of these can be singly ascribed to a single

factor [88]. However, the combination of location and host plant seems to be significantly involved in microbiome composition [4]. Nevertheless, there are remarkable overlaps between the phyllosphere microbiome composition of diverse plants irrespective of geographical location, plant genetics, and other factors [74, 84].

The most dominant fungal classes, Dothideomycetes and Sordariomycetes, are commonly detected in high numbers in the phyllosphere [74, 84, 88]. Differences in fungal composition between the plants were more visible compared to bacteria, and this was statistically significant. Similar to bacterial composition, the plants shared some ASVs, notably Cladosporiaceae, Corynesporascaceae and Mycosphaerellaceae. The commonly detected fungal families, Cladosporiaceae, Didymellaceae and Nectriaceae, were also found in our study. We observed less fungal diversity than bacterial diversity, which is consistent with other reports of phyllosphere epiphytes [74, 88].

The taxonomic composition of bacterial isolates via culture was specific and unique for each plant species. We note that 71 (56%) and 38 (40%) of the isolates characterized from *E. lateriflora* and *F. thonningii*, respectively, were Pseudomonadota belonging to the species *Escherichia coli*. Phenotypic examination demonstrated that 38 out of 41 of these isolates from *E. lateriflora* produced both acid and gas from lactose at 44°C, strongly suggesting that their origin is recent fecal contamination from warm-blooded animals. All but one of the *E. coli* isolates from *F. thonningii* did not show these properties, suggesting that they are likely of environmental origin, preferably called type 2 *Escherichia* sp., and may indeed be stable members of the phyllosphere. This unique ecological succession on the leaves presumably indicates that height is a factor to consider in the type of organisms that colonize the phyllosphere microbiome, as *F. thonningii* is a tree, while *E. lateriflora* is a shrub. At least some mammalian-associated *E. coli* lineages are known to be associated with diminished phyllosphere abundance [89], which may account for the relatively smaller number of non-*E. coli* isolates in this study compared with other culture-based phyllosphere studies. Dominance of Enterobacteriaceae other than *Pantoea* sp. in the phyllosphere, although relatively unpopular, has been reported. Enterobacteriaceae, including antimicrobial resistant *Shigella* and *Enterobacter*, were found to dominate the phyllosphere of arugula (*Eruca sativa*), a commonly consumed vegetable in Graz, Austria, with resultant implications for the health of those who consume it raw [90]. A study of banana phyllospheres in Uganda similarly reported an overdominance of enteric bacteria in the banana pseudostems [91]. The overdominance of Enterobacteriaceae may be linked to the ubiquitous use of organic manure in agriculture, and as the average annual temperature is approximately 26°C and 27°C in Uganda and Nigeria, respectively, enteric bacteria will be able to thrive for extended periods. Additionally, birds perching on trees may produce droppings that may introduce enteric organisms into phyllospheres.

All non-*E. coli* isolates belonged to three phyla, Pseudomonadota, Actinomycetota and Bacillota, which are common on plants [67, 92, 93]. The majority of isolates from *E. lateriflora* belonged to the families Comamonadaceae (Pseudomonadota), Enterobacteriaceae (Pseudomonadota) and Pseudomonadaceae (Pseudomonadota), and those from *F. thonningii* belonged to the families Enterobacteriaceae (Pseudomonadota) and Bacillaceae (Bacillota). Other less dominant families include Brevibacteriaceae

(Actinomycetota), Micrococcaceae (Actinomycetota), Xanthomonadaceae (Pseudomonadota), Microbacteriaceae (Actinomycetota), Sphingomonadaceae (Pseudomonadota) Staphylococcaceae (Bacillota) and Methylobacteriaceae (Pseudomonadota). Thirteen and nineteen genera were represented on *E. lateriflora* and *F. thonningii*, respectively, while 29 isolates, designated 'unknown', could not be classified into any genera based on the cut offs as set by Kim et al., (2014). Fourteen (48.3%) of these isolates were Burkholderiales, and work to characterize and report these potential novel species is ongoing. Isolates belonging to all three phyla, Pseudomonadota, Actinomycetota and Bacillota, were detected in both *E. lateriflora* and *F. thonningii*. However, the composition of the phyllosphere at lower classification levels is unique to each plant. The Bacillota genera *Bacillus*, *Lysinibacillus* and *Staphylococcus* were found in both plants but in higher proportions in *F. thonningii*. Similarly, *Pseudomonas* was detected in higher proportions in *E. lateriflora*. Important plant-associated taxa, including *Pantoea* and *Xanthomonas*, were found only in *F. thonningii*. Actinomycetales, represented only by *Brevibacterium*, were isolated exclusively in *E. lateriflora*, while Micrococcales were isolated exclusively in *F. thonningii*. Overall, *E. lateriflora* and *F. thonningii* shared seven bacterial genera and differed by four and nine unique genera, respectively. Several factors, including host plant intrinsic characteristics, plant location, environmental factors and microbiome interactions, influence phyllosphere microbiome composition [94–96]. Of these factors, the host plant is the most significant, as evident from the similarity in the phyllosphere microbiome of identical plants across different locations, and may account for the differences observed in the present study [4, 97–99]. Some of the isolates identified are dynamic in their interactions with plants. *Pseudomonas oryzihabitans*, for instance, could be pathogenic, causing stem and leaf rot in Chinese muskmelon [100] and rice panicle blight and grain discolouration [101]. Despite being a plant pathogen, *P. oryzihabitans* can also be beneficial and has been shown to promote plant growth and prevent colonization by pathogenic fungi and nematodes [102–104]. Other plant pathogens identified include *Pantoea allii*, *Xanthomonas perforans* and *Xanthomonas euvesicatoria* [105–107].

There was a contrast in the abundance of taxa by culture and culture-independent methods, and this is usually the case [63]. However, there were overlaps in the dominant organisms detected by both methods, including Sphingomonadaceae, Enterobacteriaceae, Bacillaceae, Pseudomonadaceae and Xanthomonadaceae.

The majority of the genera identified in this study have been reported to be found in other plants. [68, 69, 108–110]. However, at the species level, only a few species have been reported on plants. *Pseudomonas taiwanensis*, for instance, was first classified after isolating it from soil [111], while *Pantoea allii* was first classified after isolation from onion plants [105]. Actinomycetota, represented in this study by *Microbacterium*, *Brevibacteria* and *Micrococcus*, has been reported [112, 113] to produce secondary metabolites with diverse activities, including antimicrobial activity and promotion of plant growth. Similarly, the genera *Pseudomonas* and *Pantoea* consist of members exhibiting antagonistic properties [13, 68] and are commonly known to coexist with plants in a symbiotic relationship. The occurrence of these genera on *E. lateriflora* and *F. thonningii* analysed in this study could contribute positively to the antimicrobial activity of the plant. Indeed, Egamberdieva et al., (2017) reported a correlation between the

proportion of antagonistic bacteria and the antimicrobial properties of two plants that were discordant for medicinal activity. The medicinal plant employed in the above study (*Hypericum perforatum*) housed a significant population of antagonistic bacteria compared to its nonmedicinal counterpart, *Ziziphora capitata*. As has been reported previously [32, 114], hexane, ethyl-acetate and methanol extracts of *E. lateriflora* and *F. thonningii* show activity against type cultures of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Citrobacter freundii*, as they did in this study. Phyllosphere isolates obtained from both plants showed wider zones of inhibition to the extracts of both plants, whereas only a handful were susceptible to ciprofloxacin and/or chlorocresol. In the same vein, phyllosphere isolates were typically inhibited by the plant extracts, as shown by the MICs, and not by chlorocresol. MIC values were lower for the *E. lateriflora* extract than for *F. thonningii* across board. However, one *Xanthomonas perforans*, one *Micrococcus luteus* and two strains of *Microbacterium testaceum* all isolated from *F. thonningii* had higher MICs to the *E. lateriflora* extract.

