Specific Microbiome Signatures under the Canopy of Mediterranean Shrubs

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Abstract

Background: Shrub encroachment (SE) is a phenomenon in which grasses and herbaceous vegetation are replaced by woody shrubs. The progressive spread of shrubs represents a form of land cover change that is widespread in arid and semi-arid grassland ecosystems. Many previous studies have highlighted the effects of SE on soil respiration rates and nutrient storage, but little is known about its belowground effects. While previous work considered shrubs to be non-species specific or as a single intervening species, we selected an *Ampelodemos mauritanicus* grassland and six coexisting shrubs (i.e. *Pistacia lentiscus* L., *Juniperus phoenicea* L., *Myrtus communis* L., *Rosmarinus officinalis* L., *Olea europaea* L., and *Euphorbia dendroides* L.) to investigate the effects of their encroachment on soil microbiota. We used high-throughput sequencing, coupled with soil chemical analyses and litter using 13C CPMAS NMR spectroscopy.

Results: Results showed a strong influence of shrub canopy on bacterial and fungal community diversity, species richness and overall community composition in the soil. Litter chemistry was dominated by O-alkyl-C, with the highest content in *Ampelodesmos* and *Euphorbia*, but richer of aromatic C in *Pistacia* and *Rosmarinus*. Bacterial diversity was highest under *Juniperus* and *Euphorbia*, while lowest under *Rosmarinus* and grassland. Conversely, fungal diversity was highest under *Olea* and *Euphorbia*, while lowest under *Myrtus* and grassland. Moreover, soil C and N contents were highest under *Olea*, *Pistacia* and *Myrtus* compared to the other canopies. In addition, grassland and *Rosmarinus* had the highest Fe content. Furthermore, increased co-occurrence network size and connectivity were recorded under shrubs compared to the grassland matrix.

Conclusions: Our results suggest that the individual effect of each shrub on the grassland matrix depends mainly on the chemical properties of the shrub litter, which alters the chemical profile of the soil and, in cascade, shapes the associated microbiota.

Introduction

Grassland ecosystems occupy approximately 41% of the Earth's land surface (Reynolds et al., 2007), and play an important role in global biogeochemical, hydrological, and energy cycles (Huang et al., 2018). However, in 10–20% of these ecosystems, shrub encroachment (SE) is occurring (Reynolds et al., 2007). SE is a phenomenon in which grasses and herbaceous vegetation are replaced by woody shrubs (Sankaran et al., 2004). SE is a form of land cover change that is widespread in arid and semi-arid grassland ecosystems (Eldridge et al., 2011). This phenomenon has been shown, thus, to significantly alter the landscape, microclimate, and above- and belowground biological processes (Dong et al., 2014). Several studies have suggested many possible causes for SE (Ratajczak et al., 2012), including the increase in atmospheric CO₂ (Wigley et al., 2010), climate change (D’Odorico et al., 2010), nitrogen deposition (Kochy & Wilson, 2001), changes in fire regime (Van Auken, 2000), and overgrazing (Briggs et al., 2005).

The global encroachment at the expense of grasses is predicted to increase in the next years, as woody plants have multiplied, in many parts of the world, over the past 100 years (Kulmatiski & Beard, 2013). Woody plant encroachment is often a conservation concern (Van Auken, 2000) because it alters open canopy ecosystem functions and processes (Eldridge et al., 2011), such as total primary productivity, decomposition rates, nutrient availability, and soil carbon dynamics (Guido et al., 2017). The resulting resource distribution, also called “islands of fertility” (Schlesinger et al., 1990), could favour the growth of other woody species because of the shrub nurse effect (Callaway, 2007), thus creating a positive feedback that could lead to an irreversible woody encroachment process (Du et al., 2016). The formation of fertility islands under woody plant canopies involves several ecological processes. First, the extensive root system of woody plants is able to extract nutrients from the depth of the subsoil and the interspaces that are deposited under the canopy trough litterfall (Gherardi et al., 2013). The formation of island of fertility is especially evident under nitrogen-fixing woody species that accumulate large amount of N and P in soil through litterfall (Facelli & Brock, 2000). The formation of island of fertility is dependent on litterfall amount, litter chemical traits, litter decay rate and associated accumulation of soil.
organic carbon. For example, Stinca et al., (2015) reported that colonisation of the nitrogen fixing *Genista aethensis* over bare volcanic soil resulted in a sharp increase in C, N and P stocks in the topsoil in few decades. On the other hand, invasion of *Juniperus virginiana* into lower-lying grasslands in the western USA has been shown to alter the amount and distribution of C and N stocks in soil and plants (McKinley & Blair, 2008). As a result, the soil under the canopy of woody plants become the preferred site for plants, animals and microorganisms, whose metabolism increases soil C and N and further enriches soils in the understory (Dean et al., 1999). In addition, shrub canopy reduce wind speed, allowing atmospheric dust, wind- and water-transported nutrients, detritus, and seeds to accumulate beneath woody canopies (Eldridge et al., 2011), resulting in further enhancing fertile island effect (Reynolds et al., 1999).

Understanding the consequences of SE is important (Ratajczak et al., 2012). However, most studies on the effects of SE have focused mainly on vegetation (Hu et al., 2015), soil chemical cycling (Eldridge et al., 2015), and microbial biomss and enzymatic activities (Eldridge et al., 2015). Instead, little attention has been paid to the effects on soil microbiota composition and diversity (Yannarell et al., 2014). Plants are known to influence soil microbial communities both through the quantity and quality of their aboveground litter and through root exudates released into the soil that feed heterotrophic soil microorganisms, leading to overall shifts in the community composition of soil (Wallenstein et al., 2007). In this context, SE has been shown to significantly increase bacterial biomass (Yannarell et al., 2014) and alter fungal community composition (Bragazza et al., 2015). Therefore, dynamic and complex feedback mechanisms exist between aboveground vegetation and the belowground microbial community (Xiang et al., 2014). Moreover, microbial species are actors in the plant-soil feedback that can alter the outcome of plant competition and drive the process of plant community succession (Bever, 2003; Idbella et al., 2021). For this reason, SE may have lasting consequences for grassland ecosystem restoration and management, as shifts in microbiota composition may facilitate long-term succession from grassland to shrub/forest ecosystems (Yannarell et al., 2014).

