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Climatic impacts on the bacterial community profiles of cork oak soils

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ABSTRACT

Climate changes comprise increasing global temperature and water cycle deregulation (precipitation storms and long dry seasons). Many affected ecosystems are located within the Mediterranean basin, where cork oak (*Quercus suber* L.) is one of the most important forest ecosystems. Despite cork oak tolerance to drought, the decrease of water availability and increase of temperature is causing a serious decline of cork oak populations. In the present work, the bacterial community of cork oak soils was assessed by metabarcoding using *Illumina Miseq*. Soils from seven independent cork oak forests were collected along a climate gradient. In all forest soils, *Proteobacteria* and *Actinobacteria* were the richest and more abundant bacteria. *Acidobacteria* also presented a high relative abundance, and *Chloroflexi* was a rich phylum. The soil bacterial community diversity and composition was strongly affected by the climatic region where cork oak resides and specific bacterial taxa were differently affected by precipitation and temperature. Accordingly, cork oak bacterial communities clustered into three distinct groups, related with humid, sub-humid and arid/semi-arid climates. Driest and warmer forests presented more diverse bacterial communities than humid and coolest forests. However, driest climates presented more homogenous bacterial communities among forests than humid climates. Climate (mainly precipitation) revealed to be the strongest driver leading to significant variations of bacterial community profiles. The most impacted bacterial taxa by climatic variables were *Proteobacteria*, in particular *Gammaproteobacteria* and *Deltaproteobacteria*, *Chloroflexi*, and *Firmicutes*. Humid forests presented mainly *Acidobacteria* as good indicators of climate, whereas *Actinobacteria* members were better indicators for arid forests (mainly *Gaiellales* and *Frankiales*). Some indicator species for different climate conditions were members of the bacterial core of cork oak stands (7% of the total bacterial community). Taken together, different microbiomes were selected by the climate conditions in cork oak stands along a climate gradient and might provide the key to forest sustainability in times of global warming.

1. Introduction

Mediterranean forest is considered as one of the major global biodiversity hotspots, due to its rich biodiversity, comprising many endemic species that are being threatened by anthropogenic and climate challenges (Pausas and Millán, 2019). These forests are mainly composed by broadleaved evergreen tree species (holm – *Quercus ilex* and cork oak – *Quercus suber*; Valavanidis and Vlachogianni, 2011). Cork oak displays an important economic input for the Mediterranean countries, in particular for the Iberian Peninsula that presents the largest cork oak forest area, which results in 80% of annual cork production (50% of which in Portugal). Climate changes, such as increased temperatures and reduction of water availability, are currently posing a challenge to cork oak forests (reviewed by Reis et al., 2017; Maghnia et al., 2019). An adaptation of cork oak populations to drier and warmer conditions is expectable (Varela et al., 2015), but the decline of existing populations have been described all over Mediterranean basin (reviewed by Reis et al., 2017).

From the huge diversity of microbes present in cork oak soils, fungal communities are those more related with water transfer and increasing water availability to plants. This is mostly due to the ability of ectomycorrhizal fungi to become associated to cork oak roots, promoting water and nutrients transfer (Reis et al., 2017). However, bacteria also play an important ecological role on forest soils. Besides being important for decomposition of organic matter, N fixation, mineral weathering and consequently the release of inorganic nutrients (Lladó

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et al., 2017), soil bacteria also indirectly affect the water availability to plants. Specific groups of bacteria, such as plant growth promoting rhizobacteria (PGPR) and mycorrhiza helper bacteria (MHB) play an indirect role on plant development by promoting mycorrhizal symbiosis and the biological control of plant pathogens (Frey-Klett et al., 2007; Backer et al., 2018). Comparing to fungi, fewer studies have been conducted regarding bacterial adaptation to drought, although bacterial communities have been reported to change with precipitation levels (Felsmann et al., 2015), drought (Bastida et al., 2019) and season (López-Mondéjar et al., 2015).

For facing the upcoming climate changes, the microbial community present in the soil of cork oak forests has been recognized as one of the main drivers for forest sustainability (Reis et al., 2017; Maghnia et al., 2019). Different soil layers are enriched with microbes displaying different lifestyles. Bulk soil is highly enriched in oligotrophic bacteria, able to grow under low substrate concentrations due to a higher substrate utilization efficiency. As a result, oligotrophs are well-adapted to thrive on poor nutrient substrates and low moisture contents (Ho et al., 2017). Bacterial communities dominated by oligotrophs often display a high level of spatial heterogeneity and patchiness of activity, as well as a high abundance of mineral weathering bacteria (Lladó et al., 2017). In contrast, copiotrophs are recognized as fast growing microorganisms that prefer rich nutrient substrates but are sensitive to low moisture contents. Copiotrophic bacteria are more abundant in the rhizosphere, due to the higher availability of C in the soil through the rhizodeposition of plant and fungal exudates (Ho et al., 2017). For this reason, the activity of extracellular enzymes and microbial abundance (10^8 to 10^{10} bacterial cells g^{-1}) are enhanced in the rhizosphere in comparison to those in bulk soil (Finzi et al., 2015).

Mediterranean plant species have been correlated with diverse climatic variables, such as precipitation, evaporation and temperature (Suc, 1984). In the particular case of cork oak, stands density is closely related to water availability (Joffre et al., 1999). Portuguese cork oak stands comprise two different forest systems depending on tree density. High density forests (about 400 trees/ha; *sobreirais*) are typically found in northern and central Portugal, whereas low density stands (60–100 trees/ha; *montados*) are more common in the southern and drier regions. Climate variables, such as temperatures, precipitation, light and seasonality are major drivers of bacterial communities present on temperate forests (Lladó et al., 2017). In the present work, a picture of the bacterial community associated with cork oak soils is described, taking into consideration cork oak forests from different locations of the greatest cork producer country (Portugal), which comprises different Mediterranean climates. Taking this into consideration, we aim to answer the following research questions: i) what are the main drivers for bacterial communities present on cork oak soils? ii) which bacterial taxa are more affected by climatic variables and which can be indicators of specific climates? iii) what is the core bacterial community of cork oak stands? To the best of our knowledge, this is the first report comparing soil bacterial communities on cork oak stands from different climatic regions.