Overall, the data do not support our hypothesis that phyllosphere bacteria on leaves are resistant to the antimicrobial secondary metabolites extracted from plants. While it is possible that the chemical nature of antimicrobial principles is altered during extract preparation, it is more probable that secondary metabolites produced by healthy plants are embedded within the tissues of the plants, and as such, bacteria leaving on the surfaces of the plants may not have direct contact with such metabolites unless the plant is injured. Our findings also point to the possibility that antimicrobial resistance to active plant metabolites may be uncommon in nature, pointing to the potential of active principles to yield therapeutics less likely to be compromised by antimicrobial resistance. As only two plants were evaluated in this study, the generalizability of our findings is presently unknown and should be the subject of future research.

Conclusions

This study has identified the bacteria associated with the leaves of *E. lateriflora* and *F. thonningii*, which are important components of the plant, among which are organisms that have probably not been described previously. The phyllosphere bacteria of both plants are dominated by Enterobacteriaceae by culture and *Sphingomonas* by amplicon sequencing. This study has also shown that culturable components of plant microbiomes can be inhibited by plant extracts. It is, however, noteworthy that *F. thonningii* isolates are inhibited by higher concentrations of the extract from this plant than are *E. lateriflora* isolates, pointing to the possibility that antimicrobial principles may shape the microbiome. This hypothesis is supported by the observation that each of the plants we studied has a distinct, narrowly overlapping culturable phyllosphere. As only culture and amplicon sequencing methods were employed in this study, we may have missed some important components of the phyllosphere: those unculturable under the conditions employed and those that may be negatively biased by the primers used for amplicon library preparation. However, the study has provided insight into the composition of the phyllosphere, which includes some plant-associated taxa as well as some probable new species. Although we did not sample the plants to saturation, we provide baseline data for more in-depth studies on the phyllosphere of these plants and other medicinal plants.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable

Availability of data and material: The assembled 16S rRNA gene sequences generated from cultured isolates are available under an NCBI Bioproject with accession numbers OQ396403-OQ396569. Sequences from the targeted metagenomics (fungal ITS and bacterial 16S rRNA genes) are available under the NCBI SRA database BioSample accession PRJNA1036807-
<https://www.ncbi.nlm.nih.gov/sra/PRJNA1036807>

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Author Contributions: AOO, MEC and INO conceptualized and designed the study. AOO and DC prepared materials, collected data and performed data analyses. AOO prepared the first draft and revisions. MEC, GB and INO critically reviewed the article. MEC, GB and INO supervised the study. All authors read and approved the final article draft.

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References

1. Lindow SE, Brandl MT. MINIREVIEW Microbiology of the Phyllosphere. Appl Environ Microbiol. 2003;69:1875–83.
2. Vorholt JA. Microbial life in the phyllosphere. Nat Publ Gr [Internet]. Nature Publishing Group; 2012;10:828–40. Available from: <http://dx.doi.org/10.1038/nrmicro2910>
3. Yang C, Crowley DE, Borneman J, Keen NT. Microbial phyllosphere populations are more complex than previously realized. PNAS. 2001;98:3889–94.
4. Knief C, Ramette A, Frances L, Alonso-blanco C, Vorholt JA. Site and plant species are important determinants of the Methylobacterium community composition in the plant phyllosphere. ISME J [Internet]. Nature Publishing Group; 2010;4:719–28. Available from: <http://dx.doi.org/10.1038/ismej.2010.9>

5. Köberl M, Schmidt R, Ramadan EM, Bauer R, Berg G. The microbiome of medicinal plants: diversity and importance for plant growth , quality , and health. *Front Microbiol.* 2013;4:1–9.
6. Schmidt R, Köberl M, Mostafa A, Ramadan EM, Monschein M, Jensen KB, et al. Effects of bacterial inoculants on the indigenous microbiome and secondary metabolites of chamomile plants. *Front Microbiol.* 2014;5:1–11.
7. Perreault R, Laforest-Lapointe I. Plant–microbe interactions in the phyllosphere: facing challenges of the anthropocene. *ISME J. Springer US;* 2021;1–7.
8. Sauer S, Dlugosch L, Kammerer DR, Stintzing FC, Simon M. The Microbiome of the Medicinal Plants *Achillea millefolium* L . and *Hamamelis virginiana* L . *Front Microbiol.* 2021;12:1–19.
9. Strobel G, Daisy B. Bioprospecting for Microbial Endophytes and Their Natural Products. *Microbiol Mol Biol Rev.* 2003;67:491–502.
10. Strobel G, Daisy B, Castillo U, Harper J. Natural Products from Endophytic Microorganisms . *J Nat Prod.* 2004;67:257–68.
11. Gunatilaka L. Natural Products from Plant-Associated Microorganisms: Distribution, Structural Diversity, Bioactivity, and Implications of their Occurrence. *J Nat Prod.* 2006;69:509–26.
12. Chandra S. Endophytic fungi: novel sources of anticancer lead molecules. *Appl Microbiol Biotechnol.* 2012;95:47–59.
13. Egamberdieva D, Wirth S, Behrendt U, Ahmad P. Antimicrobial Activity of Medicinal Plants Correlates with the Proportion of Antagonistic Endophytes. *Front Microbiol.* 2017;8:1–11.
14. Miller KI, Qing C, Sze DMY, Roufogalis BD, Neilan BA. Culturable Endophytes of Medicinal Plants and the Genetic Basis for Their Bioactivity. *Microb Ecol.* 2012;64:431–49.
15. Burkill HM. The useful plants of west tropical africa [Internet]. 6th ed. Royal Botanic Gardens, Kew; 1994. Available from: <https://www.cabdirect.org/cabdirect/abstract/20087206660>
16. Usman M., Sule M., Gwarzo M. Toxicological studies of aqueous root extract of *Euphorbia lateriflora* (Schum and Thonn) in rats. *J Med Plants Stud.* 2014;2:58–62.
17. Kheyrodin H, Ghazvinian KH. The Toxicity Material Extraction From *Euphorbia* Species. *Iran Agric Res.* 2013;31:66–73.
18. Emmanuel N. Ethno medicines used for treatment of prostatic disease in Foumban, Cameroon. *African J Pharm Pharmacol* [Internet]. 2010;4:793–805. Available from: <http://www.academicjournals.org/ajpp/PDF/pdf2010/November/Emmanuel.pdf>
19. Egharevba HO, Carew O, Kunle OF. Phytochemical and Pharmacognostic Analysis of *Ficus thonningii* Blume Leaves for Monograph Development. *Int J Basic Appl Sci.* 2015;4:94–100.
20. Orwa C, Mutua A, Kindt R, Jamnadass R, Anthony S. *Ficus thonningii* Blume *Ficus thonningii* Blume. Agroforestry Database a tree Ref Sel Guid version 40 [Internet]. 2009. p. 1–5. Available from: <http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp>
21. Aniagu SO, Nwinyi FC, Akumka DD, Agbani EO, Dzarma S, Ajoku GA, et al. Short - term toxicity studies of *Ficus thonningii* Blume (Moraceae) leaf extract in rats. *Int J Food Sci Technol.* 2008;43:456–63.

