Uluslararası Tarım Araştırmalarında Yenilikçi Yaklaşımlar Dergisi
International Journal of Innovative Approaches in Agricultural Research 2023, Vol. 7 (4), 514-534
https://doi.org/10.29329/ijiaar.2023.630.12

Original article

Determination of the Bacterial Community in Soils Associated with Rare Wild Leguminous Species *Cicer Montbretii* Jaub. & Spach and *Lupinus albus* L. in Strandzha Natural Park

Mariana Petkova [a, *], Maryia Sabeva [b] & Nurettin Tahsin [c]

[a] Agricultural University Plovdiv, Faculty of Plant Protection and Agroecology, Department of Microbiology and Environmental Biotechnology, Plovdiv 4000, Bulgaria
[b] Institute of Plant Genetic Resources "Konstantin Malkov", Department of Plant Genetic Resources, Sadovo 4122, Bulgaria
[c] Agricultural University, Faculty of Agronomy, Department of Crop Science, Plovdiv 4000, Bulgaria

Abstract

The soil formation in Strandzha Mountain is influenced by the particular combination of the climate's unique forest tree vegetation, the extraordinary variety of root and soil-forming rocks, the hilly low-mountainous relief with significant fragmentation, a densely located hydrographic network with short slopes and dominant exposures. The diversity of soil microorganisms is crucial for plant growth and development and it makes it possible to understand in detail the plant-microbial interactions. The objectives of this study were to determine soil bacteria associated with rare wild leguminous species *Cicer montbretii* Jaub. & Spach (Constantinople chickpeas) and *Lupinus albus* L. (white lupinus) in Strandzha National Park. A new locality of Cicer montbretii Jaub was marked nearby village of *Brodilovo*. *L. albus* was found in saline-alkaline soil (A1) and yellow earth podzolic soils (A2) around the village of Brodilovo and the Great Pazvlak area. *C. montbretii* was found to grow on cinnamon forest soils (B1) and siliceous red soil (B2). A study was conducted by physio-chemical analyses and by assessing 16S rDNA metagenomics technique used to generate a total of 126,837 reads from the samples. The most significant number of observed species 2249 was found in soils saline-alkaline soil (B1) soil. According to that result, the higher diversity indices were calculated in the also in B1 soil. The α-diversity analysis reported yielded similar Shannon indices ranging from 8,322 in B2 to 9,337 in B1. The analyses revealed that B2 yellow earth podzolic soil, unique for Strandzha, has the largest composition with Proteobacteria 44% and the lowest in Actinobacteria 20%. Opposite, in A1 saline-alkaline soil have the richest composition of Actinobacteria 52% and the poorest in Proteobacteria 23%. C. montbretii was found in neutral A2 and B2 soils, while L. ablus prefer acidic A1 and B1 soils. The determination of the microbiological status of the soils associated with Constantinople chickpeas and white lupinus and the annual monitoring of the species in Strandzha Park will determine the methods for the most effective maintenance and storage outside their habitats.

Keywords: NGS, *Cicer montbretii* Jaub. & Spach, *Lupinus ablbus* L., expedition, Strandzha Nature Park.

Received: 09 October 2023 * **Accepted:** 17 December 2023 * **DOI:** https://doi.org/10.29329/ijiaar.2023.630.12

* **Corresponding author:**

Mariana Petkova, Agricultural University Plovdiv, Faculty of Plant Protection and Agroecology, Department of Microbiology and Environmental Biotechnology, Plovdiv 4000, Bulgaria.
Email: mpetkova@au-plovdiv.bg

INTRODUCTION

The territory of the Strandzha Natural Park falls into the Mediterranean soil area of Europe. The park is part of the Strandzha province, characterized by brown forest soils along the crests of the hills instead of on the slopes and wetter soils.

Within the boundaries of Strandzha Park is the only distribution of yellow soils and red soils in Bulgaria. The development of the yellow podzolic soils under the southern Euxine forest vegetation distinguishes them from the soils of Mediterranean Europe and reveals their natural connections with the low valleys of the Caucasus. Typical for Bulgaria and Europe is the weathering of the soil-forming rocks to alumino-silicate minerals. The yellow podzolic soils are formed by the additional weathering of the rocks, common in humid and warm subtropical areas. In the conditions of greater humidification and increased temperature, rapid decomposition of organic matter occurs and deep washing of bases and silicates from the soil profile. For this reason, yellow podzolic soils are strongly acidified and enriched with aluminium and iron oxides, giving them their characteristic yellow colour. Two soil subtypes are common - ordinary and podzolic.

Microbial communities provide valuable data for studying the basic processes taking place in the environment (Derry et al. 1999). Functional diversity and community structure of micro-organisms in three arctic soils as determined by sole-carbon source-utilization. Microorganisms are present in all habitats and are the first organisms to respond to chemical and physical changes occurring in the soil. Since soil microorganisms are involved in the soil formation processes, changes in microbial communities are often an indicator of changes in the functional structure of the respective ecosystem (Dokic et al., 2010; Kent and Triplett, 2002). Cluster analysis was used to construct a cluster tree to examine the similarity between different samples. The Unweighted Pair-Group Arithmetic Mean (UPGMA) method is a hierarchical clustering method widely used in ecology to classify samples.

This paper reports the results obtained from an expedition survey of several localities of *C. monbretii* and *L. albus* in Strandja Nature Park. Due to increasing human activity, many wild plants may become extinct in the near future. Faced with this enormous prospect, plant conservationists must take advantage of every available technique (Karron et al., 1991; Petkova et al. 2023). The present study aims to reliably establish the relationship between microbial diversity and the geographic distribution of wild legume species. The variety of micro-organisms in the rhizosphere is essential to ecology, as it enables a detailed understanding of plant-microbe interactions. Microorganisms play a major role in the functions of ecosystems, which makes them unique due to the different microbial composition and is directly related to plant species and their abundance.

MATERIALS AND METHODS

Expedition survey

The scientific team involved in the project conducted three expeditions in 2022. When surveying the deposits of the studied species, the first expedition was conducted at the end of April, when the studied species were in the phase of mass flowering and beginning of bean formation. The second survey was carried out in the early ripening phase. In the third expedition, the survey was conducted in the full maturation phase. Seeds of both species were collected – *C. montbretii* and *L. albus* in quantity according to the provisions of Strandzha Natural Park. A new locality of *Cicer montbretii* was marked around village of Brodilovo, Strandzha Natural Park. Brodilovo is in a fertile valley ringed by mountains, located at (42°5′N27°51′E), 56 meters above sea level, close to the Black sea.

Physico-chemical and microbiological analysis of the rhizosphere soil in the habitats of Cicer montbretii and Lupinus ablus

Soil samples were taken from the rhizosphere layer of plant's roofs (around 2-5 cm) and were cleaned from the roots, pebbles and other impurities. For greater accuracy of the research, an average sample is taken, i.e. the sample obtained from the soil taken from the two diagonals of the forest plots with deposits is averaged. Soil sampling for determination of pH, electrical conductivity, organic carbon, organic matter, C/N ratio, total nitrogen, available nitrogen and microbiological status was performed in the Department of Agrochemistry and Soil Science of the Agricultural University – Plovdiv as published by Petkova et al. 2023 according to following the Kjeldahl Method (Amin & Flowers, 2004). Soil pH and electrical conductivity were determined water: bulk soil suspension at the ratio of 1:1 (v/w) using a pH meter and a conductivity meter, respectively, according to Deribeeva's method (1986, ISO 10390:2005).

Isolation and purification of DNA from soil and metagenomic sequencing

DNA extraction and purification were obtained using the above-described DNA extraction method from (A1) soils saline-alkaline soil around the village of Brodilovo associated with *L. ablus*, (A2) and yellow earth podzolic soils from the village of Brodilovo associated with *C. montbretii* and *L. ablus*, (B1) cinnamon forest soils and (B2) siliceous red soil the Great Pazvlak area and amplification of PCR products for the 16S region with 16SV34 primers (CCTAYGGGRBGCASCAG, GGACTACNNGGGTATCTAAT) was successful for the fifth samples. The amplicon was sequenced on an Illumina double-end platform to produce 250 bp raw double-end reads (Raw PE) and then pooled and preprocessed to obtain clean tags (Krstić Tomić et al. 2023). Chimeric sequences in the pure markers were detected and removed to get the effective markers that could be used for subsequent analysis as published by Chopkova et al. 2023 and Krstić Tomić et al. 2023. Metagenomic sequencing was done at Novogene (Cambridge, UK). Library preparation was done with the Nextera DNA Flex kit (Illumina)

following a standard procedure. The exact amount of PCR products from each sample was pooled, A-tailed and further ligated with Illumina adapters. The amplicon was sequenced on an Illumina double-end platform to generate 377 bp raw double-end reads (Raw PE) and then pooled and preprocessed to obtain clean tags.