2. Material and methods

2.1. Cork oak stands and sample collection

Five independent geographic locations (Fig. S1) were selected based on local weather conditions and water availability levels (Portuguese Sea and Atmosphere Institute), previous information of cork oak stands (Varela and Eriksson, 1995), and local Emberger indexes that define the corresponding Mediterranean climates (Rego and Rocha, 2014; Reis et al., 2018; Table S1). Based on annual precipitation means, *Penedo-Gerês* (PG, 120.7 mm) and *Herdade da Contenda* (HC, 46.5 mm) comprised the extreme conditions. Two independent forests were sampled in each of these locations (PG-ER and PG-RC; HC-CT and HC-MA). Other three locations displaying intermediate precipitation levels were also sampled [*Limãos* (LI, 772.8 mm), *Alcobaça* (AL, 651.6 mm), and *Grândola* (GR, 735.6 mm)]. Using climatic data during the sampling year [annual precipitation, maximal and minimal temperatures of the hottest and coldest months], the corresponding Emberger indexes (Q) were determined according to Tate and Gustard (2000). The sampled forests were separated into four distinct Mediterranean climates: humid (PG, $Q = 186.6$), sub-humid (LI, $Q = 88.9$; AL, $Q = 102.7$), semi-arid (GR, $Q = 77.5$) and arid (HC, $Q = 43.5$). The same soil samples had been previously used for assessing ectomycorrhizal communities (through root tips barcoding) in these cork oak stands (Reis et al., 2018).

Soils sampling was conducted on the seven cork oak forests during the autumn (November and December) of 2013, using the procedure described by Reis et al. (2018). Five independent healthy trees, separated at least 30 m from each other, were selected. After removing the uppermost layer of soil that comprised plant litter and other organic material (litter, ~0.5 to 1 cm depth; organic, ~1–3 cm depth; mineral, ~3–5 cm depth), three soil cores (8 cm of diameter and 12 cm in depth) were collected under the middle of the cork oak canopy, in three tree trunk directions. Soils were stored at 4 °C until processing. In total, 105 soil cores (7 forests × 5 trees × 3 cores) were collected. Each soil core was sieved twice, through a 5- and then 10-mesh size (corresponding to sieve openings of 4 mm^2 and 2 mm^2, respectively) and stored at −80 °C up to DNA extraction.

In order to determine soil pH, samples were homogenized by mixture, dried at 40 °C, during 2 to 3 days, and sieved through a 10-mesh. After being mixed with deionized water (1 g to 2.5 ml), the supernatant pH was measured with a glass combination electrode. Soil granulometric analysis was performed using sieve analysis and *SediGraph 5100* software to determine grain size distribution in soil fractions. The percentage of sand, silt and clay was used for soil texture classification, using the soil texture triangle for Portugal (Gomes and Silva, 1962).

2.2. DNA extraction and Illumina sequencing of soil bacteria

Soil DNA was extracted from each soil sample (250 mg) using *PowerSoil DNA isolation* kit (MO BIO Laboratories), according to the instructions provided by the supplier, but using water for elution instead of EDTA. DNA samples were mixed together (400 ng from each replicate) and frozen as a single sample for each forest plot. At the end, a total of 35 DNA samples were sequenced.

The composition of microbial communities from samples was assessed by amplicon sequencing with the *Illumina MiSeq* platform. The DNA was processed according to Illumina instructions to generate *Nextera XT* paired-end libraries (2 × 250 bp) and the 16S rRNA gene was sequenced with primers targeting the hypervariable region V3-V4 (forward primer: 5′ tcgtcggcagcgtcagatgtgtataagagacagcctacgggnggcwgcag 3′; reverse primer: 5′ gtctcgtgggctcggagatgtgtataagagacaggactachvgggtatctaatcc 3′), according to Klindworth et al. (2013). The generated amplicon covered a region of approximately 460 bp.

2.3. Read processing and data analysis

Read pairs from each sample were trimmed with *Sickle* (Joshi and Fass, 2011) for a minimum phred-score of 20. *Vsearch* v2.3.2 (Rognes et al., 2016) was used to merge the trimmed read pairs into single sequences with a minimum overlap region of 20 bp and by allowing 2 mismatches. The resulting sequences were truncated and filtered to 400 bp, in order to i) remove low quality regions derived from the reverse reads and ii) to generate sequence datasets uniform in length for the following steps of the pipeline. The number of identified high-quality reads in all forests was similar (Table S2). De-replication, removal of chimeric sequences and clustering with an identity threshold of 97% were performed using *Vsearch* (Rognes et al., 2016). Taxonomic classification was assigned by using the ribosomal RNA gene reference

database *SILVA, version 123* (Quast et al., 2013). Unclassified sequences and low abundance taxa (< 5 reads in all replicates from a given forest) were filtered from the operational taxonomic unit (OTU) tables before downstream analysis. For the analysis of microbial profiles and to mitigate biases due to differences in the sampling depth, *QIIME 1.9.1* (Kuczynski et al., 2011) was used to subsample all datasets for an even number of sequences (16,868 reads, found in a soil sample from GR forest, Table S2).