22. Coker ME, Emikpe BO, Adeniyi BA, Budale BA. The anti-inflammatory potential , heamatological and histological changes induced in rats due to the administration of methanolic extracts of *Ficus thonningii* leaves. *African J Pharm Pharmacol*. 2009;3:273–6.
23. Musabayane CT, Gondwe M, Kamadyaapa DR, Chuturgoon AA, Ojewole JAO. Effects of *Ficus thonningii* (Blume) [Moraceae] stem-bark ethanolic extract on blood glucose, cardiovascular and kidney functions of rats, and on kidney cell lines of the proximal (LLC-PK1) and distal tubules (MDBK). *Ren Fail*. 2007;29:389–97.
24. Njoroge GN, Kibunga JW. Herbal medicine acceptance, sources and utilization for diarrhoea management in a cosmopolitan urban area (Thika, Kenya). *Afr J Ecol*. 2007;45:65–70.
25. Bah S, Diallo D, Dembélé S, Paulsen BS. Ethnopharmacological survey of plants used for the treatment of schistosomiasis in Niono District, Mali. *J Ethnopharmacol*. 2006;105:387–99.
26. Moshi MJ, Otieno DF, Mbabazi PK, Weisheit A. The ethnomedicine of the haya people of bugabo ward, Kagera region, north western Tanzania. *J Ethnobiol Ethnomed*. 2009;5:24.
27. COUSINS D, HUFFMAN M. Medicinal properties in the diet of gorillas: an ethno-pharmacological evaluation. *Afr Study Monogr* [Internet]. 2002;23:65–89. Available from: <http://repository.kulib.kyoto-u.ac.jp/dspace/handle/2433/68214>
28. Alawa JP, Jokthan GE, Akut K. Ethnoveterinary medical practice for ruminants in the subhumid zone of northern Nigeria. *Prev Vet Med*. 2002;54:79–90.
29. Koné WM, Atindehou KK, Terreaux C, Hostettmann K, Traoré D, Dosso M. Traditional medicine in North C^{ote}-d' Ivoire: screening of 50 medicinal plants for antibacterial activity. *J Ethnopharmacol*. 2004;93:43–9.
30. Usman A, Abdulrahman FI, Usman A. Qualitative phytochemical screening and in vitro antimicrobial effects of methanol stem bark extract of *Ficus thonningii* (Moraceae). *African J Tradit Complement Altern Med*. 2009;6:289–95.
31. Ndukwe IG, Bello AI, Habila JD, John C. Phytochemical and antimicrobial screening of the crude petroleum spirit and methanol extracts of the stem bark , leaves and roots of *Ficus thoningii* (blume). *African J Biotechnol*. 2007;6:2645–9.
32. Coker ME, Oaikhen A. Antimicrobial Activity of the crude extracts and fractions of *Ficus thonningii* (Blume) on Isolates from Urinary Tract Infections. *J Med Act Plants*. 2020;9:310.
33. Adesida SA, Abioye OA, Bamiro BS, Amisu KO, Badaru SO, Coker AO. Staphylococcal bacteraemia among human immunodeficiency virus positive patients at a screening center in Lagos, Nigeria. *Beni-Suef Univ J Basic Appl Sci*. Springer Science and Business Media LLC; 2017;6:112–7.
34. Oyelana OA, Durugbo EU, Olukanni OD, Ayodele EA, Aikulola ZO, Adewole AI. Antimicrobial Activity of *Ficus* Leaf Extracts on Some Fungal and Bacterial Pathogens of *Dioscorea rotundata* from Southwest Nigeria. *J Biol Sci*. 2011;11:359–66.
35. Dangarembizi R, Erlwanger KH, Moyo D, Chivandi E. PHYTOCHEMISTRY , PHARMACOLOGY AND ETHNOMEDICINAL USES OF *FICUS THONNINGII* (BLUME MORACEAE): A REVIEW School of Physiology , Faculty of Health Sciences , University of the Witwatersrand , 7 York Road , Parktown ,

- Johannesburg , Republic of South Africa ,. *African J Tradit Complement Altern Med*. 2013;10:203–12.
36. Falade MO, Akinboye DO, Gbotosho GO, Ajaiyeoba EO, Happi TC, Abiodun OO, et al. In Vitro and In Vivo Antimalarial Activity of *Ficus thonningii* Blume (Moraceae) and *Lophira alata* Banks (Ochnaceae), Identified from the Ethnomedicine of the Nigerian Middle Belt. *J Parasitol Res*. 2014;2014:1–7.
 37. Probst AJ, Auerbach AK, Moissl-eichinger C. Archaea on Human Skin. *PLoS One*. 2013;8:e65388.
 38. Köberl M, Müller H, Ramadan EM, Berg G. Desert Farming Benefits from Microbial Potential in Arid Soils and Promotes Diversity and Plant Health. *PLoS One*. 2011;6:e24452.
 39. White TJ, Bruns T, Lee S, Taylor J. Amplification and Direct Sequencing of Fungal Ribosomal Rna Genes for Phylogenetics [Internet]. *PCR Protoc*. Academic Press, Inc.; 1990. Available from: <http://dx.doi.org/10.1016/B978-0-12-372180-8.50042-1>
 40. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A*. 2011;108:4516–22.
 41. Lundberg DS, Yourstone S, Mieczkowski P, Jones CD, Dangl JL. Practical innovations for high-throughput amplicon sequencing. *Nat Methods*. 2013;10:999–1002.
 42. Fitzpatrick CR, Lu-Irving P, Copeland J, Guttman DS, Wang PW, Baltrus DA, et al. Chloroplast sequence variation and the efficacy of peptide nucleic acids for blocking host amplification in plant microbiome studies. *Microbiome*. *Microbiome*; 2018;6:141–54.
 43. Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Mol Ecol*. 1993;2:113–8.
 44. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J*. 2011;17:10–2.
 45. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 2019;37:852–7.
 46. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13:581–3.
 47. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: A versatile open source tool for metagenomics. *PeerJ*. 2016;2016:1–22.
 48. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res*. 2013;41:590–6.
 49. Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, et al. The SILVA and “all-species Living Tree Project (LTP)” taxonomic frameworks. *Nucleic Acids Res*. 2014;42:643–8.
 50. Abarenkov K, Nilsson H, Larsson K-H, Alexander IJ, Eberhardt U, Erland S, et al. The UNITE database for molecular identification of fungi – recent updates and future perspectives. *New Phytol*.

2018;186:281–5.

