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The Interplay Between Forest Succession and Soil Properties Shapes Microbial Community Dynamics After Land Abandonment

S. C. Panico[1,2] | G. L. Sciabbarrasi[1,3] | A. Foscari[1] | A. Tomao[1] | L. Orzan[1,3] | G. Alberti[1,2] | G. Incerti[1,2]

[1]Department of Agrifood, Environmental and Animal Science, University of Udine, Udine, Italy | [2]National Biodiversity Future Center, Palermo, Italy | [3]Department of Life Sciences, University of Trieste, Trieste, Italy

Correspondence: S. C. Panico (speranza.panico@uniud.it)

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ABSTRACT

This study investigates the response of soil microbial community to spontaneous afforestation—a natural rewilding process that has been progressing for decades across national and European landscapes following land abandonment. The research was conducted at two Italian sites: the Foreste Casentinesi National Park (CF) and Julian Prealps (JP). In both areas, five successional stages (I–V) of afforestation were identified based on historical orthophotos (1954–2020) and replicated across four chronosequences. Topsoil samples (0–10 cm) were analyzed for soil properties, including pH, bulk density, organic carbon, total nitrogen, and water content. Soil microbial communities were characterized through environmental DNA extracted from fine soil fractions, followed by DNA metabarcoding using ITS and 16S rRNA gene markers to target fungi and bacteria, respectively. We hypothesized that: (i) topsoil properties change along the afforestation dynamics, thereby controlling the pattern of soil microbiota α-diversity; (ii) microbial β-diversity differs between the two study areas as a result of the interplay between afforestation dynamics and soil properties. Results showed that topsoil became increasingly acidic and less compact (i.e., lower bulk density), accompanied by a rise in organic matter content. However, these trends were modulated by site-specific variability. Microbial α-diversity generally peaked at intermediate successional stages and declined in late forests. Bacterial communities were dominated by *Verrucomicrobiota* and *Pseudomonadota*, while fungal communities were dominated by *Ascomycota* and *Basidiomycota*, the latter increasing as afforestation progressed. β-diversity differed markedly between the two areas. In CF, but not in JP, a significant successional trajectory was evident, particularly for fungi. Bacterial community composition in CF was most associated with soil pH, and fungal composition with soil water content, while in JP multiple soil properties explained community variation. In conclusion, successional effects on soil microbiota were most evident in the more homogeneous environment of CF, whereas in the heterogeneous JP area, site-specific filtering driven by high variability in soil properties predominated.

1 | Introduction

Forest ecosystems are major reservoirs of biodiversity and provide numerous ecosystem services, including erosion control, water regulation and carbon (C) sequestration (Brockerhoff et al. 2017). Since the 1950s, many mountainous and remote areas across Europe have experienced strong depopulation and shifts in rural land-use practices, leading to the abandonment of agricultural and pastoral lands and, consequently, to land-cover changes driven by the spontaneous regrowth of shrublands and woodlands (MacDonald et al. 2000; Bätzing 2015; Mantero et al. 2020). This process, resulting from natural vegetation succession following land abandonment has promoted widespread spontaneous afforestation (Fuchs

Highlights

- Spontaneous afforestation increases soil organic carbon while lowering bulk density and pH.
- Microbial α-diversity follows contrasting successional trends between bacteria and fungi.
- Fungal communities show clearer successional shifts than bacteria.
- Afforestation effects on soil microbiota emerge mainly under homogeneous soil conditions.

et al. 2015). While the global forest area continues to decline due to deforestation and other anthropogenic pressures, many European countries, including Italy, have recorded a constant increase in forest cover (FAO 2020), a trend that is expected to continue in the coming decades (Navarro and Pereira 2012; Cimini et al. 2013; Malandra et al. 2018; Perpiña Castillo et al. 2018). According to the European Environment Agency (EEA), forests currently cover 41% of Europe's mountainous areas, where most natural grassland habitats are also found (EEA 2010). These mountain ecosystems host diverse vegetation types and exhibit pronounced ecological gradients of afforestation, making them biodiversity hotspots. At the same time, they represent effective Nature-Based Solutions (NBSs) for climate change mitigation, through their capacity to adsorb and store carbon dioxide (CO_2) in both biomass and soil (Frei et al. 2024).

Since soil microorganisms are key drivers of nutrient cycling, organic matter decomposition and C storage, thereby contributing substantially to the overall ecosystem functioning and health (Delgado-Baquerizo et al. 2017). Understanding how the soil microbial communities assemble, and how their structure and diversity change during the afforestation process, is therefore essential for providing soil-based ecosystem services and informing sustainable management practices.

The response of soil microbiota to afforestation varies according to the successional stage and the associated changes in soil properties along this ecological gradient. From early to mid- and late-succession stages, bacterial diversity generally increases due to rising environmental heterogeneity, while fungal diversity tends to increase from early to mid-stages and decline again in late or mature forests (Shang et al. 2021). Along these successional gradients, plant cover and dominant tree species can strongly influence soil microbial communities by altering soil pH and nutrient composition through differences in soil organic matter (OM) quality, organic C content (C_{org}), carbon-to-nitrogen ratio (C:N) and other fundamental soil properties such as soil bulk density (BD) and water content (W) (Horvat et al. 2025; Panico et al. 2025; Zhou et al. 2024). These abiotic factors act as major drivers of soil microbiota structure and diversity, playing a crucial role in the assembly of bacterial and fungal communities throughout forest development.

Liu et al. 2018 found that key bacterial and fungal taxa respond differently to environmental changes following grasslands afforestation: among bacteria, *Actinobacteria*, *Bacteroidetes* and *Nitrospirae* showed significant shifts in relative abundance, while the dominant fungal phyla often shifted from *Ascomycota* to *Basidiomycota*, with greater effects on diversity than on richness. At a more local scale, microbial community composition is further modulated by plant community composition and structure, which can significantly influence both diversity and composition, often leading to distinct bacterial and fungal assemblages (Labouyrie et al. 2023). Although several studies have identified forest type as a key determinant of soil microbial composition (Bach et al. 2008; Hackl et al. 2004), our understanding of how natural afforestation gradients influence soil microbiota remains limited, particularly in mountainous regions where spontaneous forest regrowth has been ongoing for decades. Moreover, to date, few studies have explored β-diversity patterns arising from local community assembly processes and regional species pools along latitudinal gradients (Zhang et al. 2020).

In this study, we investigated two mountain areas of Italy: the Julian Prealps (JP) in northern Italy and the Casentinesi Forest (CF) in central Italy which, since the 1950s, have undergone progressive depopulation due to socio-economic changes, leading to the abandonment of agricultural land and pastures (Agnoletti et al. 2022). In each area, four replicated chronosequences were established, encompassing five stages of spontaneous afforestation following grassland abandonment, ranging from still managed grassland (I) to afforested areas since at least 70 years (V). At each stage, topsoil (0–10 cm) was analysed for pH, bulk density (BD), water content (W), soil organic C (C_{org}), total nitrogen (N) content and soil C stock (C_{stock}) calculated on the whole sampling depth. Microbial communities were characterized by soil DNA metabarcoding targeting bacteria and fungi. We hypothesized that: (i) topsoil properties change along the afforestation dynamics, thereby shaping the pattern of microbial α-diversity; (ii) microbial β-diversity differs between the two study areas as a result of the interplay between afforestation dynamics and soil properties.