Data were processed using QIIME software v.1.9.1 (http://qiime.org/), as published by Caporaso et al. 2010. The first stage of 16S rRNA gene analysis involves quality control of sequences to exclude from analysis those less than 200 nucleotides in length, with a quality score of less than 25, with misread primer sequences and multiplex identifiers, extensive homopolymeric repeats (more than 8 nucleotides) and unidentified nucleotides. Chimeric sequences in clean tags were removed to obtain efficient tags that were used for subsequent analysis (Martin, 2011; Haas et al. 2011; Edgar, 2013).

OTUs were selected at greater than 97% similarity. Scores of ACE and Chao indices and Shannon and Simpson diversity indices were calculated using the Mothur program (Turak et al. 2017; Abell et al. 2005). Alpha diversity metrics summarize the structure of an ecological community by measuring the number of taxonomic groups along with group abundance, as published by Willis (2019). Alpha diversity was analyzed using six indices, including observed species, ACE, Shannon, Simpson, Chao1, and good cover, and calculated using QIIME (Version 1.9.1, http://qiime.org/1.9.1/) and displayed with R software (Version 2.15.3) (Navas-Molina et al. 2013; Zhang et al. 2019). The heat map based on weighted Unifrac and unweighted Unifrac distances was analyzed with R software (Version 2.15.3). Beta diversity represents the explicit comparison of microbial communities based on their composition. Beta-diversity metrics thus assess differences between microbial communities (Carvalho et al. 2016). To compare the microbial communities between each pair of community samples, a square matrix of the “distance” or “dissimilarity” was calculated to reflect the difference between certain samples as unweighted Unifrac and weighted Unifrac distance with R software (Version 2.15.3). The same R software was used to find the differences in dominant taxa among the three groups of samples at each taxonomic rank, the top 10 taxa with the mean abundance of the three groups of samples at each taxonomic rank were selected to generate a triple plot. OTU comparisons were performed using the Venn diagram package (Zhang et al. 2019). All tests of significance were two-tailed and values of $p < 0.05$ were considered statistically significant. In our 16S information analysis process, the input file of PCA is the beta-diversity distance matrix, which is the difference value matrix composed of the OTU abundances of two samples.

RESULTS AND DISCUSSION

Discription of Cicer montbretii and Lupinus albus habitat

With the help of the experts from Strandzha Natural Park, a new locality of *Cicer montbretii* was marked around village of Brodilovo. The deposits are located in an oak forest, on leached cinnamon-

forest soil. Constantinople chickpeas is represented by single plants and by groups of 5, 10, and 20 plants, scattered on the slope between the stream and the oak forest, as well as in open places (Fig. 1A). The number of flowers are from 2 to 5, rarely single, in loose racemes in the axils of the leaves. The bean pots are smooth, wide, oblong, brown, with 3 - 4 seeded. The seeds are globose, brown or black.

A locality of *Lupinus* ssp. was also discovered in the immediate vicinity, in an area above the village of Brodilovo, by a stream at an altitude of 92 meters. The *Lupinus* species is near an oak forest (Fig. 1B). It is located in an open rocky area with bushes of the family *Oleaceae*, *Phyllirea latifolia*. The species has a stem height of about 30 cm, upright, branched, covered with white hairs. The leaves have a palmate shape. The flowers are alternately arranged, with a pale blue color. The bean is oblong, fibrous, pinched, with 4-6 seeds. The seeds are flattened, white, and smooth. The main accompanying species in the locality of both species are *Sedum anglicum, Cancalis platicarpus, Aegilops cylindrica, Aegilops geniculate, Avena sp., Cynosurus echinatus, Trifolium angustifolium, Hypericum perforatum, Althea rosea, Ajuga laxmannii, Therucrium lamiifolium* is listed in the Red Book of threatened extinction species.

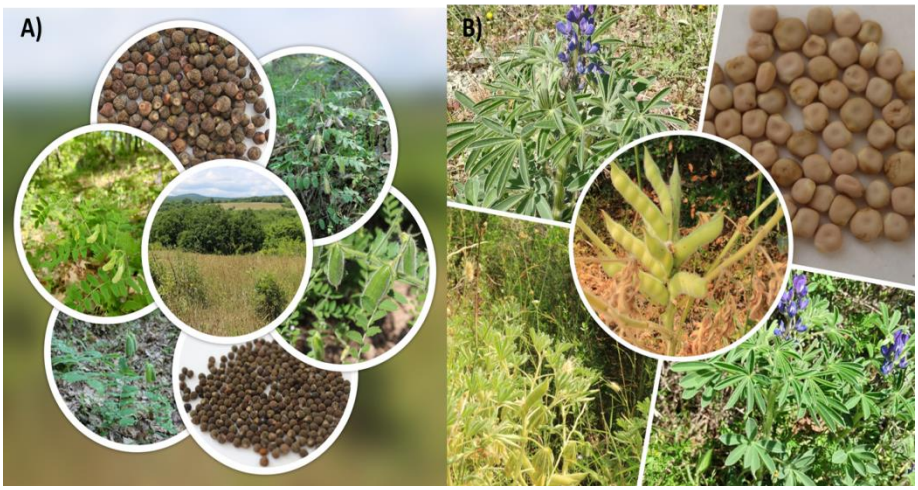


Figure 1. Pictures from *Cicer montbretii* and *Lupinus albus* new habitat found around village of Brodilovo.

Physico-chemical and microbiological analysis of the rhizosphere soil in the habitats of Cicer montbretii and Lupinus ablus

Soil pH values are important not only for the physiology of microbial cells but also for nutrient availability. Many of the nutrient soil resources have pH solubility around the neutral point. Although most microorganisms grow over a relatively wide range of pH, their activity is maximal in a neutral environment. In a neutral environment, the activity of enzymes is activated, in the processes of entry of substances into the cell, etc. In this sense, the studied soils have a close to neutral reaction and a better representation of the main groups of microorganisms can be expected (Dokic et al, 2010; Kent and

Triplett, 2002). pH of A1 and B1 was close to acidic with values 5.97 and 5.60, respectively. A2 and B2 are neutral soils (Table 1).

The measurement of soil electrical conductivity indicates the content of readily soluble salts. This is a way of tracking the movement of available forms of nutrients in the soil profile and their spatial accessibility to the plant root system. The data from the present study show that all the studied soils have a very low electrical conductivity of 46 – 92 μS/cm.

The obtained results on the content of total nitrogen in the investigated forest soils from the Strandzha Natural Park (Table 1) show that it varies from low in both Brodilovo soils A1 and A2 (50.32 mg/ 1000 g of soil and 55.60 mg/ 1000 g soil) to average stocked at B1 and B2 with values of 68.05 and 59.86 mg/ 1000 g soil, respectively. The lowest content of NH4 was reported in B1 and the highest in sample A1. The amount of NO_3 ranged from 16.35 mg/ 1000 g of soil in B1 to 28.30 mg/ 1000 g of soil in B2.

All the rhizosphere soils are characterized by low phosphorus content, measured as P_2O_5 mg/ 1000 g of soil. Similar to the results of Petkova et al. 2023, the highest value of P_2O_5 content 4.53 was found in the B1-cinnamon forest soil from the roots of C. montbretii. Cinnamon forest soils (B1) and (B2) siliceous red soil in the Great Pazvlak area also has a high supply of potassium, while in the samples of the remaining soils (Table 1).

Table 1. Physicochemical parameters and agrochemical analysis of soils.

Physicochemical parameters			Agrochemicals analysis		
Soil Type	pH	Electrical conductivity μS/cm	N mg/1000 g	P_2O_5 mg/100 g	K_2O mg/100 g
A1	5.97	46	NH_4 – 35.73 NO_3 – 23.57 Total N – 50.32	2.50	18.34
A2	6.04	77	NH^4 – 49.32 NO_3 – 27.25 Total N – 55.60	3.66	42.86
B1	5.60	92	NH_4 – 18.23 NO_3 – 16.35 Total N – 68.05	4.53	60.43
B2	6.51	72	NH_4 – 22.92 NO_3 – 28.30 Total N – 59.86	3.82	70.29

Metagenomic analyses

After quality filtering, a total of 507,350 (average 92523 per sample) sequence reads were clustered into OTUs (average 2264 per sample) and the result is shown in figure 2. The remaining sequences (unique tags: 11447) were not related to any known bacterial sequences in the public database. These results indicate that the sequencing depth is sufficient to capture a respectable number of observed bacterial species. The A1 and B1 soils had the highest number of species observed while having advanced bacterial diversity compared to the other soils.