2.4. Statistical and ecological data analysis

The bacterial community analysis was performed considering all the five soil replicates from each forest and OTUs with at least 5 reads in all replicates from a given forest (*OTUs ≥5 reads*, Table S2), in order to remove rare species and undersampling that could disturb the following analyses. The composition of bacterial communities was evaluated by collapsing OTUs at different taxonomic levels (phylum, class, order, family and genus). Differences in relative bacteria abundance or richness were evaluated for each edaphoclimatic variable by one-way ANOVA (followed by the Bonferroni correction) with *Excel* tools. For evaluating the diversity of bacterial communities, using either OTUs without any filtering (*OTUs*), OTUs with a filtering of > 5 reads in each sample from each forest (*OTUs ≥5 reads*) and OTUs merged to taxa-level (*collapsed OTUs*), computational indexes that combine both relative abundance and diversity were used (Magurran, 2004). Alpha indexes [Simpson (*D*), Shannon (*H'*)] were determined by the *diversity()* function of the package *vegan v2.4-1* from *R*, whereas *Species Diversity and Richness - version 5* (Pisces Conservation Ltd. Lymington, UK; 2014) was used for rarefaction curves (Henderson and Seaby, 2007). To determine differences on bacterial richness and diversity among forests, a Kruskal-Wallis chi-squared test was performed, followed by pairwise Wilcoxon test between groups, using the *R* package *stats v3.5.2*. The *Community Analysis Package - version 5* (Pisces Conservation Ltd. Lymington, UK; 2014) was used to evaluate changes in the microbial community through analysis of similarity (ANOSIM) using Bray-Curtis distance matrix with *OTUs ≥5 reads* dataset (Clarke and Gorley, 2015). Correlation between community structure (Bray-Curtis distance matrix) and Euclidean distances was determined using the Mantle test of the *Microsoft Excel* add-in program *XLSTAT* (version 2017, Addinsoft, New York, USA).

All subsequent analyses were performed with *R version 3.4.3* (R Core Team, 2017). For detecting differences in the abundance of genera, OTUs identified up to genus level were collapsed and used for determining significance of differences between samples using the *F-test wrapper mt()* function from the *phyloseq version 1.16.1* package (McMurdie and Holmes, 2013), with a Bonferroni correction for multiple pairwise comparisons. A heatmap of differentially abundant genera between samples was created with packages *stats version 3.4.2* and *gplots version 3.0.1* (Warnes et al., 2016). Dendrograms were made through hierarchical clustering based on the calculated Canberra distance with UPGMA agglomeration method. Species composition across samples/forests was visualized using non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity indexes of square-transformed reads from *OTUs ≥5 reads* dataset (Oksanen et al., 2012). The ordination plots were created using the *metaMDS()* function followed by the *plot()* command of the vegan package. For identifying the main factors affecting the composition of bacterial communities, environmental variables were fitted to the ordination plots using the *envfit ()* function of the same package. R^2 or the *goodness-of-fit* values and their significances were calculated using 999 permutations. The evaluated continuous factors were the climatic variables (temperature and precipitation values), climatic regions where the cork oak forests are included (as evaluated by *Q* index), and soil pH. The evaluated categorical factors were forest system (*sobreiral/montado*), forest use for grazing animals (deer and wild boar/domesticated livestock, pasture), human disturbance (mainly caused by tillage), vegetation cover (particularly *Fabaceae* presence), and soil texture.

For depicting the core bacterial community of cork oak forests, *OTUs ≥5 reads* dataset was used to detect the prevalent OTUs that were present in all forests (35 samples), as well as in different climates (humid – PG-ER, PG-RC; sub-humid/semi-arid – AL, LI, GR; Arid – HC-CT, HC-MA). Species indicator analysis (Dufrêne and Legendre, 1997) was used to identify indicator OTUs for cork oak forests/climates and was implemented by the *indval()* function in the *R* package *labdsv* (Roberts, 2010). The association of certain OTUs to cork oak stands/climate was measured by *IndVal* index that varies from 0 to 1, in which *IndVal* > 0.5 represents the most constant and specific species. The OTUs with significant *IndVal* indexes were represented in a heatmap with dendrograms, created as described above.

3. Results and discussion

3.1. Bacterial communities structure in cork oak soils from different climates

A set of high quality reads (1,116,477) from different soil samples were clustered into 7429 OTUs (Table S2). Rarefaction curves suggest that all forest soils were well-sampled and could give information about bacterial communities (Fig. S2). In order to identify bacterial signatures/patterns associated with different forests, only OTUs that were present in all five replicates of each forest were considered for further analysis. The resulting 5329 OTUs belonged to 36 *Bacteria* phyla, 109 classes and 442 families (Table S2; Fig. S3A). *Proteobacteria* (126 families), *Actinobacteria* (54 families) and *Chloroflexi* (51 families) were the richest phyla. Among the *Proteobacteria* phylum, *Alphaproteobacteria* and *Deltaproteobacteria* classes were the most diverse (comprising 36% and 29% of *Proteobacteria* families, respectively), followed by *Betaproteobacteria* and *Gammaproteobacteria* classes (17% and 14%, respectively). Within the *Actinobacteria* phylum, which includes many plant beneficial microorganisms (Barka et al., 2015), 56% of families belong to the *Actinobacteria* class, being *Frankiales* the most diverse order comprising seven families. *Ktedonobacteria* class (19 identified families) was the richest from the *Chloroflexi* phylum. When considering the number of identified OTUs, a significant increase was found as water availability decreased ($p < 0.05$; Figs. 1 and S4). While wettest forests (PG-ER and PG-RC) comprised 243 and 287 identified bacterial families, driest forests (HC-CT and HC-MA) included 323 and 308 families, respectively (Table S2).

