

Leaf Microbiome Differences Between Winter (Invasive) and Summer (Non-invasive) Annual Growth forms of *Anthemis cotula* L.: Insights into Invasiveness

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Abstract

Background Biological invasions are major drivers of global change, and plant-associated microbiomes are increasingly recognized as potential mediators of invasive success. While belowground microbial associations have been widely explored, the role of aboveground endophytes in shaping plant performance and ecological strategies remains poorly understood—particularly in seasonally dynamic mountain ecosystems.

Anthemis cotula L., a widespread invader in the Kashmir Himalaya, occurs in two distinct growth forms: a winter annual that is highly invasive and a summer annual that is non-invasive. This unique intraspecific contrast offers an opportunity to assess how microbiome composition may vary with seasonal and ecological strategy, while controlling for phylogenetic differences. We hypothesized that seasonal shifts in leaf endophytic communities would parallel the contrasting ecological strategies of the two growth forms, thereby offering insights into possible relationships between microbiome structure and plant performance in a mountain environment.

Results Sequencing revealed 420 bacterial species and 3,036 fungal OTUs spanning both winter and summer annuals. Alpha-diversity patterns were contrasting: the non-invasive summer annual harboured higher bacterial richness, Shannon diversity, and evenness, while invasive winter annuals showed notably greater fungal diversity. Beta-diversity analyses and ordination (NMDS, PERMANOVA) demonstrated strong seasonal and growth form-specific structuring, with clear separation and little overlap between bacterial communities of winter vs. summer forms, and more pronounced fungal diversity within winter. Differential abundance testing (ANCOM-BC2) identified enrichment of stress-tolerant bacteria (e.g., *Massilia*, *Nocardioides*) and cold-adapted or mutualistic fungi (e.g., *Coniosporium*, *Cladosporium*) in winter annuals, while summer forms were associated with bacterial taxa supporting growth in milder conditions (e.g., *Methylobacterium*). These contrasting patterns indicate that the invasive winter annual's microbiome, though less diverse for bacteria and richer for fungi, may be compositionally structured in ways that could support nutrient uptake, abiotic stress tolerance, and competitive performance under harsher conditions.

Conclusion This study shows that *A. cotula* harbors distinct, seasonally structured leaf microbiomes across its winter and summer growth forms. The invasive winter annual is associated with a functionally enriched bacterial and a more diverse, stress-tolerant fungal community, potentially supporting resilience and early competitive advantage. By relating microbial composition to plant life-history variation, our findings suggest that aboveground microbiome seasonality is an underexplored but potentially important aspect of plant–environment interactions in mountain ecosystems.

Background

Plant-associated microbiomes, comprising bacteria, fungi, archaea, and viruses, are increasingly recognized as integral determinants of plant health, fitness, and ecological success [1–3]. These microbial communities inhabit diverse plant compartments, occurring externally as epiphytes or

internally as endophytes [4]. Through complex and context-dependent interactions, microbiomes can act as mutualists, commensals, or pathogens, thereby shaping host physiology and influencing ecological outcomes. In the context of plant invasions, they are thought to play dual roles. The Enemy Release Hypothesis (ERH) posits that invasive plants escape co-evolved pathogens in novel ranges, allowing them to reallocate resources toward growth and reproduction [5–7]. In contrast, the Enhanced Mutualism Hypothesis (EMH) suggests that invaders may form novel or strengthened beneficial associations, improving their competitive ability and stress tolerance [8]. While extensive research has focused on belowground microbial communities, particularly rhizosphere-associated bacteria and fungi, the ecological significance of aboveground endophytic microbiomes—especially leaf-associated communities—remains poorly understood in the context of plant invasions [9–11]

Leaves represent a primary interface between plants and their environment. They are critical for photosynthesis and gas exchange but also experience strong fluctuations in temperature, light, and moisture across seasons. Endophytic microbes residing within leaves can directly influence host physiology by enhancing photosynthetic efficiency, conferring stress tolerance, modulating secondary metabolite production, and providing protection against herbivory and pathogens [12–14]. Yet most microbiome research has focused on root or soil systems [15], leaving the seasonal dynamics, taxonomic composition, and functional potential of leaf-associated microbiomes largely unexplored—particularly in invasive species. This knowledge gap is critical because leaf-associated microbes may directly determine plant survival and performance during ecologically challenging periods such as winter or early growth stages.

In the Kashmir Himalaya, *Anthemis cotula* L. (stinking chamomile or dog fennel), native of Europe, is highly invasive and is now considered as a global invader (Adhikari et al., 2020). It presents a unique natural experiment since in Kashmir Himalaya two ecologically distinct annual forms: a highly invasive winter annual and a non-invasive summer annual occur [17]. The winter annual overwinters as a rosette, retains leaf tissue through harsh conditions, and exhibits early-season growth and reproductive success, whereas the summer annual has a shorter life cycle, is restricted to milder conditions, and shows limited spread and competitiveness [18]. These contrasting life-history strategies provide an exceptional system to explore how differences in phenology are reflected in leaf-associated microbiomes, and whether these microbial communities contribute to differential invasiveness. Despite their potential ecological significance [19], the role of leaf-associated microbiomes in promoting invasive success remains largely speculative. Key gaps in current knowledge include the diversity, composition, and temporal dynamics of leaf microbiomes in invasive species; the extent to which bacterial and fungal communities respond differently to seasonal or life-history shifts; and the functional contributions of these microbes to host survival, stress tolerance, and competitive ability. Addressing these gaps is essential for advancing our understanding of plant–microbe interactions in invasion ecology, particularly under seasonally variable environments.

Here, we present the first comprehensive characterization of bacterial and fungal leaf endophytes in both the invasive winter annual and non-invasive summer annual of *A. cotula*. Using high-throughput

sequencing, rarefaction and diversity analyses, and multivariate approaches, we investigated whether bacterial and fungal communities differ in composition, richness, diversity, and taxon-specific abundance between seasonal forms. We hypothesized that, owing to limited co-evolution with local microbes, the leaf microbiome of *A. cotula* would (i) be dominated by opportunistic and generalist taxa, (ii) differ in overall diversity and structure between invasive and non-invasive forms, and (iii) exhibit distinct seasonal patterns reflecting each form's life-history strategy.

By addressing these questions, our study provides novel insights into the seasonally structured leaf microbiomes of an invasive species and its non-invasive counterpart. It highlights how dynamic microbial assemblages may contribute to host fitness, survival, and ecological success, offering a mechanistic link between microbial ecology and plant invasiveness. Importantly, this work advances our understanding of the functional and ecological significance of aboveground endophytes, a relatively understudied yet potentially critical component of plant invasion biology, and emphasizes the need to consider temporal dynamics in plant–microbe interactions for predicting and managing biological invasions.

Methodology

Anthemis cotula L., belonging to the family Asteraceae, is a noxious invasive weed with far-reaching ecological consequences worldwide (War et al., 2023). Commonly known as stinking chamomile or dog fennel, this hardy annual herb is native to Europe but has successfully established across North America, Australia, and Asia owing to its high adaptability and prolific seed production (Adhikari et al., 2020). In the Kashmir Himalaya, *A. cotula* has emerged as one of the most infamous alien plant species, outcompeting native flora and invading a wide range of ecosystems (Adhikari et al., 2020). Its ruderal life history enables it to rapidly colonize agricultural lands, roadsides, and disturbed habitats, where it displaces native species and disrupts local ecosystem functioning (Reshi et al., 2011; Adhikari et al., 2020).

Sampling

Plant material used in this study was obtained from wild populations of *Anthemis cotula* L., an invasive alien species in Kashmir Himalaya. The species is not listed under any national or international protection frameworks (e.g., CITES) and is not considered threatened or endangered. As the study involved sampling of a non-protected, invasive species from publicly accessible sites, no specific permissions were required for collection. Voucher specimen of *A. cotula* identified by Zafar A Reshi has been deposited in Kashmir University Herbarium (KASH) with accession number 9338-KASH.

To examine seasonal differences in the leaf endophytic microbiomes of *Anthemis cotula* L., we conducted systematic sampling across three sites in the Kashmir Himalaya (Supplementary Table S1). The study sites—Daksum (Anantnag, 2,345 m amsl), University of Kashmir (Srinagar, 1,540 m amsl), and

Tangmarg (Baramulla, 2,000 m amsl)—span a broad elevational gradient and represent distinct ecological zones of the region.

For each growth form (winter annual and summer annual), 18–20 healthy, fully expanded leaves were randomly collected from individual plants at each site during May–June, ensuring uniformity in phenological stage. To avoid bias arising from temporal variation, pre-winter individuals were marked in advance, and all sampling was carried out simultaneously across sites. Three independent replicate samples were collected per growth form per site, yielding a total of nine samples for the summer annual and nine for the winter annual forms. All collections were performed using sterile gloves and aseptic techniques. Leaf samples were immediately placed in sterile polyethylene bags, transported on ice to the laboratory, and processed within 24 hours to minimize alterations in microbial community composition. Field collections were conducted in accordance with institutional, national, and international guidelines governing research on wild plant species. Species identity was confirmed by Dr. Z.A. Reshi using standard taxonomic keys.

To ensure characterization of only endophytic communities, leaf samples underwent standardized surface sterilization. About five leaves per plant were rinsed under running water, immersed in 70% ethanol for 3 minutes, followed by 1.5% sodium hypochlorite (NaOCl) for 1 minute, and finally rinsed three times with sterile double-distilled water [20]. Sterilization efficacy was confirmed by plating the final rinse water on nutrient agar; only samples showing no microbial growth were processed further. Extraction blanks and PCR negatives were included throughout and handled according to standard protocols to monitor potential contamination [21, 22]. For the purposes of this study, we adopted the definition of [23] which describes endophytes as microbes that can be isolated from surface-disinfested plant tissues or extracted from within the plant, and that do not visibly harm the host. By applying this definition and validating sterilization efficacy, our analyses specifically targeted the endophytic leaf microbiome.

DNA extraction and quality assessment

Genomic DNA was extracted from surface-sterilized leaf tissue using the DNeasy® PowerMax® Soil DNA Isolation Kit (Qiagen, Mo Bio Laboratories, UK), following the manufacturer's protocol with minor modifications [24, 25]. DNA integrity was verified via 0.8% agarose gel electrophoresis, and purity and concentration were assessed spectrophotometrically (OD 260/280). Samples with OD values between 1.7 and 1.9 were retained. High-purity DNA was further cleaned using AMPure XP magnetic beads (Agencourt, Beckman Coulter) [26].

PCR amplification and library preparation

The bacterial 16S rRNA gene (V3–V4 region) and fungal ITS2 region were amplified to target bacterial and fungal communities, respectively. For bacteria, primers 341F (5'-CCTACGGGAGGCAGCAG-3') and

785R (5'-GACTACGGGTATCTAATCC-3') were used (Klindworth et al., 2013) [27] while fungal ITS2 was amplified using ITS3mixF (5'-CAHCGATGAAGAACGYRG-3') and ITS4R (5'-TTCCTSCGCTTATTGATATGC-3') [28]. Successful amplification was confirmed via agarose gel electrophoresis. PCR products were purified, barcoded, and pooled in equimolar concentrations for library preparation according to Illumina MiSeq protocols. Library quantification was performed using Qubit, and final pooled libraries were sequenced on the Illumina MiSeq platform using 2 × 300 bp paired-end chemistry [29].

Sequence processing and OTU assignment

Raw FASTQ reads were quality-checked using FastQC and processed in QIIME v1.9.1 (Caporaso et al., 2010). High-quality paired-end reads were merged, denoised, and clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity [30]. Chimeric sequences were removed during clustering. Taxonomic assignment of bacterial OTUs was performed against the SILVA 138 database [31], while fungal OTUs were classified using the UNITE database [32] with a closed-reference approach.

Diversity analyses

All downstream analyses were conducted in R version 4.1.2 (R Core Team, 2022). OTU tables were rarefied to a uniform sequencing depth using `rarefy_even_depth()` in the `phyloseq` package [33]. Rarefaction curves were visualized with `ggrare()` from the `ranacapa` package [34]. Alpha diversity was assessed using Hill numbers ($q = 0$ for richness, $q = 1$ for Shannon diversity, and $q = 2$ for Simpson diversity) via sample-size-based interpolation and extrapolation with the `iNEXT` package [35]. Confidence intervals were generated with 999 bootstrap replicates, and diversity curves were visualized with `ggiNEXT()`.