2 | Materials and Methods

2.1 | Study Areas: The Julian Prealps and the Casentinesi Forest

The study areas are located in two regions of northern and central Italy—Friuli Venezia Giulia and Tuscany and sampling sites were established in two mountain areas: the Julian Prealps (46°20′16.8″ N 13°19′04.8″ E) and the Casentinesi Forest (43°50′36″ N 11°47′28″ E). The four sites are located in areas belonging to the beech forest vegetation series (Blasi and Rosati 2010) and share a common history of significant depopulation beginning in the 1950s, which led to the widespread abandonment of grasslands, meadows, pastures and cultivated land (Malandra et al. 2018; Agnoletti et al. 2022). In detail, the northernmost site, located in Friuli Venezia Giulia region, is dominated by European ash (*Fraxinus excelsior* L.) and sycamore maple (*Acer pseudoplatanus* L.), with additional presence of hop hornbeam (*Ostrya carpinifolia* Scop.), chestnut (*Castanea sativa* Mill.), and hazel (*Corylus avellana* L.). In Tuscany site, the dominant species are Turkey oak (*Quercus cerris* L.) and European beech (*Fagus sylvatica* L.), with less

frequent occurrences of hop hornbeam, sycamore maple, and wych elm (*Ulmus glabra* Huds.) The two study areas (i.e., Julian Prealps and Casentinesi Forest) and the four chronosequences sampled are represented in Figure S1 in which an illustrative example of the study sites across the five afforestation stages is also reported. The detailed composition of the dominant vegetation and tree density across afforestation stages for both study areas is provided in Supplementary Figure 2. The soil at both study areas is classified as Dystric Cambisol, with loamy texture.

2.2 | Experimental Design, Soil Sampling and Analysis

Four chronosequences were established within the two study areas using historical georeferenced digital orthophotos documenting the spatial progression of afforestation following grassland abandonment over the past 70 years. For each chronosequence, five stages were selected to represent distinct secondary successional stages, according to the year of the oldest orthophotos in which forest was detectable: grassland (I, forest not detected), early (II, 2020), intermediate (III, 2000), intermediate-late (IV, 1978), and late (V, 1954) afforestation.

At both study areas, for each of the five stages of each of the four chronosequences, one 13 m-radius plot was installed. Hence, the four plots at equal stage are considered as randomized replicates. After removing surface litter and the forest floor layers, at each plot four cores (5 cm diameter, up to 35 cm of depth) were collected at the four cardinal directions using a soil auger (Eijkelkamp, NL). Each core was subdivided into two samples: a top layer up to 10 cm depth and a deep layer. The top soil samples were pooled resulting in a total of 40 topsoil pooled samples (4 replicated plots × 5 afforestation stages × 2 study areas) then used to assess pH, W, C_{org}, and N following standard protocol (Panico et al. 2025). Soil bulk density (BD) was assessed on 3 replicated 100 cm^3 Kopecky's rings (Blake and Hartge 1986) collected from both layers at each plot. C_{stock} was calculated according to the following equation:

$$C_{stock} = \sum_{i=1}^{2} BD_i \times T_i \times C\,\%$$

where for each of the two *i*-th layers, BD_i is the bulk density (in g cm^{-3}), T_i is the layer thickness (in cm) and $C\%$ is the soil C percent content ($g_C\ g_{soil}^{-1} \times 100$).

For environmental DNA (eDNA) extraction, topsoil was collected using sterilised gloves and spatulas, placed in sterile 50 mL Falcon tubes, kept on ice during transport and stored at −20°C in the laboratory. To prevent cross-contamination, soil corers were sterilized with 90% ethanol and rinsed with deionized water between samples.

2.3 | Soil eDNA Extraction and Metabarcoding

Within a week from the sampling, total soil genomic DNA was extracted from 0.5 g of each of the 40 topsoil samples using the Power Soil DNA Isolation Kit (QIAGEN Laboratories Inc., Solana Beach, USA) according to the manufacturer's instructions. DNA extracts from the three within-plot replicates were pooled in equal proportions to obtain a single composite sample per site. DNA concentration and purity were measured using a Thermo Scientific PicoDrop 1000 spectrophotometer with OD260 and OD280, and the quality was verified by 1% agarose gel electrophoresis. Negative controls were included in each extraction batch and amplified to detect contaminants.

Library preparation followed a two-step PCR protocol: an initial amplification with locus-specific primers, followed by a second PCR to attach flow-cell adapters and unique indices (NexteraXT Index Kit, FC-131-1001/FC-131-1002). PCR conditions were: 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. Each sample was amplified in triplicate (20 μL per reaction). Bacterial libraries targeted the V3–V4 region of the 16S rRNA gene using primers 341F (5′-CCTACGGGNBGCASCAG-3′) and 805R (5′-GACTACNVGGGTATCTAATCC-3′) (Klindworth et al. 2013), while fungal libraries targeted the ITS1 region of EukaryausingprimersITS1(5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′-GCTGCGTTC TTCATCGATGC-3′) (Martin and Rygiewicz 2005; White et al. 1990). Although the combined use of ITS1 and ITS2 may improve taxonomic coverage, both regions are widely recognized as suitable for characterizing fungal communities and capturing ecological patterns, despite region-specific biases (Blaalid et al. 2013; Nilsson et al. 2019). The choice between ITS1 and ITS2 largely depends on study objectives and primer availability; however, both markers may recover complementary components of fungal diversity (Bazzicalupo et al. 2013).

PCR products were prepared for sequencing and run on an Illumina NovaSeq 6000 (250-bp paired-end mode) following IGATech's standard protocols (Udine, Italy). Sequence processing, including quality filtering, denoising, merging, and chimera removal, was performed in R (dada2 v1.22.0) after primer trimming (Callahan et al. 2016). This pipeline produced highly specific amplicon sequence variants (ASVs), capable of detecting single-nucleotide differences (Callahan et al. 2017). Taxonomic classification used the SILVA (r138) and UNITE (v2019) databases for bacteria and fungi, respectively (Glöckner et al. 2017; Nilsson et al. 2019; Glöckner et al. 2017), via the RDP Classifier (Wang et al. 2007). Raw sequencing data are publicly available in the NCBI GenBank Archive under accession number PRJNA1159178.

2.4 | Data Elaboration and Statistical Analyses

All the data are presented aggregated by successional stage (I-V) for the two study areas. The effect of successional stage on physicochemical soil properties was tested within each area using one-way analysis of variance (one-way ANOVA), after preliminarily checking that the assumptions of normality and homoscedasticity were met, followed by Tukey post hoc ($p < 0.05$).