Considering taxa relative abundance, *Proteobacteria* (31% of total reads), *Actinobacteria* (22%) and *Acidobacteria* (16%) were the most abundant detected phyla, whereas 27 phyla (74 families) individually registered < 1% of total identified reads in all samples from the seven forest soils (Fig. S3B). Analysis of the ten most abundant taxa (phylum or class) in each cork oak forest revealed the same bacterial pattern (Fig. 2). Similar profiles have revealed a high abundance of these three phyla in most forest soils (Lladó et al., 2017). A strong predominance by *Proteobacteria* was indeed detected in cork oak forests from the Mediterranean region (Sardinia, Italy), using a combination of culture-based and molecular techniques (Bevivino et al., 2014). These results are corroborated by meta-analysis studies that have attributed to *Proteobacteria* a central role on forests rhizosphere (Hawkes et al., 2007). However, different results were obtained when using the bulk soil of *Quercus* forests, accessed by 454-pyrosequencing, where *Acidobacteria* revealed to be the dominant phylum (40 to 50% of identified sequences by López-Mondéjar et al., 2015). The use of culture-dependent or independent strategies for surveying microbial communities and sampling of either bulk soil or soil containing superficial cork oak roots (as in the present work), could explain these contrasting results. The high availability of C provided by the tree roots and mycorrhizal hyphal exudates (Finzi et al., 2015) could determine a higher abundance of copiotrophic taxa that grow in conditions of elevated C availability and exhibit faster growth rates (Eilers et al., 2010; Lladó and Baldrian,

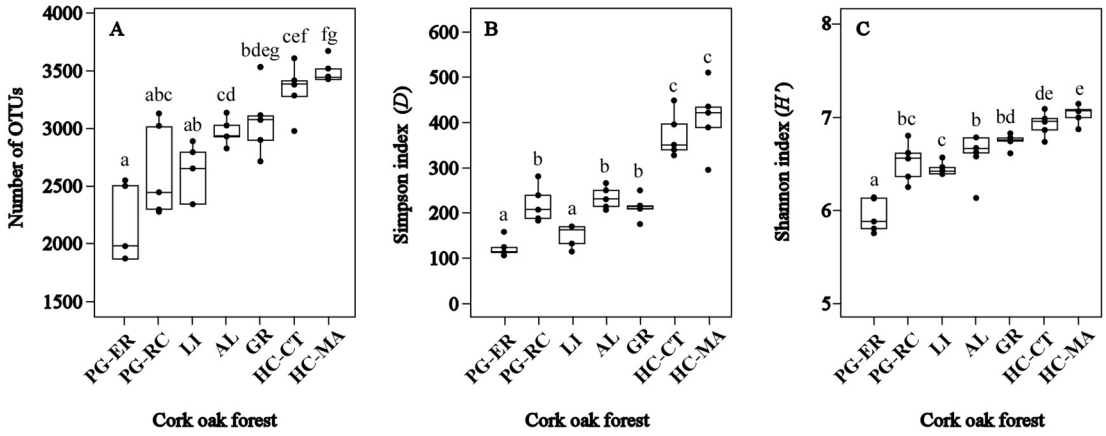


Fig. 1. Diversity analysis of bacterial communities from the seven sampled cork oak forests. Box plots graphically represent the number of OTUs (S), Simpson (*D*) and Shannon (*H'*) indexes. The significance of differences observed between alpha-diversity metrics for each soil was tested with the Kruskal-Wallis chi-squared test, followed by pairwise Wilcoxon test between groups. Different letters denote statistically significant differences at $p < 0.05$. Each site is referred by their code: PG - *National Park of Peneda-Gerês* (PG-ER - *Ermida*; PG-RC – *Rio Cabril*); HC – *Herdade da Contenda* (HC-CT – *Contenda*; HC-MA – *Monte Asparão*); LI – *Limãos*; AL – *Alcobaça*; GR - *Grândola* (see Table S1 for more details).

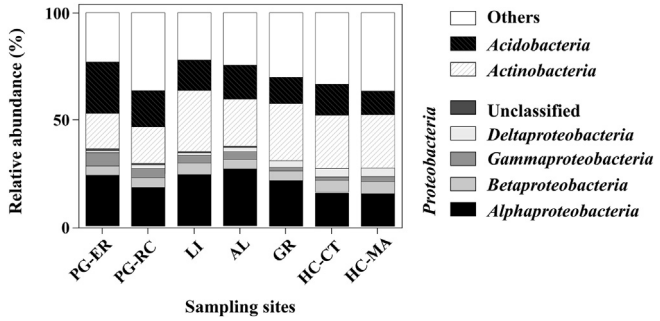


Fig. 2. Relative abundance of bacterial phyla/classes identified in all cork oak forest soils. Relative abundances are presented considering OTUs with at least 5 reads in all replicates from a given forest (*OTUs ≥5 reads* dataset). Each site is referred by their code, as used in Fig. 1.

2017; Lladó et al., 2017). Therefore, an enrichment on taxa that are known to include many copiotrophic members (as *Proteobacteria*; Trivedi et al., 2013) could be related with the presence of many residual roots, as was the case of our sampled soil cores. This is in agreement with other reports describing differences in the bacterial composition of bulk or rhizospheric soils (Lladó et al., 2018). Within *Proteobacteria*, *Alphaproteobacteria* was the most conspicuous identified class in cork oak soils, representing 21% of total reads (65% of *Proteobacteria* reads, Fig. S3B). Within this class, the *Rhizobiales* order (comprising 30% of *Proteobacteria* reads) was the most abundant taxa, mainly including reads assigned to the *Bradyrhizobiaceae* family (11%). The most dominant *Proteobacteria* genus was *Afipia* (comprising almost all *Bradyrhizobiaceae* found) and *Sphingomonas* (9% of *Proteobacteria* reads). Within *Actinobacteria* phylum, *Actinobacteria* was the most abundant class (14% of total reads, 61% of *Actinobacteria* phylum reads). *Acidobacteria* class comprised > 96% of *Acidobacteria* phylum reads (16% of total reads) and the most relevant genus was *Acidothermus* (a member of *Frankiales*) with 5% of total reads. Taken together, these results reveal that cork oak forests are highly abundant on taxa described to include many symbiotic and free-living nitrogen fixing members (Sellstedt and Richau, 2013; Garrido-Oter et al., 2018; Lladó et al., 2017).