As for the study of the influence of SE on the composition of the soil microbial community, very little evidence was found in the literature, since they all consider shrubs as non-species specific or as a single encroached species. For example, Xiang et al., (2019) showed that the encroachment of *Caragana microphylla* into a grassland dominated by *Cleistogenes songorica* induced significant changes in soil bacterial community composition. Similarly, Ding et al., (2020) showed that the encroachment by *Vaccinium fragile* into a grassland dominated by *Eulalia pallens* significantly restructured the diversity and composition of soil bacterial and fungal communities. On the other hand, Yannarell et al., (2014) showed that the encroachment by four different species on the grassland Remnant Hill Prairies significantly altered both bacterial and fungal communities without selecting species-specific signatures under the canopy of each woody plant. However, most of the previous studies compared only a single woody species with the surrounding open vegetation. Here, our study aimed to investigate the influence of six coexisting Mediterranean shrubs (i.e. *Pistacia lentiscus*, *Juniperus phoenicea*, *Myrtus communis*, *Rosmarinus officinalis*, *Olea europaea*, and *Euphorbia dendroides*), over *Ampelodomos mauritanicus* grassland, on the soil microbiota under their canopies. Specifically, we combined soil and litter chemistry analyses with next-generation sequencing techniques to determine how the soil microbiota is shaped by the canopy of Mediterranean shrubs. Our hypothesis states that litter chemistry and canopy structure are evidently different between shrubs, resulting in a specific microbial fingerprint. This specific effect is thought to be derived from the specific chemical characteristics of the litter of the shrubs as it falls and decomposes (De Marco et al., 2011), leading to different changes in soil chemistry and also microbial composition. The aim of this study was therefore to provide basic information and novel insights into the environmental selection of soil microbial communities by each of the most abundant species-specific shrubs in a Mediterranean SE ecosystem. Specific aims were:

1. to assess the “island of fertility” effect under the canopy of different Mediterranean shrubs;
2. to describe the bacterial and fungal microbiota associated with the different shrub species;
3. to explore the link between soil chemistry and bacterial and fungal microbiota.
Material And Methods

Study site description

The study was conducted in Cape Palinuro shrubland site (40°01’35 ”N 15°16’30 ”E), located in southwestern Italy, about 40 miles southwest of the city of Salerno (Fig. 1). This area is located in a hot Mediterranean/dry summer subtropical climate zone characterised by mild with moderate seasonality. Summers are dry and hot due to the dominance of high-pressure subtropical systems, while winters have moderate temperatures and changeable, rainy weather due to the polar front. This climate is typical of the western sides of continents between 30° and 45° latitudes. Vegetation is adapted to dry summers and it is fragrant and oily, making it susceptible to fire (http://www.cape-palinuro.climatemps.com). The site is characterized geomorphologically with limestone rocks overlying clay soils with abundant rock outcrops. The elevation of the study area is 185 m a.s.l., and the average annual temperature is 16.7°C. In winter, temperatures average is 13.3°C during the day and drop to 7.9°C overnight; in spring, temperatures reach 17.6°C, mostly in the afternoon, while during the night-time it drops to 11.2°C; in summer, average maximum temperature is 27.1°C and average minimum is 19.7°C. The average annual precipitation is 789.8 mm.

Soil sampling

Within the study area, six shrubs were selected for soil sampling, including five evergreens: *Pistacia lentiscus* L., *Juniperus phoenicea* L., *Myrtus communis* L., *Rosmarinus officinalis* L. and *Olea europaea* L., and one deciduous: *Euphorbia dendroides* L. (Fig. 1). Soil samples were also collected in the grassland soil dominated by *Ampelodesmos mauritanicus* L., a perennial, fire-prone tall grass that dominate in the matrix between shrubs (Incerti et al., 2013). For each shrub, three replicates were randomly selected for soil sample collection. In April 2019, 21 samples were collected independently: 7 samples (6 samples under the canopy of each shrub + one sample for the grassland) with 3 replicates. Shrub's size was 135 ± 22 cm for *Euphorbia*, 163 ± 74 cm for *Olea*, 173 ± 59 cm for *Pistachia*, 126 ± 9 cm for *Juniperus*, 164 ± 8 cm for *Rosmarinus*, and 117 ± 11 cm for *Myrtus*. Soil samples were collected by a 5 cm diameter soil corer, at a depth of 10 cm after removal of above-ground litter, in four randomly selected points under each canopy. Subsequently, soil was pooled and sieved (2 mm mesh) on site resulting in a single composite sample for each canopy replicate. The samples were stored in sterile plastic bags and labelled. Before every sampling operation, the soil corer was thoroughly cleaned and sterilised to avoid between samples contamination. After collection, samples were then divided into two fractions: one fraction was kept at 4°C to investigate soil chemical properties; the other fraction was stored at − 20°C and used for molecular analysis.

Litter collection and chemical analysis

Under each canopy, leaf litter was collected with net traps during the period of maximum leaf fall from three randomly selected individuals of each shrub species. Freshly collected litters were dried in a ventilated chamber at 30°C until they reached a constant weight and then stored at room temperature. Litters were then characterized for total C and N content by flash combustion of microsamples (5 mg of litter) using a CN soil elemental analyser (Flash EA2000 Thermo). Proximate cellulose and lignin content were quantified as acid-hydrolysable fraction and acid-unhydrolyzable materials, respectively (Gessner, 2005). In addition, leaf materials were characterized by $^{13}$C cross-polarization magic angle spinning (CPMAS) nuclear magnetic resonance (NMR) (Kögel-Knabner, 2002) obtained in the solid-state and under the same conditions, which allowed a comparative analysis of the resulting spectra. The spectrometer used was a Bruker AV-300 equipped with a 4 mm wide-bore MAS probe (Bonanomi et al., 2013). The spectral ranges and corresponding C types were identified as described by Bonanomi et al., (2013): 0–45 ppm = alkyl-C (characteristic of lipid waxes, cutins and microbial products); 46–60 ppm = methoxyl- and N-alkyl-C (characteristic of amino acids and lignin components); 61–90 ppm = O-alkyl-C (characteristic of carbohydrates and polysaccharides); 91–110 ppm = di-O-alkyl-C (anomeric C1 of celluloses, tannin and lignin components); 111–140 ppm = H- and C-substituted aromatic C (mainly associated with polyphenols,
lignin and tannin components); 141–160 ppm O-substituted aromatic C (phenolic and O-aryl-C, characteristic of phenols, lignin and tannin components); 161–190 ppm carboxyl-C (characteristic of organic acids, amides, esters).

**Soil chemical analyses**

Soil samples were dried in a ventilated chamber at room temperature until a constant weight was reached. The soil was analyzed for 16 parameters that included the most important factors for root development, i.e., total organic carbon, pH, total nitrogen, and macro- and micronutrients important for plant growth. Specifically, the following parameters were measured: soil electrical conductivity (EC) and pH, were determined in 1:5 and 1:2.5 soil-water suspensions, using a conductivity meter and a pH meter, respectively (Czekala et al., 2016). Total nitrogen was determined by the Kjeldhal method (Czekala et al., 2016), while phosphorus was assessed by the molybdovanadate phosphate method (AOAC, 1990). Water content and organic matter content were determined by weight loss at 105°C for 24 h and 550°C for 8 h, respectively (Silva et al., 2014). Potassium, magnesium, iron, manganese, calcium, sodium, copper and zinc were determined by flame atomic absorption spectroscopy (Peters et al., 2003). Total limestone is determined by the weight method against a strong acid, the attack of the limestone leads to a gas release of CO₂, the volume of which is measured (LANO: NF ISO 10693). Finally, the chloride content (Cl) in the soil was determined by the volumetric method described by Meldrum and Forbes (1928).