Beta diversity and community structure

Community differentiation between winter and summer annual forms was assessed using Hellinger-transformed OTU data and PERMANOVA (`adonis2()` in the `vegan` package) [36]. Taxonomic composition at multiple levels (phylum, class, order, family, genus) was visualized as relative abundance bar plots using `ggplot2` and `phyloseq`.

Differential abundance analysis

To identify taxa exhibiting significant seasonal shifts, OTU counts were aggregated and analysed at multiple taxonomic levels—phylum, class, order, family, and genus—using ANCOM-BC2 (Analysis of Compositions of Microbiomes with Bias Correction 2), with stage (winter vs. summer annual) included as a fixed effect [37]. Taxa present in fewer than 10% of samples or with fewer than 1000 reads were excluded. ANCOM-BC2 accounts for library size differences, compositionality, and variance bias, and Holm's method was applied to adjust p-values for multiple testing. Structural zeros, representing taxa

absent in a group, were automatically detected. Log fold changes (LFCs) and standard errors were estimated for each taxon, and significance was assessed at $\alpha = 0.05$. While analyses at the phylum level did not reveal any significant fold changes, differential abundance was observed at class, order, family, and genus levels.

Genus-level analysis was useful given the limited taxonomic resolution of 16S rRNA and ITS amplicons. While OTU-level analyses provide maximum resolution, they frequently contain sparse counts and zeros, which can reduce analytical robustness. Aggregating at the genus level strikes a balance between interpretability, statistical power, and ecological relevance, capturing meaningful taxonomic and ecological patterns while minimizing noise. OTU-level results can be provided in supplementary materials if required.

Results

Bacterial OUT richness across growth forms

Sequencing of leaf-associated microbiomes of *Anthemis cotula* yielded 55,763 high-quality bacterial reads from 18 samples (393–5,807 per sample) and 1,495,722 fungal reads (770,889 from winter annuals and 724,833 from summer annuals). Bacteria spanned 13 phyla, 29 classes, 50 orders, 115 families, 246 genera, and 420 species Table 1).

Table 1
Taxonomic richness of bacterial taxa in winter and summer annuals. Values represent the number of distinct taxa recorded within each microbial group and growth form.

Taxonomic Rank	Winter annual	Summer annual	Total
Phylum	12	10	13
Class	25	24	29
Order	41	40	50
Family	99	96	115
Genus	194	163	246
Species	310	260	420

The upset plot for bacteria (Fig. 1) indicates that winter annuals consistently exhibited greater richness of both bacterial and fungal taxa compared to summer annuals. Among bacteria, 310 OTUs were detected in winter and 260 in summer, with 150 shared, 160 unique to winter, and 110 unique to summer. Proteobacteria dominated across both forms, often exceeding 50% of reads and reaching > 80% in some samples.

The upset plot summarizing the fungal OTU (Operational Taxonomic Unit) richness across winter annual and summer annual samples (Fig. 2) depicts that winter annual samples exhibited a higher total number of fungal OTUs, with 2,109 detected, compared to 1,592 OTUs in summer annual samples. Within winter annuals, the OTUs were distributed as follows: 665 OTUs represent taxa unique to winter annuals, 1,444 OTUs were shared, and 927 OTUs were also present in summer annuals. These patterns highlight higher fungal diversity in winter annuals, while also revealing a consistent set of OTUs found throughout both seasonal groups.

Community composition

Rarefaction curves

Rarefaction curves depicting bacterial species richness as a function of sequencing depth are presented for both winter annual and summer annual samples (Supplementary Fig. S1). Across all samples, species richness of bacteria increased rapidly with increasing sequencing effort, reaching a plateau at higher sequence depths. Notably, summer annual samples generally exhibited higher bacterial species richness compared to winter annual samples at comparable sequence sample sizes. For summer annual samples, the richness values approached or exceeded 90 species in several instances (e.g., LM32, LM31, LM3, LM22), while winter annual samples plateaued at lower richness values, rarely exceeding 65 species per sample. This pattern indicates a greater overall diversity in microbial communities during the summer annual period.

The rarefaction analysis for fungi (Supplementary Fig. S2) demonstrated that species richness was markedly higher in winter annual communities compared to summer annuals, as indicated by the higher asymptotic values of the rarefaction curves in the winter group. In both seasonal groups, species accumulation curves approached a plateau at higher sequencing depths, suggesting that sampling effort was adequate to capture the bulk of local fungal diversity. Notably, among-site variability was observed within each group, with some winter annual sites (PL22, PL21) reaching peak richness values near 850, whereas the highest richness in summer annual sites (LM22, LM21) remained below this threshold. These results highlight pronounced seasonal and spatial differences in fungal diversity across the study sites.

Bacterial composition

Bacterial communities in winter annual samples were strongly dominated by Proteobacteria, with this phylum constituting more than 75% of the total community in all samples. In contrast, the relative abundance of Actinobacteria and Bacteroidetes was lower in winter annuals compared to summer annuals, where these phyla were more prominent (Fig. 3). Minor phyla such as Firmicutes, Fusobacteria, and others appeared only sporadically and did not exceed 10% in any sample. These patterns indicate a marked seasonal shift at the phylum level, characterized by a greater phylum-level diversity and increased relative abundances of Actinobacteria and Bacteroidetes in summer annuals, although Proteobacteria remained the consistently dominant phylum across both periods.

At the class level, summer annual samples were predominantly composed of Alphaproteobacteria, often making up more than 50% of the community in several samples. As the season progressed, Betaproteobacteria, Bacteroidia, and Actinobacteria increased in relative abundance, contributing to higher community diversity (Fig. 4). Other classes, including Flavobacteriia, Clostridia, and Bacilli, were present at moderate or low levels, highlighting pronounced heterogeneity within summer annuals. In winter annuals, Betaproteobacteria and Bacteroidia became the dominant classes, especially in specific samples, while classes such as Gammaproteobacteria and Sphingobacteriia remained stable but less abundant. The presence of minor classes was generally sporadic and of low relative abundance. At the order level, Rhizobiales dominated summer annual samples, frequently accounting for over half of the local community. In contrast, winter annual samples exhibited increased dominance of Enterobacteriales, with some samples showing abundances surpassing 75% (Supplementary Fig. S3). Additional orders, such as Burkholderiales, Bacillales, Clostridiales, and Sphingobacteriales, increased in winter annuals, pointing to greater taxonomic diversity during this period.

Family-level analysis revealed that Methylobacteriaceae were the most abundant in summer annual samples, particularly in certain sites (Supplementary Fig. S4). Increased numerical importance of Enterobacteriaceae, Flavobacteriaceae, Sphingobacteriaceae, Microbacteriaceae, and Nocardaceae was observed in other samples, and winter annuals showed a clear shift toward greater prevalence of Enterobacteriaceae and Flavobacteriaceae. Several other families, such as Sphingobacteriaceae and Micrococcaceae, showed variable representation, further supporting increased taxonomic turnover between seasons. Genus-level examination showed that *Acinetobacter* was dominant in summer annual samples, sometimes exceeding 70% relative abundance. However, winter annuals displayed increased proportions of genera like *Bifidobacterium*, *Kocuria*, and *Cetobacterium* (Fig. 5). Additional genera including *Flavobacterium*, *Brevundimonas*, *Pseudomonas*, and *Sphingomonas* contributed to enhanced diversity, while many samples contained a substantial "Others" fraction, reflecting the high degree of heterogeneity in community composition.

Fungal OTU richness across growth forms

A pronounced diversity of fungal taxa was observed across both winter annual and summer annual samples, with distinct trends evident at each major taxonomic rank (Table 2). At the phylum level, a total of 14 fungal phyla were recorded overall, with winter annuals contributing 13 phyla and summer annuals 11 phyla. This pattern of higher richness in winter annuals continued at the class (33 vs. 30), order (74 vs. 66), and genus (194 vs. 187) levels, indicating greater fungal community complexity in winter annuals compared to summer annuals. Family-level counts were similar for both seasons (140 in winter and 139 in summer), while the overall total reached 177 fungal families when both groups were combined.

Species-level richness showed a clear seasonal distinction: winter annuals harbored 2,109 species, whereas summer annuals contained 1,592 species, combining to a total fungal richness of 3,036 distinct species. These results suggest substantial turnover and unique contributions of fungal taxa by each seasonal type, with winter annuals exhibiting consistently higher diversity at most taxonomic levels.

Table 2
Taxonomic richness of fungal taxa in winter and summer annuals. Values represent the number of distinct taxa recorded within each microbial group and growth form.

Taxonomic Rank	Winter annual	Summer annual	Total
	Fungi	Fungi	Fungi
Phylum	13	11	14
Class	33	30	37
Order	74	66	81
Family	140	139	177
Genus	194	187	264
Species	2109	1592	3036

Fungal composition

Across both winter and summer annual samples, fungal communities were overwhelmingly dominated by Basidiomycota and Ascomycota. In winter annuals, Basidiomycota formed over 60–70% of the total abundance, with Ascomycota as the next most prevalent group.

Other phyla, such as Mortierellomycota and Mucoromycota, appeared only in very low abundances, while Chytridiomycota, Entomophthoromycota, and Glomeromycota were detected sporadically at minor levels. In summer annuals, Basidiomycota remained dominant in most cases, but certain samples exhibited reduced Basidiomycota with a concomitant rise in Ascomycota, indicating substantial seasonally-driven shifts in community composition.

At finer taxonomic resolution, Agaricomycetes consistently represented the dominant fungal class in both seasonal groups, comprising over half the community in winter annuals and slightly less in summer annuals, where Leotiomycetes and Dothideomycetes became more prominent. A broad array of orders, led by Agaricales and a diversity of other Basidiomycete and Ascomycete orders (e.g., Pleosporales, Polyporales, Tremellales), structured the communities (Supplementary Fig. S5).. Notably, the "Others" category, representing rare or less abundant orders, contributed substantially, especially in summer annuals, demonstrating high compositional heterogeneity.

Family-level results underscored marked temporal and spatial variation. Agaricaceae dominated some summer annual samples, while Strophariaceae and Pleosporaceae showed greater importance in winter annuals. Tricholomataceae and several other families contributed variably across all samples (Supplementary Fig. S6). At the genus level, sites were frequently dominated by one or a few genera (e.g., Agaricus, Leucoagaricus, Coprinopsis, Coprinellus, Penicillium), with a large portion of the community often assigned to "Others," highlighting both unclassified and rare genera and reinforcing evidence for pronounced seasonal and spatial turnover in community structure.

In summary, the data reveal pronounced differences in both bacterial and fungal community composition, characterized by changes in the relative abundance and diversity of dominant and minor taxa at all examined taxonomic levels.

Differential abundance of taxa

Bacteria

Differential abundance analysis using ANCOM-BC2 was performed to identify bacterial taxa exhibiting notable seasonal shifts between summer and winter annual samples. Notably, no significant differential abundance was observed at the phylum level, whereas analyses at the class, order, family, and genus levels revealed significant log fold changes (LFC), underscoring the value of finer taxonomic resolution for detecting seasonal shifts in bacterial communities. At the class level, Coriobacteriia and Alphaproteobacteria were significantly enriched in summer annual samples, showing positive log fold changes (LFCs) of 2.10 and 1.64, respectively. In contrast, Clostridia, Deltaproteobacteria, Gammaproteobacteria, and Betaproteobacteria exhibited significant negative LFCs, indicating higher relative abundance in winter annuals (Fig. 9). Changes at the order and family levels also revealed marked seasonal differences in bacterial community composition (Supplementary Figs. S7, S8). For instance, Rhizobiales and Bacillales increased in summer, whereas Burkholderiales and Pseudomonadales were more abundant in winter. At the family level, Methylobacteriaceae and Propionibacteriaceae were enriched during summer, while Oxalobacteraceae and Sphingomonadaceae were associated with winter assemblages. These finer taxonomic shifts complement the patterns seen in class- and genus-level analyses and are documented in the supplementary figures.

At the genus level, Methylobacterium, Propionibacterium, Sphingomonas, Bacteroides, and Nocardiods were found to be positively associated with summer annuals, while Massilia showed a strong negative association, indicating enrichment in winter samples (Fig. 8). Collectively, these results highlight pronounced and consistent seasonal restructuring of bacterial assemblages across multiple taxonomic levels, reflecting dynamic ecological responses to seasonal environmental changes. The detailed class and genus level results presented herein emphasize key taxa driving these patterns, while supplementary figures provide a comprehensive overview of order- and family-level shifts.