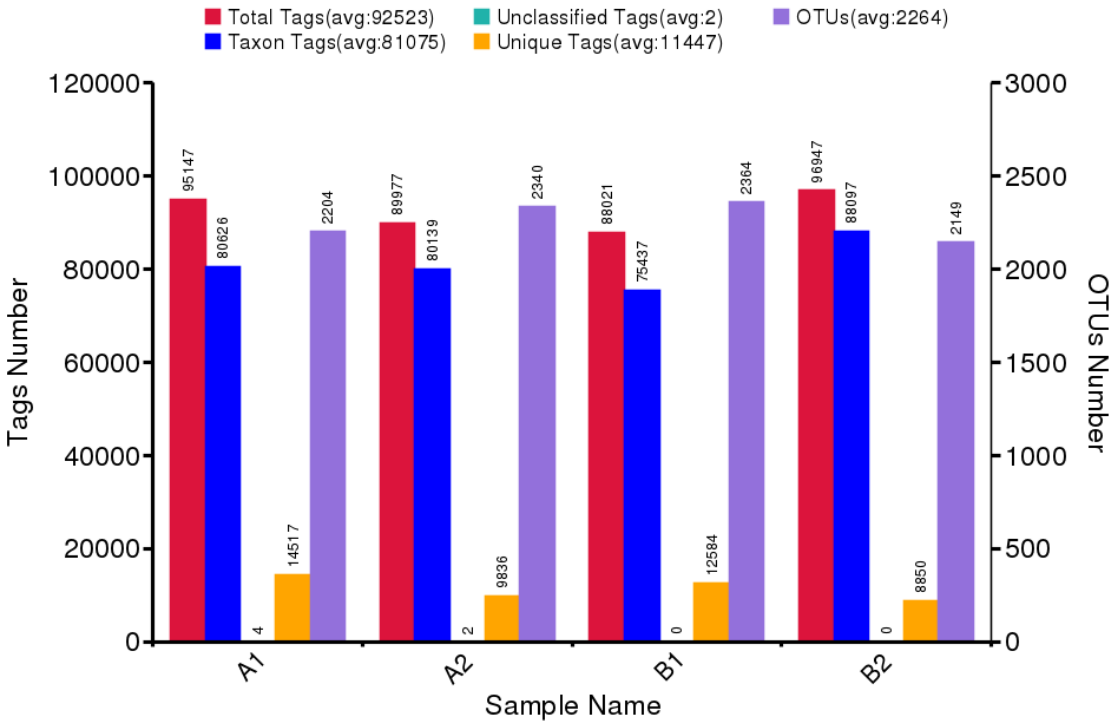


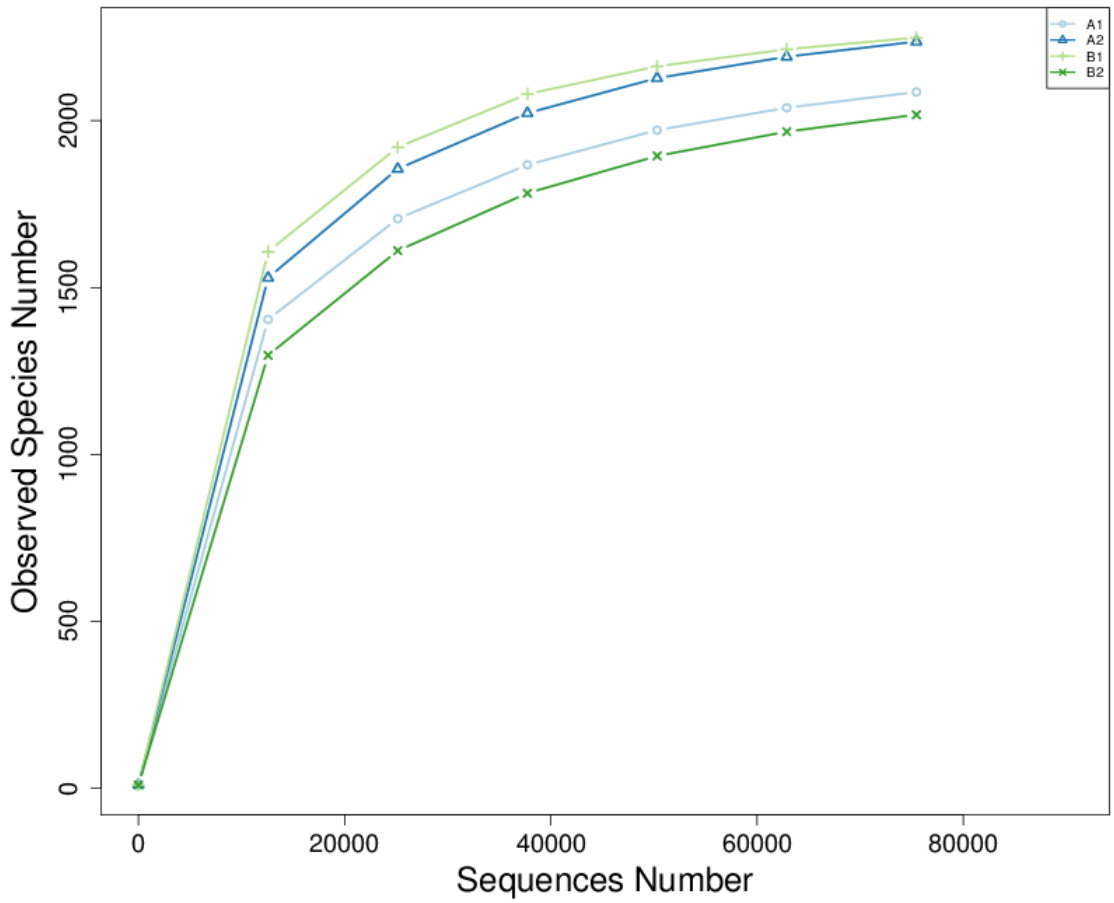
Figure 2. OTUs number of each soil sample OTUs as follow (A1) soils saline-alkaline soil around the village of Brodilovo associated with *L. ablus*, (A2) and yellow earth podzolic soils from the village of Brodilovo associated with *C. montbretii* and *L. ablus*, (B1) cinnamon forest soils and (B2) siliceous red soil the Great Pazvlak area.

Rarefaction curves and Rank abundance curves of quantitative diversity are widely used to indicate the biodiversity of samples. The rarefaction curve is created by selecting an arbitrary amount of sequence data from the samples, then counting the number of species they represent (the number of OTUs) (Chopkova et al. 2023). Rarefaction curves can directly reflect the rationality of the sequencing data volume and indirectly reflect the richness of the microbial community in the samples. If the curve

is steep, many species remain to be discovered. Rarefaction curves of four different soils are shown in Figure 3, and B1 showed bigger richness, which was higher than A2, A1, and B2.

The abundance curves in figure 3B are used to sort according to the number of sequences included in sequencing analysis. Similar to the analysis of microbial community richness, the relative abundance of species in the soils form Brodilovo region, where steeper slopes reflect greater dominance. Conversely, flatter slopes in B1 and B2 indicate greater uniformity according to Whittaker, 1969.

A)



B)