**Soil DNA extraction and amplification**

The microbiome of homogenized soil samples under each canopy was analyzed by Illumina high-throughput sequencing. The DNeasy PowerSoil kit (Qiagen) was used to extract the microbial DNA from 2 g of each homogenized soil. Bacterial and fungal diversity were assessed by high-throughput sequencing of the amplified V3-V4 regions of the 16S rRNA gene (~ 460 bp) and ITS1-2 (~ 300 bp). PCR was carried out with primers S-D-Bact-0341-b-S-17/S-D-Bact0785-a-A-21 (Berni Canani et al., 2017) and BITS1fw/ B58S3- ITS2rev (Bokulich & Mills, 2013) using conditions reported in the original studies. For bacterial primers S-D-Bact-0341-b-S-17 (5’-CTTACG GGGCGGCGAGC-3’) and S-D-Bact-0785-a-A-21 (5’-GAC TACGHGTTATCTAATCC-3’), PCR conditions were: 25 cycles of 95°C for 3 min, 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, 72°C for 5 min and held at 4°C. For fungal primers BITS1fw (5’-ACTTGGGAGGATCA-3’) and B58S3-ITS2rev (5’-GAGATCCRTTGYTRAAAGTT-3’) PCR conditions were: 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and a final extension of 72°C for 5 min. PCR products were purified with the Agencourt AMPure beads (Beckman Coulter, Milan, IT) and quantified using an AF2200 Plate Reader (Eppendorf, Milan, IT). Equimolar pools were obtained and sequencing was carried out on an Illumina MiSeq platform, yielding to 2× 250 bp, paired-end reads.

**Sequence data analysis**

Demultiplexed fastq files were processed using the DADA2 package (version 1.16.0 pipeline) (Callahan et al., 2016) in R software (4.0.4) (Team, 2016). DADA2 provides better taxonomic resolution than other methods because it retains unique sequences and calculates sequencing error rates rather than clustering to 97% similarity (Hugerth & Andersson, 2017). The resulting taxonomic units are referred to as amplicon sequence variants (ASVs) rather than operational taxonomic units (OTUs). For bacterial sequences, forward and reverse reads were trimmed to 250 bp, primer sequences were removed from all reads, and filter parameters used were the following: maxN = 0, maxEE for both reads = 2, truncQ = 2 (MaxEE corresponds to the maximum expected errors, TruncQ represents the parameter that truncates reads on the first occurrence of a quality score less than or equal to two, and MaxN is the maximum number of ‘N’ bases accepted). Error rates were estimated by learnErrors using nearly 4 million reads. Sequences were dereplicated using derepFastq with default parameters and exact sequence variants were resolved using the dada algorithm. The RemoveBimeraDenovo function was then used to remove chimeric sequences. For fungal sequences, the pipeline was pre-empted by a preliminary step of trimming adapter sequences and low-quality ends (< Q20) using Cutadapt software (Martin, 2011). For both the bacterial and fungal datasets, reads with more than three errors in the forward reads and five errors in the reverse reads were removed. Taxonomy was then assigned using assignTaxonomy based on the SILVA (v132) and UNITE (v7) databases for
bacterial and fungal communities, respectively (Quast et al., 2013; Nilsson et al., 2019). Chloroplast and Streptophyta contaminants and singleton ASVs were removed, and relative abundances of the other taxa were recalculated.

**Statistical analysis and data visualization**

Plotting was performed using PRIMER 7 software (Primer-E Ltd, Plymouth; UK). Alpha diversity metrics were calculated. Heatmaps were created to assess variation in community composition at lowest taxonomic levels. Heatplots were used clustering variables according to the results of an index of association similarity matrix and samples according to Bray-Curtis dissimilarity. The 50 most abundant taxa in the fungal and bacterial communities are shown in the heatplots. A resemblance matrix calculated based on Bray-Curtis dissimilarity was used to perform non-metric multidimensional scaling (nMDS) to assess variation in species composition under the different investigated canopies for the bacterial and fungal communities. The significance of changes in composition of the two communities analysed were tested by PERMANOVA (999 permutations), using the shrub species as fixed factor. The significance of variation in the alpha diversity metrics of the two communities was assessed along with the soil and litter chemical characteristics using the ANOVA test, and the means were pairwise separated using the post-hoc Tukey test. The level of significant differences was assessed at P < 0.05. All statistical analyses were performed using STATISTICA 13.3 software.

Furthermore, we analysed functional group variation for the fungal community, identifying putative fungal functional groups as well as their trophic modes using FUNGuild (Nguyen et al., 2016). The core microbiome was identified by constructing Venn diagrams for 7 sets (i.e., 6 canopies + the grass) for bacterial and fungal community using R software and the VennDiagram package (Chen & Boutros, 2011). Co-occurrence networks incorporating communities containing bacteria and fungi were based on single ASVs and generated to assess co-occurrence or potential interactions between species. To reveal microbiome complexity and potential interrelationships among microbial community members, network analyses were carried out for communities at the seven different sampled canopies. Recent studies have shown that network inference techniques are useful for deciphering microbial relationships from co-occurrence patterns (Barberán et al., 2012). For the purpose of focusing on the most commonly occurring ASVs and decreasing the effects of rare ones, only the most abundant 50 ASVs were analysed for each bacteria and fungi. The pairwise correlations between the ASVs were calculated using the Spearman correlation in R (version 3.3.2 and Hmisc package 4.0–1). Based on the statistical analysis, only strong and significant (Spearman's r > 0.6 or r < −0.6 and p < 0.05) correlations were considered. The network visualization was made using Gephi (version 0.9.2, Bastian & Jacomy, 2009). Each edge represents a robust and significant correlation and each node represents an ASV. A set of integrative metrics were calculated and compared to describe the network topology. For example, the average number of neighbours explains the complex pairwise connections and the average path length describes node distribution. Number of modules, defined as clusters of nodes that form coherent structural subsystems of interacting units, and modularity were measured according to the Louvain algorithm within Gephi.