Fungi

Differential abundance analysis of fungal taxa using ANCOM-BC2 demonstrated significant seasonal shifts across multiple taxonomic levels. At the class level, Archaeorhizomycetes and Malasseziomycetes were enriched in summer annual samples, showing positive log fold changes (LFCs), while Glomeromycetes were significantly more abundant in winter annuals (Fig. 10). Significant changes were also identified at the order and family levels (see (Supplementary Figs. S9, S10). Orders such as Hymenochaetales, Microthyriales, and an unidentified order showed positive LFCs for summer, while Glomerales was enriched in winter. At the family level, Onygenales_fam_incertae_sedis was more abundant in summer, whereas Entolomataceae, Glomeraceae, and Stachybotryaceae were enriched in

winter annuals. At the genus level, *Coniosporium* increased markedly in summer, whereas *Entoloma* exhibited a strong negative LFC, indicating enrichment in winter samples (Fig. 10).

Together, these results indicate that both bacterial and fungal microbiomes of *A. cotula* exhibit strong seasonal restructuring. Bacterial communities are richer in summer but dominated by fewer taxa in winter, whereas fungal communities are richer and more evenly distributed in winter. Such dynamic turnover may underlie the ecological success and invasiveness of this species.

Alpha diversity

Bacteria

Using Hill numbers that offer a unified framework to quantify alpha diversity by accommodating different sensitivities to species frequencies, we evaluated alpha diversity in bacterial and fungal communities of winter and summer annual samples to assess how seasonal variation influences taxonomic richness and evenness in these ecosystems. The Fig. 11 presents alpha diversity estimates for bacterial communities associated with winter and summer annual growth forms. At order $q = 0$, representing observed species richness, summer annual samples exhibited higher diversity, reaching up to 244 species for extrapolated sample sizes, compared to winter annuals, which peaked at 239 species. The rarefaction and extrapolation curves showed a clear separation between the two growth forms, with summer annuals maintaining a consistently higher richness across the entire gradient of individuals. Winter annuals, however, still supported substantial species diversity, with rarefied richness near 189 and extrapolated richness approaching 239 species.

For order $q = 1$, which accounts for both richness and relative abundance (Shannon diversity), summer annuals again demonstrated elevated diversity, with values rising to 48 for extrapolation and 46 for rarefaction. Winter annual samples displayed slightly lower diversity, with the corresponding values at 30 (extrapolation) and 29 (rarefaction), indicating that the summer annual fungal communities not only contained more species but also exhibited greater evenness in species abundances.

At order $q = 2$, emphasizing the influence of dominant taxa (Simpson diversity), both growth forms converged to similar and lower diversity estimates. Summer annuals plateaued at 9 for both rarefaction and extrapolation, while winter annuals reached 13 throughout the range of individuals analyzed. This result suggests that, despite differences in richness and evenness at lower orders, a few highly abundant taxa dominate both communities when considering Simpson diversity. Overall, alpha diversity analyses using Hill numbers reveal that fungal communities in summer annual samples are richer and more even, while winter annuals are characterized by slightly higher dominance of individual taxa.

We also conducted sample completeness and coverage-based rarefaction/extrapolation (R/E) analyses to verify whether sequencing depth adequately captured bacterial diversity and allowed reliable comparisons between growth forms. Sample coverage curves (Supplementary Fig. S11) demonstrate that sequencing depth was sufficient to capture the majority of bacterial diversity present in both growth

forms. For both summer annual and winter annual samples, rarefied and extrapolated sample coverage values rapidly approached 1.0 as the number of individuals increased, indicating that nearly all detectable bacterial taxa were sampled, and there is a minimal chance of overlooking important rare taxa. The coverage-based rarefaction and extrapolation (R/E) curves (Supplementary Fig. S12) depicting a robust assessment of bacterial alpha diversity between winter and summer annual samples, standardized by sample completeness rather than raw sample size, revealed that at all three Hill diversity orders (0, 1, and 2), summer annual samples consistently displayed higher species richness (order 0) and Shannon diversity (order 1), suggesting more diverse and evenly distributed bacterial communities during this period. Specifically, species richness estimates at full coverage nearly approached 244 for summer annuals compared to approximately 239 for winter annuals. Shannon diversity followed a similar pattern with summer annuals reaching 48 compared to 30 in winter annuals. For Simpson diversity (order 2), which emphasizes dominant taxa, winter annual samples showed slightly higher values (13) than summer annuals (9), indicating a stronger dominance by fewer taxa in winter. These trends persisted across rarefied and extrapolated estimates, confirming adequate sampling depth and reinforcing the ecological inference that summer annual conditions support richer and more even bacterial communities, while winter annuals are characterized by higher dominance of particular taxa.

Fungi

The fungal alpha diversity in winter and summer annual samples, estimated using rarefaction and extrapolation of Hill numbers (orders 0, 1, and 2) is displayed in Fig. 12. At order 0, representing observed species richness, winter annual samples harboured substantially higher fungal diversity, with extrapolated richness reaching 2,106 OTUs compared to 1,593 in summer annuals. Rarefied richness values closely matched these trends, highlighting the robustness of the estimates across sampling depths.

For Shannon diversity (order 1), which accounts for richness and relative abundance, winter annuals remained more diverse, attaining values of 48 compared to just 15 for summer annuals. This indicates both greater species numbers and a more even distribution of abundances in winter annual communities. At order 2 (Simpson diversity), which emphasizes dominant taxa, winter annuals exhibited almost threefold higher diversity (11) relative to summer annuals (4). This indicates that not only do winter annual fungal communities contain more taxa, but they are also less dominated by a small set of highly abundant species.

Collectively, these results demonstrate pronounced seasonal differences in fungal community structure, with winter annual samples supporting markedly greater richness and evenness, and reduced dominance by individual taxa compared to summer annual samples.

Like bacteria, we carried out sample completeness assessments and coverage-based rarefaction and extrapolation (R/E) analyses for fungi as well to evaluate the adequacy of sequencing depth and to enable reliable diversity comparisons between growth forms. Sample coverage curves (Supplementary Fig. S13) indicated that sequencing depth was sufficient for both winter and summer annual fungal

communities, with rarefied and extrapolated coverage values swiftly approaching 1.0 as sequencing effort increased. This confirms that the majority of fungal diversity was captured and minimizes potential bias from undersampling.

The coverage-based rarefaction and extrapolation (R/E) curves for summer and winter annuals using three orders of diversity ($q = 0, 1, 2$) are shown in Supplementary Fig. S14). At full sample coverage (coverage = 1), which represents near-complete sampling, winter annuals consistently show higher species diversity than summer annuals across all three diversity metrics. Specifically, winter annuals exhibit approximately 2106 species for $q = 0$ (species richness), compared to 1593 for summer annuals. For $q = 1$ (Shannon diversity), winter annuals have a value of 48 versus 15 for summer annuals, and for $q = 2$ (Simpson diversity), the values are 11 and 4, respectively. These differences are most pronounced at high sample coverage, highlighting that the observed patterns reflect true ecological differences rather than differences in sampling effort. In summary, winter annuals demonstrate not only greater species richness but also higher evenness and dominance diversity, suggesting a more diverse and evenly structured community compared to summer annuals.

Beta diversity and seasonal differentiation

Bacteria

Multivariate analyses revealed strong seasonal structuring of both bacterial and fungal communities using Bray–Curtis distances on Hellinger-transformed data.

The PERMANOVA results for bacterial community composition (Table 3) indicate significant influences of growth form, site, and their interaction on bacterial assemblages. Growth form explained 28.0% of the variation in bacterial community structure ($R^2 = 0.280$), with a statistically significant pseudo-F value of 8.685 ($p < 0.0001$). This suggests distinct bacterial assemblages between winter annual and summer annual groups. Site accounted for 26.8% of the variation ($R^2 = 0.268$) with a significant effect (pseudo-F = 4.150, $p < 0.0001$), indicating spatial heterogeneity in bacterial communities across sampling locations.

The interaction term (growth form $\times$ site) explained the largest portion of the variation at 33.8% ($R^2 = 0.338$) and exhibited the highest pseudo-F (17.717, $p < 0.0001$). This suggests that the influence of growth form on bacterial community composition varies substantially depending on site, reflecting complex spatially heterogeneous seasonal dynamics.

Table 3
Summary results of PERMANOVA (adonis2) testing the effects of growth form, site, and their interaction on bacterial community composition.

Factor	Df	R ²	F	p-value
Bacteria				
Growth form	1	0.280	8.685	< 0.0001
Site	2	0.268	4.150	< 0.0001
Growth form × Site	2	0.338	17.717	< 0.0001

The NMDS (Non-metric Multidimensional Scaling) ordination plot (Fig. 13) illustrates the compositional dissimilarity in bacterial communities between winter annual and summer annual samples. The results reveal a clear and pronounced separation between winter annual and summer annual bacterial communities along the NMDS axes, with no overlap between the Fig. 13. Non-metric multidimensional scaling (NMDS) ordination plot illustrating the compositional differences of microbial communities between winter annual (red) and summer annual (blue) samples. Each point represents an individual sample, with ellipses denoting 95% confidence intervals around group centroids

two ellipses. All summer annual samples are grouped tightly to the left side of the plot (lower NMDS1 values), while winter annual samples cluster to the right (higher NMDS1 values). This distinct clustering demonstrates that bacterial community composition is strongly differentiated by season, which is further supported by the low stress value (0.078), indicating an excellent representation of the ecological dissimilarity in two dimensions. Within each group, samples are closely aggregated, suggesting high internal similarity and low beta diversity among sites of the same seasonal growth form, while the spatial separation between groups highlights pronounced turnover in bacterial taxa between winter and summer periods. Overall, the NMDS analysis provides compelling evidence of seasonal shifts in bacterial community structure, with winter and summer annual growth forms hosting discrete assemblages of bacteria that are minimally shared across the seasonal divide.

A principal coordinates analysis (PCoA) is presented (Supplementary Fig. S15) which further corroborates the findings from NMDS by illustrating a clear and notable compositional separation between winter annual and summer annual samples along the first two principal coordinates. The ordination analysis shows that the first axis explains 37.09% and the second axis 24.33% of the total variance in the dataset, together accounting for approximately 61.42% of the variation in community composition.

Fungi

The PERMANOVA results for fungal community composition (Table 4) indicate significant effects of growth form, site, and their interaction on fungal assemblages. Growth form alone explained 28.6% of the variation in community composition ($R^2 = 0.286$), with a highly significant pseudo-F statistic of 11.50

($p = 0.0001$), revealing distinct fungal assemblages between winter annual and summer annual groups. Site also accounted for a substantial 36.6% of the variation ($R^2 = 0.366$) and was statistically significant (pseudo- $F = 7.35$, $p = 0.0001$), indicating spatial heterogeneity in fungal communities across sampling locations.

Table 4
Summary results of PERMANOVA (adonis2) testing the effects of growth form, site, and their interaction on fungal community composition.

Factor	Df	R ²	F	p-value
Growth form	1	0.286	11.50	0.0001
Site	2	0.366	7.35	0.0001
Growth form × Site	2	0.331	115.15	0.0001

Importantly, the interaction between growth form and site explained an even larger portion of variation ($R^2 = 0.331$) with a very high pseudo- F value of 115.15 ($p = 0.0001$), demonstrating that the effect of growth form on fungal assemblages varies notably among sites. This suggests complex seasonal-spatial dynamics where fungal community responses to growth form environmental conditions differ depending on the locality.

The NMDS (Non-metric Multidimensional Scaling) ordination plot (Fig. 14) visualizes the variation in fungal community composition between winter annual and summer annual samples. In contrast to the complete separation observed in bacterial NMDS, the fungal ordination indicates that the ellipses indicating the 95% confidence intervals for each group exhibit some overlap, suggesting partial compositional similarity between some winter and summer annual fungal communities.