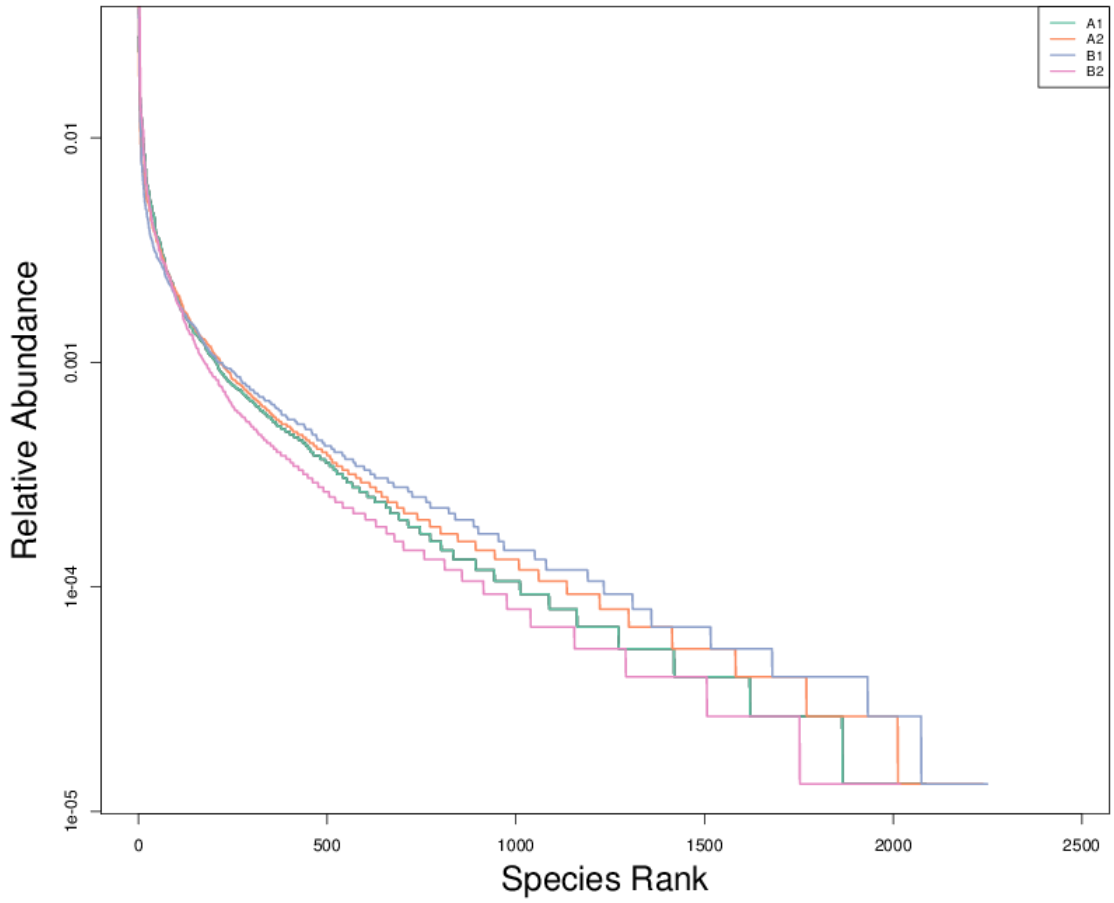


Figure 3. A) Rarefaction curves of four different soils from Strandzha Natural Park. B) Rank Abundance curves. The sequences number is on the X-axis and the observed OTUs number is on the Y-axis. For the Rank abundance curves, each curve represents a single sample, plotted by OTU relative abundance on the Y-axis and the OTU abundance rank on the X-axis.

Relative microbial diversity

According to the results of the taxonomic annotations, the relative abundance of taxa in the phylum is illustrated below in Fig 4.

The highest amount of Actinobacteria (52%) was found in the soils saline-alkaline soil around the village of Brodilovo associated with L. ablus (A1) while in the other soils, the percentage ranges from 20% to 24%. conversely, the amount of Proteobacteria and Acidobacteria increases in A2, B1, and B2 soils compared to A1 soil (Fig. 4) The highest amount (44%) of Proteobacteria was observed in B2-siliceous red soil and the smallest in A1-soils saline-alkaline soil. Firmicutes and Nitrospirota are presented in insignificant quantities around 1-2% in those soils.

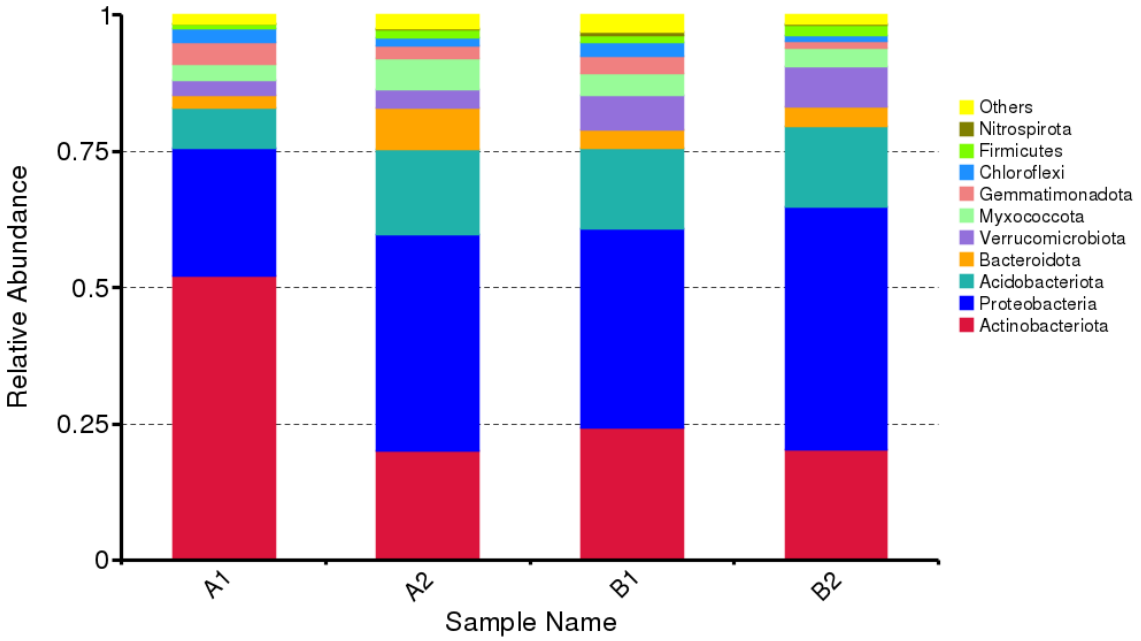


Figure 4. Relative abundance of taxa at the phylum level. A1) soils saline-alkaline soil around the village of Brodilovo associated with *L. ablus*, (A2) and yellow earth podzolic soils from the village of Brodilovo associated with *C. montbretii* and *L. ablus*, (B1) cinnamon forest soils and (B2) siliceous red soil the Great Pazvlak area.

Three major bacterial classes were identified and among them, Actinobacteria (20-52%) and Alphaproteobacteria were the most dominant group (17-25%), Gammaproteobacteria (6-23%), and Acidobacteria (8-16%) (Figure 4 and 5).

In saline-alkaline soil (A1) around the village of Brodilovo associated with L. ablus dominated microbial species belonged to *Pseudonocardia*, *Amycolatopsis*, *Streptomyces* spp., *Solirubrobacter*, and *Nocardioides*. *Actinomycetes* comprise an important group with great potential to synthesize bioactive secondary metabolites (Fig. 5). The saline-alkaline soil refers to the soil type which has the simultaneous existence of high salt concentration and a higher pH in the soil. Nitrogen-fixing bacteria *Beijerinckii* (2%) was found together with Bradyrzhizobium (5%). There are limited reports on salt-tolerant azotobacters. Generally, one would not expect a high abundance of acetobacter in halophilic environments which are unfavourable for the growth of bacteria according to Steila and Pond, (1989). *Beijerinckii* and *Bradyrzhizobium* are involved in symbiotic bradyrhizobia and play an important role in legume growth and development under nitrogen-limiting conditions via their active conversion of

atmospheric N2 into plant-assimilable ammonium using the nitrogenase enzyme complex (Wongdee et al. 2023; Dixon and Kahn, 2004).

In yellow earth podzolic soils from the village of Brodilovo associated with *C. montbretii* and *L. ablus* observed distribution of the Proteobacteria 40%, Acidobacteria 16% and Actinobacteria 20%. The greater abundance from the phylum Proteobacteria are sequenced bacteria from genera *Solibacter, Phenylobacterium*, and *Reyranella*. Actinomycetes are presented by Mycobacterium and *Acidothermus* around 2%. *Acidobacteria* are the most abundant in A2 soil and include the genera *Solibacter* 3%, *Bryobacter* 2%, Granulicella 1%. These results demonstrated that at the genus level, we observed that the relative abundance of certain bacterial genera was affected by both plants *C. montbretii* and *L. ablus* distribution. *Solibacter* is known to produce enzymes that break down organic carbon (Pearce et al. 2012). Zhang reported in 2018 that the genera *Bryobacter, Candidatus Solibacter,* and *Bradyrhizobium* dominated in the excessively managed forest soils, and their relative abundance was significantly higher than in the ecologically managed forest soils. Thus yellow earth podzolic soils which are unique for the European forest soils are well-preserved soils.