**Results**

**Litter and soil chemistry**

Leaf litter chemistry varied largely among shrub species and the grass *Ampelodesmos* (Table S4 & Table 1). *Ampelodesmos* had the highest cellulose content and C/N and lignin/N ratios, while it had the lowest N content and relatively low lignin content. On the other hand, the highest lignin content was found in *Rosmarinus* litter, which also had high N content and the lowest cellulose content. *Pistacia* had relatively high contents of all proximate parameters except N content, which was relatively low. *Juniperus* had very low N content, and thus the highest C/N and lignin/N ratios. *Euphorbia* leaf litter, on the other hand, had the highest N content and the lowest C/N and lignin/N ratios. Finally, *Myrtus* leaf litter recorded the lowest lignin content among all other canopies and thus a very low lignin/N ratio.
Table 1
Chemical traits of the studies shrubs and the dominant perennial plant in the grassland (*Ampelodesmos mauritanicus*).

| Elemental and proximate parameters | Euphorbia | Juniperus | Myrtus | Olea | Pistacia | Rosmarinus | Ampelodesmos |
|-----------------------------------|-----------|-----------|--------|------|----------|------------|--------------|
| Cellulose (%)                     | 14.2b     | 13.0c     | 15.8bc | 10.6d | 17.9b    | 8.6d       | 27.3a        |
| Lignin (%)                        | 13.2d     | 23.8b     | 10.6e  | 16.3c | 23.9b    | 41.4a      | 18.1c        |
| N (%)                             | 2.1a      | 0.7d      | 1.2c   | 1.7b  | 1.1c     | 1.9a       | 0.5d         |
| C / N                             | 20.5d     | 69.3a     | 41.1b  | 31.2c | 45.4b    | 22.8d      | 77.6a        |
| Lignin / N                        | 6.3d      | 33.0a     | 8.7d   | 16.3c | 21.7b    | 21.6b      | 34.5a        |
| **13C-CPMAS NMR-derived parameters** |          |           |        |      |          |            |              |
| Carboxylic C – 161–190 ppm        | 9.8a      | 4.4c      | 6.2b   | 5.6c  | 5.3c     | 6.9b       | 4.8c         |
| O-substituted aromatic C – 141–160 ppm | 4.4d  | 4.8cd     | 5.7c   | 3.6e  | 9.3a     | 7.4b       | 4.0d         |
| di-O-alkyl C – 91–110 ppm         | 10.8bc    | 9.7cd     | 11.4b  | 8.9d  | 12.6b    | 11.1a      | 14.9a        |
| O-alkyl C – 61–90 ppm             | 44.8b     | 38.4c     | 42.4b  | 39.1c | 34.7c    | 37.5c      | 55.7a        |
| Methoxyl C – 46–60 ppm            | 8.1a      | 6.2b      | 6.2b   | 8.3a  | 4.5c     | 5.6bc      | 4.6c         |
| Alkyl C – 0–45 ppm                | 17.4d     | 27.5a     | 19.2c  | 24.9b | 21.5bc   | 19.9c      | 6.7e         |

Values are mean of five replicates. Different letters within each column indicate significant difference (Duncan test, P < 0.05).

The 13C-CPMAS NMR data showed that *Euphorbia* leaf litter had the highest content of carboxylic C, followed by *Rosmarinus* and *Myrtus*, while the others, including *Ampelodesmos*, had significantly low values. The O-substituted aromatic C fraction was highest in *Pistacia* and lowest in *Ampelodesmos* and *Euphorbia*. Moreover, *Pistacia* and *Rosmarinus* showed the highest H-C substituted aromatic C content while the lowest was found in *Euphorbia*. However, *Ampelodesmos* and *Rosmarinus* had the highest di-O-alkyl C and O-alkyl C fractions, while the lowest methoxyl C and alkyl C fractions were recorded for *Ampelodesmos* alone. In addition, *Euphorbia* and *Olea* had the highest methoxyl C fraction, and *Juniperus* was the one that contained the highest alkyl C fraction content instead.

Soil chemical parameters showed significant variation among canopies (Table S5 & Table 2). Clustering of the canopies, by D1 Euclidean distance, based on chemical parameters resulted in 5 clusters for the six studied shrubs and the grass, *Ampelodesmos*, separating *Euphorbia, Myrtus, Juniperus, Grassland-Rosmarinus* and *Pistacia-Olea* (Fig. S2). In details, *Euphorbia* and *Myrtus* showed the highest soil pH compared to grassland and other canopies. In addition, *Euphorbia* soil proved to enclose the highest total limestone by a maximum of 4.11% and soil potassium by a maximum of 1.27 g/kg. Electrical conductivity, chlorides and sodium were higher under *Olea* and *Myrtus*, while significantly lower values were
measured under the rest of the canopies, with an exception for sodium under Juniperus. The organic C and total N contents were higher under Myrtus, Pistacia and Olea than under the rest of the canopies. In addition, the highest phosphorus (P) and Mg contents were found in the soils under Euphorbia and Myrtus canopies. The distribution of Ca, Cu, Zn and Mn was different among the canopies. Fe content was found to be significantly higher only in the grassland and under Rosmarinus compared to other canopies, while the lowest value was found under Euphorbia canopy.

Table 2
Chemical analysis of the soils collected under the canopy of plant species in the studied shrubland as well as the grassland.

| Parameters             | Grassland | Juniperus | Rosmarinus | Pistacia | Olea  | Myrtus | Euphorbia |
|------------------------|-----------|-----------|------------|----------|-------|--------|-----------|
| pH                     | 5.62c     | 6.00c     | 5.82c      | 6.32b    | 6.34b | 6.74a  | 6.93a     |
| Water content (%)      | 5.82c     | 11.19b    | 6.31c      | 9.13b    | 9.42b | 10.46b | 16.99a    |
| Total limestone (%)    | 0.87b     | 1.75b     | 1.39b      | 0.82b    | 0.67b | 1.11b  | 4.11a     |
| Electrical conductivity (mS/cm) | 0.31b   | 0.26b     | 0.21b      | 0.34b    | 0.42a | 0.47a  | 0.31b     |
| Chlorides Cl (g/Kg)    | 0.21a     | 0.15bc    | 0.13bc     | 0.18b    | 0.278a| 0.30a  | 0.087c    |
| Sodium Na₂O (g/Kg)     | 0.25b     | 0.40a     | 0.19c      | 0.31b    | 0.43a | 0.43a  | 0.26c     |
| Organic Carbon (%)     | 12.48b    | 11.90b    | 11.53b     | 16.6a    | 16.27a| 16.17a | 9.80b     |
| Total Nitrogen (%)     | 0.50b     | 0.47b     | 0.42b      | 0.77a    | 0.85a | 0.99a  | 0.70a     |
| P (mg/Kg)              | 20.47b    | 21.73b    | 12.55c     | 20.6b    | 20.88b| 35.20a | 39.89a    |
| K (g/kg)               | 0.35c     | 0.65b     | 0.44bc     | 0.51b    | 0.53b | 0.55b  | 1.27a     |
| Mg (g/kg)              | 0.57b     | 0.91a     | 0.46b      | 0.89ab   | 0.97a | 1.20a  | 1.32a     |
| Ca (g/kg)              | 3.76b     | 3.91b     | 2.63b      | 9.01a    | 8.65a | 10.92a | 8.40a     |
| Cu (mg/Kg)             | 1.37b     | 1.62b     | 1.43b      | 2.04a    | 2.52a | 1.87a  | 2.05a     |
| Zn (mg/Kg)             | 8.08c     | 3.96d     | 5.53d      | 17.95b   | 18.68ab| 22.59a | 4.69d     |
| Mn (mg/Kg)             | 26.66b    | 30.84ab   | 25.48b     | 43.98a   | 39.82a| 27.77b | 13.29c    |
| Fe (mg/Kg)             | 56.78a    | 29.80b    | 44.57a     | 23.25b   | 30.00b| 23.23b | 5.21c     |

Different letters within each parameter indicate significant differences (Duncan test, $P < 0.05$).