Microbial α-diversity was analysed on rarefied samples at the minimum sequencing depth for the smallest samples (bacteria: 130,305 reads for JP and 87,621 reads for CF; fungi: 74,008 reads for JP and 227,194 reads for CF). Richness (R), Shannon (H) and

Pielou's evenness (J) indices were calculated for bacterial and fungal communities using the R package "vegan" (v. 2.6–4) (Oksanen et al. 2022; R Core Team 2022). Because α-diversity data did not meet the assumptions of normality and homoscedasticity, two-way non-parametric ANOVA (Scheirer–Ray–Hare) was performed using the *rcompanion* package (Mangiafico 2025) to test the main and interactive effects of study area (2 levels) and successional stage (5 levels) on richness (Amplicon Sequence Variants (ASVs) counts), Shannon's entropy (H′) and Pielou's evenness (J). Dunn's post hoc test with Benjamini-Hochberg adjustment was applied for pairwise comparisons ($p < 0.05$).

ASVs relative abundance was calculated as the proportion of reads for each ASV relative to the total reads per sample. ASVs assigned to the same species were merged, summing their relative abundances. Taxa with relative abundances lower than 0.01% across all samples were removed and phyla of dubious rank or known to occur in non-soil environments (e.g., candidate phylum SAR324 Marine Group B) were excluded.

Venn diagrams were used to explore shared taxa at phylum and genus levels and unique taxa between the two study areas. Stacked bar plots displayed the relative abundance of microbial communities at each successional stage.

β-diversity was assessed using Bray–Curtis dissimilarity and visualized with Principal Coordinate Analysis (PCoA) to evaluate compositional changes across afforestation stages (I, II, III, IV, and V) and study areas (JP and CF). Differences were tested using two-way permutational ANOVA (PERMANOVA) through *adonis2* function in the "vegan" package and permutational multivariate analysis of dispersion (PERMDISP). Pairwise comparisons among communities were performed using the *permanova_pairwise* function with 999 permutations. Variation partition analysis was used to quantify the contribution of soil properties to β-diversity variation across successional stages within each study area. Finally, Spearman's correlation was calculated between each bacterial and fungal taxon (phylum or genus level) and soil parameters, and results were visualized using heatmap. All statistical analyses were performed in R studio version 4.4.

3 | Results

3.1 | Influence of Successional Dynamics on Soil Characteristics at the Two Study Area

Most of the soil properties changed significantly across different successional stages in JP, also showing large within-stage variation, while in CF, only C:N and W varied significantly, and all parameters were less variable as compared to JP (Figure 1). In detail, C_{org} and C_{stock}, as well as C:N and N content, significantly increased in JP following afforestation, generally showing minimum and maximum values at the initial (I) and final (V) stages, respectively (Figure 1, Table S1). Out of these 4 variables, only C:N also varied significantly in CF, with a trend substantially analogous to that observed in JP, although with values generally higher as compared to JP (5.97–10.52 vs. 9.92–14.66, Figure 1). Soil pH showed sub acidic values in both areas at most stages, with a significant decrease to acidic values at the V stage in JP, while not varying significantly in CF. Correspondingly, BD changes in CF were not statistically significant, while in JP we observed a significantly higher value at the initial stage (I) as compared to the minimum at the final (V) one (Figure 1, Table S1). Finally, W increased in both areas, although with significantly higher values in CF than JP at most afforestation stages (Figure 1).

3.2 | Soil DNA Metabarcoding

All 120 bacterial (16S) and fungal (ITS) libraries were successfully sequenced. Following the abundance filtering, 1050 and 958 bacterial taxa and 1665 and 1320 fungal taxa were retained in JP and CF, respectively (Table S3). Of these, 131 bacterial and 926 fungal taxa in JP, and 120 and 758 in CF, respectively, were identified at species level. On average, sequence retention was 95.3% ± 1.5% for bacterial reads and 97.7% ± 1.1% for fungal reads across all replicates.

3.3 | Soil Microbiota α-Diversity Across Study Sites

The α-diversity of soil microbiota showed interesting similarities and differences between the two study areas. In particular, genetic richness (R) in bacterial communities consistently decreased at the final V stage in both areas, but R dynamics along the afforestation gradient did not show other significant variations in JP, while in CF it followed a hump-shaped trajectory (Figure 2). Moreover, at almost all stages R was higher in JP compared to CF for bacteria, showing the opposite trend for fungi (Figure 2). H′ and J of bacterial communities followed substantially the pattern of R across afforestation stages in both areas, although between-areas differences were limited to stages I and III. For fungal communities, H′ and J followed a hump-shaped trajectory in JP, with peaks at stage III also higher than in CF, where they did not vary significantly across stages (Figure 2).

3.4 | Comparative Analysis of Microbial Community Composition

Venn diagram analysis revealed that, for bacteria, a total of 27 phyla were observed across the two study areas, of which 24 were shared and 3 were unique to JP (Figure 3). At the genus level, 546 bacterial genera were detected, with 331 shared between study areas, 128 unique to JP, and 87 unique to CF. For fungi, 18 phyla were observed, with 15 shared, one unique to JP and two unique to CF (Figure 3). At the genus level, 826 fungal genera were identified, comprising 367 shared, 324 unique to JP and 135 unique to CF.

At phylum level, the bacterial community was dominated by *Verrucomicrobiota* and *Pseudomonadota*, whose combined relative abundances exceeded 55% and 45%, respectively, in both areas (Figure 4). In CF soils, *Pseudomonadota* increased along the succession, while *Acidobacteriota* consistently represented more than 10% of the total, reaching up to 17% in JP. *Actinobacteria* accounted for 15%–20% in CF, peaking at latest (IV and V) stages, whereas in JP, their abundance decreased progressively in favour of *Verrucomicrobiota* and minor phyla, including *Nitrospirota*, *Myxococcota, Bacteroidota* and *Chloroflexi*, which together represented around 5% of the total