In cinnamon forest soils associated with *C. montbretii* (B1) are dominated by actinomycetes from genera Microthrichales, Gaiella, and Streptomyces and acid bacteria from genera *Vicinamibacter* and *Blastocatella*. It was confirmed that *Gaiella* and *Streptomyces* (affiliated with phylum Actinobacteria) were core genera responsible for substrate mineralization (Jeewani et al. 2021). Phylum Proteobacteria is presented by *Rhodoplanes* and *Caulobacter.*

L. albus was grown on siliceous red soil in the Great Pazvlak area where the soil was occupied by phylum Proteobacteria. *Burkholderia* 13%, Sphingomonas 4%, *Bradyrhizobium* 5%, and *Nitrosomonas* 1% were the most abundant genera from the phylum *Actinobacteria*. Actinobacteria had a higher frequency of OTUs related to *Acidothermus* rather than *Micromonospora* and *Nocardia.*

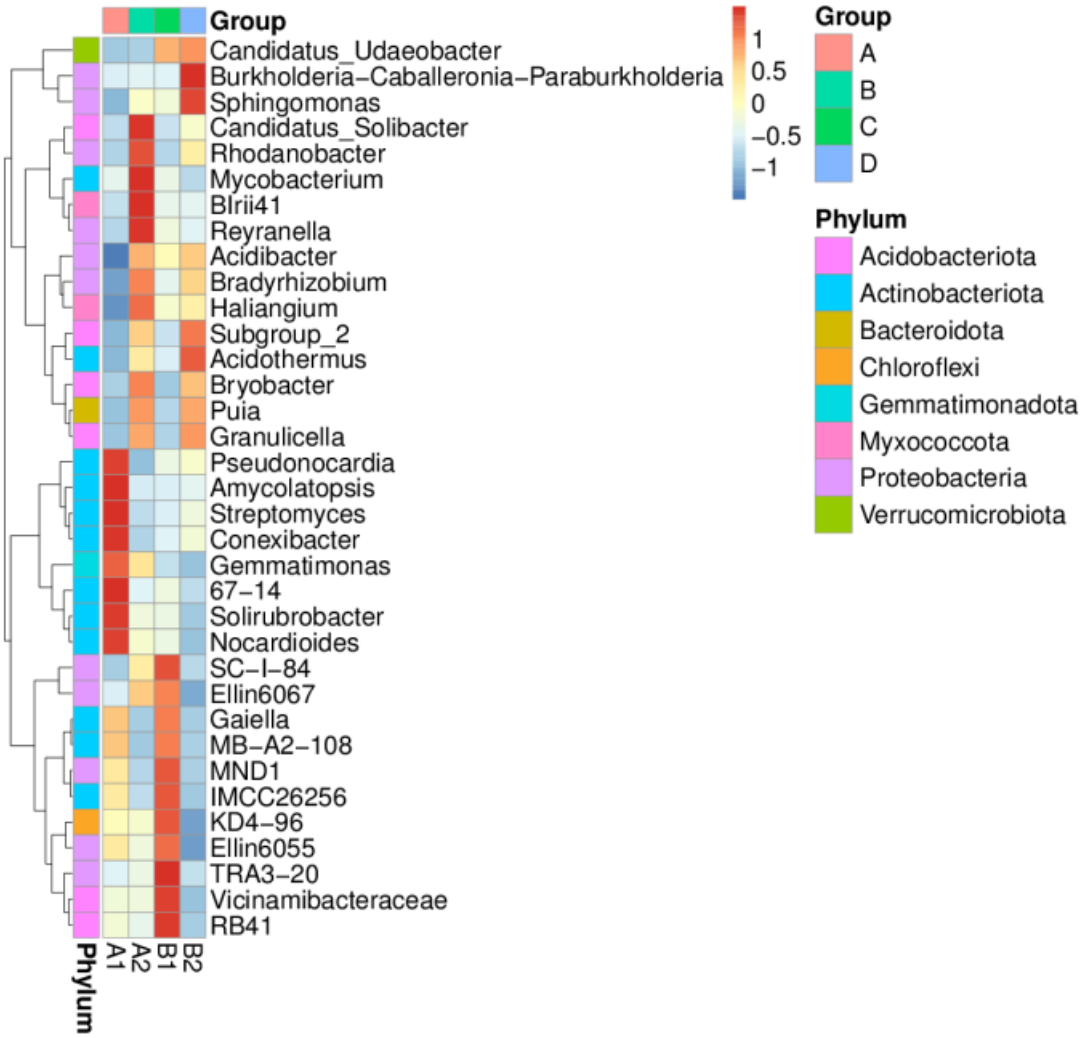


Figure 5. Taxonomic abundance cluster heatmap. (A1) soils saline-alkaline soil around the village of Brodilovo associated with *L. ablus*, (A2) and yellow earth podzolic soils from the village of Brodilovo associated with *C. montbretii* and *L. ablus*, (B1) cinnamon forest soils and (B2) siliceous red soil the Great Pazvlak area.

Alpha diversity

In microbial ecology, analyzing the alpha diversity of amplicon sequencing data is a common first approach to assessing differences between environments (Willis, 2019). Diversity indices (Simpson and Channon) and richness indices (Ace and Chao1) were calculated to estimate the alpha diversity of the bacterial communities in the oat soils and oat and vetch intercropping soils (Table 2). Table 2 shows the richness and diversity of bacterial communities in the four Starndzha soils. The lowest number of

prokaryotic species observed was found in the B2 red soil 2018, followed by the A1 soil 2086 species. The highest number of 2249 observed bacterial species was found in cinnamon soil B1 (Table 2).

Table 2. Indices of alpha diversity of Strandzha soils

Sample name	observed_species	shannon	simpson	chao1	ACE	goods_coverage	PD_whole_tree
A1	2086	8.929	0.995	2184.821	2198.988	0.997	162.643
A2	2237	9.130	0.994	2340.704	2348.650	0.997	173.377
B1	2249	9.337	0.995	2355.469	2321.428	0.998	166.408
B2	2018	8.322	0.988	2160.692	2171.487	0.996	153.686

Difference in alpha diversity indices between groups

Boxplots were formed to analyze the difference in alpha diversity indices between soil groups. T-test, Wilcox and Tukey tests were performed to analyze the significance of differences between groups. Boxplots based on the number of observed species and Shannon indices are shown as follows in Figure 6. The comparative characteristic of this mathematical dependence shows that the number of species in A1 and B2 are similar and therefore these soils are characterized by the same ecological indices, which are based on comparable physicochemical parameters. A2 and B1 also show similar blot box positions and therefore are characterized with analogous parameters and the number of microbial species.

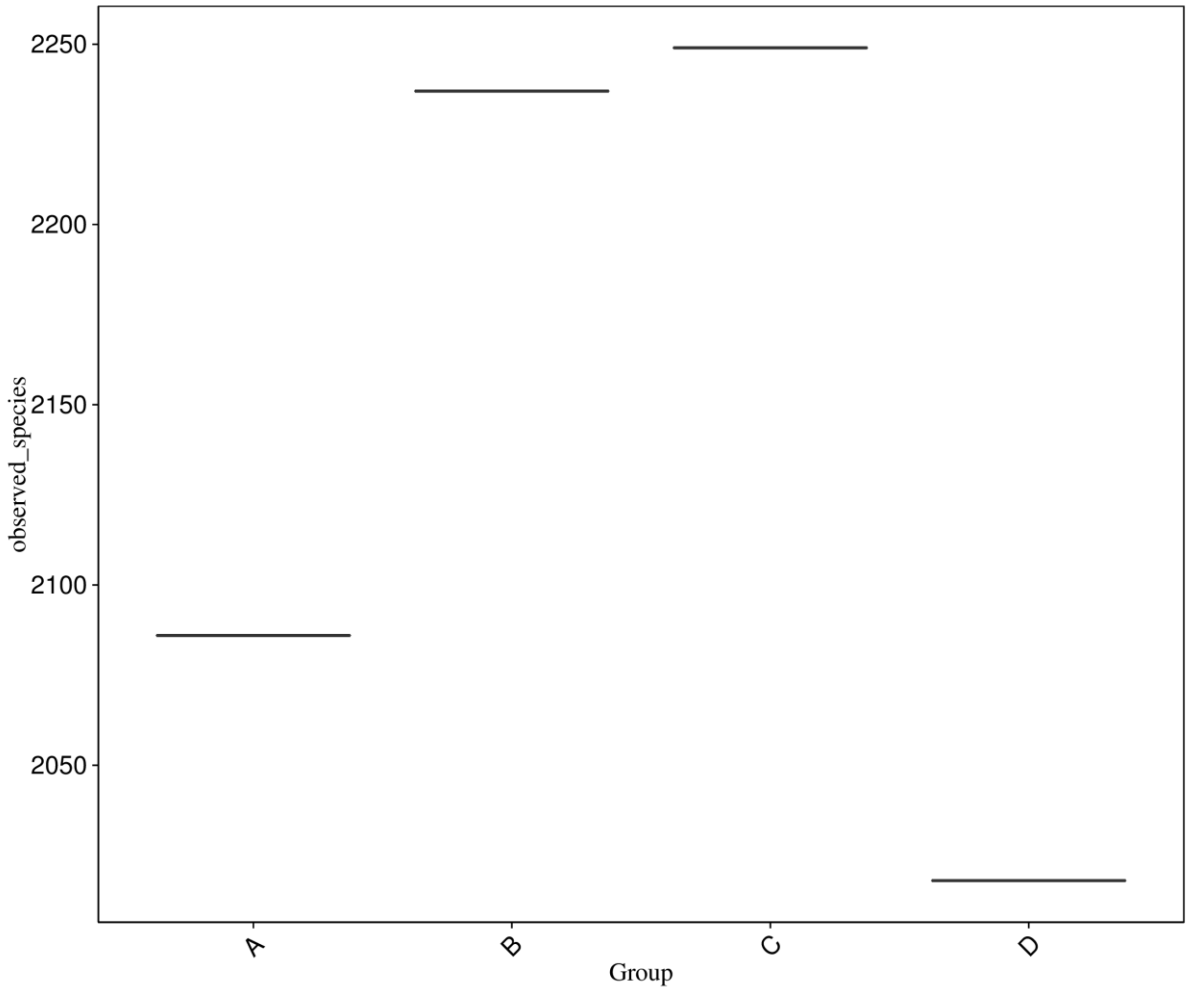


Figure 6. Boxplots to analyze the difference in alpha diversity indices between groups. (A) soils saline-alkaline soil around the village of Brodilovo associated with *L. ablus*, (B) and yellow earth podzolic soils from the village of Brodilovo associated with *C. montbretii* and *L. ablus*, (C) cinnamon forest soils and (D) siliceous red soil the Great Pazvlak area.