51. Chong J, Liu P, Zhou G, Xia J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat Protoc* [Internet]. Springer US; 2020;15:799–821. Available from: <http://dx.doi.org/10.1038/s41596-019-0264-1>
52. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One*. 2013;8.
53. Yashiro E, Spear RN, Mcmanus PS. Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere. *J Appl Microbiol*. 2011;110:1284–96.
54. Mateos M, Castrezana SJ, Nankivell BJ, Estes AM, Markow TA, Moran NA. Heritable endosymbionts of *Drosophila*. *Genetics*. 2006;174:363–76.
55. Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. *J Computational Biol*. 2000;7:203–14.
56. Kim M, Oh HS, Park SC, Chun J. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol*. 2014;64:346–51.
57. Hall BG. Building Phylogenetic Trees from Molecular Data with MEGA. *Mol Biol Evol*. 2013;30:1229–35.
58. Letunic I, Gmbh BS. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res*. Oxford University Press; 2021;49:293–6.
59. Kamel MM. Thermotolerant Coliform and *Escherichia coli* Detection and Enumeration Through Multiple Tube Fermentation. *J Med Sci*. 2006;6:125–30.
60. Dockins WS, McFeters GA. Fecal coliform elevated temperature test: a physiological basis. *Appl Environ Microbiol*. 1978;36:341–8.
61. Harborne JB. *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*. 2nd ed. Chapman and Hall; 1984.
62. Bao L, Cai W, Zhang X, Liu J, Chen H, Wei Y, et al. Distinct Microbial Community of Phyllosphere Associated with Five Tropical Plants on Yongxing Island, South China Sea. *Microorganisms*. 2019;7:1–17.
63. Chelius MK, Triplett EW. The diversity of archaea and bacteria in association with the roots of *Zea mays* L. *Microb Ecol*. 2001;41:252–63.
64. Taffner J, Erlacher A, Bragina A, Berg C, Moissl-Eichinger C, Berg G. What Is the Role of Archaea in Plants? New Insights from the Vegetation of Alpine Bogs. *mSphere*. 2018;3.
65. Moissl-Eichinger C, Pausan M, Taffner J, Berg G, Bang C, Schmitz RA. Archaea Are Interactive Components of Complex Microbiomes. *Trends Microbiol* [Internet]. Elsevier Ltd; 2018;26:70–85. Available from: <http://dx.doi.org/10.1016/j.tim.2017.07.004>
66. Müller H, Berg C, Landa BB, Auerbach A, Moissl-Eichinger C, Berg G. Plant genotype-specific archaeal and bacterial endophytes but similar *Bacillus* antagonists colonize Mediterranean olive trees. *Front*

- Microbiol. 2015;6:1–9.
67. Knief C, Chaffron S, Stark M, Innerebner G. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J.* 2012;6:1378–90.
 68. Chiellini C, Maida I, Emiliani G, Mengoni A, Fabiani A, Bif S, et al. Endophytic and rhizospheric bacterial communities isolated from the medicinal plants *Echinacea purpurea* and *Echinacea angustifolia*. *Int Microbiol.* 2014;17:165–74.
 69. Mocali S, Bertelli E, Di F, Mengoni A. Fluctuation of bacteria isolated from elm tissues during different seasons and from different plant organs. *Res Microbiol.* 2003;154:105–14.
 70. Jackson CR, Randolph KC, Osborn SL, Tyler HL. Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. *BMC Microbiol.* 2013;13:1–12.
 71. Rastogi G, Sbodio A, Tech JJ, Suslow T V, Coaker GL, Leveau JHJ. Leaf microbiota in an agroecosystem: Spatiotemporal variation in bacterial community composition on field-grown lettuce. *ISME J. Nature Publishing Group;* 2012;6:1812–22.
 72. Thapa S, Prasanna R, Ranjan K, Velmourougane K, Ramakrishnan B. Nutrients and host attributes modulate the abundance and functional traits of phyllosphere microbiome in rice. *Microbiol Res [Internet]. Elsevier;* 2017;204:55–64. Available from: <http://dx.doi.org/10.1016/j.micres.2017.07.007>
 73. Lamb TG, Tonkyn DW, Kluepfel DA. Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue¹. *Can J Microbiol.* 1996;42:1112–20.
 74. Sohrabi R, Paasch BC, Liber JA, He SY. Phyllosphere Microbiome. *Annu Rev Plant Biol.* 2023;74.
 75. Li PD, Zhu ZR, Zhang Y, Xu J, Wang H, Wang Z, et al. The phyllosphere microbiome shifts toward combating melanose pathogen. *Microbiome [Internet]. BioMed Central;* 2022;10:1–17. Available from: <https://doi.org/10.1186/s40168-022-01234-x>
 76. Innerebner G, Knief C, Vorholt JA. Protection of *Arabidopsis thaliana* against Leaf-Pathogenic *Pseudomonas syringae* by *Sphingomonas* Strains in a Controlled Model System †. *Appl Environ Microbiol.* 2011;77:3202–10.
 77. Chen X, Wicaksono WA, Berg G, Cernava T. Bacterial communities in the plant phyllosphere harbour distinct responders to a broad-spectrum pesticide. *Sci Total Environ [Internet]. The Author(s);* 2021;751:141799. Available from: <https://doi.org/10.1016/j.scitotenv.2020.141799>
 78. Bao L, Cai W, Cao J, Zhang X, Liu J, Chen H, et al. Microbial community overlap between the phyllosphere and rhizosphere of three plants from Yongxing Island , South China Sea. *Microbiol Open.* 2020;1–18.
 79. Lundberg DS, Pedro R De, Pramoj P, Ayutthaya N, Karasov TL. Contrasting patterns of microbial dominance in the *Arabidopsis thaliana* phyllosphere. *PNAS.* 2022;119:1–11.
 80. Adi Wicaksono W, Reisenhofer-Graber T, Erschen S, Kusstatscher P, Berg C, Krause R, et al. Phyllosphere-associated microbiota in built environment: Do they have the potential to antagonize human pathogens? *J Adv Res [Internet]. Cairo University;* 2023;43:109–21. Available from: <https://doi.org/10.1016/j.jare.2022.02.003>

81. Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, et al. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc Natl Acad Sci U S A*. 2009;106:16428–33.
82. Sahu KP, Patel A, Kumar M, Sheoran N, Mehta S, Reddy B, et al. Integrated Metabarcoding and Culturomic-Based Microbiome Profiling of Rice Phyllosphere Reveal Diverse and Functional Bacterial Communities for Blast Disease Suppression. *Front Microbiol*. 2021;12:1–21.
83. Qin C, Tao J, Liu T, Liu Y, Xiao N, Li T, et al. Responses of phyllosphere microbiota and plant health to application of two different biocontrol agents. *AMB Express* [Internet]. Springer Berlin Heidelberg; 2019;9:42. Available from: <https://doi.org/10.1186/s13568-019-0765-x>
84. Bashir I, War AF, Rafiq I, Reshi ZA, Rashid I, Shouche YS. Phyllosphere microbiome: Diversity and functions. *Microbiol Res* [Internet]. Elsevier GmbH; 2022;254:126888. Available from: <https://doi.org/10.1016/j.micres.2021.126888>
85. Li J, Jin MK, Neilson R, Hu SL, Tang YJ, Zhang Z, et al. Plant identity shapes phyllosphere microbiome structure and abundance of genes involved in nutrient cycling. *Sci Total Environ* [Internet]. Elsevier B.V.; 2023;865:161245. Available from: <https://doi.org/10.1016/j.scitotenv.2022.161245>
86. Liu J, Zhang W, Liu Y, Zhu W, Yuan Z, Su X, et al. Differences in phyllosphere microbiomes among different *Populus* spp. in the same habitat. *Front Plant Sci*. 2023;14:1–12.
87. Meyer KM, Porch R, Muscettola IE, Vasconcelos ALS, Sherman JK, Metcalf CJE, et al. Plant neighborhood shapes diversity and reduces interspecific variation of the phyllosphere microbiome. *ISME J*. Springer US; 2022;16:1376–87.
88. Brandl MT, Mammel MK, Simko I, Richter TKS, Gebru ST, Leonard SR. Weather factors, soil microbiome, and bacteria-fungi interactions as drivers of the epiphytic phyllosphere communities of romaine lettuce. *Food Microbiol* [Internet]. Elsevier Ltd; 2023;113:104260. Available from: <https://doi.org/10.1016/j.fm.2023.104260>
89. Williams TR, Moyne AL, Harris LJ, Marco ML. Season, Irrigation, Leaf Age, and *Escherichia coli* Inoculation Influence the Bacterial Diversity in the Lettuce Phyllosphere. *PLoS One*. 2013;8:1–14.
90. Cernava T, Erlacher A, Soh J, Sensen CW, Grube M, Berg G. Enterobacteriaceae dominate the core microbiome and contribute to the resistome of arugula (*Eruca sativa* Mill.). *Microbiome*. *Microbiome*; 2019;7:1–12.
91. Rossmann B, Müller H, Smalla K, Mpiira S, Tumuhairwe JB, Staver C, et al. Banana-associated microbial communities in Uganda are highly diverse but dominated by Enterobacteriaceae. *Appl Environ Microbiol*. 2012;78:4933–41.
92. Finkel OM, Burch AY, Lindow SE, Post AF, Belkin S. Geographical Location Determines the Population Structure in Phyllosphere Microbial Communities of a Salt-Excreting Desert Tree †. *Appl Environ Microbiol*. 2011;77:7647–55.
93. Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Malfatti S, Tremblay J, et al. Defining the core *Arabidopsis thaliana* root microbiome. *Nature*. 2012;488:86–90.