Microbial diversity

Significant variation in the two indices (Shannon diversity and species richness) was found for bacterial and fungal diversity. Compared with the grassland, bacterial species richness was significantly higher in the soil under the canopy of Euphorbia and significantly lower under Rosmarinus, while slight, although not significant, variations were observed for the other canopies (Fig. 2a). The Shannon index of bacteria was significantly higher in the soil under the canopy of Juniperus and Euphorbia compared to the canopy of Rosmarinus and the grassland. On the other hand, no significant variation was found in fungal species richness, while the Olea showed the highest Shannon diversity index of the soil fungi, which showed a statistical significance compared to Myrtus and grassland soils (Fig. 2b).

Bacterial community composition
At the phylum level, considerable significant variation was found among shrubs in the bacterial community (Fig. 3a, Table S1). All shrubs harboured mainly Actinobacteria under their canopies, ranging from 30.1% in Rosmarinus to 40.3% in Olea, except for Pistacia, which harboured equally Actinobacteria and Proteobacteria, with a proportion of 25.4% each. On the other hand, the lowest abundance of Proteobacteria was found in the grassland soil at 15.7% compared to 20.9% – 25.4% among the other canopies. Acidobacteria, on the other hand, was most abundant in the grassland soil at 18.5% followed by Pistacia at 15.8% and less than 10% among the other canopies. In addition, the highest levels of Verrucomicrobia were found in Myrtus canopy (13.2%), followed by Pistacia (8.7%) and the remaining canopies with less than 5%. On the contrary, Planctomycetes was higher in Rosmarinus (20.2%) compared to the other canopies (<13%).

Moreover, the Venn diagram (Fig. 4a) confirmed that the canopies of Euphorbia, Juniperus, and Pistacia are the ones that include the highest number of unique ASVs, where they attained 47, 34, and 31 ASVs, respectively. While the lowest number of unique ASVs was found under the canopy of Rosmarinus and Olea. However, 219 ASVs were determined as the core bacterial microbiota of the studied microbiomes.

At the lowest taxonomic level (Fig. 5), the clustering of the canopies, based on the 50 most frequent ASVs, is highly distinct using the Bray-Curtis similarity index across different shrubs of the grassland (P = 0.001; Table S2), where it revealed five different clusters for the six canopies and the grass. Rosmarinus soil is clearly separated from the other samples by a difference of 40% (cluster A). Cluster B includes Pistacia soil, separated from the rest by a difference of 35%, while grassland soil is separated from the rest by a difference of 25% (Cluster C). All the other canopies have more than 80% similarities. The main driving ASVs of the clustering were 1) Acidobacteria subgroup_6, which was more abundant under Pistacia soil compared to the other canopies, 2) Rubrobacter, which was highly abundant under the canopy of Rosmarinus, grassland and Olea, while very rare under the canopy of Pistacia, 3) Candidatus Udaeobacter, which was abundant only under Myrtus canopy, followed by grassland and Pistacia, 4) Isosphaeraceae, which was abundant under Rosmarinus, less abundant under other canopies and almost absent under Pistacia canopy, and 5) WD2101_soil_group ASV belonging to Planctomycetes, which was highly abundant under Rosmarinus and less abundant under the rest of the canopies.

The nMDS analysis of the microbial community distribution according to the chemical parameters (Fig. S3a) showed a strong positive correlation between Fe and the distribution of grassland by its bacteria. However, Rosmarinus and Olea were positively correlated with Cu, Na, total N, Mg, and Cl; and negatively correlated with OC, total limestone, P, Mn, Ca, and Zn; with an opposite pattern for the bacterial distribution of Pistacia and Myrtus, which was positively correlated with OC, P, and total limestone and negatively correlated with total N, Na, and Mg. Finally, Juniperus and Euphorbia showed a high negative correlation with Fe, which was the main reason for their bacterial community distribution.

**Fungal community composition**

The fungal community showed a clear variation among the shrub canopies and also in comparison with the grassland (Fig. 3b, Table S1). Specifically, all the soils studied were dominated by the phylum Ascomycota, with abundance ranging from 60.5% in the grassland soil to 80.3% in Myrtus and Rosmarinus. However, the highest proportion of the phylum Basidiomycota was found in the grassland soil (25.1%), followed by Juniperus, Euphorbia and Myrtus with ~16%, while in the other canopies their abundance did not exceed 10%. On the other hand, the phylum Chytridiomycota was found with an abundance of 5.7% under the canopy of Olea, and less than 2% in the rest of the canopies, with the exception of Myrtus, in which it was completely absent. In addition, Pistacia showed exclusivity in harbouring the Mortierellomycota.
phylum (15.4%), followed by *Euphorbia* (10.9%), while it was almost absent under the *Rosmarinus* canopy. Finally, the phylum *Zoopagomycota* was present in only 2.4% of the *Pistacia* soil, and the phylum *Rozellomycota* was found only in *Juniperus, Euphorbia* and *Pistacia* at a very low abundance.

Moreover, the Venn diagram showed that all the canopies have enclosed a high amount of exclusive ASVs compared to the bacterial community (Fig. 4b). The highest number of unique ASVs was found under the grassland (i.e., 46) and the lowest was found under *Euphorbia* canopy (21) and *Myrtus* (22). However, the core microbiota, responsible for the most frequent ASVs, was represented by 115 ASVs.