Despite this overlap, the clustering of samples within each group indicates generally high intragroup similarity and lower intergroup similarity, mirroring significant compositional divergence by season. The separation between group centroids remains marked, highlighting robust seasonal changes in community composition. The low stress value (0.025) confirms that the NMDS solution provides an accurate two-dimensional representation of the observed ecological dissimilarities. This subtle overlap in fungal communities contrasts with the more complete seasonal segregation found in bacterial assemblages and may reflect a higher proportion of shared or transitional taxa, or greater functional redundancy in fungi across winter and summer annual growth forms. Overall, the NMDS results demonstrate strong, though not absolute, seasonal differentiation in fungal community composition, emphasizing both distinctiveness and some continuity across growth forms.

The PCoA analysis (Supplementary Fig. S16) further corroborates the NMDS results by demonstrating clear compositional differentiation between winter annual and summer annual fungal communities. The first and second principal coordinates explain 32.13% and 20.73% of the variation in fungal community composition, respectively, together accounting for over 52% of the total variance observed. This

ordination reinforces the distinct seasonal structuring of fungal assemblages detected in the NMDS analysis.

Discussion

Our study reveals, for the first time, pronounced seasonal divergence in both bacterial and fungal leaf microbiomes of *Anthemis cotula*, with clear distinctions between its invasive winter annual form and non-invasive summer annual form. This finding underscores the ecological complexity of plant–microbe interactions and suggests that differences in microbial assemblages may be a key, previously overlooked mechanism contributing to invasiveness. Seasonal shifts in microbial composition, richness, and evenness reflect dynamic restructuring, with bacterial communities being more diverse during summer and fungal communities richer in winter. This contrasting temporal pattern suggests that bacterial and fungal endophytes may occupy complementary ecological niches, potentially conferring year-round advantages to the invasive winter annual [38, 39].

The observed turnover in bacterial OTUs, 160 unique to winter and 110 unique to summer annual forms, suggests that microbial communities are not merely subsets of one another but undergo substantial reassembly across seasons [40, 41]. For the winter annual, which overwinters as a rosette and is ecologically aggressive, this microbial turnover likely buffers the host against harsh conditions, enabling early-season growth and competitive dominance. Dominance of Proteobacteria in both seasons, with summer enrichment of Actinobacteria and Bacteroidetes, suggests that environmental conditions (e.g., temperature, humidity, and changing leaf physiology) drive phylum-level redistribution in the phyllosphere [38, 42]. For fungi, the higher richness and evenness in winter, dominated by Basidiomycota, align with reports that winter fungal communities may be more resilient to harsh conditions, potentially supporting host survival through decomposition, nutrient cycling, or stress tolerance [43, 44].

Alpha diversity patterns highlight complementary ecological strategies: the non-invasive summer annual harbours bacterial communities with maximized richness and evenness, perhaps enhancing nutrient acquisition under milder conditions [41]. In contrast, the invasive winter annual concentrates its fungal richness and evenness during winter, potentially reinforcing stress tolerance and pathogen defense under harsh conditions [45]. Our beta-diversity analyses further reveal strong seasonal segregation, emphasizing the importance of temporal niche partitioning in structuring plant-associated microbial assemblages [42].

Log fold-change analyses reveal distinct taxon-specific enrichment patterns that correspond to the functional needs of each growth form. In the non-invasive summer annual, bacterial taxa such as *Methylobacterium*—known for phytohormone production and metabolic flexibility—are enriched, potentially supporting growth during mild conditions [46–48]. Conversely, the invasive winter annual is enriched with taxa such as *Massilia* and *Nocardoides*, which may enhance stress tolerance, pathogen suppression, and nutrient cycling in cold conditions [49]. For fungi, winter enrichment of *Entoloma* and Pleosporaceae members in the invasive form may further enhance host defence or facilitate nutrient

acquisition thereby promoting its invasion [19, 50, 51]. These taxon-specific shifts suggest that the invasive winter annual is associated with a microbiome composition that may confer greater stress resilience and resource-use efficiency, potentially contributing to its ecological success.

By integrating bacterial and fungal perspectives, our study indicates that seasonal microbiome dynamics may facilitate year-round niche expansion in the invasive winter annual of *A. cotula*. The complementary pattern of bacterial summer richness and fungal winter stability gives the winter annual functional redundancy and diversity across seasons, buffering environmental stress and enabling rapid spring emergence and competitive dominance [39, 41]. In contrast, the summer annual's microbiome appears tuned to short-term growth under milder conditions, offering less year-round support. These insights suggest that microbiomes are not passive passengers but active contributors to plant life-history success and invasiveness. Infact, Bashir et al. (2024) demonstrated that leaf endophytic microbes in *A. cotula* not only enhance plant growth but also provide pathogen protection, effectively acting as hidden facilitators of invasion.

This study is among the first to comprehensively assess seasonally structured leaf microbiomes across invasive and non-invasive forms of the same plant species, with parallel focus on both bacterial and fungal communities. The observed seasonal partitioning and taxon-specific associations underscore the importance of considering temporal ecological context in understanding host–microbe relationships [40]. Future work employing functional metagenomics or transcriptomics could help identify microbial traits that potentially contribute to invasion success by linking community composition with metabolic or functional pathways across seasons. Such insights may ultimately inform biocontrol or management approaches by distinguishing microbial consortia associated with invasiveness or resilience in non-invasive forms. However, a limitation of the present work lies in the absence of experimental validation directly linking the leaf microbiome to the invasiveness of the pre-winter growth form, which warrants future experimental investigation to establish causality.

Conclusion

The present study provides one of the first comprehensive, seasonally resolved examinations of bacterial and fungal leaf microbiomes in the invasive annual *Anthemis cotula*, revealing striking temporal shifts in microbial community structure across its growth forms. By distinguishing the opposing seasonal peaks in bacterial and fungal diversity— with bacteria most diverse in summer annuals and fungi prevailing in leaves of winter annuals—our work uncovers the dynamic and complementary ecological roles these microbial groups play in supporting host adaptability.

The integration of high-resolution taxonomic profiling and multivariate community analyses demonstrates robust, site- and growth form-dependent restructuring of these microbiomes, underscoring the flexibility with which invasive plants harness microbial associations to thrive in fluctuating environments. Notably, our findings move beyond cataloguing microbial richness, instead

providing direct evidence that seasonal microbiome turnover is likely a key factor in invasion success, conferring year-round resilience to *A. cotula*.

This work has broader relevance, offering a conceptual framework for exploring how temporal partitioning among distinctly structured microbiomes may facilitate plant adaptation across climatic regimes—a perspective valuable not only for invasion biology but also for understanding native plant ecology and managing global changes in plant–microbe interactions.

Declarations

Supplementary information OUT data of both bacteria and fungi used in the present study has been shared as supplementary data.

Data availability statement The sequences have been deposited in NCBI Sequence Read Archive (SRA) on 9/03/2023 with Bio project accession number PRJNA1000036.

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Author contribution statement IB and ZAR conceptualized the research problem, IB undertook field sampling and processing of leaf samples for DNA extraction and analysis. ZAR statistically analysed the data and prepared tables and figures. IB wrote the first draft of the manuscript and ZAR revised and edited the first draft.

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Code availability Not applicable.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Figures

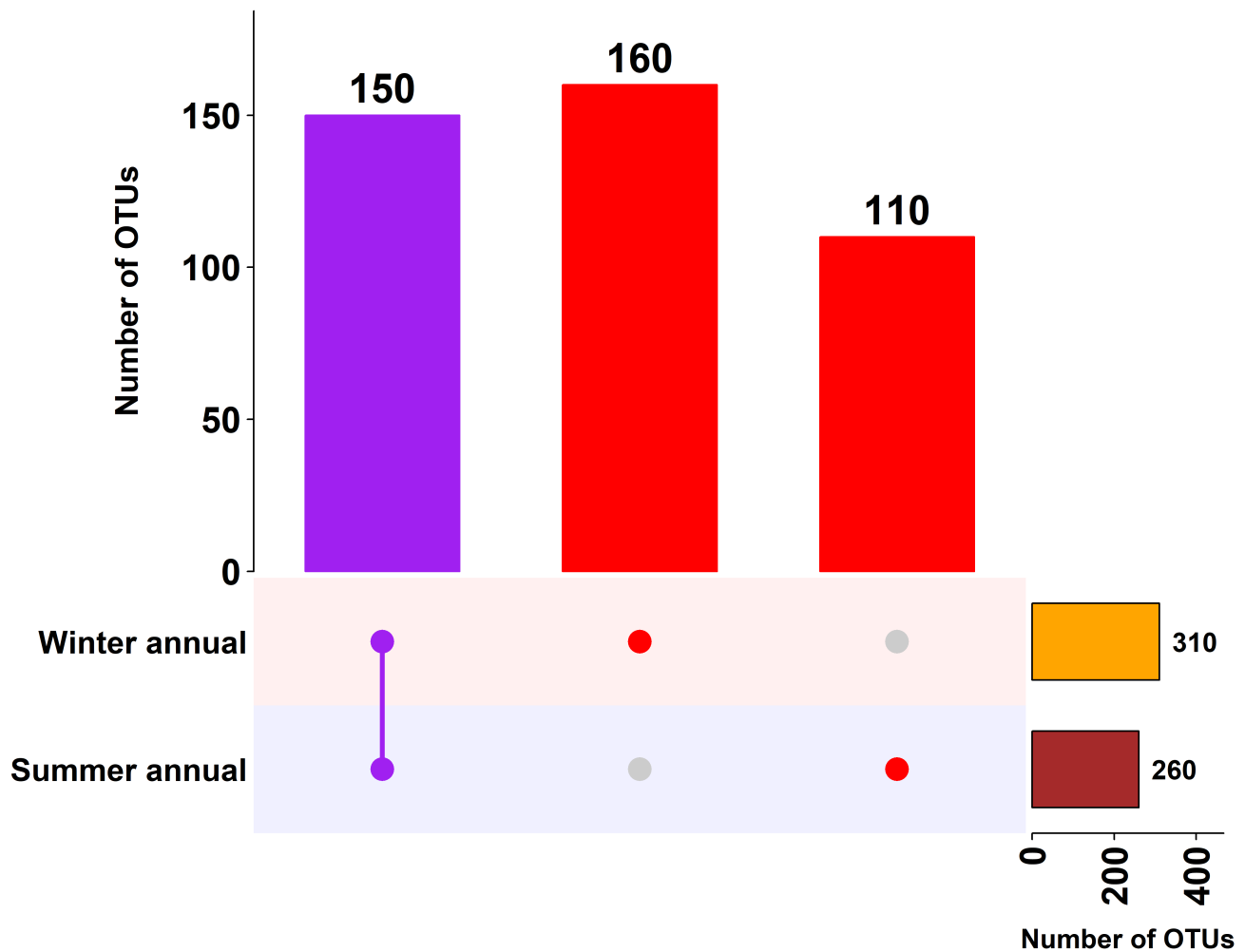


Figure 1

Upset plot showing the distribution of bacterial OTUs (operational taxonomic units) shared and unique among winter annual and summer annual samples.