94. Zhu Y, Xiong C, Wei Z, Chen Q, Ma B, Zhou S, et al. Impacts of global change on the phyllosphere microbiome. *New Phytol.* 2022;234:1977–86.
95. Eldridge DJ, Travers SK, Val J, Ding J, Wang T, Singh BK, et al. Experimental evidence of strong relationships between soil microbial communities and plant germination. *Journall Ecol.* 2021;1–11.
96. Singh P, Santoni S, This P, Peros J-P. Genotype-Environment Interaction Shapes the Microbial Assemblage in Grapevine ' s Phyllosphere and Carposphere: An NGS Approach. *Microorganisms.* 2018;6.
97. Abdelfattah A, Wisniewski M, Schena L, Tack AJM. Experimental evidence of microbial inheritance in plants and transmission routes from seed to phyllosphere and root. *Environ Microbiol.* 2021;23:2199–214.
98. Shakir S, Zaidi SS, Vries FT De, Mansoor S. Trends in Genetics Plant Genetic Networks Shaping Phyllosphere Microbial Community. *Trends Genet [Internet]. Elsevier Ltd;* 2020;1–11. Available from: <https://doi.org/10.1016/j.tig.2020.09.010>
99. Zhou S, Li H, Giles M, Neilson R, Yang X, Adams R. Microbial Flow Within an Air-Phyllosphere-Soil Continuum. *Front Microbiol.* 2021;11:1–10.
100. Li JF, Zhou GH, Wang T, Lin T, Wang YW, Zhu PK, et al. First Report of *Pseudomonas oryzihabitans* Causing Stem and Leaf Rot on Muskmelon in China. *Plant Dis.* 2021;2713.
101. Hou Y, Zhang Y, Yu L, Ding X, Liu L, Wang L, et al. First Report of *Pseudomonas oryzihabitans* Causing Rice Panicle Blight and Grain Discolouration in China. *Plant Dis.* 2020;3055.
102. Vagelas I, Gowen SR. CONTROL OF FUSARIUM OXYSPORUM AND ROOT-KNOT NEMATODES (MELOIDOGYNE SPP.) WITH PSEUDOMONAS ORYZIHABITANS. *Pakistan J Phytopathol.* 2012;1:32–8.
103. Cantabella D, Dolcet-sanjuan R, Solsona C, Vilanova L, Torres R, Teixido N. Optimization of a food industry-waste-based medium for the production of the plant growth promoting microorganism *Pseudomonas oryzihabitans* PGP01 based on agro-food industries byproducts. *Biotechnol Reports.* 2021;32.
104. Agelas IK V, Embroke BP, Owen SRG, Avies KGD. The control of root-knot nematodes (*Meloidogyne* spp .) by *Pseudomonas oryzihabitans* and its immunological detection on tomato roots. *Nematology.* 2007;9:363–70.
105. Brady CL, Goszczynska T, Venter SN, Cleenwerck I, Vos P De, Gitaitis RD, et al. *Pantoea allii* sp . nov ., isolated from onion plants and seed. *Int J Syst Evol Microbiol.* 2011;61:932–7.
106. Kyeon M, Son S, Noh Y, Kim Y, Lee H, Cha J. *Xanthomonas euvesicatoria* Causes Bacterial Spot Disease on Pepper Plant in Korea. *Plant Pathol J.* 2016;32:431–40.
107. Abrahamian P, Klein-Gordon JM, Jones JB, Vallad GE. Epidemiology , diversity , and management of bacterial spot of tomato caused by *Xanthomonas perforans*. *Appl Microbiol Biotechnol. Applied Microbiology and Biotechnology;* 2021;
108. Ikeda AC, Bassani LL, Adamoski D, Stringari D, Cordeiro VK, Glienke C, et al. Morphological and genetic characterization of endophytic bacteria isolated from roots of different maize genotypes.

- Microb Ecol. 2013;65:154–60.
109. Jin H, Yang X-Y, Yan Z-Q, Liu Q, Li X-Z, Chen J-X, et al. Characterization of rhizosphere and endophytic bacterial communities from leaves, stems and roots of medicinal *Stellera chamaejasme* L. Syst Appl Microbiol [Internet]. Elsevier GmbH.; 2014;1–28. Available from: <http://dx.doi.org/10.1016/j.syapm.2014.05.001>
 110. Gagne-Bourgue F, Aliferis KA, Seguin P, Rani M, Samson R, Jabaji S. Isolation and characterization of indigenous endophytic bacteria associated with leaves of switchgrass (*Panicum virgatum* L .) cultivars. J Appl Microbiol. 2013;114:836–53.
 111. Wang L, Tai C, Wu Y, Chen Y, Lee F, Wang S, et al. *Pseudomonas taiwanensis* sp . nov ., isolated from soil. Int J Syst Evol Microbiol. 2010;60:2094–8.
 112. Qin S, Xing K, Jiang J, Xu L. Biodiversity , bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria. Appl Microbiol Biotechnol. 2011;89:457–73.
 113. Pugh ND, Jackson CR, Pasco DS. Total bacterial load within *Echinacea purpurea*, determined using a new PCR-based quantification method, is correlated with LPS levels and in vitro macrophage activity. Planta Med. 2013;79:9–14.
 114. Coker ME, Oaikhena AO, Ajayi TO. Antimicrobial activity of extracts and fractions of *Euphorbia lateriflora* (Schum. and Thonn) on microbial isolates of the urinary tract. Saudi J Biol Sci [Internet]. King Saud University; 2021;28:4723–31. Available from: <https://doi.org/10.1016/j.sjbs.2021.04.086>

