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Metagenomic characterization of soil bacterial communities in young chestnut orchards in northern Portugal

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Soil microorganisms play a crucial role in biogeochemical cycles and are key drivers of soil productivity. However, the soil bacterial community associated with the European chestnut (*Castanea sativa* Mill.) remains poorly characterized. In this sense, this study aimed to characterize the soil bacterial community in young chestnut orchards in northern Portugal and investigate its dynamics throughout the year. Soil samples were taken in three different periods of the year (spring, summer, and autumn) from two young chestnuts orchards located in Parada (41°38'12.53"N, 6°42'42.94"W) and Salgueiros (41°54'12.73"N, 7°01'40.95"W) with elevations of 740 and 1008 m, respectively. Soil DNA was extracted, and the 16S rRNA amplicons were sequenced using the Illumina MiSeq platform. Overall, the bacterial core of chestnut orchards predominantly consisted of four main phyla: *Proteobacteria*, *Acidobacteriota*, *Actinobacteriota*, and *Chloroflexi*, which were consistently present in both study sites and across all seasons. However, the phylum *Chloroflexi*, known for its metabolic and phenotypic diversity, exhibited the highest prominence at higher altitudes during autumn. *Acidobacteriales* and *Acidobacteriaceae* (Subgroup 1) of *Acidobacteriota*, as well as *Rhizobiales*, *Sphingomonadaceae*, *Rickettsiale*, and *Micropespsaceae*, *Burkholderiales*, and *Xanthomonadales* of *Proteobacteria*, as well as *Thermoleophila* and *Acidimicrobia* of *Actinobacteriota* were dominant across all seasons and in both study sites. This study provides valuable insights into the bacterial community associated with chestnut species and contributes to monitoring potential ecosystem changes resulting from climate change.

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Metagenomic characterization of soil bacterial communities in young chestnut orchards in northern Portugal

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Abstract

Soil microorganisms play a crucial role in biogeochemical cycles and are key drivers of soil productivity. However, the soil bacterial community associated with the European chestnut (*Castanea sativa* Mill.) remains poorly characterized. In this sense, this study aimed to characterize the soil bacterial community in young chestnut orchards in Northern Portugal and investigate its dynamics throughout the year. Soil samples were taken in three different periods of the year (spring, summer, and autumn) from two young chestnuts orchards located in Parada ($41^0$38'12.53" N; $6^0$42'42.94" W) and Salgueiros ($41^0$54'12.73" N; $7^0$01'40.95" W) with elevations of 740 and 1008 m, respectively. Soil DNA was extracted, and the 16S rRNA amplicons were sequenced using the Illumina MiSeq platform. Overall, the bacterial core of chestnut orchards predominantly consisted of four main phyla: Proteobacteria, Acidobacteriota, Actinobacteriota, and Chloroflexi, which were consistently present in both study sites and across all seasons. However, the phylum Chloroflexi, known for its metabolic and phenotypic diversity, exhibited the highest prominence at higher altitudes during autumn. Acidobacteriales and Acidobacteriaceae (Subgroup 1) of Acidobacteriota, as well as Rhizobiales, Sphingomonadaceae, Rickettsiale, and Micropespsaceae, Burkholderiales, and Xanthomonadales of Proteobacteria, as well as Thermoleophila and Acidimicrobia of Actinobacteriota were dominant across all seasons and in both study sites. This study provides valuable insights into the bacterial community associated with chestnut species and contributes to monitoring potential ecosystem changes resulting from climate change.

Keywords: Chestnut, bacteria, 16S rRNA, Illumina MiSeq

Introduction

Soil microorganisms are key factors in biogeochemical cycles and are important drivers of soil productivity. Changes in precipitation, temperature, and soil moisture patterns can significantly impact microbial communities, thereby influencing crop productivity. Therefore, understanding the structure of microbial communities and how they vary over time and space due to climate change is of utmost importance. In recent years, the European chestnut tree (*Castanea sativa* Mill.) has faced various stress factors including climate variations and increased incidence of pests and diseases (Fraga et al., 2011, Freitas et al., 2021). However, the soil bacterial community associated with European chestnut remains poorly characterized. Metagenomics is a valuable tool for analyzing the genomes of microbial communities in the environment samples, without prior cultivation (Prayogo et al., 2020). In this sense, this study aimed to characterize the bacterial community in the soil of young chestnut orchards in Northern Portugal and investigate its dynamics throughout the year using a large-scale approach based on high-throughput 16S rRNA sequencing.