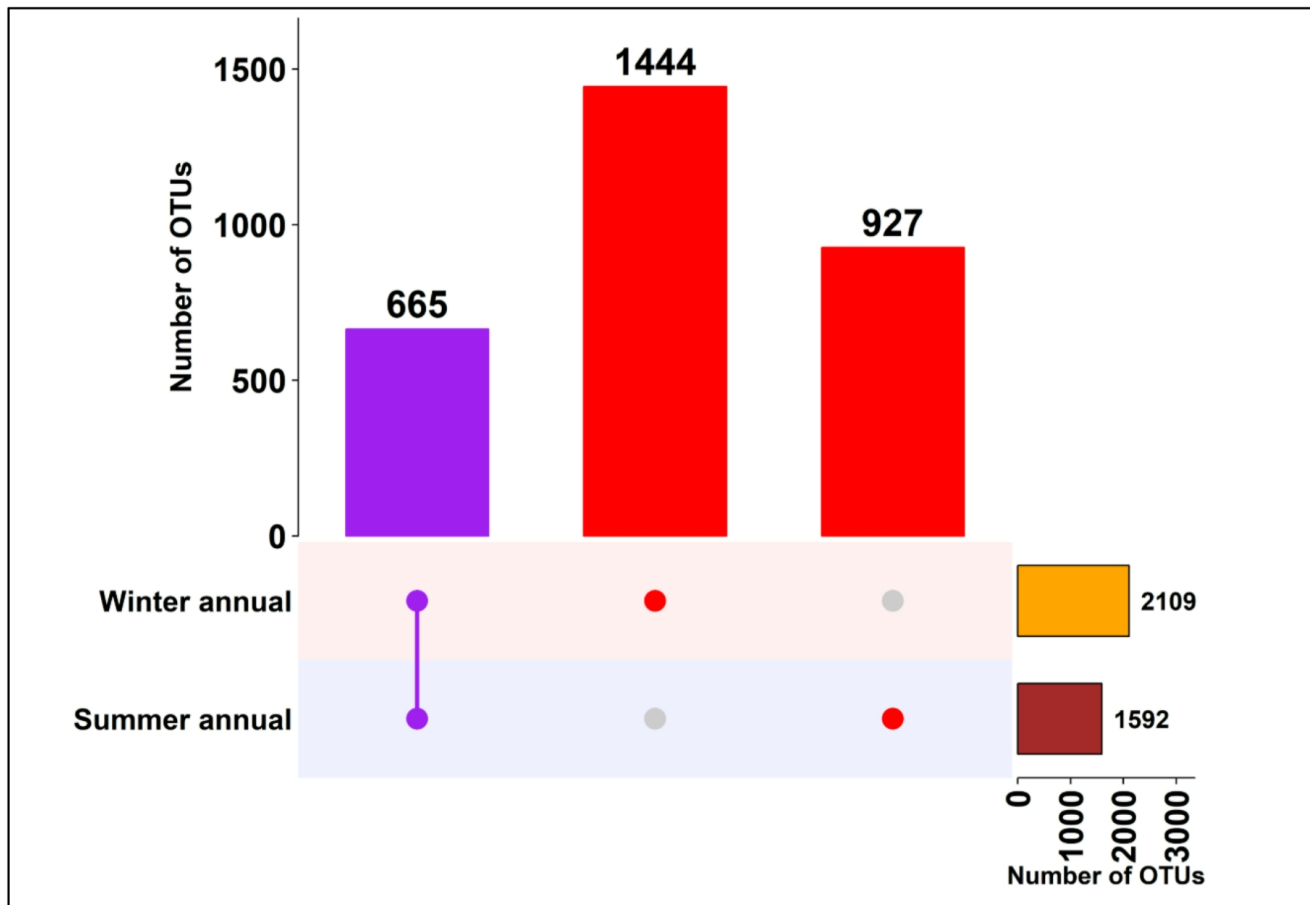


Figure 2

Upset plot showing the distribution of fungal OTUs (operational taxonomic units) shared and unique among winter annual and summer annual samples.

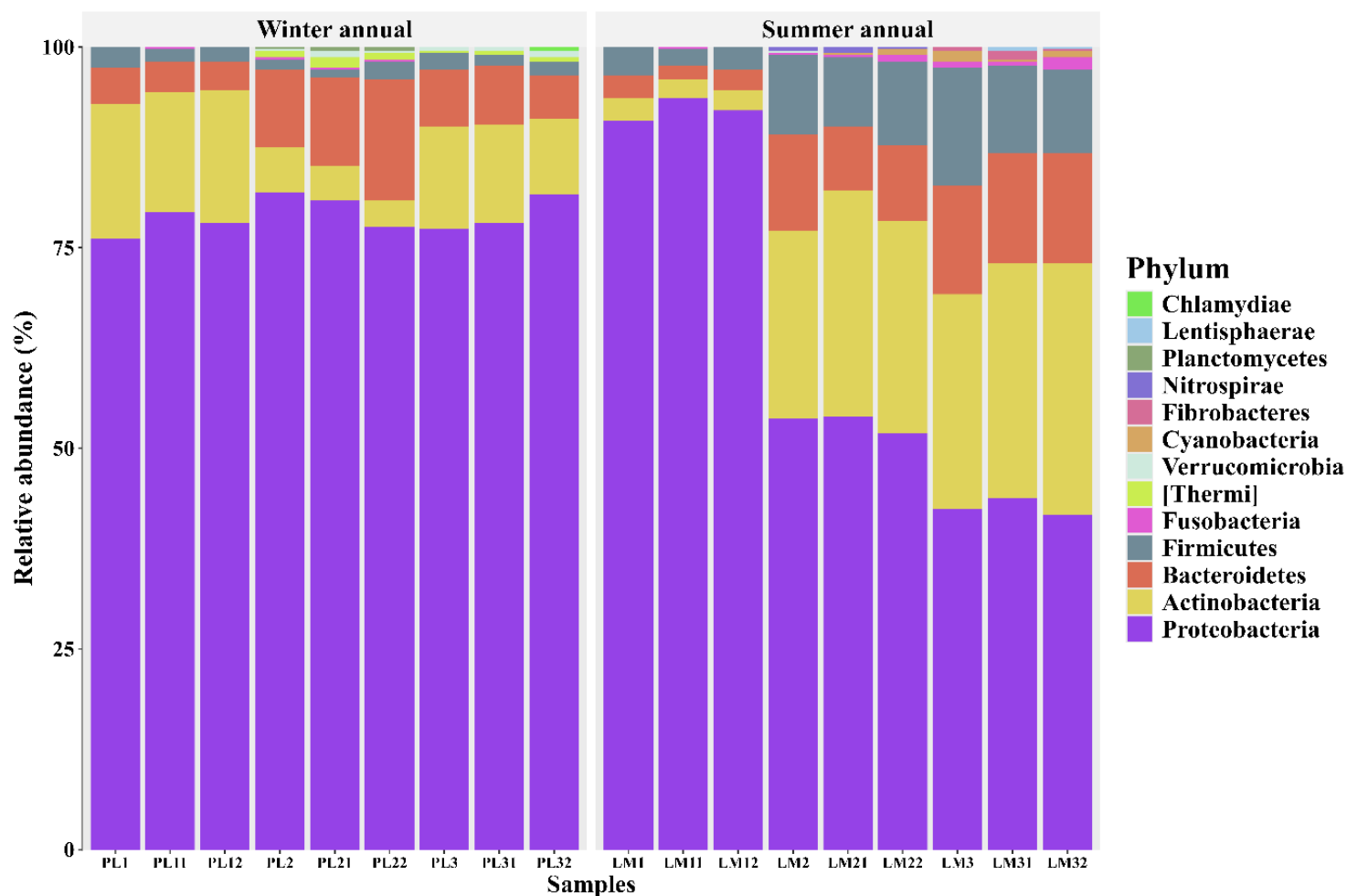


Figure 3

Stacked bar plot displaying the relative abundance of bacterial phyla across winter annual and summer annual samples.

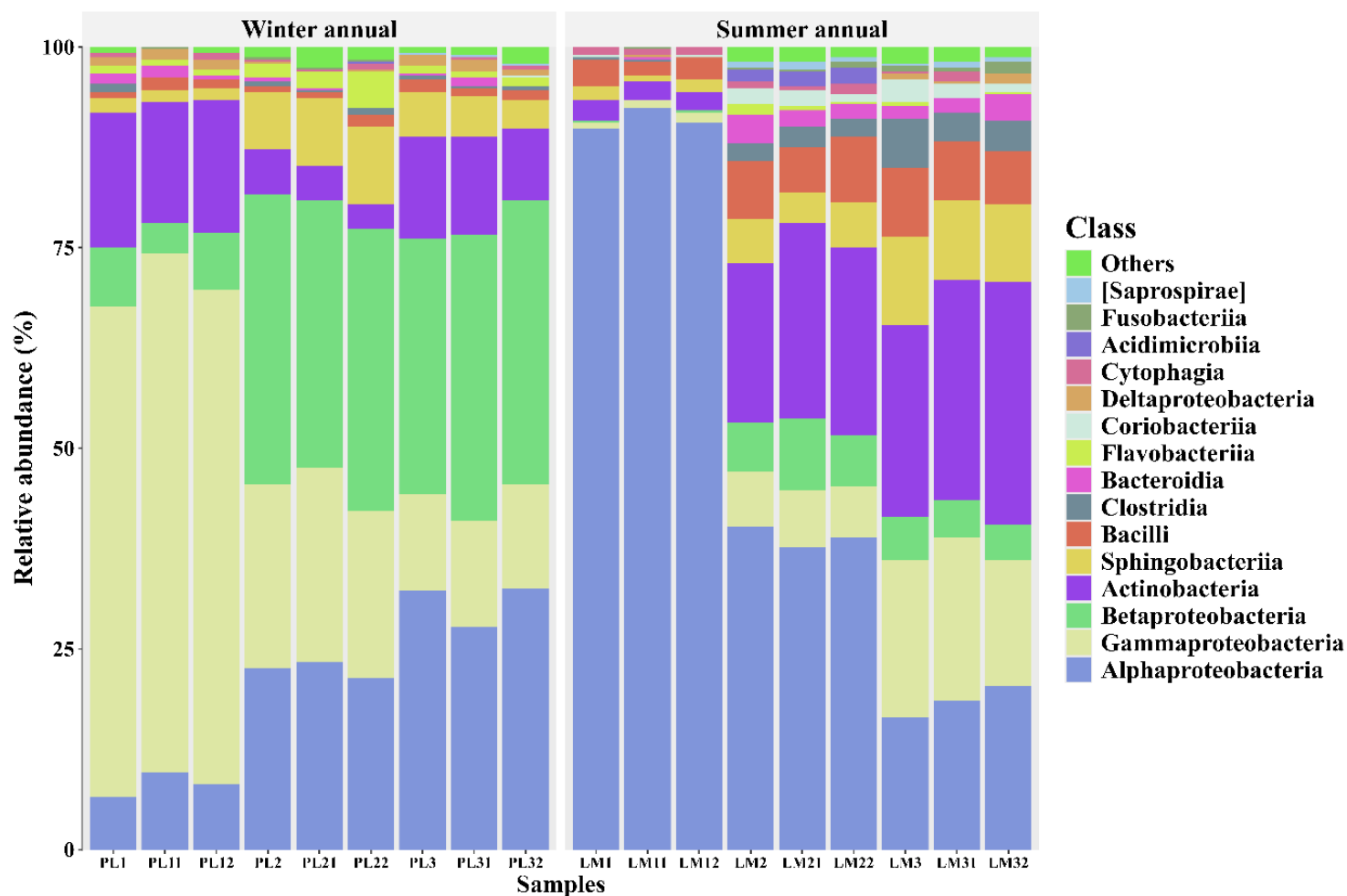


Figure 4

Stacked bar plot displaying the relative abundance of bacterial classes across winter annual and summer annual samples.