Figures

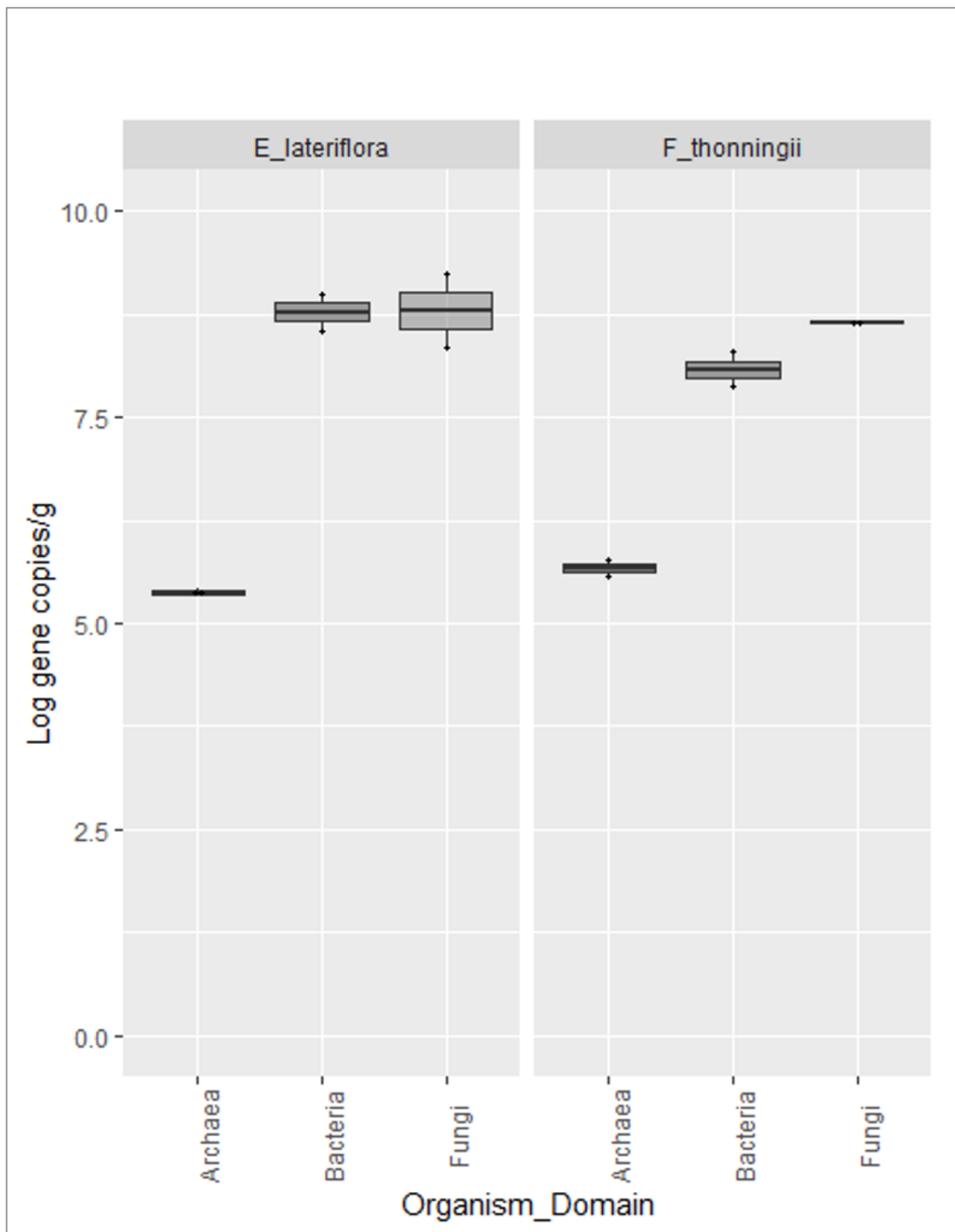


Figure 1

qPCR estimation of the abundance of archaeal 16S rRNA, bacterial 16S rRNA and fungal ITS genes in the community DNA of *E. lateriflora* and *F. thonningii*. Gene copies were normalized to the weight of DNA extraction starting plant material.

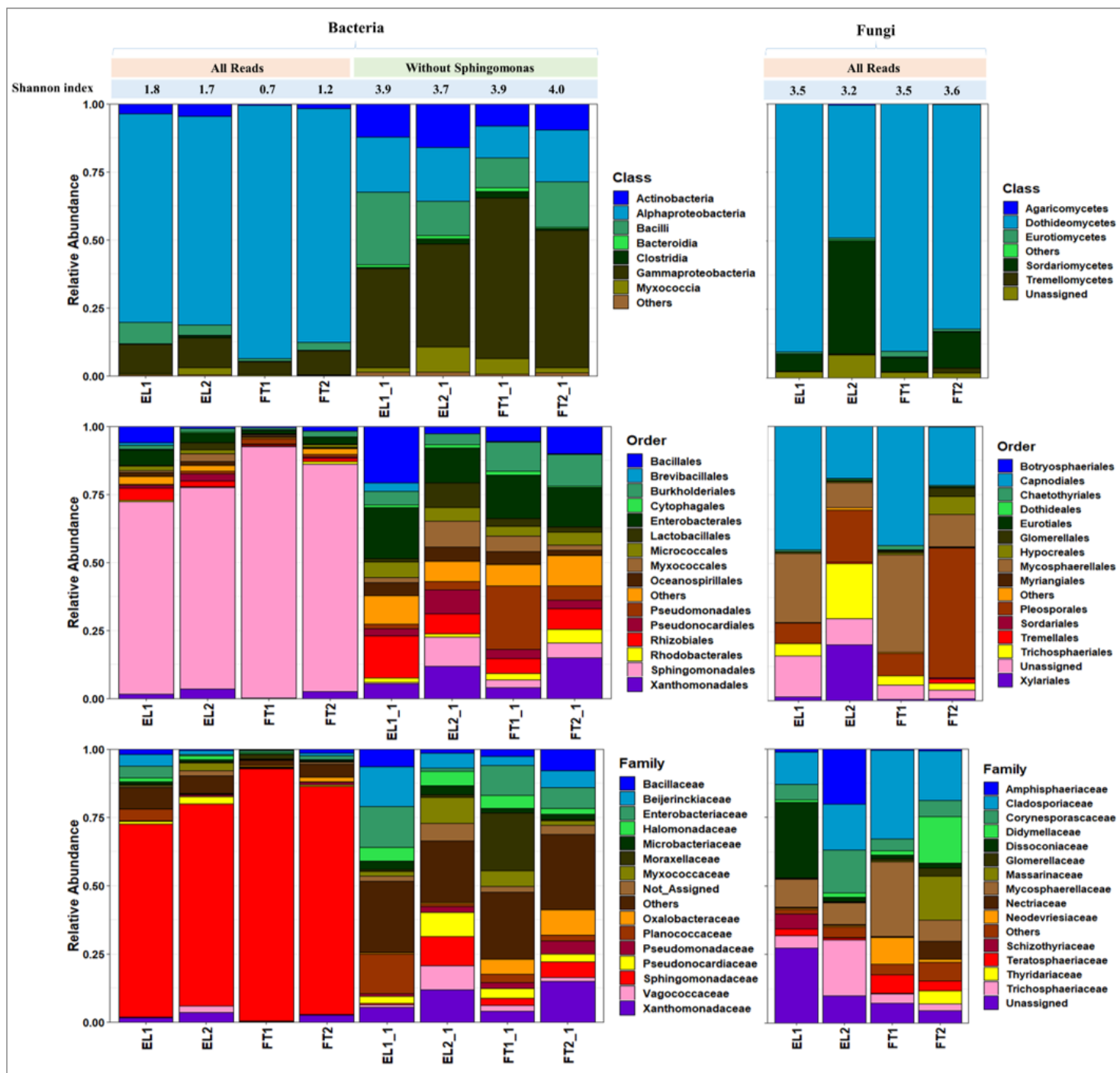


Figure 2

16S rRNA- and ITS-based bacterial and fungal diversity, composition and abundance in the phyllosphere of *E. lateriflora* and *F. thonningii*. Sample labels EL1 and EL2 represent technical replicates of *E. lateriflora*, while FT1 and FT2 represent technical replicates of *F. thonningii*. The bacterial samples EL1_1, EL2_1, FT1_1, and FT2_1 represent subsamples from which all ASVs of the genus *Sphingomonas* have been excluded.