At the lowest taxonomic level (Fig. 6), clustering of the canopies, based on the 50 most abundant ASVs, using the Bray-Curtis similarity index showed clear representation of variation among the fungal community across different shrubs and grassland (P = 0.001; Table S2). This clustering resulted in 7 separated clusters. The first cluster (A) representing *Myrtus*, which is separated from the rest by 70% of dissimilarity, followed by cluster B where *Juniperus* is separated from the rest by almost 65% of dissimilarity; while the rest of the canopies are separated by more than 50% of dissimilarity. The heatplot shows that the main ASVs responsible for the observed clustering differ from one canopy to another, comprising a group of genera instead of a specific one (Fig. 6). For example, the group that includes the genera *Penicillium, Gibberella, Helicoma*, and *Pilidium* is responsible for separating *Myrtus* from the rest because they are more abundant under its canopy. In addition, the genera *Didymella, Sarocladium, Phaeosphaeria, Xylariales* and *Russoella*, all belonging to the phylum *Ascomycota*, distinguish the *Rosmarinus* canopy from the rest. In addition, the overall diversity of *Juniperus* was associated with a group of ASVs that included *Stemphylium, Agaricomycetes, Mortierella* and other *Ascomycota* genera. While the grassland was characterized mainly by *Claviceps* genus belonging to the *Ascomycota*.

As revealed by FUNGuild analysis (Fig. S1), the distribution patterns of dominant ecological guild functions were more similar between *Pistacia, Rosmarinus* and the grassland. However, each plant canopy was found to host one or more guilds exclusively or more frequently compared to the others. For example, algal parasites are present only under the canopy of *Euphorbia* and *Pistacia*, however, bryophyte parasites are more abundant under the canopy of *Euphorbia*, while they are almost absent in the canopy of *Rosmarinus* and the grassland. In addition, ectomycorrhizal fungi were present under all canopies with low abundance except in grasslands where they were almost absent.

As for the distribution of fungal community, *Pistacia* had the highest positive correlation with Ca (Fig. S3b). However, Fe was responsible for the distribution of grassland and *Rosmarinus* with the highest positive correlation observed, while the large negative correlation between Fe and the fungal community of *Myrtus* was responsible for their distribution. In addition, P, Zn and OC were negatively correlated with grassland, *Rosmarinus* and *Olea* and positively correlated with *Myrtus*. Finally, the distribution of the fungal community of *Juniperus* was due to its strong negative correlation with Ca.

**Microbial co-occurrence**

We constructed two co-occurrence networks dividing samples from the grassland and the shrubland areas (Fig. 7) and calculated seven topological parameters to assess the interactions between the ASVs in the two networks (Table S3). The microbial network of the grassland area contained 91 nodes and 1946 edges, while the network of the shrubland area contained 97 nodes and 1159 edges. The proportions of positive ASVs correlations in the microbial networks of the grassland and shrubland areas were 81.9% and 99.7%, respectively. Network centralization, network heterogeneity, and characteristic path length values were significantly higher in the shrubland than in the grassland area. Compared with the shrubland, the network density, and clustering coefficient values were much higher in the grassland. Microbial assemblages in the two groups exhibited modular structures, and the modularity was notably higher in the shrubland (Fig. 7b1) than in the grassland area (Fig. 7a1). Nodes with high degrees, high closeness centrality, and low betweenness centrality were considered keystone taxa. In the grassland, 37.4% of the top 13 keystone taxa were *Ascomycota*, 18.7%
were Actinobacteria, and 14.3% were Proteobacteria. In the shrubland, Ascomycota, Actinobacteria and Proteobacteria represented 39.2%, 17.5% and 13.4% of the top 13 keystone taxa, respectively.

**Discussion**

**Grassland matrix**

Our proximate and $^{13}$C NMR analysis show that litter of *Ampelodesmos*, the dominant perennial grass in the monitored grassland, contained the highest levels of cellulose, di-O-alkyl-C, and O-alkyl-C compounds, while the levels of lignin, N, carboxyl-C, O-substituted aromatic C, methoxyl-C, and alkyl-C were the lowest. This litter type decomposes relatively quickly on the ground (Bonanomi et al., 2019), but is also highly flammable and most of it remains in the plant tussock, a condition that promotes the occurrence of fire events in this grassland (Incerti et al., 2013). Previous studies have shown the significant and diverse effects of plant litter on soil physio-chemical properties and also on microbial communities (Schneider et al., 2012; Ping et al., 2019). Since litter input could affect soil ecological processes, including soil C and N cycling via litter decomposition, we observed that soil under *Ampelodesmos* had correspondingly lower levels of organic carbon, total nitrogen, and P, while soil Fe content was highest. This is probably due to the occurrence of summer fires, which allow low accumulation of litter and OC in the soil profile. Moreover, we generally found low bacterial and fungal taxonomic richness and diversity in the grassland soil. These results could be explained by the low chemical diversity of the litter combined with low structural heterogeneity, resulting in low trophic specialisation that does not allow the coexistence of different saprotrophic communities.

In addition, we found high abundance of Acidobacteria and Actinobacteria at the phylum level, whereas the abundance of Proteobacteria was low compared to the soil under shrub canopies. Proteobacteria are generally considered to be more copiotrophic, while Acidobacteria are considered oligotrophic in soil (Leff et al., 2015). Fierer et al., (2007) described Acidobacteria as oligotrophs that prefer poor soils with lower carbon availability. Therefore, their high proportion in grassland could be explained by the low C and N contents in the soil. In addition, the Verrucomicrobia phylum has been shown to utilise a variety of carbon compounds and may be closely linked to carbon cycling (Fierer et al., 2013), making it more adaptable to oligotrophic environments in soils (Noll et al., 2005). Indeed, our results are in agreement with those of Qiu et al., (2020), who found that the proportion of Verrucomicrobia was significantly lower in grasslands than in shrublands at different soil depths.

On the other hand, we found the highest abundance of Basidiomycota and the lowest of Ascomycota compared to the soil under shrub canopies. This is surprising considering the low lignin content of *Ampelodesmos* litter. The low abundance of Ascomycota in grasslands compared to shrub canopies suggests strong litter selection, as they are considered important decomposers in the early and intermediate stages of litter succession (Boanomi et al., 2019). Therefore, abundance of Ascomycota is likely promoted by more complex plant litter (Purahong et al., 2016). In detail, the genus *Trechispora*, which belongs to the phylum Basidiomycota, was found only in grassland soils. This saprophytic fungus is involved in wood decomposition processes, and its occurrence in grasslands rather than under shrub canopies is consistent with the results of Kirker et al., (2017), who found higher abundance of wood decomposition fungi for the exposed areas compared to the unexposed areas, and many of these species are well-studied wood decay fungi or at least the genera, with the exception of *Trechispora* spp, which is more abundant on decaying wood debris on the forest floor than on solid wood. Finally, we recorded a high abundance of the genus *Claviceps* in the grassland soil, whereas it was almost absent under all shrub canopies. This result suggests a rather specific association between *Ampelodesmos* and *Claviceps*, which are known for the ergot disease infecting ~200 species of wild and cultivated grasses (Boestfleisch et al., 2015).