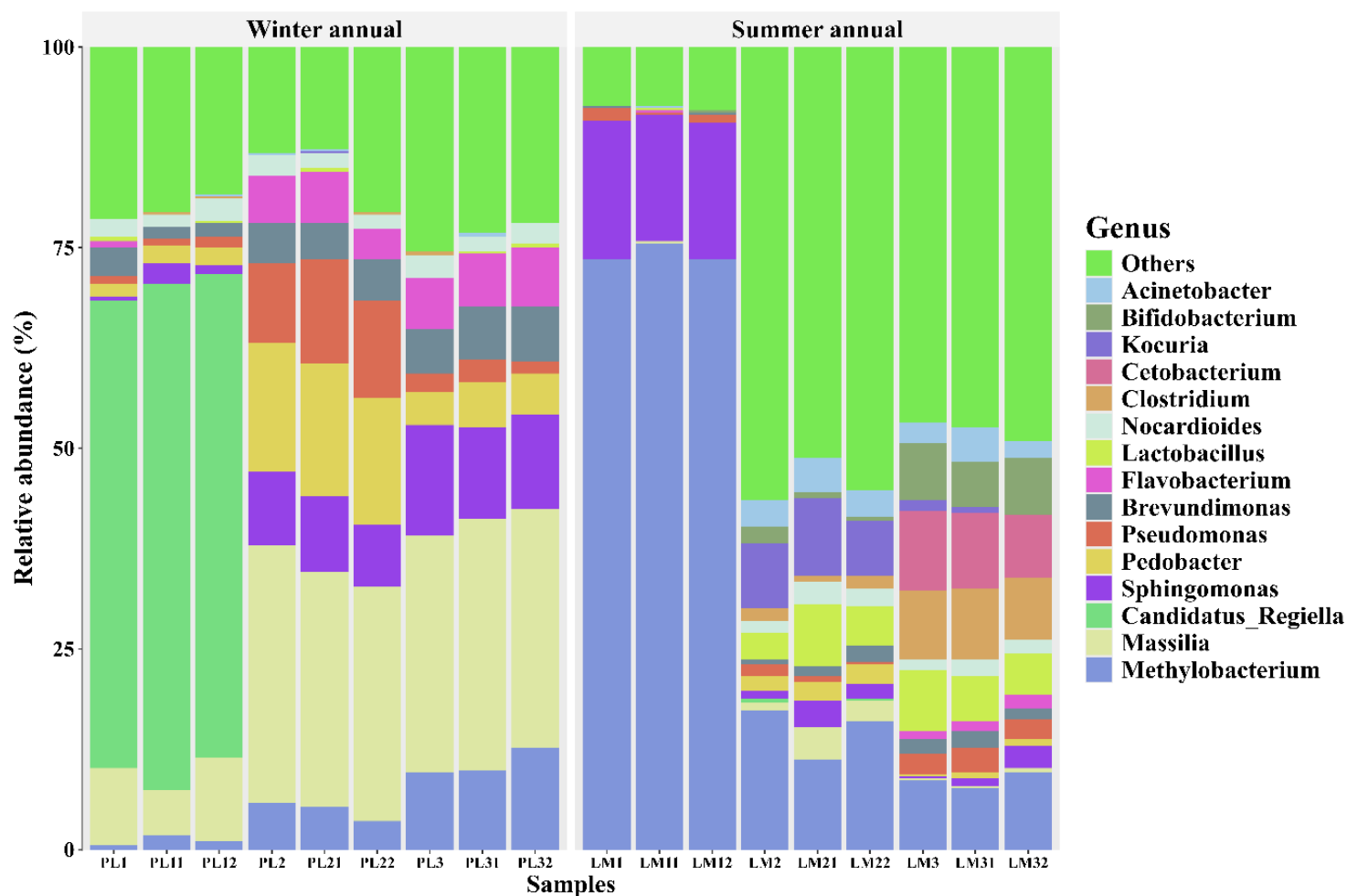


Figure 5

Stacked bar plot displaying the relative abundance of bacterial genera across winter annual and summer annual samples.

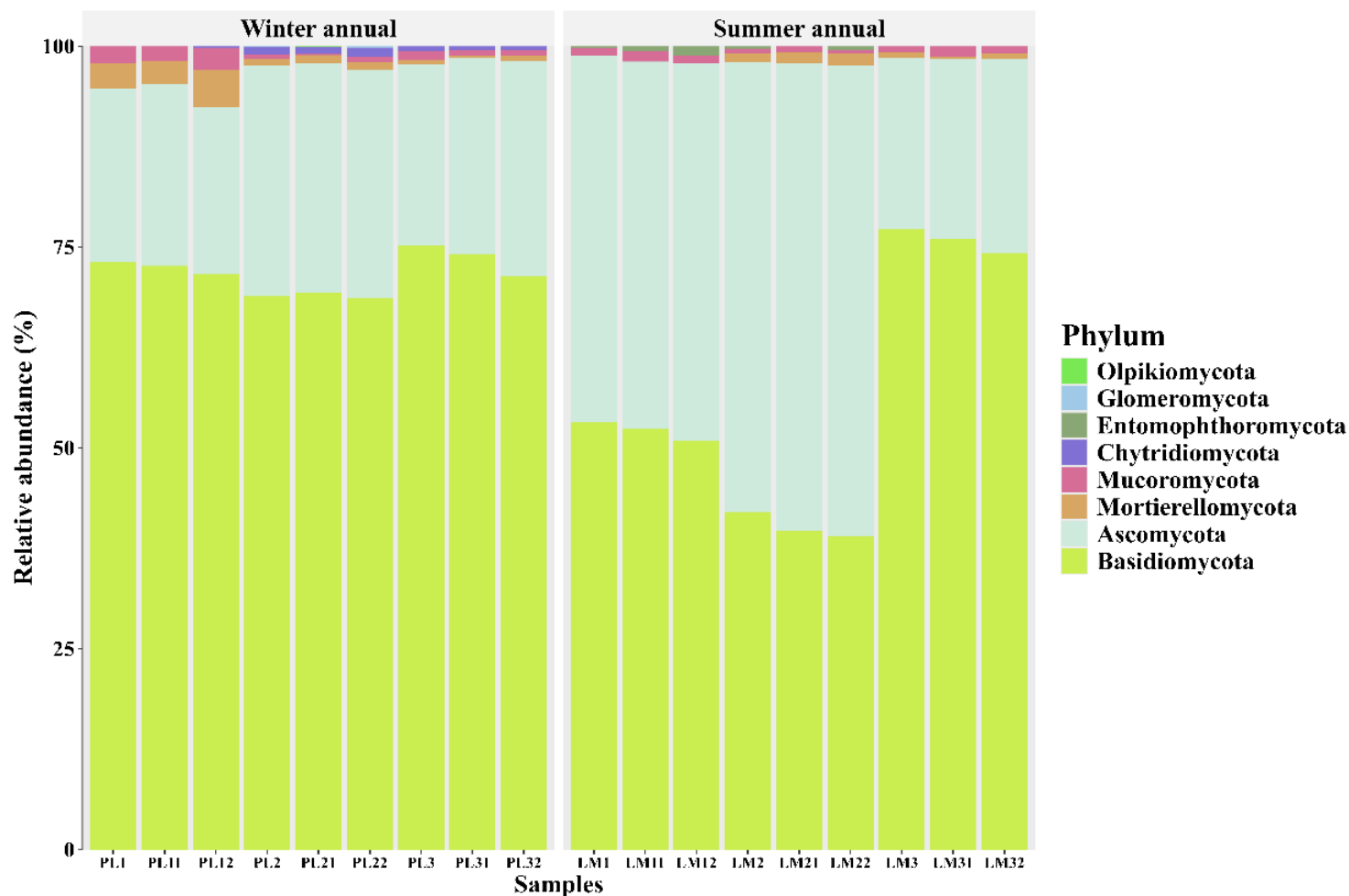


Figure 6

Stacked bar plot displaying the relative abundance of fungal phyla across winter annual and summer annual samples.

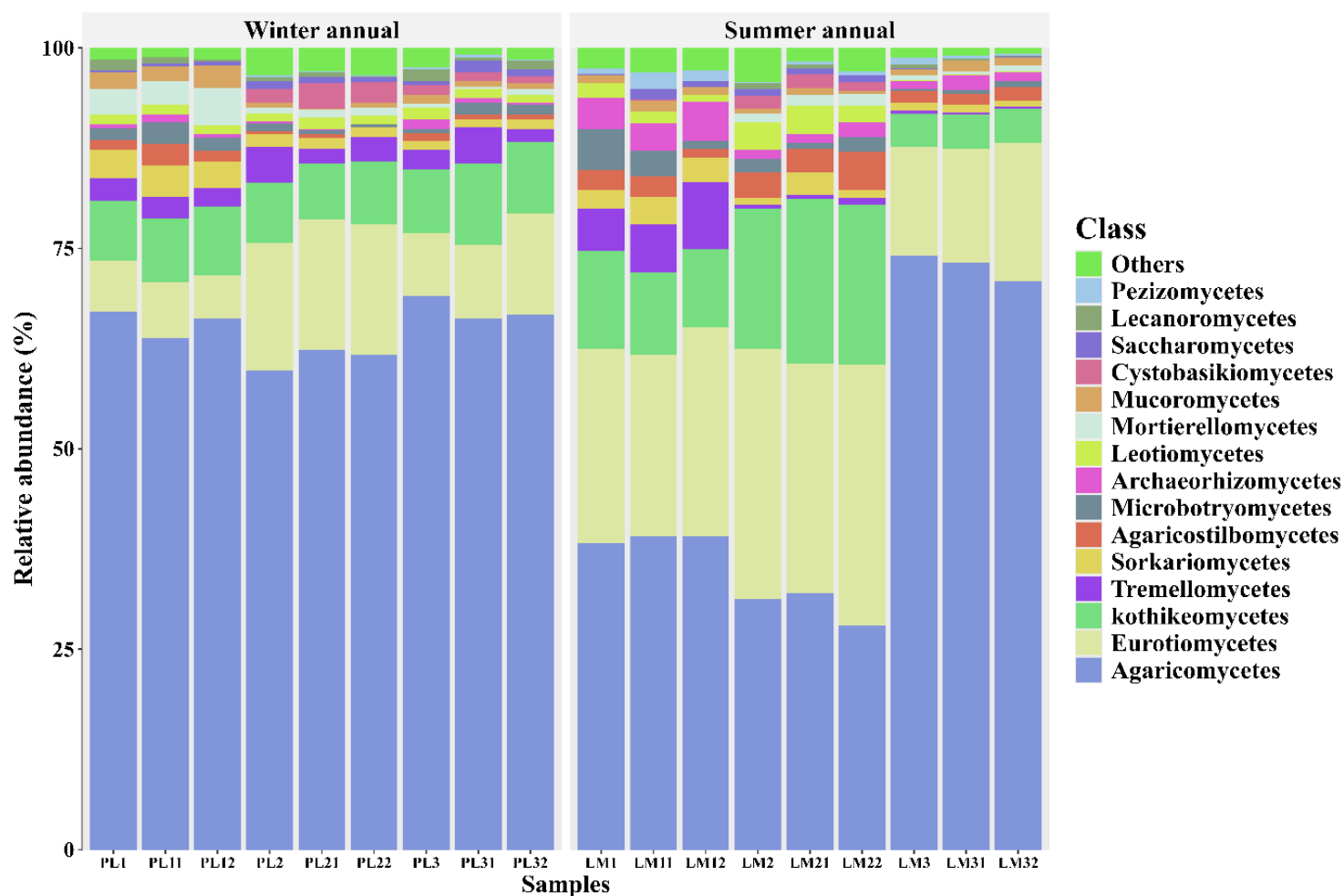


Figure 7

Stacked bar plot displaying the relative abundance of fungal class across winter annual and summer annual samples.

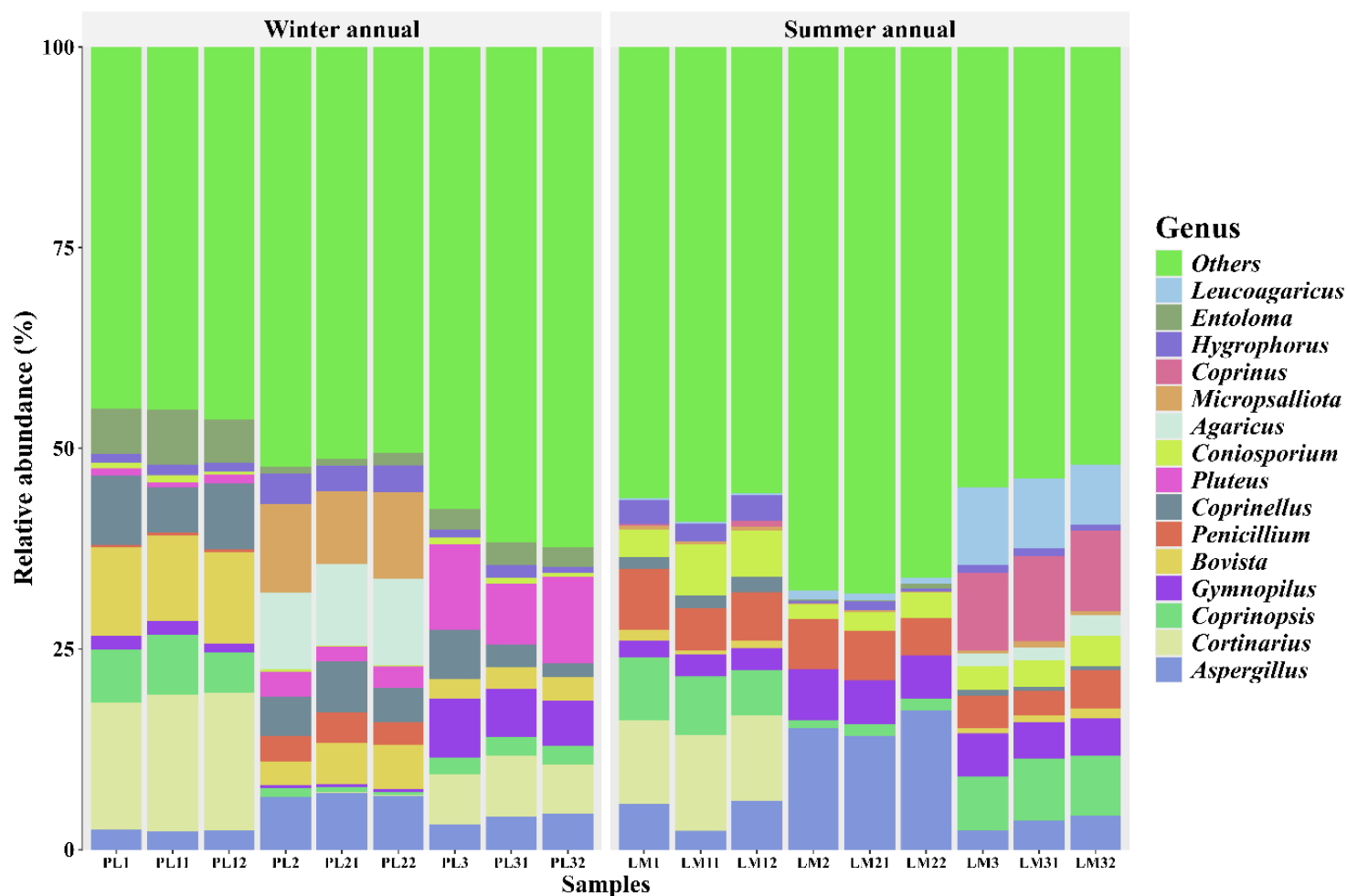


Figure 8

Stacked bar plot displaying the relative abundance of fungal genera across winter annual and summer annual samples.