Material and Methods

Study sites

The study was carried out in two three-year-old young chestnut orchards located in Northern Portugal, specifically in Parada (41°38'12.53" N; 6°42'42.94" W) and Salgueiros (41°54'12.73" N; 7^{0}01'40.95" W) at elevations 740 and 1008 m, respectively. The average annual rainfall in Parada is approximately 821.1 mm, while in Salgueiros it is around 1215.6 mm. The average annual temperature in the region is 12.6 °C, with the highest recorded temperature in August reaching 39.5 °C, and the lowest recorded temperature in February being -11.6 °C (IPMA I.P., 2021). Both soils are derived from schist and are classified as Dystric Leptosols. Before the establishment of the orchards, the soil in Salgueiros was previously used for cereal cultivation, while the soil in Parada was previously occupied by a pine forest.

These two chestnut orchards, labeled as 2 (Salgueiros) and 4 (Parada) in Figure 1, are part of a demonstration chestnut orchard network established by the GO_ClimCast project led by the Portuguese Chestnut Association-Refcast.

Figure 1. Location of the chestnut orchards in Salgueiros, Vinhais (number 2), and Parada, Bragança (number 4), under study, inserted into the ClimCast chestnut orchard network (Carneiro et al. 2022).

Soil sampling

Soil samples were taken from chestnut orchards in three different periods of the year: spring, summer, and autumn. A total of 15 soil samples were taken from each chestnut orchard with five samples collected during each sampling period (n = 3 seasons x 5 replicates = 15 samples). The samples were collected at a distance of 50 cm from the chestnut trunk, at a depth of 0-20 cm, excluding the topmost dry layer of approximately 5 cm. After collection, the samples were transported to the laboratory in a portable cooler bag, sieved, and then stored in a refrigerator until analysis. Chemical parameters of the soil such as pH, organic carbon, total nitrogen, extractable phosphorus, and extractable potassium were analyzed in the samples. For the analysis of the bacterial community, 3 random independent samples were considered for each sampling period in each chestnut orchard (n = 3 seasons x 3 replicates = 9 samples). These samples were analyzed individually.

Chemical analysis

The soil was analyzed for pH (H_2O) using a soil-to-solution ratio of 1:2.5 (w/v). Soil organic carbon was determined by the Walkley-Black method and total nitrogen by the Kjeldahl method. Extractable phosphorus and potassium were analyzed using the Egner-Riehm method as described by van Reeuwijk (2002).

Bacterial Community Analysis

DNA for metagenomic analysis was extracted from 18 soil samples collected across two sites and three different seasons (n = 2 sites x 3 seasons x 3 replicates = 18 samples) using the FastDNA® SPIN Kit for soil (MP Biomedicals, USA). DNA extractions were performed

according to the manufacturer's protocols. The extracted DNA was then stored at – 20 °C until further use. The concentration and quality of the extracted DNA were determined using a Qubit spectrophotometer (Thermo Scientific, USA) and agarose gel electrophoresis with a 1% gel, respectively. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were sequenced by Illumina MiSeq platform. The libraries were generated independently from each sample, without pooling DNA. Only an equimolar pool of all libraries for sequencing was prepared at a concentration of 4nM and loaded onto the MiSeq plataform. The NGS metagenomic analysis was performed at the Nucleotide Sequencing Service at the University of Salamanca, Spain.

Bioinformatic data processing

The sequencing quality of each individual sample was validated with FASTQC software (Andrews, 2010). A read filtering process was performed based on quality parameters using the R package DADA2 (Callahan et al., 2016), with reads trimmed at 230 bases for the left chain and 220 bases for the right chain. Subsequently, an independent analysis of the reads was carried out for bacteria detection. Taxonomic assignment was performed using the DADA2 package and the SILVA database (version 138) for bacteria (Callahan et al., 2016; Quast et al., 2013). The R package phyloseq and the Krona software (McMurdie and Holmes, 2013; Ondov et al., 2011) were employed to generate result tables of results and visualizations. Additionally, alpha diversity indices (Shannon-Wiener and Simpson) and beta diversity index (Bray-Curtis) were employed to assess the diversity of the bacterial community within the two chestnut orchards. Non-metric multidimensional scaling (NMDS) graphs were generated using the ordinate function of phyloseq package, employing Bray-Curtis distance.