3.2. Cork oak driest forests present more diverse and homogeneous bacterial communities

Bacterial communities were compared between forests by computation of diversity indexes (Figs. 1 and S4). PG-ER forest systematically presented the lowest values for alpha (*D* and *H'*) diversity indexes ($p < 0.05$, when compared with GR, HC-CT and HC-MA), while the highest values were shared between the driest forests (HC-MA, HC-CT and GR). Indeed, bacterial communities were significantly more diverse across a gradient of water availability, from the rainiest forests (PG-ER and LI) with less diversity to the driest forests (HC-CT, HC-MA, $p < 0.05$). In fact, bacterial richness and diversity have been reported to increase as water potential decreases and soils become drier (Carson et al., 2010). Under these conditions, the low pore connectivity in soil promotes bacterial diversity by limiting the strength of competitive interactions, while favoring the bacterial coexistence. Drier soils prevent motile bacteria from exploiting nutrient resources, thus protecting less motile species from competition and extinction (Carson et al., 2010; Vos et al., 2013). These reasons should have increased the bacterial diversity in semi-arid/arid climates (HC-CT, HC-MA, and GR), where reduced precipitation level is associated with high temperatures.

A different bacterial community profile was obtained for the soil of each cork oak forest (Fig. 2). Within *Proteobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* were differently abundant among cork oak stands, displaying rainiest forests (PG-ER and LI) a higher abundance than driest ones (HC-CT and HC-MA; $p < 0.001$). An opposite situation was found for *Deltaproteobacteria* that exhibited more abundance in dry samples [HC-MA ($p < 0.05$), HC-CT ($p < 0.05$) and GR ($p < 0.01$)] when compared with humid samples. The climate effect of the soil microbiome structure is underlined by the effect of climatic/soil parameters, especially precipitation and temperature, on the abundance of specific bacterial phyla/classes (Table 1). While in humid soils, the transport of resources to microbes is described to be promoted by water diffusion, in dry soils the resources become more limited to microbes (Schimel, 2018). Probably due to the reduction in C allocation under drier conditions, taxa comprising many copiotrophic members (such as *Alphaproteobacteria* and *Gammaproteobacteria*; Philippot et al., 2010) were less abundant in the driest climates (Fig. 2). *Gammaproteobacteria* abundance was indeed positively correlated with humid climates (high *Q* index) displaying higher precipitation and lower temperatures (Table 1). The most abundant classes of *Proteobacteria* (including *Alphaproteobacteria*, but also *Betaproteobacteria*) were not disturbed by the climatic conditions occurring in soil sampling sites, which agrees with the findings of Felsmann et al. (2015) that described a greater resilience and tolerance to drought of well-represented bacteria. Opposite results - higher abundance and a strong correlation with driest climates (low *Q* index) exhibiting less precipitation and high temperature - were found for *Deltaproteobacteria*. This bacterial class is typically enriched in oligotrophic members that thrive under poor C conditions (Trivedi et al.,

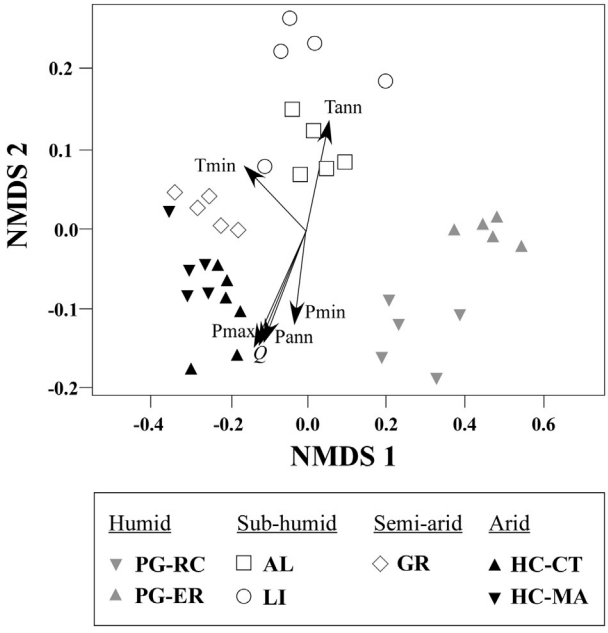
Table 1
Effect of different edaphoclimatic variables on the relative abundance of specific taxa (A) and families richness (B) in all seven sampled forests. Variables comprised precipitation and temperature [average from past 30 years (aver.), from the wettest/hottest month (max) and from the driest/coldest month (min) of the sampling year], Emberger index (*Q*) and soil pH. Differences in relative bacteria abundance or richness were evaluated for each edaphoclimatic variable by one-way ANOVA followed by Bonferroni correction (*F*-values displayed).

	Precipitation			Temperature			*Q*	pH
	Aver.	Max	Min	Aver.	Max	Min		
A. Bacterial relative abundance								
Proteobacteria	−10.4**	−10.5**	−5.5*	7.2*	2.4	−1.7	−11.6**	8.9**
Alphaproteobacteria	0.8	0.5	1.6	−0.6	−5.8	−1.3	3.7	0.2
Betaproteobacteria	−2.9	−3.0	−1.1	1.4	1.4	−1.5	−4.0	0.7
Gammaproteobacteria	38.9***	35.1***	45.1***	−41.0***	−27.2***	−1.4	50.4***	−9.3**
Deltaproteobacteria	−22.3***	−19.5***	−38.3***	26.6***	56.8***	7.2*	−34.6***	6.4*
Actinobacteria	1.8	2.1	0.0	−0.1	−0.2	12.0**	3.1	0.1
Acidobacteria	−3.2	−3.7	0.0	0.4	−0.1	−34.2***	−4.3*	0.2
Chloroflexi	13.7***	14.0***	5.7*	−8.4**	−1.4	5.1*	15.5***	−6.5*
Planctomycetes	4.9*	5.0*	3.2	−4.9*	0.0	1.0	2.9	−6.6*
Verrucomicrobia	−4.5*	−4.7*	−1.5	2.6	0.3	−3.6	−4.5*	5.4*
Bacteroidetes	−2.5	−2.5	−0.8	1.1	0.3	−3.4	−4.8*	−1.6
Firmicutes	11.0**	10.9**	5.2*	−6.3*	−4.0	2.4	16.7***	−2.8
B. Bacterial richness	−19.19***	−17.56***	−27.12***	22.23***	32.98***	4.58*	−22.96***	8.96**

* Statistical significance at $p < 0.05$.
** Statistical significance at $p < 0.01$.
*** Statistical significance at $p < 0.001$.