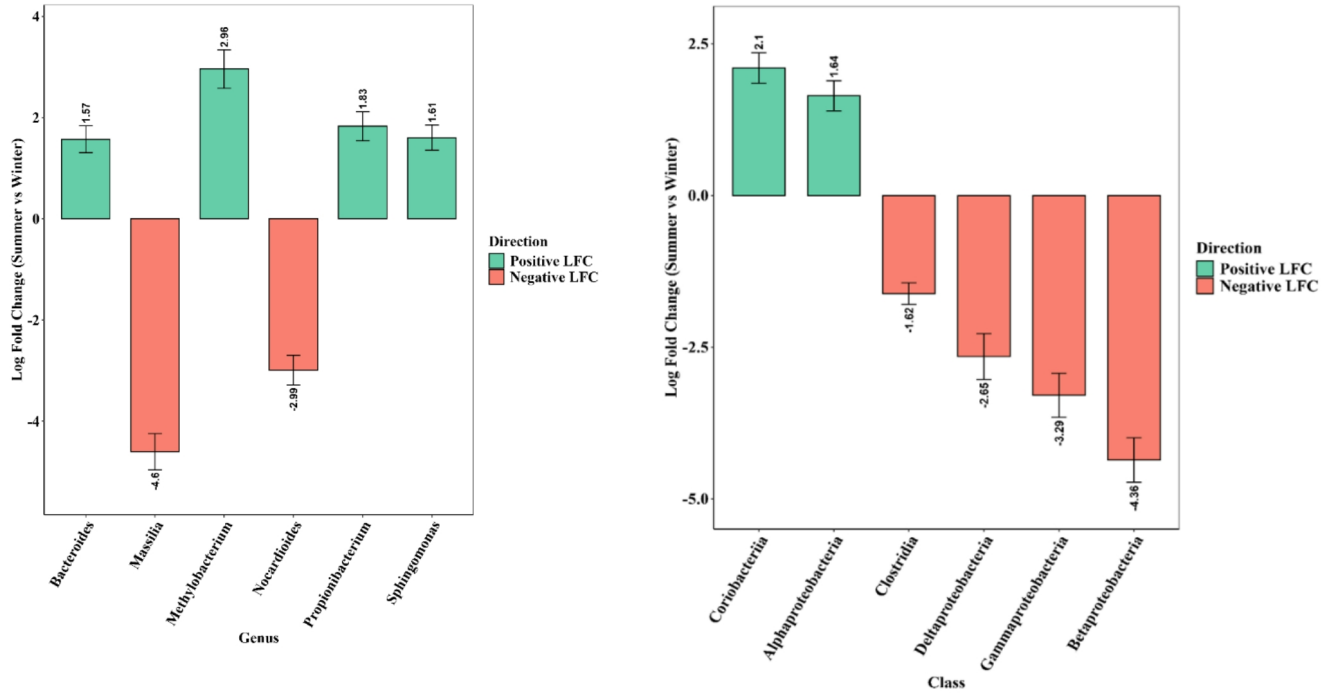


Figure 9

Bar plots illustrating the log fold change (summer vs. winter annuals) in the relative abundance of significantly dominant bacterial genera (left) and classes (right). Positive log fold changes (green bars) indicate genera enriched in summer annuals, while negative values (red bars) represent those more abundant in winter annuals. Error bars denote standard errors of the log fold change.

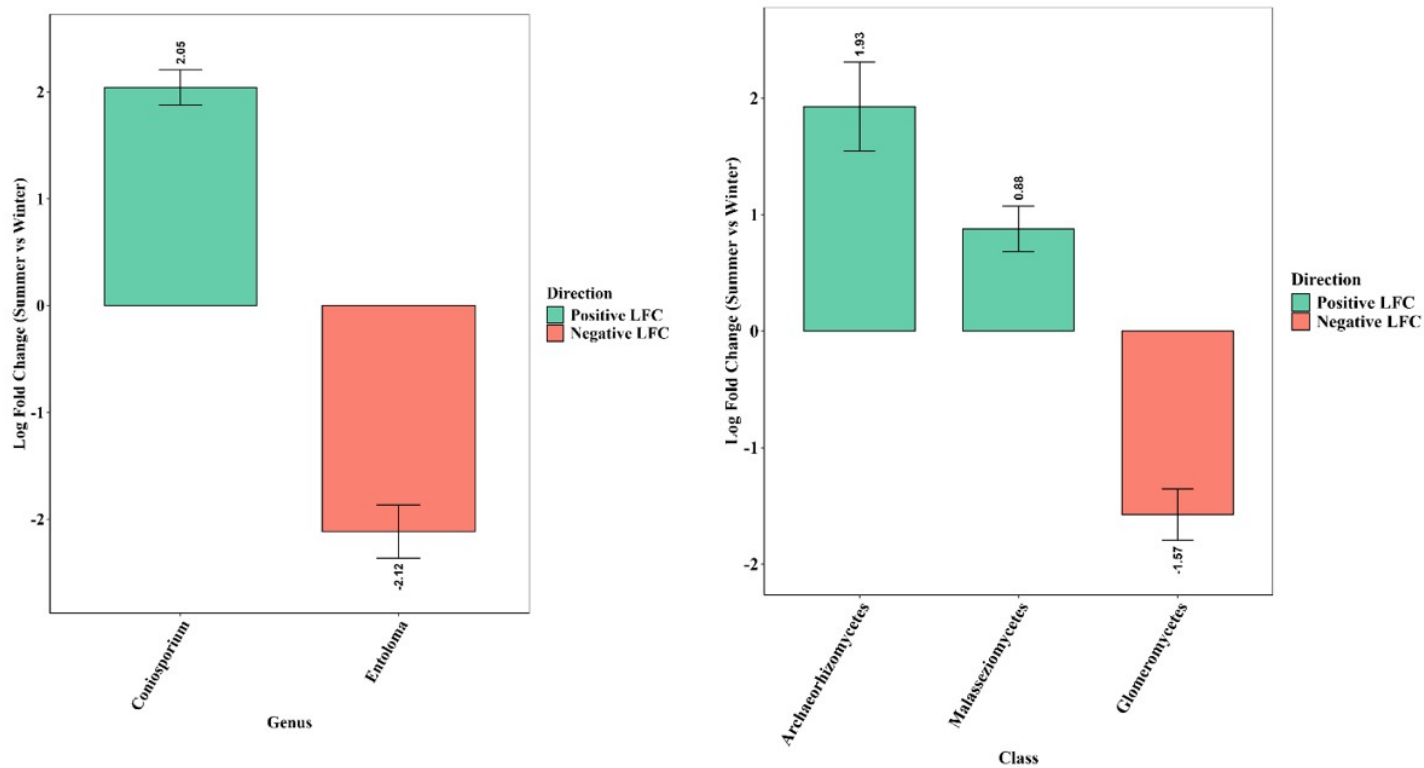


Figure 10

Bar plots illustrating the log fold change (summer vs. winter annuals) in the relative abundance of significantly dominant fungal genera (left) and classes (right). Positive log fold changes (green bars) indicate genera enriched in summer annuals, while negative values (red bars) represent those more abundant in winter annuals. Error bars denote standard errors of the log fold change.

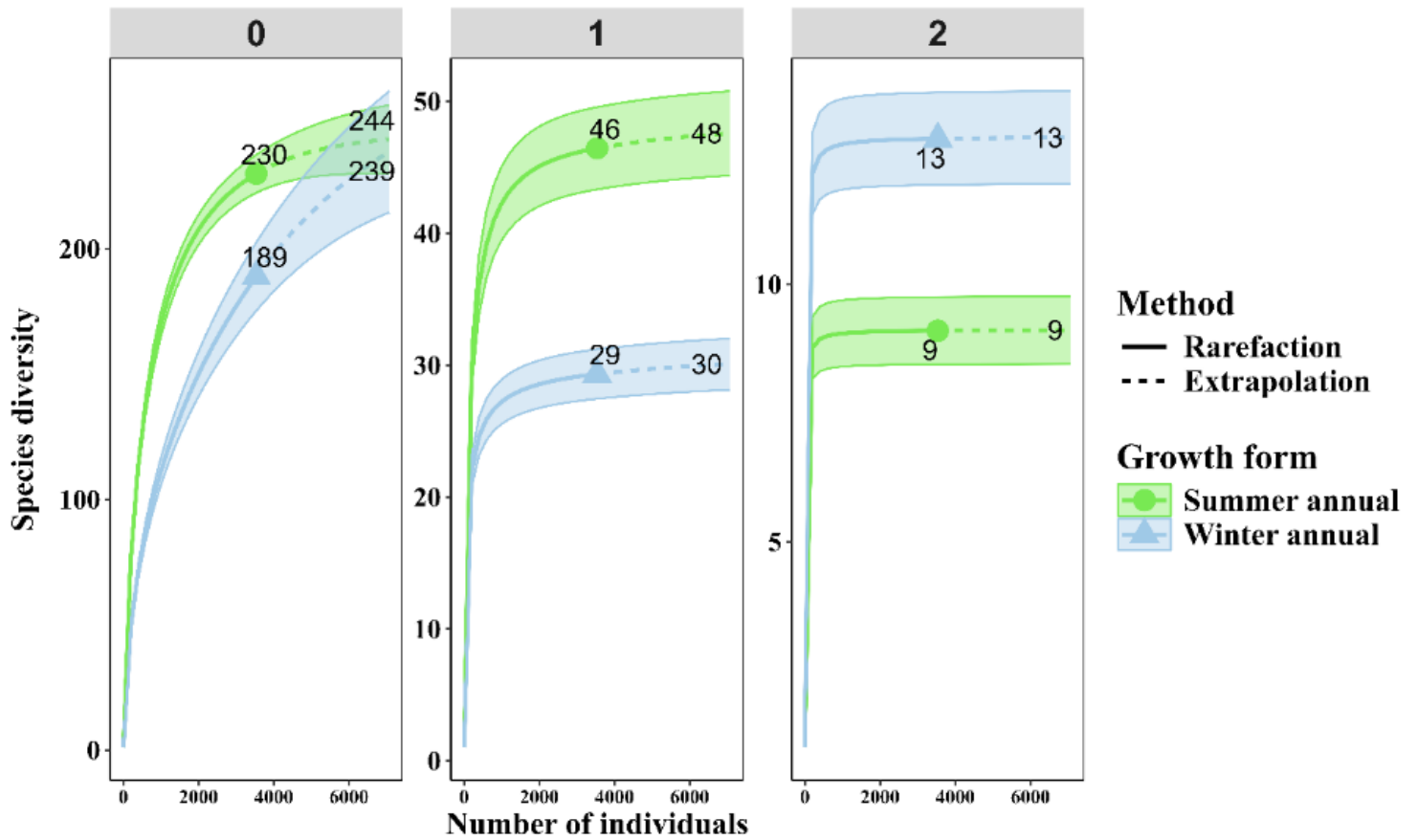


Figure 11

Alpha diversity of bacterial communities in winter and summer annual samples, quantified using Hill numbers of orders 0 (species richness), 1 (Shannon diversity), and 2 (Simpson diversity).