Venn and Flower diagram

According to the result of the clustering analysis, operational taxonomic units were normalized and used to analyze both the common and unique information of different samples (groups), and on this basis the Venn and Flower diagram was generated in Figure 7.

The diagram in Figure 7 compares the bacterial diversity in the different Strandzha soils. A total of 5783 OTUs were differentially investigated at different stages of composting. A total of 1244 OTUs were shared by all four soils. The greatest overlap was observed at 181 species between soils from Brodilovo region (A) saline-alkaline soil around the village of Brodilovo associated with L. ablus and (B) yellow earth podzolic soils from the village of Brodilovo associated with C. montbretii and L. ablus. In the comparison of soils associated with C. montbretii, 105 were common species and the sum of

shared species with the remaining samples was found to be 1624, which is 6.47%. The lower unique OTUs number of 126 was found in the siliceous red soil in the Great Pazvlak area (D) soil and the highest in cinnamon forest soils (C). This revealed that the number and species that showed changes in growth and development across all four developmental stages were distinctive.

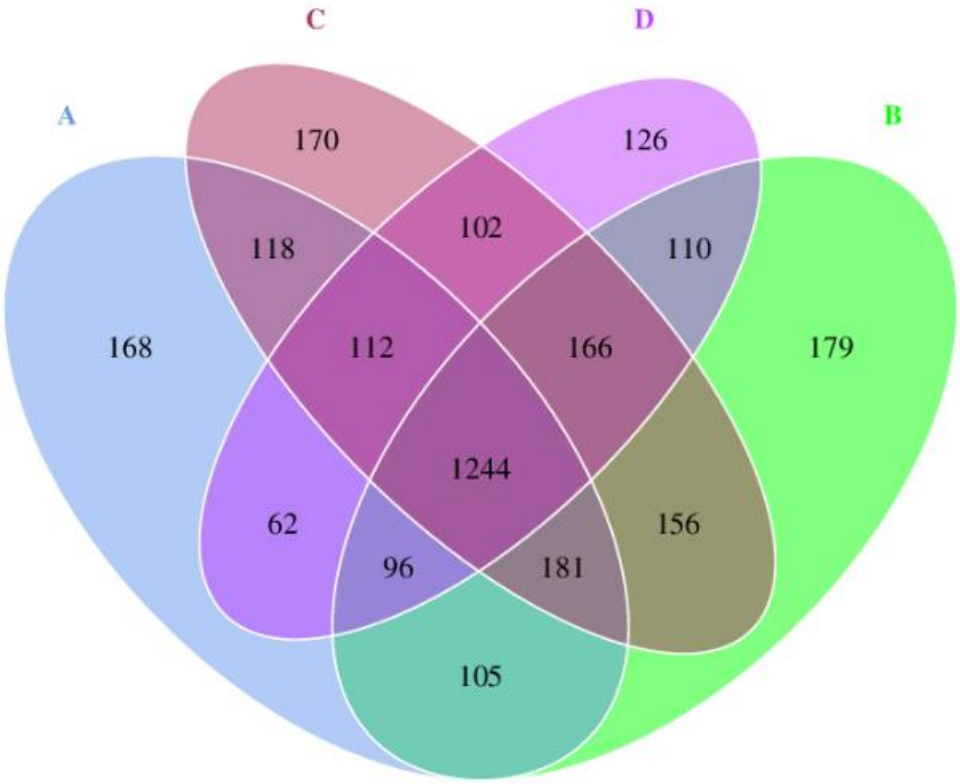


Figure 7. Venn diagram of four Stradzha soils associated with wild legume species (A) soils saline-alkaline soil around the village of Brodilovo associated with *L. ablus*, (B) and yellow earth podzolic soils from the village of Brodilovo associated with *C. montbretii* and *L. ablus*, (C) cinnamon forest soils and (D) siliceous red soil the Great Pazvlak area.

Triple plot

To find the differences in dominant taxa among the three sample groups at each taxonomic rank, the top 10 taxa with the average abundance of the three sample groups at each taxonomic rank were selected to generate a ternary schedule. The ternary plot in Figure 8 was built using (A) soils saline-

alkaline soil around the village of Brodilovo associated with *L. ablus*, (B) and yellow earth podzolic soils from the village of Brodilovo associated with *C. montbretii* and *L. ablus*, (C) cinnamon forest soil share OTUs indicating a strong influence of climate, mineral composition of the rock and relief. Each circle represents an OTU, and its size represents relative abundance. Bacterial communities at the phylum level showed that Proteobacteria, Actinobacteria, and Acidobacteria were most strongly affected by plants, soil type, and environmental factors.

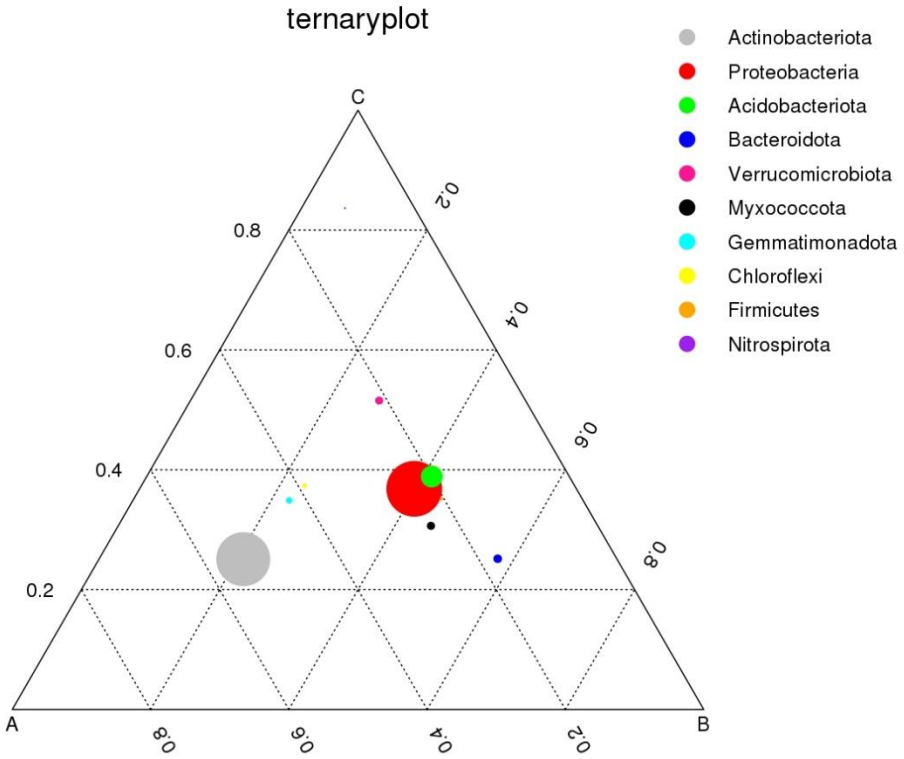
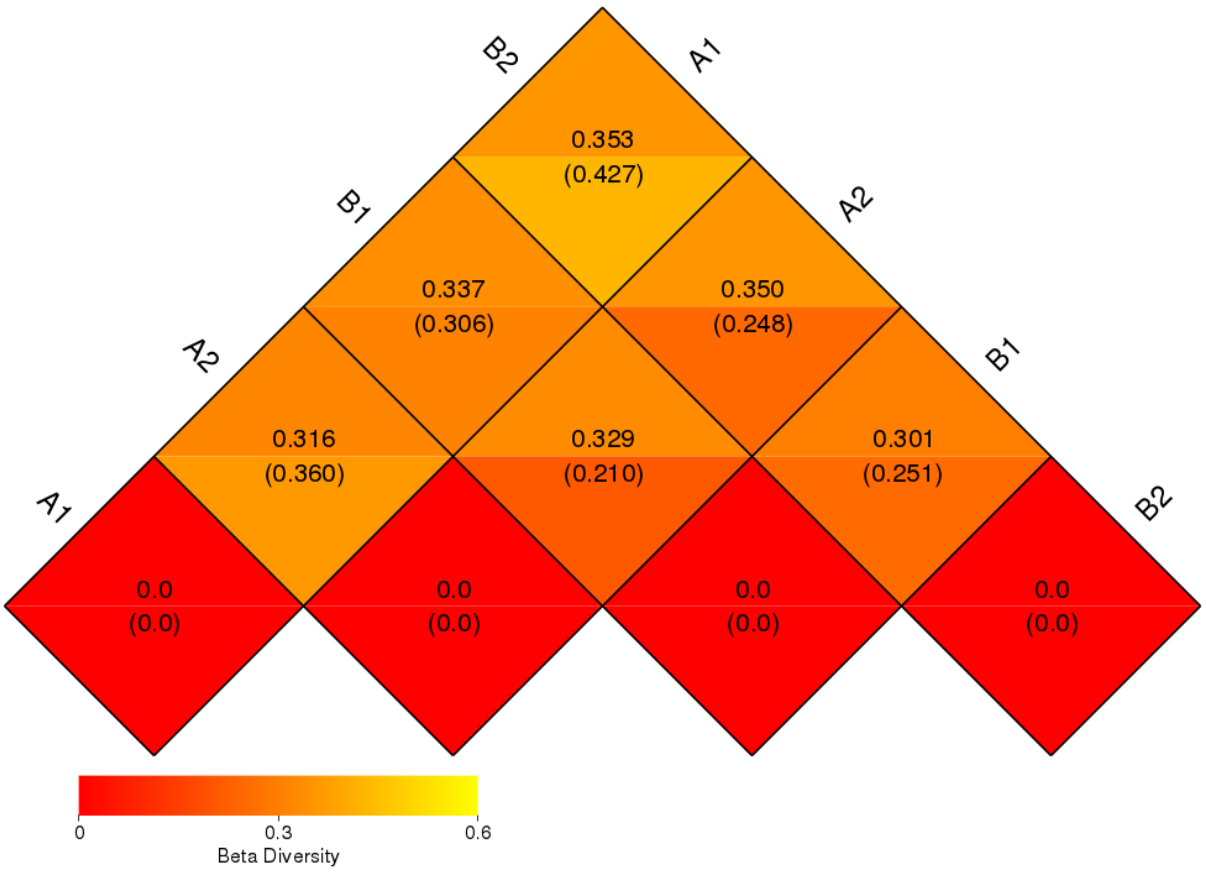