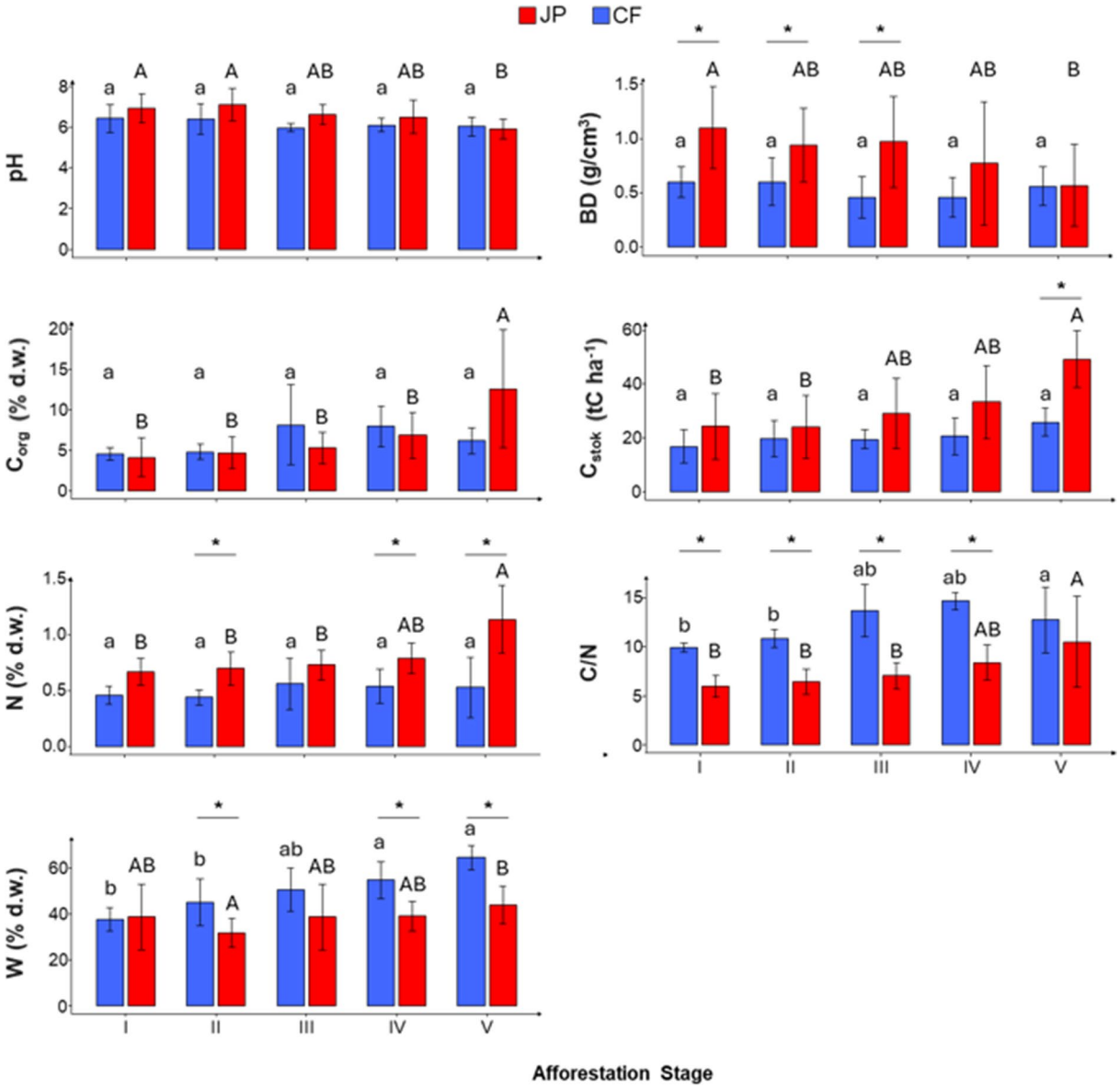

FIGURE 1 | Bar plot of basic soil properties at the two study areas along the afforestation dynamics. Data refer to mean ± standard error ($N = 4$) of soil pH, bulk density (BD), organic carbon (C_{org}%) and total nitrogen (N%), organic carbon stock (C_{stock}), carbon-to-nitrogen content (C/N) and water content (W) at 5 different afforestation stages (I-V) at Julian Prealps (JP, in red) and Casentinesi Forest (CF, in blue). Different letters within each study area indicate statistically significant stage-dependent differences for each soil property. Asterisks indicate significantly higher values at equal stage between the two study areas (Tukey post hoc test after two-ways ANOVA, $p < 0.05$). For detailed results, see Tables S1 and S2.

(Figure 4). *Planctomycetota* constituted around 10% at all stages in CF, slightly decreasing with the succession, while increasing from 5% to 10% at JP. *Firmicutes* were more abundant in CF at the early (II) stage (9%) but remained low (1%–2%) across the following ones in JP, hence showing a decreasing trend with the afforestation process (Figure 4). All other bacterial phyla contributed negligibly.

Fungal community in both study areas was dominated by *Ascomycota* and *Basidiomycota*. In CF, these phyla exceeded 90% of the relative abundance, whereas in JP, they ranged from 70% at the initial stage to 80% at the late one. *Mortierellomycota* were more abundant in JP, peaking at the II and III stages. *Chytridiomycota* also contributed significantly in JP, declining along the successional gradient. *Rozellomycota* showed a similar pattern in JP, peaking at II with a relative abundance of 15% and decreasing thereafter. *Mucoromycota* remained below 5% in late stage (V) in both areas. All other fungal phyla accounted for negligible abundance.

3.5 | β-Diversity of Microbial Communities Across Study Areas and Successional Stages

The β-diversity of soil biota revealed clear differences between bacterial and fungal communities in the two study areas, with greater compositional variability observed at JP compared to CF (Figure 5A,B).

PERMANOVA analysis (Table S4) confirmed a significant effect of study area on both bacterial ($F = 33.867$, $p < 0.0001$) and fungal communities ($F = 12.138$, $p < 0.0001$), suggesting the presence of