2013). Other bacterial taxa that were highly affected by climatic variables were *Chloroflexi* and *Firmicutes* that presented a strong positive correlation with water availability, in agreement with previous studies (Ochoa-Hueso et al., 2017; Naylor and Coleman-Derr, 2018).

When taking into consideration the bacterial communities present in all soil samples, a non-metric multidimensional scaling (NMDS) analysis, performed based on Bray-Curtis dissimilarity coefficient (Kruskal's stress = 0.074; Fig. 3), revealed that bacterial communities grouped differently according to the climate where cork oaks reside. Three distinct clusters were formed, related with the humid, sub-humid and arid/semi-arid climates. The forest replicates for the most divergent Mediterranean climate scenarios (HC and PG) displayed related bacterial communities, although arid replicates (HC-CT and HC-MA) revealed to be more similar between each other than humid (PG-ER and PG-RC) forests. Indeed, arid replicate forests (HC-CT and HC-MA) presented similar diversity indexes, whereas humid replicate forests (PG-ER and PG-RC) displayed significantly different *D* and *H′* indexes ($p < 0.05$; Figs. 1 and S4). Using an analysis of similarities (ANOSIM), the bacterial communities among all cork oak stands revealed to be significantly distinct ($R = 0.814$, $p < 0.001$). However, humid replicate forests (PG) presented more distinctive communities between each other ($R = 0.682$, $p < 0.01$) than driest replicate forests (HC) among them ($R = 0.476$, $p < 0.01$). A higher bacterial similarity among semi-arid/arid climates was also detected when performing an abundance heatmap with the most differently abundant genera among cork oak forests (at $p < 0.05$). A clear cluster of semi-arid/arid bacterial communities was detected, in contrast with bacterial communities from more humid climates (Fig. S5). Dissimilarity of bacterial communities among all cork oak forests was positively correlated with Euclidean distances among forests (Mantel test, at $p < 0.001$). In forests within the same climatic region, the distance between forests continued to result in significant differences between bacterial communities ($p < 0.05$), except for arid and semi-arid soils (HC-CT, HC-MA, and GR), which did not reveal to be dissimilar even being



Variables	R^2	*p*
Continuous variables		
Tmax	0.076	0.270
Tmin	**0.413**	**0.001**
Tannual	**0.278**	**0.007**
Pmax	**0.421**	**0.001**
Pmin	*0.231*	*0.025*
Pannual	**0.417**	**0.001**
Q	**0.514**	**0.001**
pH	0.179	0.054
Categorical factors		
Forest system	0.075	0.093
Pasture	*0.103*	*0.047*
Human disturb.	**0.288**	**0.001**
Fabaceae cov.	**0.566**	**0.001**
Soil texture	*0.178*	*0.037*

Fig. 3. Evaluation of the main environmental factors affecting bacterial diversity. Nonmetric multidimensional scaling (NMDS) analysis of bacterial OTUs represented by more than five reads in all replicates from each sampling site (*OTUs ≥5 reads* dataset). Clustering analysis was performed with Bray-Curtis dissimilarity measure (Kruskal's stress = 0.074). Each point represents a different sample (five samples from each forest). The environmental variables were fitted to the NMDS ordination, where the direction of arrows indicates positive correlations between continuous environmental factors and bacterial communities. Only those factors that were significantly correlated with NMDS ordination axes ($p < 0.05$) are shown. R^2 (continuous variables) or *goodness-of-fit* statistics (categorical factors) of environmental variables fitted to the NMDS ordination space are provided. Statistical differences are denoted in italics at $p < 0.05$, in bold at $p < 0.01$, and in underlined-bold at $p < 0.001$. Each site is referred by their code, as used in Fig. 1.

separated > 140 km (for comparison, humid forests were < 9 km away from each other). These results revealed that distance was not the main driver for bacterial community structure, at least in the driest climates.

The similarity results revealed a higher bacterial heterogeneity in humid climates, suggesting that common drivers could be shaping the bacterial composition in the most arid forests. In comparison to the humid forests (PG-ER and PG-RC), drier forests (HC-CT, HC-MA, and GR) have always experienced increased temperatures and reduced precipitation along time (Fraga et al., 2018), although their intensity has been increasing in the last years. Selection for more tolerant bacterial taxa may have occurred in this selective and persistent environment, where bacteria displaying different strategies to cope with drought stress and nutrient limitation would present a competitive advantage (Evans and Wallenstein, 2014; Meisner et al., 2018). This adaptation of whole bacterial communities to drier environments could have similarly shaped the communities in soil forests from semi-arid/arid climates (HC-CT, HC-MA, and GR), resulting in more similar bacterial communities than in humid soil forests. In semi-humid/humid environments (particularly, in PG-ER and PG-RC), where water availability is not limited, other selective environmental forces would have shaped the bacterial communities, leading to more heterogeneous communities as compared to arid forests.

The effect of environmental variables on the structure of bacterial communities was evaluated by vector fitting analyses to the ordination plot (Fig. 3). As expected, within the most significant drivers, *Q* index and precipitation (annual and maximal) levels presented the highest scores, in agreement with their suggested role in shaping bacterial communities in driest soils. Shifts on bacterial communities of beech forest soils were also correlated with precipitation, as those plots with low precipitation levels presented more bacterial genera then control plots (Felsmann et al., 2015). In cork oak forests, the bacterial communities were also strongly affected by other environmental variables, such as the temperature (minimum), and other factors like the plant cover and soil tillage but displaying a lower score. These drivers could play a central role in shaping the bacterial communities on sites where soil water is not limited.