Figure 8. Data in this distance matrix can be visualized with principal coordinate analysis (PCoA), principal component analysis (PCA), nonmetric multidimensional scaling (NMDS), and unweighted pair method with arithmetic means (UPGMA).

Beta-diversity and PCA were also performed to determine the differences and similarities in the distribution of prokaryotes among all soil samples by cluster analysis (Fig. 9 A). The result, quantitative data from four different soil types showed the lowest β-diversity difference between A2 and B1 (0.210), while the highest was found among A1 and B2 (0.427). Bacterial communities at the phylum level and β-diversity showed that Proteobacteria, Actinobacteria, Acidobacteria, and Bacteroidetes and were most strongly affected by soil type (Fig. 9 A).

PCA analysis can extract two coordinate axes that reflect the difference between samples to the greatest extent, so as to reflect the difference of multidimensional data on the two-dimensional coordinate map, and then reveal the simple rules in the background of complex data. If the community composition of the samples is more similar, their distances in the PCA map are closer. The results of PCA analysis based on feature consistency level are shown in Figure 10 A. PCA is a method based on Euclidean distances for applying variance decomposition to reduce the dimensionality of multivariate data. Initial principal component analysis (PCA) showed a significant percentage of the total variation of the bacterial microflora with 34.09% on the abscissa and 37.16% on the ordinate between all six soil samples. The results show that the sequences in C (A2) and D (B1) form one cluster, and A and C differ from the rest of the samples due to the influence of soil type and plants. In Figure 9B, B and D soils have similar distances and therefore have similar microbiome composition and the remaining phases differ from each other with a different probability of p<0.1.. The A, B and D have the same position on the abscissa axis. Farthest from all other samples is the microbial composition of cinnamon forest soils (Fig. 9B).

A)



B)

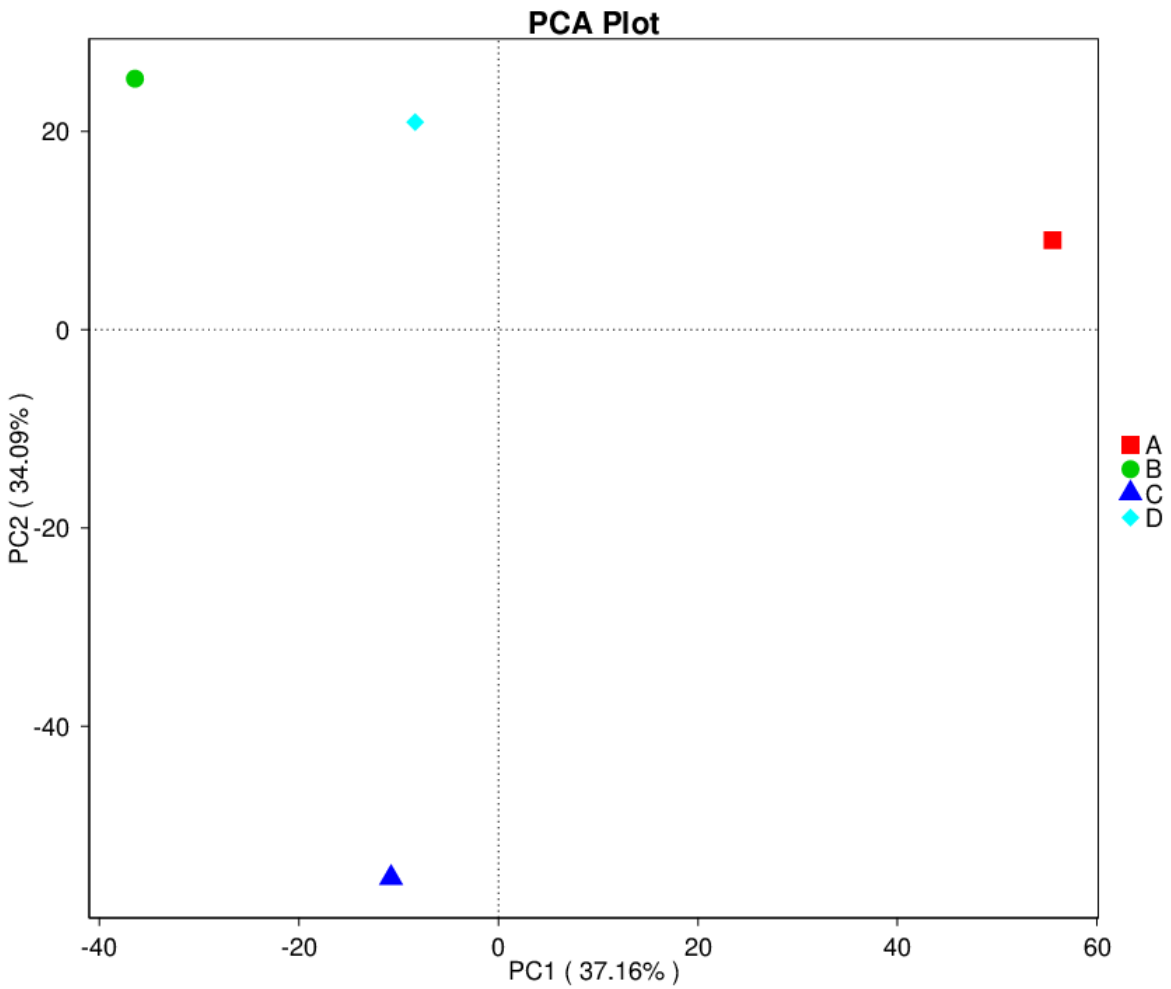


Figure 9. β-diversity and Principal component analysis PCA.

The main ideas of UPGMA are as follows. First, samples with the closest distance are grouped together to form a new node (as a new sample). A full cluster tree can be obtained until all samples are clustered together. The weighted Unifrac distance matrix and the unweighted Unifrac distance matrix were calculated before being used for UPGMA cluster analysis.