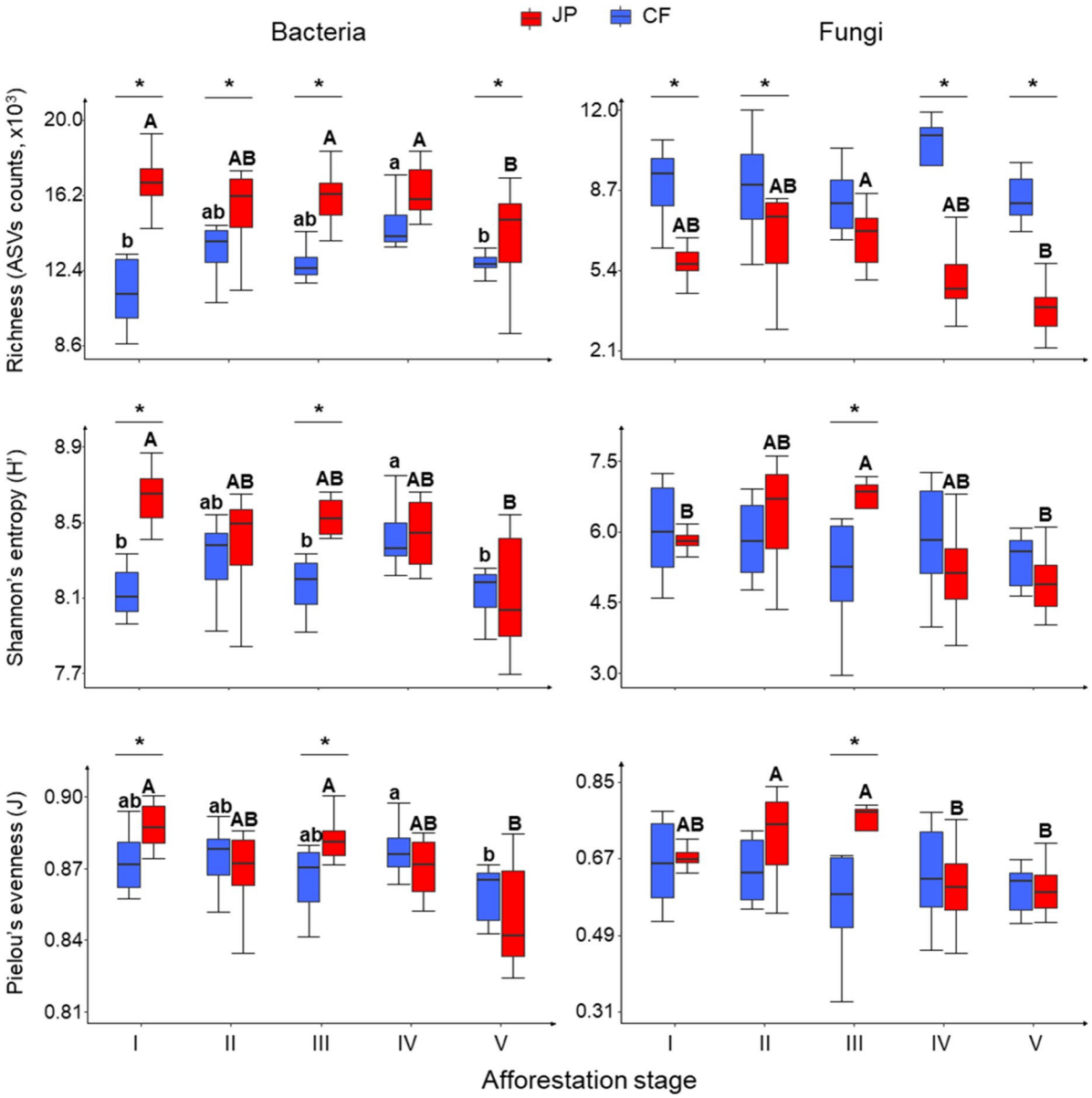

FIGURE 2 | Diversity metrics for bacterial (left) and fungal (right) communities at the Julian Prealps (JP in red) and Casentinesi Forest (CF in blue) across five successional stages (I–V). Data refer are presented as median, interquartile range (box) and minimum and maximum values (whiskers) for each series. Asterisks indicate statistically significant differences between JP and CF within the same stage. Different letters indicate stage-dependent significant pairwise differences within each study area (JP: Capital letters; CF: Small letters) ($p < 0.05$, Dunn's post hoc test with Benjamini-Hochberg correction for multiple following two-factor non-parametric Scheirer-Ray-Hare ANOVA).

a core microbial biota shaped by local environmental conditions at the regional scale.

When PERMANOVA was re-applied separately within each study area to test for successional effects, a statistically significant F value ($F = 3.214$, $p = 0.0193$) was detected only for fungal communities in CF. Bacterial communities in CF showed a borderline effect ($F = 2.092$, $p = 0.0942$), whereas in JP neither fungi nor bacteria exhibited significant variation along the forest successional gradient (Table S4). Pairwise comparisons highlighted a significant or borderline shift between initial communities and those of latest forest stages (IV and V) for fungi ($p = 0.0133$ and $p = 0.0714$, respectively), limited, for bacteria, to the I vs. IV comparison ($p = 0.0605$).

Consistent with these results, PCoA plots showed overlapping stage-related ellipses in JP, reflecting the lack of significant successional effects on community composition for both bacteria and fungi (Figure 5C,D). Differently, in CF a progressive community shift along the successional gradient was revealed, more pronounced for fungi (Figure 4E,F).

Linking β-diversity patterns to topsoil properties highlighted both shared and distinct drivers across areas and microbial groups (Table 1).

In JP, soil properties accounted for 98.9% and 93.6% of compositional variation in bacterial and fungal communities, respectively, with both groups influenced by total N, C:N and pH. Additionally,

bacterial composition was selectively shaped by C_{org}, while fungal composition was more affected by C_{stock} (Table 1). In CF, soil properties explained 50.3% and 94.7% of the total variation in bacterial and fungal composition, respectively, with bacteria responding primarily to pH and fungi to soil water content (W).

3.6 | Univariate Responses of Bacterial and Fungal Taxa to Soil Properties

The number of taxa significantly associated, either positively or negatively to soil properties was higher in JP than in CF, with 25 versus 11 significant associations for bacteria, and an even more pronounced difference for fungi, with 33 versus 6 associations, respectively (Figure 6).

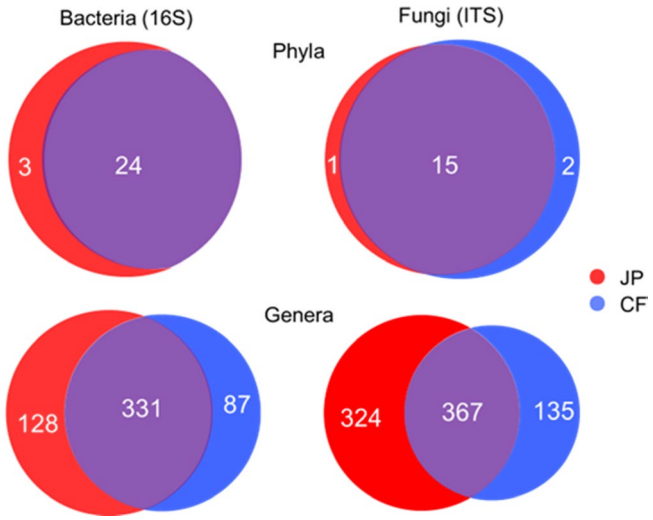

FIGURE 3 | Venn diagrams of bacterial and fungal communities at the phylum and genus levels, showing taxa shared between the study areas (violet) or unique to the Julian Prealps (JP, in red) and the Casentinesi Forest (CF, in blue).

For bacterial phyla, the majority of significant correlations in both areas involved soil pH. In CF, three positive and two negative correlations were observed, whereas in JP there were seven positive and three negative correlations. Other significant associations in CF included BD (2 positive and 1 negative), C_{stock} (1 positive and 1 negative) and W (1 positive). In JP, additional associations include one phylum positively correlated to BD, one and two negatively correlated with C_{org} and C_{stock}, respectively, five and four negatively correlated with total N and C:N and one positive correlation each with N and W (Figure 6).

For fungal phyla, in CF significant correlations were limited, with one positive association with BD, and four negative associations involving C_{org} (1), C_{stock} (1) and C:N (2), three of which were linked to *Rozellomycota*. In JP, most significant correlations involved negative associations between soil organic matter parameters (C_{org}, C_{stock}, total N and C:N) and eight fungal phyla, totalling 23 cases. *Basidiomycota* showed a contrasting pattern, displaying positive correlations with C_{org}, total N and C:N. The remaining three significant associations included two with BD (one positive and one negative) and one negative with W. Two of these associations involved *Mucoromycota*, highlighting a distinct response pattern.