**Are shrubs signatures specific?**
We hypothesized that the diversity, richness, and composition of the microbial community would be altered by each shrub canopy, possibly due to the higher amount and/or diversity of litter trapped under the shrub canopy, which would enter the soil C and N cycles (Hooper et al., 2000). Our $^{13}$C NMR analysis shows high diversity within the litter characteristics of the shrubs studied compared to the grassland. We found that the litter of the evergreen, scherollus Rosmarinus had a high content of lignin, N and di-O-alkyl C, while it had the lowest cellulose content. On the other hand, Pistacia had the highest content of O-substituted aromatic C while it had the lowest N content. Moreover, Olea had the highest value of methoxyl-C, while Myrtus litter had the lowest lignin content. Euphorbia, a deciduous species that sheds its leaves in summer to avoid drought period, had high N content associated with high carboxyl-C and methoxyl-C content. As a result, Euphorbia accumulated little OC in the soil compared to other shrubs such as Pistacia and Olea. Previous studies suggest that litter decomposition in soil can alter microbial biomass, composition and community structure by increasing substrate variability and diversity of chemical compounds, and that this can vary depending on litter quality (Meier & Bowman, 2008; Chapman et al., 2013). Accordingly, the evergreens Pistacia, Olea and Myrtus have demonstrated high C levels and low N levels in their soils, while the coniferous Juniperus and Rosmarinus enclose low C and N levels. Our results, at microbiota scale, showed that bacterial diversity was significantly higher under Juniperus and Euphorbia canopies than under Rosmarinus, while fungal diversity was significantly higher under Olea than under Myrtus. Collins et al., (2020) found that SE did not assign a “global signature” but was associated with increased, decreased, or no change in alpha microbial diversity when compared to soils from nearby herbaceous plant communities. Our data, instead, indicate that the microbiota signature among coexisting shrub species is highly specific.

The increase in Proteobacteria in shrub soils is consistent with the findings of Wallenstein et al., (2007), who found an increase in Proteobacteria in Arctic shrub soils, suggesting that these bacteria thrive in C- and nutrient-rich soils that develop under shrub canopies and exhibit copiotrophic properties. Previous work has shown that SE increases oxygenation and nutrient content in surface soil (Braganza et al., 2015), suggesting that SE may influence the distribution of bacterial life strategies in the soil, i.e. enrichment of copiotrophic and depletion of oligotrophic bacteria. Moreover, all shrubs harbored a significant amount of Actinobacteria under their canopy. In particular, the Streptomyces genus was more abundant under the Myrtus canopy compared to the other canopies and the grassland. In this context, Qiao et al., (2017) studied microbial communities in nutrient-rich soils and found that Actinobacteria were more abundant than other microbes. It is now widely accepted that the establishment of bacterial communities in soils is not random but is controlled by specific compositional rules (Rosenholtz et al., 2015), including plant species (Edwards et al., 2015). Interestingly, we found high abundance of free-living N-fixing bacteria, including the genera Allorhizobium, Bradyrhizobium, Mesorhizobium, and Neorhizobium, under the canopies of Euphorbia and Pistacia, while it was lower under the canopy of Rosmarinus. Surprisingly, the abundance of these free-living N-fixing bacteria was positively correlated with soil pH, phosphorus content and cations, while it was negatively correlated with soil Fe content, which could partially explain their distribution. Our results also showed that Rosmarinus had the highest Fe content, which assigns for it an intermediate-like level to grassland, while Euphorbia had a very low soil Fe content. Fe plays a fundamental role in all isozymes of nitrogenase, the ubiquitous enzyme involved in biological N fixation (Raymond, 2003). This contradicts the negative correlation between Fe and the abundance of N-fixing bacteria under Rosmarinus and grassland soils, which contain high amounts of mineral Fe. In this regard, it is possible that Fe is immobilized in the grassland matrix and thus become unavailable to microbes, as well as to plants.

The abundance of fungal phyla showed a marked variation among shrub canopies. Our findings are in line with earlier studies showing that the Ascomycota, which are early colonisers of litter and the major decomposers, are litter type specific (Štursova et al., 2020) and thus highly abundant under shrub canopies. The phylum Basidiomycota is generally better equipped for lignin degradation (Lundell et al., 2010); our results confirm that the highest amount of lignin was found in the litter of Rosmarinus, so the abundance of Basidiomycota is positively correlated with the lignin content in the litter of shrub canopies. Moreover, Mortierellomycota are known to be saprobic and ubiquitous, and several studies show that they have the ability to solubilize phosphorus and are associated with increasing yields and establishing symbioses.
with plants (Grządziel et al., 2019). In our study, Mortierellomycota showed significant accumulation mainly in soils under Pistacia, followed by Euphorbia, indicating the good quality of these soils due to the quality of their falling decomposed litter. In detail, our results showed that Myrtus was characterised by a group of fungi composed mainly of Penicillium (soil-inhabiting Penicillium), which are among the common producers of secondary metabolites in soil (Zhelifonova et al., 2010) and have an important function in the decomposition of organic matter (Frisvad & Samson, 2004). Park et al., (2020) found that most Penicillium species from soil are highly selective and unique to each plant. In our study, all shrubs do not form symbiosis with ectomycorrhiza, except Juniperus (Mejstrik & Cudlin, 1983), therefore, their presence under shrub canopies is in the form of free-living spores. The fact of the presence of these mycorrhizal fungi confirms the formation of islands of fertility under the shrub canopies, indicating that the soil is ready for vegetation succession. Furthermore, Euphorbia was characterised by the presence of a significant amount of weak saprophytes, including Aspergillus, Alternaria and Cladosporium, while Rosmarinus exclusively hosted the genus Didymella, which are opportunistic parasitic microorganisms that often exploit special conditions to colonise on plants and occasionally cause severe damage (Blancard, 2012). Euphorbia also exclusively harbours the genus Aureobasidium, a typical phyllospheric endophyte that is mainly found in fresh litter and rapidly disappears upon decomposition (Bonanomi et al., 2019). According to previous culture-based studies conducted on different tree species, the persistence of Aureobasidium in decomposed litter is unusual due to its limited competitive ability (Voříšková & Baldrian, 2013). Our finding, that different functional groups of litter stimulate different fungal taxa, suggests that fungi have a preference for certain litter types, probably because the ability to degrade specific organic compounds varies among taxa (Schneider et al., 2012; van der Wal et al., 2013).

Our results suggest that the composition of the fungal microbiota converges in part by the types of litter functional group enclosed by each shrub canopy (Reinhart & Callaway, 2006). This could be due to differences in chemical composition between litter types (Fanin et al., 2014; Schneider et al., 2012), with plant functional groups often playing an important role in explaining differences in chemical traits (Diaz et al., 2004). In contrast to our results for fungal communities, we did not find strong shifts in the community composition of bacteria. That the effects were particularly pronounced for fungal communities may not be surprising, considering that fungi play a key role in the degradation of more recalcitrant organic compounds (van der Wal et al., 2013). It could be that fungi are more specialised to certain litter types, while bacteria use simpler carbon compounds from litter and fungal degradation products and are therefore less responsive to different litter types. Our results are consistent with previous work showing that the addition of organic amendments to agricultural soils can influence fungal biomass and community composition in soils (Clocchiatti et al., 2020; Reardon & Wuest, 2016) and that leaf litter is associated with a unique microbiome enhancement (Asplund et al., 2018; Lin et al., 2019).