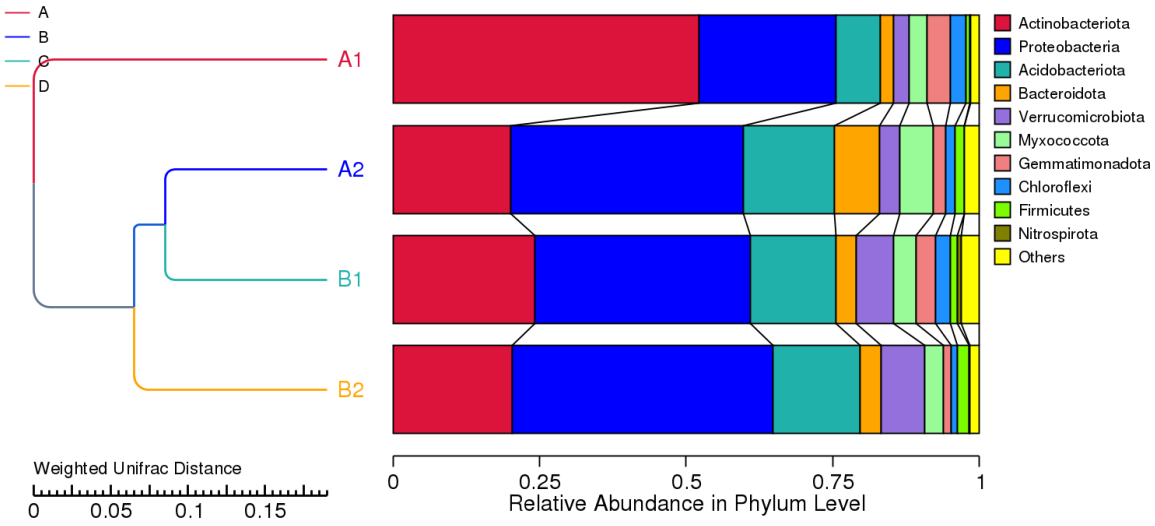
It has been reported that the wild legumes development is highly dependent on bacterial diversity, which is mainly due to a change in some important environmental factors such as temperature, pH, aeration and moisture, and properties of composting raw materials.

At phylum level A2, B1, and B2 were grouped together with a relatively similar percentage of

Actinobacteriota, Proteobacteria, and Acidobacteria, while A1 clustered alone with higher content of Actonobacteria (Figure 10). The most common to all four soils were *Proteobacteria*, *Actinobacteria*, Our results are largely in agreement with Hackl et al., 2004 and Barns et al. 1999 who published that

Proteobacteria, Actinobacteria Acidobacterium sp are common in Austrian forest soils under pine to 35% in spruce-fir-beech soils in Europe.

A)



Acknowledgments

The research presented in this paper was conducted by financial support of Centre of Research, Technology Transfer and Protection of Intellectual Property Rights at the Agricultural University of Plovdiv by the project 07-21.

REFERENCES

Amin, M., & Flowers, T. H. (2004). Evaluation of Kjeldahl digestion method. *J. Res. Science*, *15*, 159-179.

Barns, S. M., Takala, S. L., & Kuske, C. R. (1999). Wide distribution and diversity of members of the bacterial kingdom Acidobacterium in the environment. *Applied and environmental microbiology*, *65*(4), 1731-1737.

Bokulich, N.A.; Subramanian, S.; Faith, J.J.; Gevers, D.; Gordon, J.I.; Knight, R.; Mills, D.A.; Caporaso, J.G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature methods*, *7*(5), 335-336.

Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Pena, A.G.; Goodrich, J.K.; Gordon, J.I. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 2010, 7, 335.

Chopkova, V., Petkova, M., & Shilev, S. (2023). Uncovering Bacterial Diversity during Mesophilic and Thermophilic Phases of Biowaste Composting through Next-Generation Sequencing. *Applied Sciences*, *13*(5), 3111.

de Carvalho, T. S., Jesus, E. D. C., Barlow, J., Gardner, T. A., Soares, I. C., Tiedje, J. M., & Moreira, F. M. D. S. (2016). Land use intensification in the humid tropics increased both alpha and beta diversity of soil bacteria. *Ecology*, *97*(10), 2760-2771.

Derry, A.M., Staddon, W.J., Kevan, P.G. and Trevors, J.T. (1999). Functional diversity and community structure of micro-organisms in three arctic soils as determined by sole-carbon source-utilization. Biodiversity and Conservation 8: 205–221.

Dixon, R., and Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. *Nat. Rev. Microbiol.* 2, 621–631. doi: 10.1038/nrmicro954

Dokić, L., Savić, M., Narančić, T. and Vasiljević, B. (2010). Metagenomic Analysis of Soil Microbial Communities. Archives of Biological Science-Belgrade 62(3): 559-564.

Edgar, R.C. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10*, 996–998.

Edgar, R.C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods*, 10, 996.

Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 2011, 27, 2194–2200.

Gui, H., Purahong, W., Hyde, K. D., Xu, J., & Mortimer, P. E. (2017). The arbuscular mycorrhizal fungus Funneliformis mosseae alters bacterial communities in subtropical forest soils during litter decomposition. *Frontiers in Microbiology*, *8*, 1120.

Haas, B.J.; Gevers, D.; Earl, A.M.; Feldgarden, M.; Ward, D.V.; Giannoukos, G.; Ciulla, D.; Tabbaa, D.; Highlander, S.K.; Sodergren, E.; et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* **2011**, *21*, 494–504.

Hackl, E., Zechmeister-Boltenstern, S., Bodrossy, L., & Sessitsch, A. (2004). Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. *Applied and Environmental Microbiology*, *70*(9), 5057-5065.

Jeewani, P. H., Ling, L., Fu, Y., Van Zwieten, L., Zhu, Z., Ge, T., ... & Xu, J. (2021). The stoichiometric C-Fe ratio regulates glucose mineralization and stabilization via microbial processes. *Geoderma*, *383*, 114769.

Karron, J. D., Falk, D. A., & Holsinger, K. E. (1991). Breeding systems in rare plant species. *Genetics and conservation of rare plants. Oxford, UK: Oxford University Press On Demand*, 87-98.

Kent, A. D., & Triplett, E. W. (2002). Microbial communities and their interactions in soil and rhizosphere ecosystems. *Annual Reviews in Microbiology*, *56*(1), 211-236.

Krstić Tomić, T., Atanasković, I., Nikolić, I., Joković, N., Stević, T., Stanković, S., ... & Lozo, J. (2023). Culture-Dependent and Metabarcoding Characterization of the Sugar Beet (Beta vulgaris L.) Microbiome for High-Yield Isolation of Bacteria with Plant Growth-Promoting Traits. *Microorganisms*, *11*(6), 1538.

Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *Embnet. J.* **2011**, *17*, 10–12.

Navas-Molina, J. A., Peralta-Sánchez, J. M., González, A., McMurdie, P. J., Vázquez-Baeza, Y., Xu, Z.,Knight, R. (2013). Advancing our understanding of the human microbiome using QIIME. In *Methods in enzymology* (Vol. 531, pp. 371-444). Academic Press.

Pearce, D. A., Newsham, K. K., Thorne, M. A., Calvo-Bado, L., Krsek, M., Laskaris, P., et al. (2012). Metagenomic analysis of a southern maritime Antarctic soil. *Front. Microbiol.* 3:403. doi: 10.3389/fmicb.2012.00403

Petkova, M., Sabeva, M., Petrova, S., & Tahsin, N. (2023). The bacterial community structure of rhizosphere soil associated with *Cicer montbretii* Jaub. & Spach endemic to Strandzha Mountain. *Ecologia Balkanica*, *15*(1).

Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Lesniewski, R.A.; Oakley, B.B.; Parks, D.H.; Robinson, C.J. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol*. 2009, 75, 7537–7541.

Steila, D., & Pond, T. E. (1989). The geography of soils: formation, distribution, and management. Rowman & Littlefield.

Whittaker, R. H. (1969). New Concepts of Kingdoms of Organisms: Evolutionary relations are better represented by new classifications than by the traditional two kingdoms. *Science*, *163*(3863), 150-160.

Willis, A. D. (2019). Rarefaction, alpha diversity, and statistics. *Frontiers in microbiology*, *10*, 2407.

Wongdee, J., Piromyou, P., Songwattana, P., Greetatorn, T., Teaumroong, N., Boonkerd, N., Tittabutr, P. (2023). Role of two RpoN in Bradyrhizobium sp. strain DOA9 in symbiosis and free-living growth. *Frontiers in Microbiology*, *14*, 1131860.

Zhang, J.; Chen, M.; Huang, J.; Guo, X.; Zhang, Y.; Liu, D.; Wu, R.; He, H.; Wang, J. Diversity of the microbial community and cultivable protease-producing bacteria in the sediments of the Bohai Sea, Yellow Sea and South China Sea. *PLoS ONE* **2019**, *14*, e0215328.

Zhang, M., Lin, M., Cao, X., Zhao, S., Jiang, D., Wang, B., & Lin, H. (2018). Difference in pH value and nutrient and bacterial diversity in the *Carya cathayensis* forest soil under different management models. *Biodiversity Science*, *26*(6), 611-619.

Zheng, Y., Ji, N. N., Wu, B. W., Wang, J. T., Hu, H. W., Guo, L. D., & He, J. Z. (2020). Climatic factors have unexpectedly strong impacts on soil bacterial β-diversity in 12 forest ecosystems. *Soil Biology and Biochemistry*, *142*, 107699.