Statistical analysis

The soil chemical properties data were analyzed using analysis of variance (ANOVA), followed by Tukey's test in JMPv11 (SAS Institute Inc., Cary, NC, USA). A significance level of $p < 0.05$ was applied.

Results and Discussion

Table 1 shows the chemical properties of chestnut orchard soils at 0-20 cm depth. Both soils exhibited acidic pH levels and low concentrations of organic carbon and extractable phosphorus. However, the chestnut orchard in Salgueiros demonstrated significantly lower values compared to the orchard in Parada, except for total N. The variations observed in these chemical properties can be attributed to the prior land use of these soils (cereal crop vs. pine forest), as well as to specific climatic factors such as temperature and precipitation, which may influence soil microbial activity.

Table 1. Chemical properties of chestnut orchard soils.

Parameters	Parada	Salgueiros
pH (H_20)	5.84 ± 0.07[a]	5.11 ± 0.07[b]
Organic C (g kg-1)	18.99 ± 0.23[a]	11.84 ± 0.41[b]
Total N (g kg-1)	0.62 ± 0.02[b]	1.34 ± 0.16[a]
Extractable P (mg kg-1)	19.29 ± 3.61[a]	17.54 ± 1.36[a]
Extractable K (mg kg-1)	170.46 ± 5.53[a]	82.46 ± 1.27[b]

Mean values ± standard deviation. Different letters per line indicate statistically significant differences ($p<0.05$)

The phyla detected in chestnut orchard soils, displayed with the KRONA software, are presented in Fig. 2. Overall, Proteobacteria, Acidobacteriota, Actinobacteriota, and Chloroflexi are among the most abundant bacterial phyla in the soil environment of both study sites. These findings align with previous studies on bacterial community composition in orchards (Bastida et al., 2017, Jin et al., 2022; Santolamazza-Carbone et al., 2023), grasslands (Li et al., 2022) and forest ecosystems (Kiu et al 2022, Li et al., 2022). The relative abundance of these bacterial phyla varies depending on the season and the specific site. The phylum Proteobacteria ranged from 19-23% and 13-19% in low and high-altitude chestnut orchard soils, respectively. This phylum of bacteria is commonly found in soil environments and is associated with a wide range of functions related to carbon, nitrogen, and sulfur cycling (Spain et al., 2009). Within the Proteobacteria, members belonging to the α-Proteobacteria (i.e., Rhizobiales, Sphingomonadaceae, Rickettsiales, and Micropepsaceae) and γ-Proteobacteria (i.e., Xanthomonadales) were dominant across all seasons and in both types of soils.

The phylum Acidobacteriota is commonly associated with oligotrophic and acidic soils (Kielak et al., 2016; Conradie and Jacobs, 2021). In this study, this phylum represented approximately 18-21% and 15%-22% of the total bacteria in the chestnut orchards of Parada and Salgueiros, respectively. The order Acidobacteriales, which includes acidophilic or acid-tolerant, mesophilic, and psychrotolerant bacteria (Ivanova et al., 2020), was more abundant in the more acidic soil (Salgueiros), representing 51-67% of the Acidobacteriota phylum, while it comprised 52-55% in the less acidic soil (Parada). Acidobacteriaceae (Subgroup 1) exhibited relative abundance in the high-altitude soil with pH 5.

Actinobacteriota, encompassing bacteria with mycelium and a wide range of reproduction forms and metabolic versatility (Barka et al., 2016), showed variations of 10-14% in Parada and 11-23% in Salgueiros. The predominant members of this phylum were *Actinobacteria*, *Thermoleophila*, and *Acidimicrobia*. These Gram-positive bacteria that play a crucial role in the decomposition process. particularly in the degradation of recalcitrant compounds such as cellulose and lignin (Barka et al., 2016).