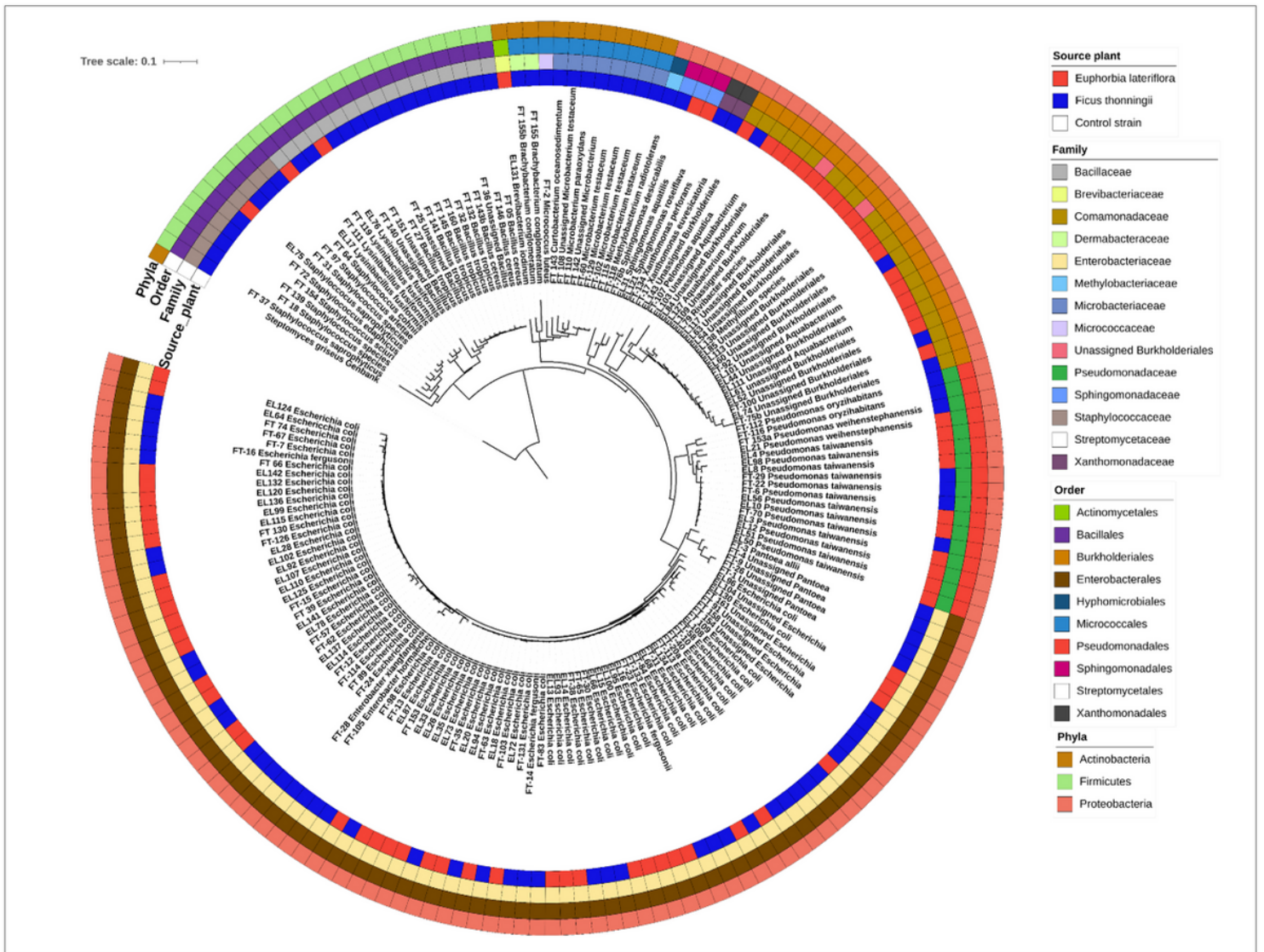


Figure 3

16S rRNA gene sequence-based phylogeny of cultured isolates from *E. lateriflora* and *F. thonningii*. Metadata bars represent host plant and phyla, order and family classification.

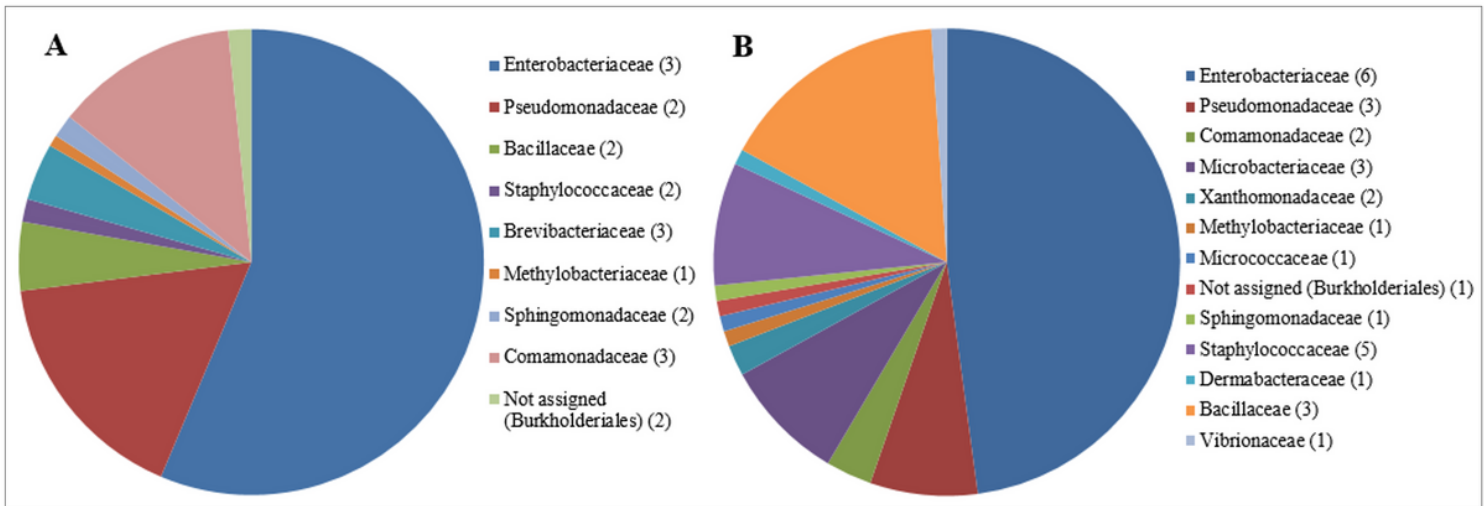


Figure 4

Distribution of Bacterial Families on (A) *E. lateriflora* and (B) *F. thonningii*. Numbers in parentheses represent different OTUs identified within the family.