3.3. Bacterial core of cork oak forests

From all 36 identified bacterial phyla on cork oak soils, 20 were shared between all sampled soils; however, when considering the identified families (442), only 33% were present in all 35 soil samples (Table S3). For determining the bacterial core of cork oak soils (also containing residual root segments), the OTUs present in all 35 soil samples from seven cork oak forests were selected, resulting in a total of 371 OTUs (Table S3). Although comprising only a small fraction of all bacterial community identified in our work (7% of the total number of identified OTUs), representing 52.6% of OTUs relative abundance, the core bacteria in the soil zone under the influence of cork oak roots could be essential for the plant tree adaptation and fitness. Accordingly, the relative abundance of core OTUs is not significantly changed among cork oak forest, except for PG-ER and arid forests ($p < 0.05$, ANOVA). Indeed, bacterial species of core microbiomes have been described as less susceptible to external disturbance than other bacteria (Toju et al., 2018) and essential for environmental adaptation of plants (Li et al., 2018). The cork oak core microbiome was dominated by *Proteobacteria* (33.2% of core OTUs and 39.7% of total reads assigned), mainly including *Alphaproteobacteria* (20.5% and 29.8%, respectively), but also *Actinobacteria* (19.1% and 24.2%, respectively) and *Acidobacteria* (19.9% and 20%, respectively). From the core bacterial community, several bacterial orders that typically inhabit soil fractions under the influence of plants were detected (*Rhizobiales*, *Burkholderiales*, *Shingomonadales* and *Pseudomonadales*, all from *Proteobacteria*; *Gaiellales* from *Actinobacteria*; Hayat et al., 2010; Vergani et al., 2017), comprising together > 54% of total bacterial core reads. Although only a part of the studied soil was in direct contact with cork oak roots, this core bacterial community is likely to be modulated by the specific influence of cork oak roots proximity. The typical dimorphic rooting habitat of cork oak, in which a superficial network root system and deep roots are responsible for the hydraulic redistribution in soil, allows cork oak trees to survive in drier climates of Mediterranean regions (Nadezhdina et al., 2008). These adaptations give cork oak trees access to groundwater (Barbeta et al., 2015), even for supplying the superficial roots during drying periods, and could play a role on core bacterial community structure. The typical ligninolytic and cellulolytic bacteria found in forest soils were also highly abundant in the core microbiome (including genera like *Sphingomonas*, *Burkholderia*, and *Pseudomonas*, all from *Proteobacteria*; *Bacillus* from *Firmicutes*; *Mycobacterium* and *Streptomyces* from *Actinobacteria*; Lladó et al., 2017), which together comprised > 12.5% of bacterial core reads.

For detecting differences on the cork oak core microbiome in each climatic region, the OTUs found in all soil samples from each climate were selected (Table S3). From the most humid to driest soils, a decrease on *Acidobacteria* ($p < 0.0001$) and *Proteobacteria* relative abundances ($p < 0.001$) were detected in core communities. In contrast, core *Actinobacteria* relative abundance increased in driest climates ($p < 0.0001$), in agreement with the relatively conserved response of bacterial communities associated to different plant species under drought (Naylor et al., 2017). In addition, the drought-resistant bacterial taxa *Chloroflexi* (Ochoa-Hueso et al., 2018) also revealed a significant increase on their relative abundance in driest climates ($p < 0.05$). These differences on core bacterial communities could be explained, not only by the nutritional requirements of bacteria (copiotrophic *vs.* oligotrophic), but also by the presence of different root exudates in soil. Drought-stressed plants are described to produce different exudate profiles able to recruit beneficial bacteria to cope with drought stress (Naylor and Coleman-Derr, 2018). Accordingly, in comparison with humid forests, cork oak driest forests presented a higher number of OTUs and relative abundances of some bacterial orders that have been previously found to be associated to plants under drought conditions, mainly *Rhizobiales*, *Burkholderiales*, *Shingomonadales* (all from *Proteobacteria*) and *Gaiellales* (from *Actinobacteria*). Other bacteria that typically thrive in dried soils, like *Myxococcales* and *Propionibacteriales*, were also increased in the driest forests. In order to evaluate which bacteria (OTU) could be indicators of specific climates, an indicator species analysis was performed with all microbiome data (*OTUs ≥5 reads* dataset). The results revealed that 28 OTUs (11 of which with an *IndVal* = 1) could be considered as indicators of humid forests, 11 (3 with *IndVal* = 1) as indicators of sub-humid forests and 21 (7 with *IndVal* = 1) indicators of arid/semi-arid forests (Fig. 4; Table S3). Many of these indicator species were present in the core cork oak microbiome (humid forests: 6 out 28; sub-humid forests: 3 out 11; arid/semiarid forests: 4 out 21), suggesting the role of the core community for the environmental adaptation of plants as proposed by Li et al. (2018). While most indicator OTUs (46%) from humid forests were *Acidobacteria* (mainly from the *Actinobacteriaceae* family), this phylum was less represented in sub-humid (18%) and arid/semi-arid forests (5%), which is in agreement with their sensitivity to drier conditions (Lladó et al., 2017; Naylor and Coleman-Derr, 2018). An opposite trend was detected concerning the number of *Actinobacteria* indicator OTUs: 43% in driest soils, followed by sub-humid (36%) and then by humid soils (25%). From *Actinobacteria*, indicator OTUs from *Frankiales* order were the most representative, being also present in the core microbiome of cork oak. These bacterial changes that occur in cork oak soils are most probably due to the plant genotype and plant metabolism under drought conditions, mainly through the production of specific root exudates, and could have impact on overall plant health and productivity (Vandenkoornhuyse et al., 2015; Naylor and Coleman-Derr, 2018).