4 | Discussion

4.1 | Responses of α-Diversity to Afforestation Dynamics and Soil Properties

Overall, our results revealed a qualitatively similar successional pattern of α-diversity across the two study areas, with a significant decline at older stages, although each area exhibited

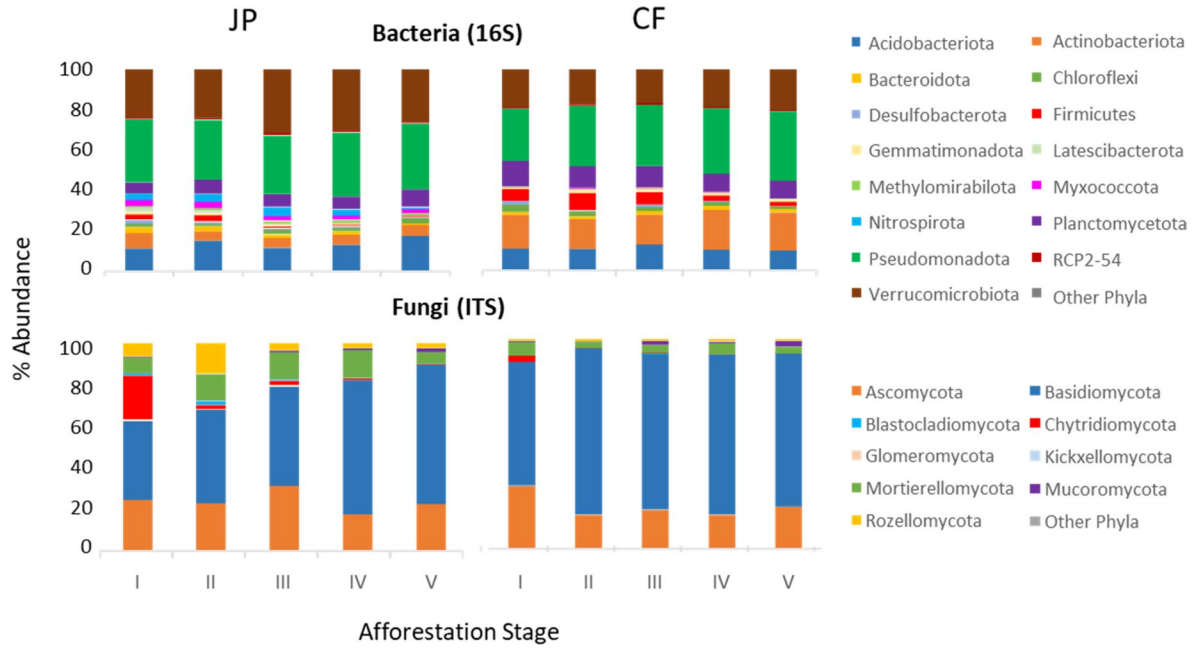

FIGURE 4 | Stacked bar plots showing the most abundant microbial phyla for bacteria (top) and fungi (bottom) at the two study areas, across the 5 stages of spontaneous afforestation (I–V).

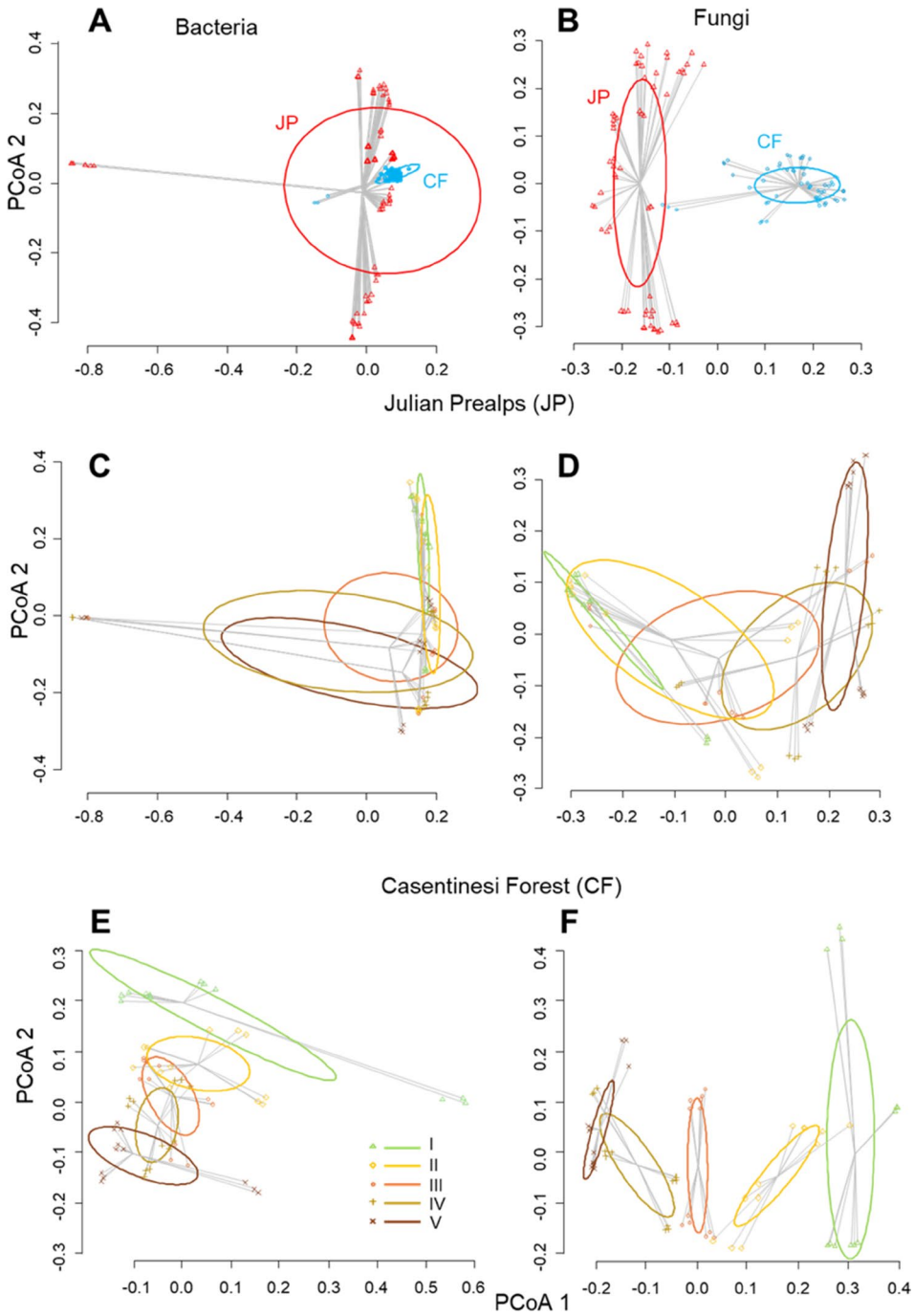

FIGURE 5 | Principal Coordinate Analysis (PCoA) of microbial community composition, shown separately for bacteria (A, C, E) and fungi (B, D, F), illustrating community variation according to study areas (A, B) and successional stages at Julian Prealps (JP) and Casentinesi Forest (CF) (C, D) and (E, F), respectively.

distinct trajectories. In JP, bacterial richness remained initially stable and then declined at the late successional stage, whereas in CF it initially increased, peaking at transitional stages. The opposite occurred for fungal richness. Additionally, lower evenness and equitability at later stages suggest a shift toward more specialized organisms adapted to a mature and stable ecosystem. This pattern likely reflects the development of more complex soil structure, higher nutrient availability, and a broader range of ecological microhabitats driven by plant selection (Lai et al. 2023).