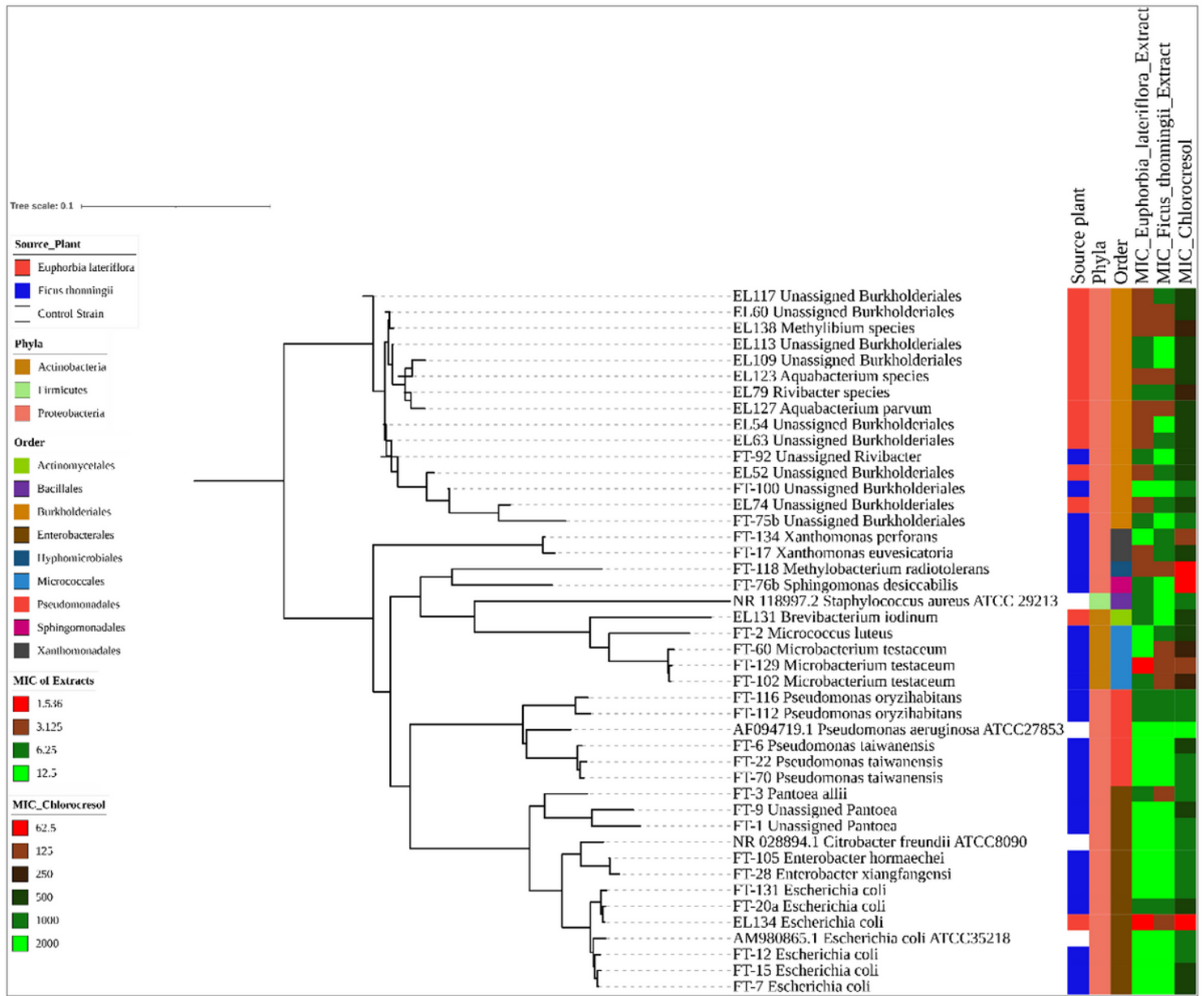


Figure 5

16S rRNA gene sequence-based phylogeny of selected cultured strains representing the different OTUs showing susceptibility to ethyl acetate extracts of *E. lateriflora* and *F. thonningii*.

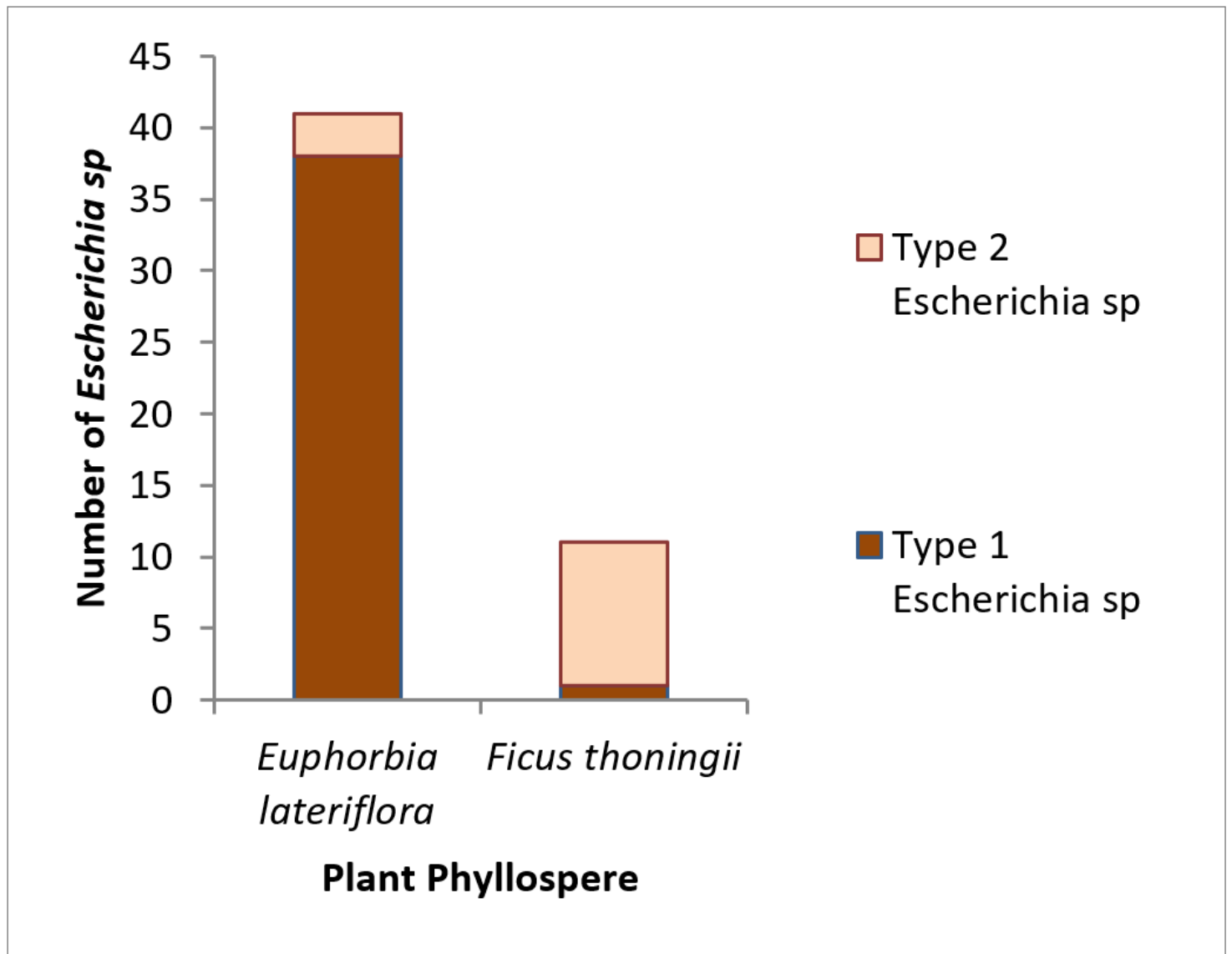


Figure 6

Distribution of Type 1 and Type 2 *Escherichia* spp. on *E. lateriflora* and *F. thonningii*.