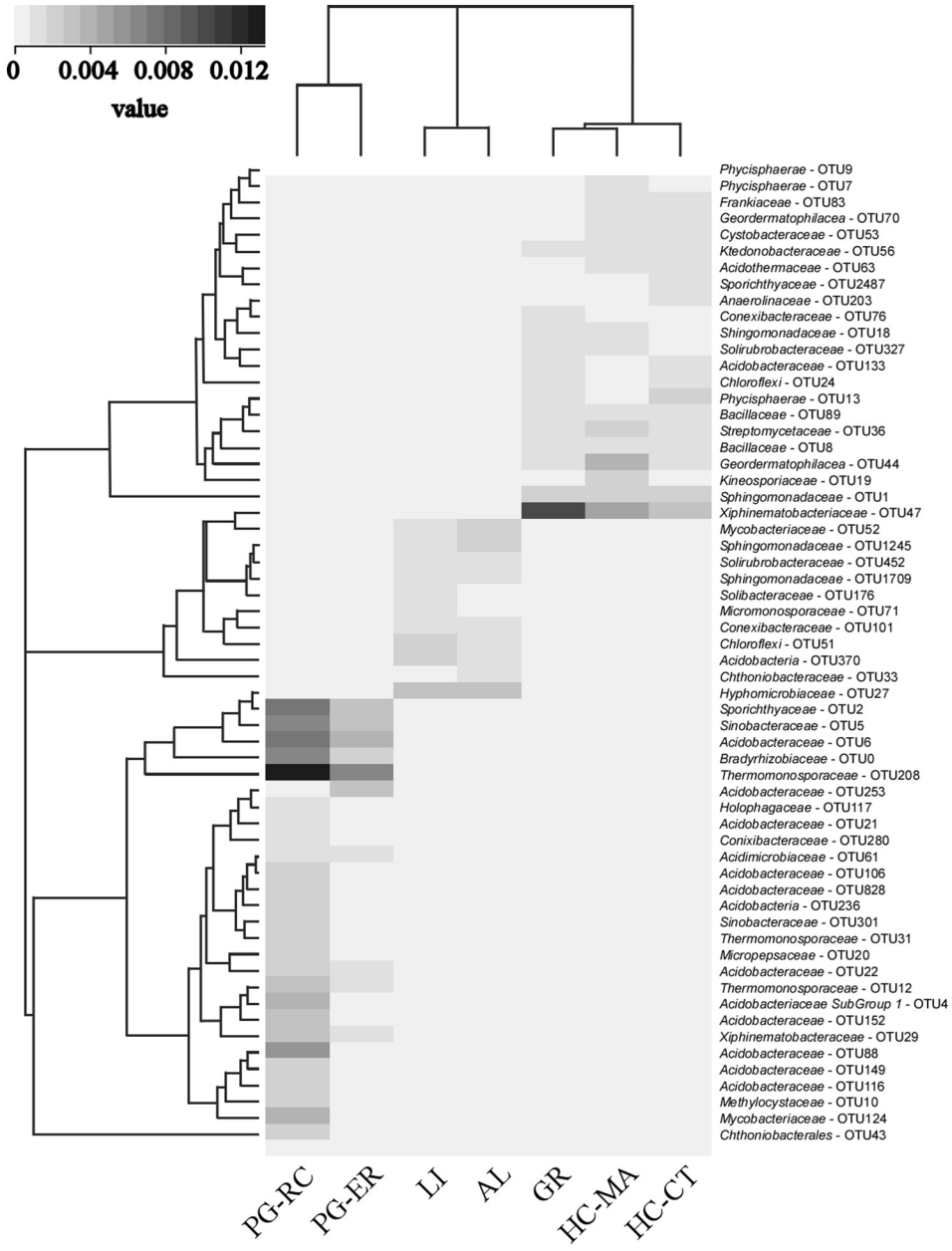


Fig. 4. Heatmap depicting the abundance of distinctive OTUs for each climate, as evaluated by species indicator analysis. Black color represents higher abundance and light grey color represents absence of related taxa. For each OTU, classification of the best BLAST hit is shown at the family level (whenever possible) or at a higher taxonomic rank. OTUs relative abundance was transformed as $\log_{10}(x + 1)$ for representation. Each site is referred by their code, as used in Fig. 1. Details about the represented OTUs and corresponding BLAST analyses are shown in Table S3.

4. Conclusions

Plants health and tolerance to climate changes are known to be closely tied to belowground microorganisms, but plants can also play an important role in the determination of soil microbiomes. In this work, we have disclosed part of the bacterial biodiversity present in cork oak forests. The diversity and composition of soil bacterial communities from cork oak forests along a climate gradient revealed that soil bacterial communities are strongly affected by the climatic regions where cork oak resides. Accordingly, the bacterial communities from humid, sub-humid and arid/semi-arid climates were clearly discriminated. This work revealed an important role of climate driving forces (especially precipitation) in shaping the soil bacterial communities, but the contribution of cork oak trees could not be underestimated. The conservation of a healthy root system could be responsible for the local shaping of bacterial communities and formation of specific microhabitats for soil microbes. The driest and warmer cork oak forests revealed more diverse soil bacterial communities, which includes bacteria taxa that are known to be typically associated to plants under drought. Furthermore, the occurrence of persistent mild drought conditions along time, should promote an adaptation of whole bacterial community to drier environments. Therefore, bacterial communities in driest cork oak forests are expected to better cope with drier and warmer climates. Not only due to the higher microbial diversity that could enhance the multiple ecosystem functions and services (Maron et al., 2018), but also due to their composition on drought-related bacteria that could help plants dealing with drought stress. This suggests a higher capacity of these bacterial communities to deal with global warming, while continuing to provide multiple ecosystem functions. Furthermore, bacteria could help plants to get prepared for facing mild drought conditions. But would bacterial communities always help plants to cope with climate changes? In a climate changing scenario, where extreme droughts will intensify the moderate but persistent drier conditions, the cork oak physiology would be severely imbalanced by water scarcity, mainly due to depletion of groundwater reservoirs (Barbeta et al., 2015). In this situation, bacteria would not be sufficient to halt forest degradation and cork oak decline. The high level of cork oak disturbance will lead to tree weakness and reduced cork

productivity, thus contributing for the decline of cork oaks.

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Data accessibility

Sequence data have been deposited in the National Centre for Biotechnology Information (NCBI) under the bioproject accession number PRJNA428525.

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