The effect of successional stage on soil properties was mostly significant in JP, which also showed higher within-stage variability as compared to CF. These findings indicate a higher topsoil heterogeneity in JP, likely reflecting the wider variation above-ground, in tree basal area and species diversity as compared to CF. Such differences in soil properties can be linked to the distinct patterns of diversity observed for bacterial and fungal communities in the two areas. Accordingly, bacterial and fungal responses to spontaneous afforestation may result from fundamental differences in their metabolic

TABLE 1 | Mean values ± standard error of basic soil properties of the two study areas Julian Prealps (JP) and Casentinesi Forest (CF) at different successional stages (S) of afforestation (G: Meadow, E: Early, M-E: Mid early; M-L: Mid late and L: Late).

Area	S	pH	BD (g/cm^3)	C (% d.w.)	SOC (tC ha^{-1})	N (% d.w.)	C/N	W (% d.w.)
JP	G	6.92 ± 0.70^{a}	1.10 ± 0.38^{a}	4.14 ± 2.34^{b}	24.34 ± 12.25^{b}	0.67 ± 0.12^{b}	5.97 ± 1.11^{b}	38.83 ± 14.32
	E	7.12 ± 0.76^{ab}	0.94 ± 0.34^{ab}	4.69 ± 1.95^{b}	24.15 ± 11.64^{b}	0.70 ± 0.15^{b}	6.44 ± 1.31^{b}	31.98 ± 6.31
	M-E	6.62 ± 0.50^{ab}	0.97 ± 0.42^{ab}	5.28 ± 1.92^{b}	29.19 ± 13.01^{b}	0.73 ± 0.13^{b}	7.04 ± 1.36^{b}	38.71 ± 14.37
	M-L	6.50 ± 0.80^{ab}	0.77 ± 0.57^{ab}	6.83 ± 2.81^{b}	33.36 ± 13.50^{b}	0.79 ± 0.14^{ab}	8.40 ± 1.81^{ab}	39.18 ± 6.45
	L	5.91 ± 0.47^{b}	0.57 ± 0.38^{b}	12.57 ± 7.27^{a}	49.25 ± 10.47^{a}	1.14 ± 0.30^{a}	10.52 ± 4.66^{a}	44.00 ± 8.18
CF	G	6.39 ± 0.74	0.60 ± 0.14	4.52 ± 0.79^{a}	16.80 ± 6.27^{b}	0.46 ± 0.08	9.92 ± 0.48^{b}	37.74 ± 5.1^{b}
	E	5.63 ± 0.03	0.60 ± 0.22	4.80 ± 0.95^{a}	19.60 ± 6.71^{b}	0.44 ± 0.07	10.83 ± 0.91^{b}	45.24 ± 10.1^{b}
	M-E	5.96 ± 0.20	0.46 ± 0.19	8.12 ± 4.91^{b}	19.50 ± 3.62^{a}	0.56 ± 0.23	13.69 ± 2.68^{a}	50.51 ± 9.32^{ab}
	M-L	6.1 ± 0.32	0.46 ± 0.18	7.96 ± 2.50^{b}	20.66 ± 6.91^{a}	0.54 ± 0.15	14.66 ± 0.9^{a}	54.85 ± 7.96^{ab}
	L	6.02 ± 0.45	0.56 ± 0.18	6.16 ± 1.58^{ab}	25.86 ± 5.30^{a}	0.53 ± 0.27	12.74 ± 3.33^{a}	64.59 ± 5.31^{a}

Note: Letters indicate statistical significance (One-way ANOVA; Tukey post hoc $p < 0.05$).
Abbreviations: BD: bulk density; N: nitrogen; OC: organic carbon; SOC: soil organic carbon; W: actual water content.

strategies and resource preferences (Panico et al. 2025) based on the niche partitioning principles (Zhou et al. 2017). In other words, the highly diverse bacterial communities in JP at the initial stages would simply indicate the availability of a wider range of niches, as related to larger variability of soil properties at this stage, and vice-versa for poorer bacterial communities in CF linked to more homogeneous soil conditions. Correspondingly, at later successional stages, under more homogeneous soil conditions, bacterial richness in CF remained stable, whereas in JP it decreased along the afforestation gradient, possibly reflecting a reduction in available ecological niches as soil conditions converge toward more uniform forest states. This interpretation is consistent with ecological theory predicting reduced niche differentiation under increasing environmental filtering. In addition, shifts in community composition may be interpreted within the copiotroph–oligotroph (r/K) framework, whereby fast-growing, resource-opportunistic (r-strategist) bacteria tend to dominate early successional or nutrient-rich conditions, while slower-growing, resource-efficient (K-strategist) microorganisms become more prevalent in mature, resource-limited systems (Fierer et al. 2007; Ho et al. 2017; Hu et al. 2023). In this context, fungal communities, often better adapted to degrade complex organic matter and tolerate acidic conditions, may gain a competitive advantage at later stages.

Our results also provide additional insight into the ecological strategies of different bacterial and fungal taxonomic groups, at least at phylum level. In JP, where soil chemical and physical properties vary widely, several bacterial phyla (e.g., *Bdellovibrionota, Desulfobacterota, Fibrobacterota, Latescibacterota, Methylomirabilota, Myxococcota, Nitrospirota, Spirochaetota*) and most fungal phyla, with the exception of *Basidiomycota*, were negatively associated to SOM parameters, indicating that their trophic niches shrink along the successional gradient. *Basidiomycota*, in contrast, thrived at the latest stages, in correspondence of highest SOM content and quality (i.e., positively associated to C_{org}, N and C:N), likely competitively excluding other groups, but also of highest tree basal area and tree density, indicative of higher substrate availability for mycorrhizal symbioses (Tomao et al. 2020). However, *Basidiomycota* were not consistently associated to other soil properties increasing across afforestation stages, such as pH. As in previous studies (e.g., Panico et al. 2025) some subgroups of *Basidiomycota* were negatively associated to this soil property, showing acidophylic behaviour, our result indicate that such phylum cannot be considered as an *unicuum* by a functionally ecological point of view, as their response along the afforestation process is likely different for different subgroups according to distinct trophic requirements, primarily considering saprophytic or symbiotic strategies. Interestingly, major bacterial groups (e.g., *Pseudomanadota*, *Verrucomicrobiota*, *Acidobacteriota* and *Actinobacteriota*) did not show uniform responses to soil properties at the phylum level, likely because these phyla include subgroups with diverse trophic requirements and fundamental niches, resulting in heterogeneous responses to environmental gradients such as soil properties (Philippot et al. 2010).