The phylum Chloroflexi, known for its metabolic and phenotypic diversity and ability to adapt to different ecosystems, demonstrated the highest prominence at high altitudes (Salgueiros) during autumn (Fig. 2F), with a relative abundance of 22%, whereas it constituted only 8% of all bacteria in the low-altitude orchard (Parada). Ktedonobacteria was the most representative class of the phylum Chloroflexi representing in autumn 70% (high altitude) and 40% (low altitude) of the total of this phylum. This class is ubiquitous and characterized by its large genome size and complex life cycle, potential active producer of bioactive compounds, and ecologically versatile (Zheng et al., 2019). This class appears predominantly in oligotrophic and extreme environments (Zheng et al., 2019), having been found in several extreme biotopes, such as volcanic, Antarctic, and caves ecosystems (Yabe et al., 2017, Kudinova et al., 2021).

The alpha diversity measures (Shannon and Simpson indices) presented in Fig. 3 indicated a lower diversity of bacterial communities in the Salgueiros soil compared to the Parada soil. This difference was particularly pronounced during the summer (SN) and autumn (SR) periods. Non-metric multidimensional scaling (NMDS) analysis, based on Bray-Curtis dissimilarities showed a clear differentiation of the bacterial microbiome between the two chestnut orchards (Fig. 3).

The differences observed between chestnut orchards in the bacterial community are likely related to previous land use and seasonal variations at each study site. These findings are consistent with prior studies that have also highlighted the sensitivity of bacterial communities to these environmental factors (Kui et al., 2021).