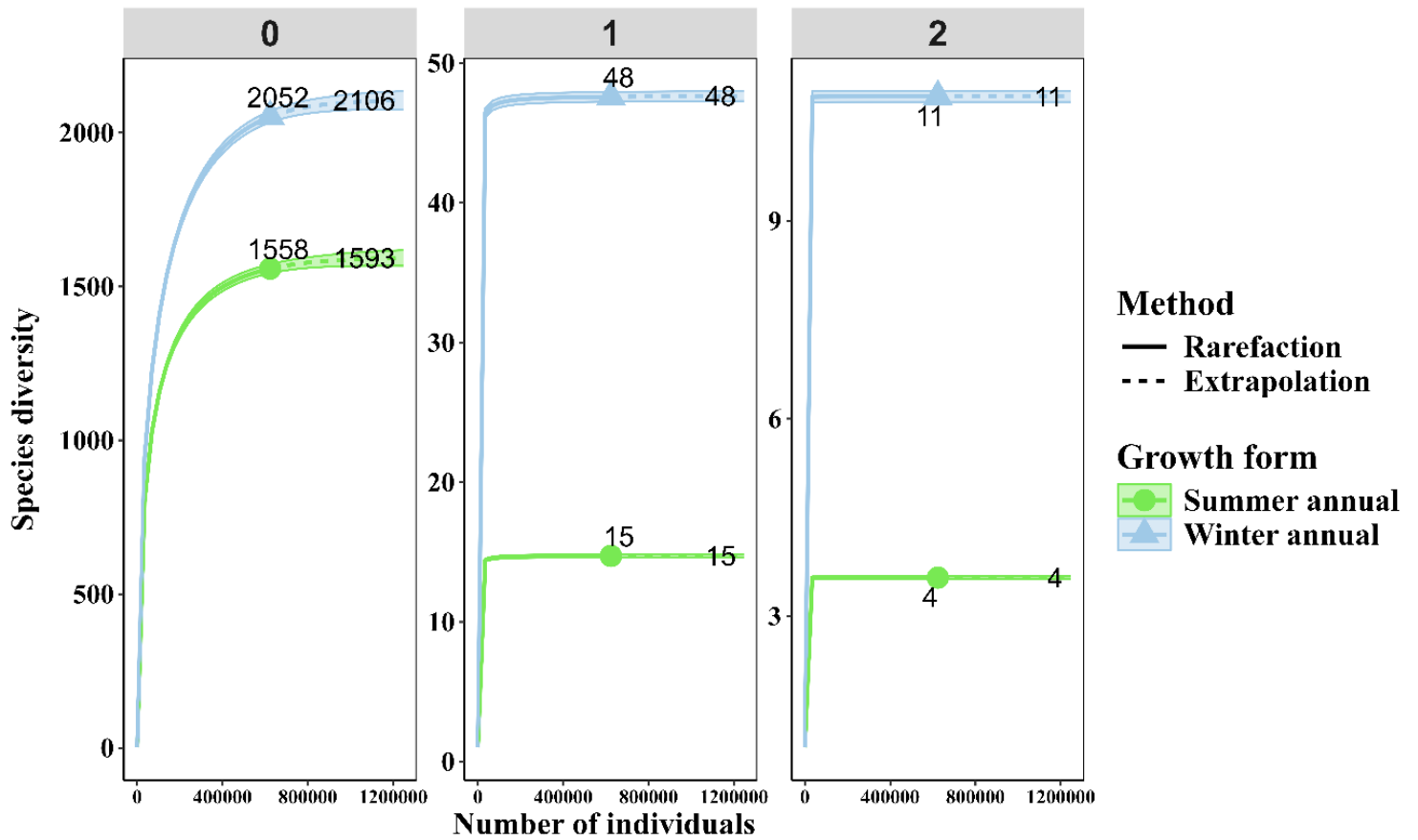


Figure 12

Alpha diversity of fungal communities in winter and summer annual samples, quantified using Hill numbers of orders 0 (species richness), 1 (Shannon diversity), and 2 (Simpson diversity).

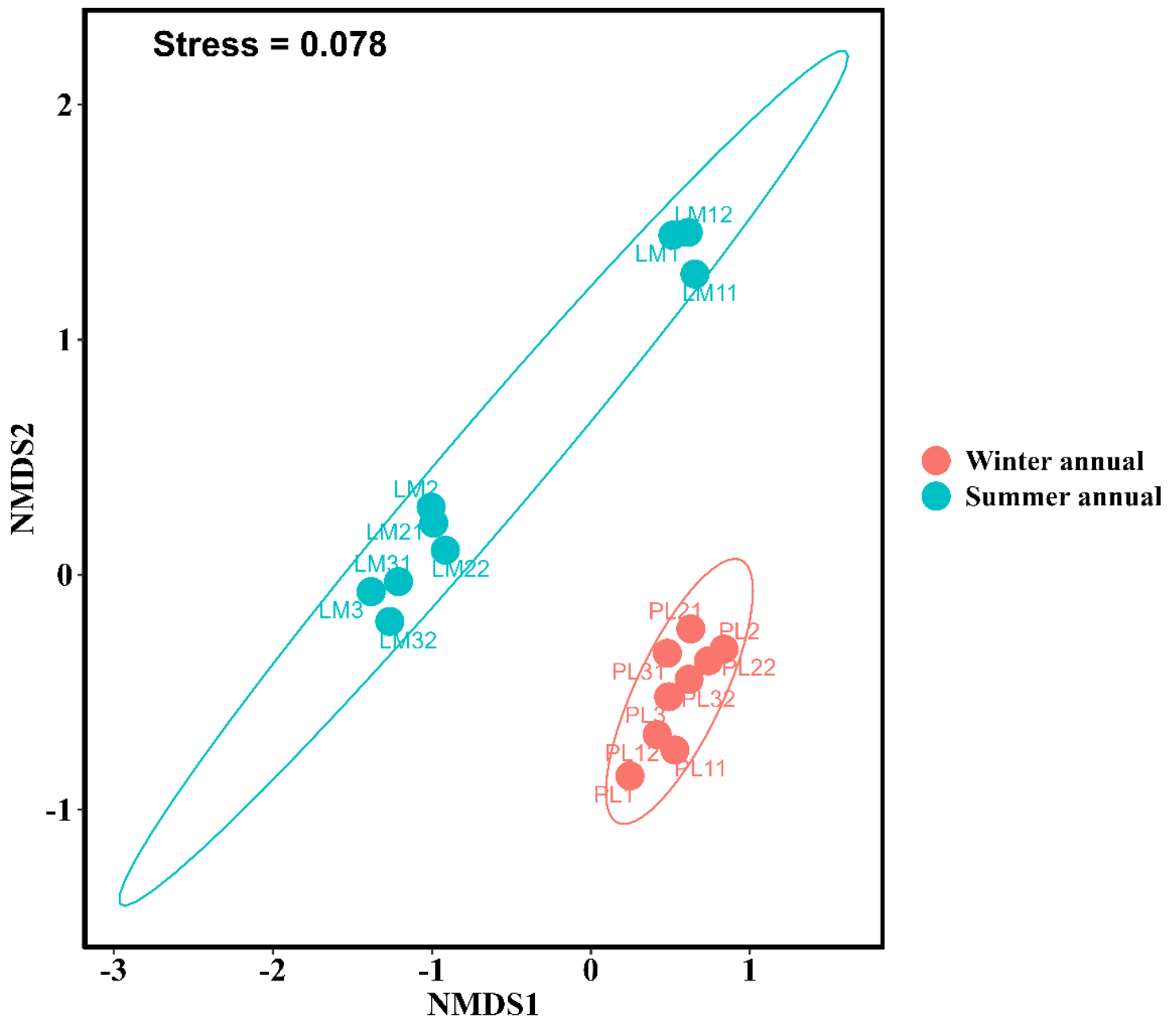


Figure 13

Non-metric multidimensional scaling (NMDS) ordination plot illustrating the compositional differences of microbial communities between winter annual (red) and summer annual (blue) samples. Each point represents an individual sample, with ellipses denoting 95% confidence intervals around group centroids

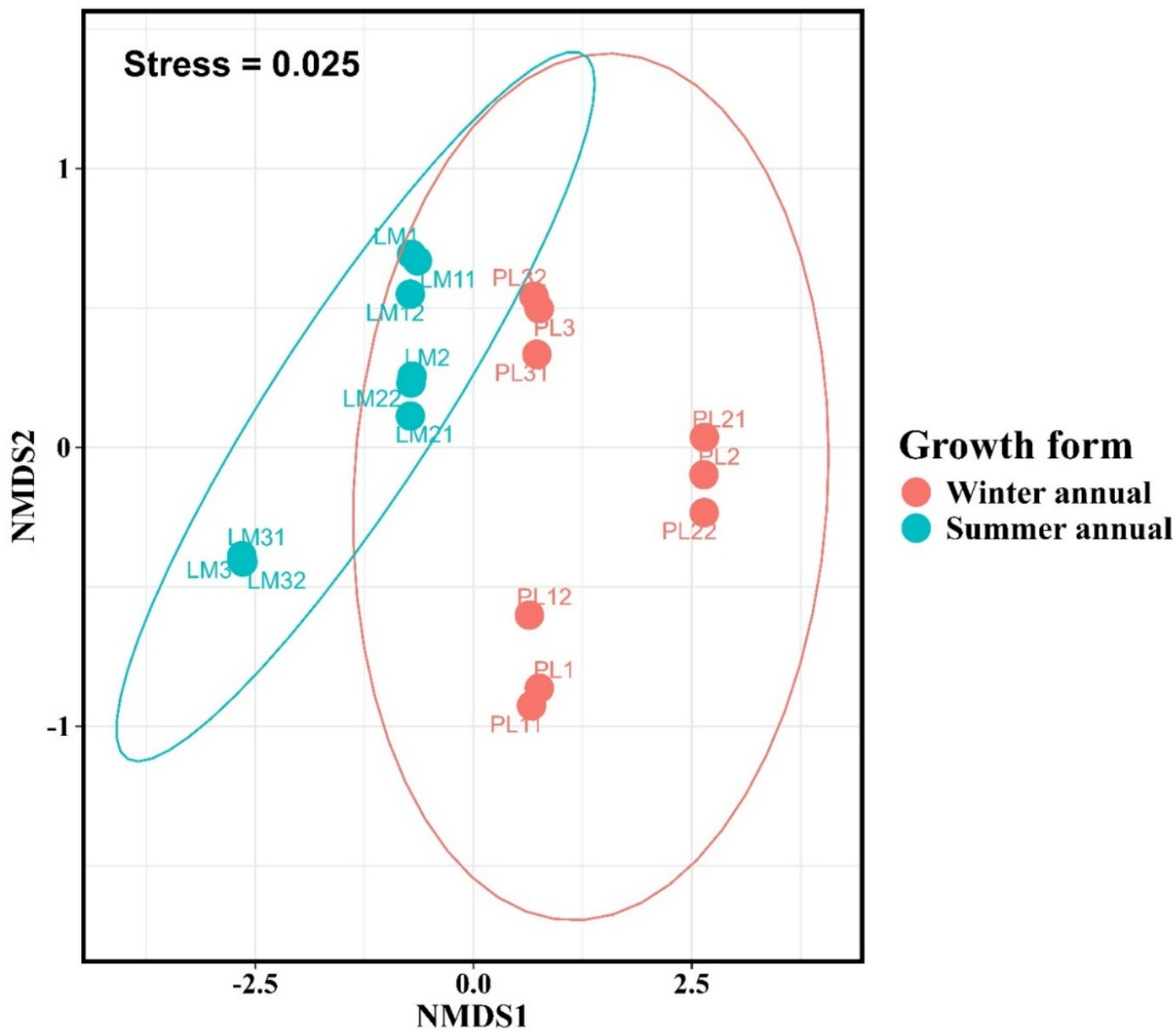


Figure 14

Non-metric multidimensional scaling (NMDS) ordination plot illustrating the compositional differences of microbial communities between winter annual (red) and summer annual (blue) samples. Each point represents an individual sample, with ellipses denoting 95% confidence intervals around group centroids

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable.docx](#)
- [SupplementaryFigures.docx](#)