Co-occurrence network of microbial community

Microbial co-occurrence patterns have been analysed to assess the rules of community assembly and interaction networks in highly complex systems (Gotelli & McCabe, 2002). Compared to that in shrubland, the microbial co-occurrence network in grassland was more complex, which might have been caused by the temporal and special continuity of connections between microbes, in contrast to shrubland, where connections might have been disrupted by the process of shrub encroachment. Previous studies have also suggested that positive correlations between nodes in co-occurrence networks of microbial communities in desert soil could be the result of functional interdependencies among microbial taxa under different environmental conditions (Neilson et al., 2017). In our study, the proportion of positive correlations was higher in the shrubland than the grassland, suggesting a high level of interdependence between microbial taxa in the shrubland. Topological parameters provide important information to understand microbial community structure (Newman, 2006; Ren et al., 2017). Higher values of network centralization and heterogeneity were observed in the shrubland than in grassland, indicate that there are many closely connected microbial modules (subnetworks/subcommunities in the overall network) in the shrubland.
Interactions between species are more frequent and intense in one module than in the rest of the community (Newman, 2006). Therefore, a small disturbance in the main module of a microbial network can have a large effect on the entire network of microbial communities (Liu et al., 2020). Moreover, higher mean degree and high closeness centrality and lower betweenness centrality could be used together to identify bacterial keystone taxa (Banerjee et al., 2018), which were mainly classified as Ascomycota, Actinobacteria and Proteobacteria in both networks. These keystone taxa could have a significant impact on microbial community structure and function (Banerjee et al., 2018). Removal of these highly connected taxa in a network would cause the collapse of the ecosystem structure and function (Saavedra et al., 2011).

**Conclusion**

Our study discovered that, under the same climate and rock type, coexisting shrubs generate specific signature of bacterial and fungal microbiota in the soil. We found that shrubs not only differentiate from the Ampelodesmsos grassland matrix, but also between shrubs belonging to different functional groups, i.e. deciduous, evergreen coniferous and evergreen sclerollus. Differences in litter chemistry probably play a key role in controlling soil chemistry and, in cascade, in shaping the soil microbiota. The observation that shrub signatures are specific is not trivial, and highlights the limitation and imprecision of the common approach, which often generalises the effect of shrubs compared to open vegetation habitats. Moreover, the presence of specific microbiota under shrub canopies is likely the result of a progressive plant-soil feedback that probably took decades to generate the observed signature. This, indeed, opens the door for further studies that would investigate the functional consequences and direction of such feedback for species coexistence as well as for litter decomposition in the context of home field advantage framework.

**Abbreviations**

SE
Shrub encroachment; CO\textsubscript{2}:Carbon dioxide; N:Nitrogen; P:Phosphorus; C:Carbon; USA:United states of America; CPMAS:Cross-polarization magic angle spinning; NMR:Nuclear magnetic resonance; Bruker AV:Bruker Avance; MAS probe:Magic Angle Spinning Probe; EC:Electrical conductivity; Cl:chloride content; DNA:Deoxyribonucleic acid; rRNA:Ribosomal ribonucleic acid; ITS:Internal transcribed spacer; PCR:Polymerase Chain Reaction; DADA:Divisive Amplicon Denoising Algorithm; ASV:Amplicon sequence variants; OUT:Operational taxonomic units; PRIMER:Plymouth Routines In Multivariate Ecological Research; nMDS:non-metric multidimensional scaling; PERMANOVA:permutational multivariate analysis of variance; ANOVA:Analysis of variance; Mg:Magnesium; Ca:Calcium; Cu:Copper; Zn:Zinc; Mn:Manganese; Fe:Iron; Na:Sodium; OC:Organic carbon;

**Declarations**

**Ethical Approval and Consent to participate**

Not Applicable

**Consent for publication**

Not Applicable

**Availability of data and materials**

Reads of the sequence data have been deposited in the NCBI Sequence Read Archive (SRA) under the bioproject Palinuro microbiome with accession no. PRJNA744707.

**Competing interests**
The authors declare that they have no competing interests

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**Authors' contributions**

MI, GB, and MZ have realized the soil collection in the field along with the monitoring of the shrubs within the grassland. MI, FDF, and GS have extracted the DNA and performed the molecular analysis. AMA and TF have done the chemical analysis of the litter and the soil. MI, SF, and GB have analysed data and written the manuscript. All authors read and approved the final manuscript.

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**Figures**

**Figure 1**

Sampling site of Capo Palinuro located in Southern Italy in Campania region (black circle on the map) on the grassland matrix, at the top of the figure, dominated by Ampelodesmos mauritanicus in which the studied shrubs have encroached; followed by the six encroached shrubs studied at the shrubland.
Figure 2

Box plots showing the variation in the Shannon diversity and species richness indices for bacterial (A) and fungal (B) communities under each plant canopy across the shrubland of Capo Palinuro. Different letters indicate significant (P < 0.05) differences in the indices under different plant canopies. The lower and upper bounds of the boxplots show the first and third quartiles (the 25th and 75th percentiles); the middle line shows the median, whiskers above and below the boxplot indicate inter-quartile range.
Figure 3

The relative abundance of various bacterial (A) and fungal (B) phyla under the canopy of each plant across the shrubland and grassland.
Figure 4

Venn diagram for bacterial (A) and fungal (B) communities show the numbers of ASVs (97% sequence identity) that were shared or not shared by the studied plant individuals depending on overlaps.

Figure 5
Heatmap showing relative abundance of the 50 most frequent Amplicon Sequence Variants (ASVs) in the bacterial community under the canopy of each plant across the shrubland and grassland. The hierarchical clustering of variables is based on Whittaker’s association index.

**Figure 6**

Heatmap showing relative abundance of the 50 most frequent Amplicon Sequence Variants (ASVs) in the fungal community under the canopy of each plant across the shrubland and grassland. The hierarchical clustering of variables is based on Whittaker’s association index.
Figure 7

Correlation base network between bacterial and fungal families within the grassland (A) and the shrubland (B) communities. Nodes with black borders correspond to fungal amplicon sequence variants (ASVs) and nodes with no black borders corresponding to bacterial ASVs. Only the top 50 frequent ASVs, for each kingdom, reported. The nodes are coloured by phylum level. Modularity networks of grassland (A1) and shrubland (B1) communities. Edges connecting nodes show either positive (light grey) or negative (red) co-occurrence relationships. Edges length represents correlation strength. The connection stands for significant correlations (P-value<0.05).

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