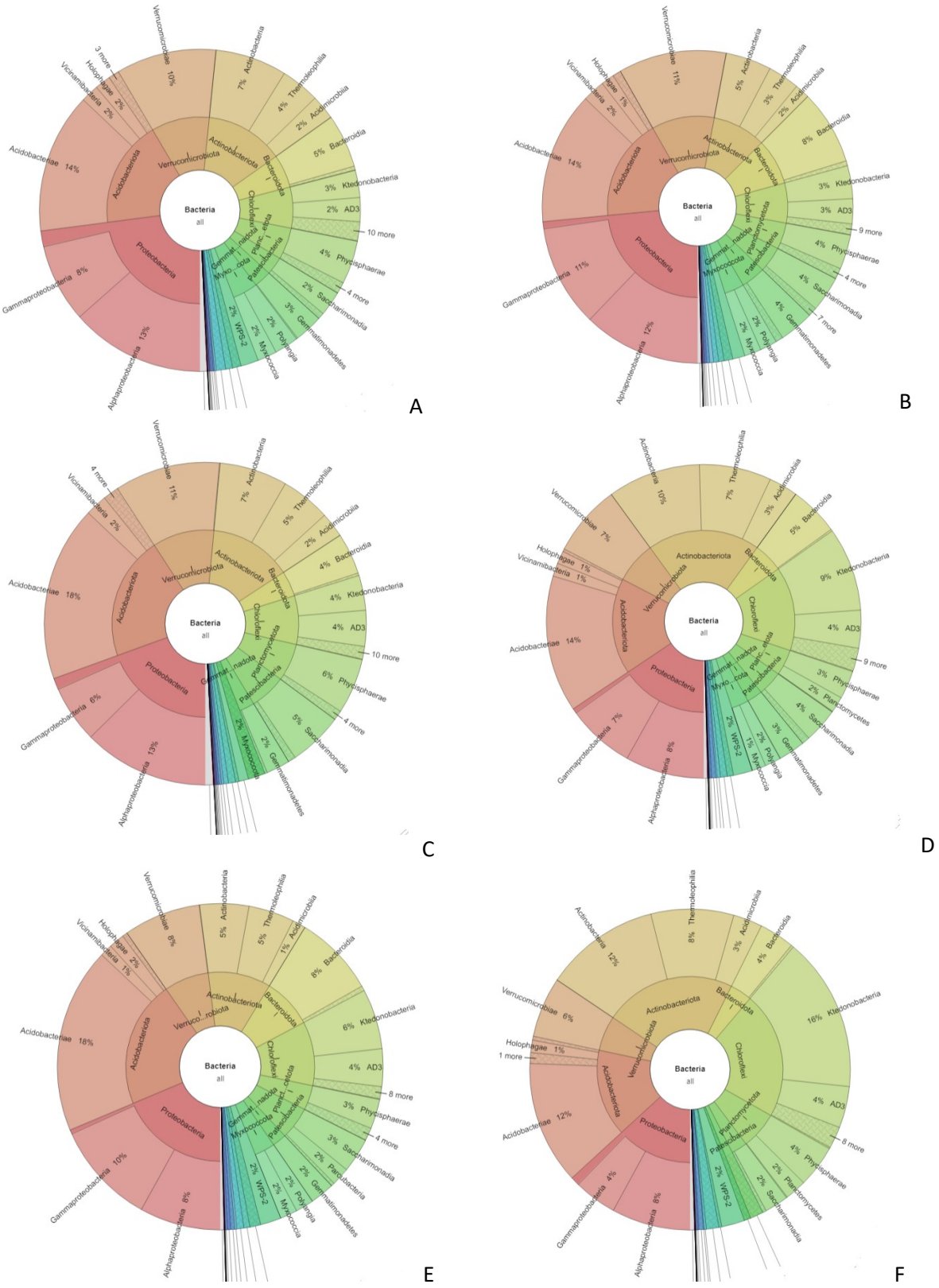

Figure 2. Krona graphical representation of taxonomic abundance in soils of chestnut orchards in Parada (Spring -A; Summer-B; Autumn -C) and Salgueiros (Spring – D; Summer E; Autumn – F).

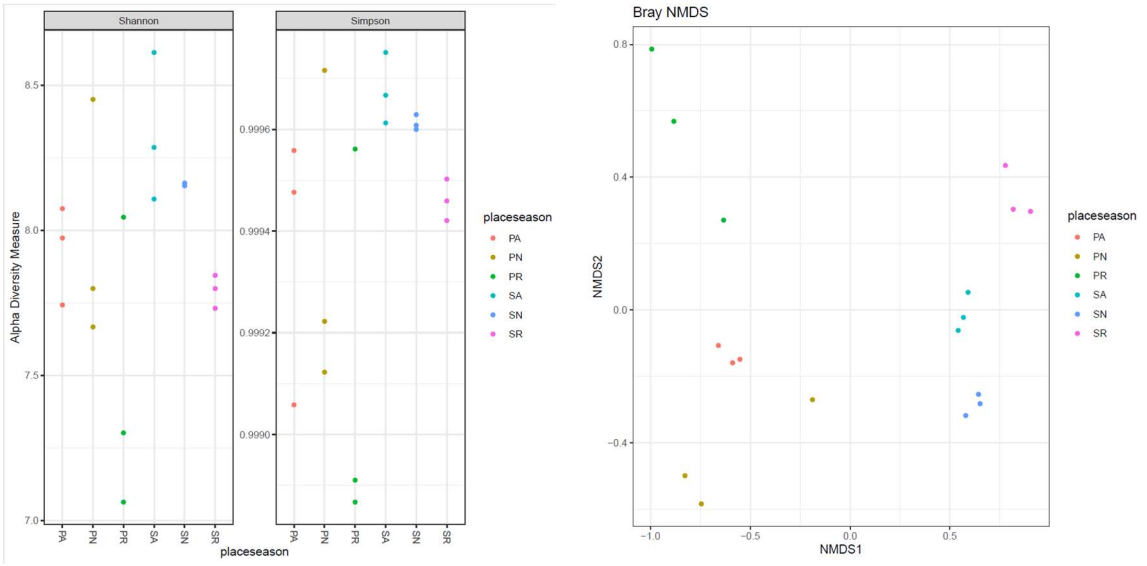

Figure 3. Alpha diversity measures (Shannon and Simpson indices) are depicted on the left, while the right shows Bray–Curtis dissimilarity-based non-metric multidimensional scaling (NMDS) ordinations of bacterial community composition (Parada (P); Salgueiros (S); A –Spring; N- Summer; R- Autumn).

CONCLUSIONS

The results of this study demonstrate that the bacterial core of chestnut orchards mainly consists of the phyla Proteobacteria, Acidobacteriota, Actinobacteriota, and Chloroflexi, which were consistently identified throughout the year. However, the relative abundance of these phyla varied depending on the season and the specific site. The Chloroflexi phylum was most prominent in the chestnut orchard located at high altitude (Salgueiros). Although the observed differences in the bacterial community between chestnut orchards suggest a correlation with previous land use and seasonal variations at each study site, it is important to note that further studies will be needed to draw more consistent conclusions. Specifically, these studies should explore how these communities will be affected by climate change and to what extent the microclimate, influenced by the trees, will impact them in the future. Nonetheless, this study serves as a valuable reference for future research in continuing to study bacterial communities in chestnut network orchards.

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