4.2 | Interactions Between Soil Properties and Afforestation in Shaping β Diversity and Community Composition

β-diversity of soil microbiota differed between the two study areas, reflecting a combination of factors operating at multiple spatial scales. Our results confirm that microbial community structure is influenced by spatial variability in controlling soil properties, as previously reported in studies linking taxonomic composition to sampling location (O'Brien et al. 2016; Labouyrie et al. 2023). In particular, afforestation dynamics had a clear effect on community composition in CF, most pronounced for fungi and only partially for bacteria. Topsoil physico-chemical properties were more homogeneous in CF than in JP, which likely contributed to this pattern.

Variation partitioning analysis revealed that bacterial communities in CF responded primarily to the increasing C content

FIGURE 6 | Heat-map showing Spearman's correlation score (ρ) between bacterial (tiop) and fungal (bottom) phyla and soil physico-chemical parameters. Negative correlations are shown in red shades and positive correlations in green shades. Statistical significance is indicated as: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

and acidity along the successional gradient, whereas fungal assemblages were largely unaffected by soil chemistry and were instead influenced by W. As soil moisture can vary over very small spatial scales due to differences in micro-topography and vegetation cover, its role is most evident where variation of other soil chemical properties is low. In contrast, in JP, Permanova results did not highlight a significant impact of successional stage on microbial communities. In this study

area, it is reasonable that the large within-stage variation in most soil properties such as C_{org}, N, C/N and pH played the controlling role on diversity and composition of microbial communities, hence possibly masking that of the afforestation dynamics. These findings interestingly suggest that the influence of afforestation on soil microbiota can clearly emerge in homogeneous ecological conditions, as those we observed in the CF study site, whereas at JP, with greater compositional heterogeneity of aboveground parameters, such as tree species composition and density, reflected in soil properties variation among replicates and within each successional stage, are the main determinant of the microbial response.

This aligns with the well-established role of edaphic factors (soil pH, moisture, temperature, and nutrient availability) in shaping microbial diversity and function (Mishra et al. 2023). Accordingly, in areas with low spatial variability, soil properties act as the main environmental filter imposing similar selective pressures across the overall regional community. This is consistent with the general hypothesis that environmental heterogeneity shapes community composition by selectively filtering taxa from the regional pool, differently across local communities, resulting in high β-diversity (Zhang et al. 2020; Nemergut et al. 2013). Under such conditions, the effect of a single ecological process at the macroscale, like spontaneous afforestation, is unlikely to produce consistent community patterns across varying local contexts. This is particularly relevant for microbial assemblages, whose turnover occurs over different temporal scales than forest dynamics and is primarily driven by factors acting at limited spatial and within chemically and physically homogenous environments.

5 | Conclusions

Our findings suggested that spontaneous afforestation of abandoned grasslands is associated with changes in topsoil properties, particularly an increase in organic matter along the successional gradient. These trends were consistent across the two study areas, although other properties, such as soil pH and bulk density, showed site-specific variability, potentially reflecting differences in local environmental conditions and vegetation characteristics.

At the community level, bacterial and fungal α-diversity generally decreased at later successional stages, which may reflect reduced niche heterogeneity compared to transitional phases. However, response patterns differed between microbial groups and study areas, highlighting the influence of both ecological strategies and local environmental variability. Several bacterial and fungal taxa showed negative associations with soil organic matter, whereas Basidiomycota increased in relative abundance at later stages, consistent with their known role in organic matter turnover.

β-diversity patterns further emphasised the role of environmental context as soil microbiota collected at Julian Prealps showed higher compositional heterogeneity correlated with high variability in soil properties, potentially masking the successional effects. By contrast, clearer successional shifts in microbial community composition were observed in the more homogeneous Casentinesi Forests, particularly for fungi.

Future studies integrating functional, temporal and above–belowground interactions are needed to further clarify the role of microbiota in the spontaneous afforestation process after land abandonment.

Author Contributions

S. C. Panico: conceptualization, investigation, writing – original draft, methodology, validation, visualization, writing – review and editing, software, formal analysis, data curation, project administration. **G. L. Sciabbarrasi:** validation, software, formal analysis, data curation, methodology. **A. Foscari:** methodology, validation. **A. Tomao:** investigation, writing – original draft, writing – review and editing, methodology, validation, data curation. **L. Orzan:** methodology, validation, investigation. **G. Alberti:** investigation, funding acquisition, writing – original draft, writing – review and editing, supervision, data curation, validation, methodology, project administration, resources. **G. Incerti:** conceptualization, investigation, writing – original draft, visualization, validation, writing – review and editing, project administration, data curation, supervision, methodology, formal analysis.

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Map of the two study areas with illustrative pictures of afforestation stages representing one of the four chronosequences sampled for the Casentinesi Forest (CF, blue frame) and Julian Prealps (JP, red frame). The four chronosequences (yellow frame) for each area are indicated by the yellow frame and each afforestation stage by different colour (I—pink, II—violet, III—light blue, IV—orange, V—brown). **Figure S2:** Mean basal area ($m^2 ha^{-1}$) and tree density ($N ha^{-1}$) at the five successional stages for the Casentinesi Forest (CF, left) and Julian Prealps (JP, right). **Table S1:** Results of Tukey's HSD post hoc tests showing the effect of successional stage (I–V) on soil physicochemical properties within each study area. Data are presented to *p* values for each pairwise comparison with significant values highlighted in bold. **Table S2:** Results of Tukey's HSD post hoc tests for significant differences between the two study areas at each successional stage (I–V) on soil physicochemical properties. Data are presented as *p* values for each comparison with Significant values highlighted in bold. **Table S3:** Counts of ASVs, unique taxa and sequences in datasets. Data represent average over three replicates for each sample, identified by study area (SA), chronosequence (CHR) and successional stage (ST), for both bacterial and fungal datasets, before (Pre) and after (Post) the filtering procedure. Filtering involved removing unique taxa with an abundance below 0.01% in all samples and merging ASVs with the same species-level taxonomic identification. The percentage of retained abundance (i.e., retained sequences) after filtering is also reported. **Table S4:** Results of PERMANOVA analyses and subsequent pair-wise post hoc comparisons of microbial community across different successional stages at the two study areas. Data are presented as *F*-values and associated *p* values for the Iain effects of study area and stage (estimated separately for each study area), along with bootstrap tests (999 permutations) for each pairwise stage comparison. Significant *p* values are highlighted in bold.