Аграрен университет – Пловдив, Научни трудове, т. LXVII, кн. 1, 2025 г.
Юбилейна научна конференция *„80 години Аграрен университет – Пловдив: Традиции срещат иновации “*
Anniversary Scientific Conference
"80 Years Agricultural University – Plovdiv:Traditions Meet Innovations"
Agricultural University – Plovdiv, Scientific Works, vol. LXVII, book 1, 2025

DOI: 10.22620/sciworks.2025.02.018

COMPARATIVE METAGENOMIC ANALYSIS OF ROOT ENDOPHYTIC AND RHIZOSPHERE MICROBIOMES IN OAT–VETCH INTERCROPPING AND VETCH MONOCULTURE SYSTEMS

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Abstract

Root endophytic and rhizosphere microorganisms are vital for ecosystem functions such as nutrient cycling, plant health maintenance, and the promotion of sustainable agriculture. In this investigation, a metagenomic strategy was employed to explore the microbial communities associated with oat (*Avena sativa* L.) and common vetch (*Vicia sativa* L.), cultivated both as monocultures and in a mixed intercropping system. Four types of microbiomes were examined: (A) root endophytic microbiome in oat–vetch intercropping, (B) root endophytic microbiome in vetch, (C) rhizosphere microbiome in oat–vetch intercropping, and (D) rhizosphere microbiome in vetch. Total DNA was extracted and subjected to next-generation sequencing of the bacterial 16S rRNA gene. The obtained data were analysed using the MG-RAST and QIIME platforms to assess taxonomic composition, alpha and beta diversity, and the relative abundance of microbial taxa. The results revealed distinct differences among the four microbiome types. The oat–vetch intercropping promoted higher microbial diversity and a more balanced community structure compared to monocultures. The enrichment of bacterial and fungal groups involved in nitrogen fixation, phosphate solubilization, and organic matter decomposition highlighted the enhanced biological activity under intercropping. Alpha diversity indices indicated greater species richness and evenness, while beta diversity analyses demonstrated clearly separated clustering patterns among the treatments. The combined metagenomic and functional analyses provide a comprehensive understanding of the dynamics of endophytic and soil microbiomes and demonstrate the potential of the oat–vetch system to enrich beneficial microbial communities, improve soil fertility, and support sustainable agroecosystem management.

Keywords: intercropping, oat (*Avena sativa*), metagenomics, microbial diversity, soil microbiome, vetch (*Vicia sativa*)

INTRODUCTION

Soil represents a complex biological system in which physical, chemical, and biological components interact to form a dynamic environment essential for plant growth and productivity. Among the biological factors, soil microorganisms play a key role in nutrient cycling, organic matter mineralisation, and maintaining agroecosystem balance (Li et al., 2024; Petkova et al., 2025). These microbial communities regulate multiple processes, including nitrogen fixation, phosphate solubilization, micronutrient mobilisation, and phytohormone synthesis, all of which determine soil fertility and resilience (Van Der Heijden et al., 2008; Philippot et al., 2013).

Sustainable soil management requires agricultural practices that combine high productivity with the preservation of biological diversity. One of the most effective approaches is intercropping, the simultaneous cultivation of two or more crops in the same field, which promotes synergistic interactions between plants and soil microorganisms (Tuna et al., 2007; Ray et al., 2025). Intercropping systems enhance resource use efficiency (light, water, and nutrients), suppress weeds and phytopathogens, and reduce the need for mineral fertilisation (Wang et al., 2021). Beyond agronomic benefits, intercropping positively affects soil microbial diversity and activity, stimulating nitrogen fixation and organic matter decomposition (Luo et al., 2016).

The combination of oat and vetch represents a classical example of a successful intercropping system. Vetch, as a leguminous species, forms symbiotic associations with Rhizobium spp., capable of fixing atmospheric nitrogen and enriching the soil with bioavailable nitrogen compounds (Peoples et al., 2009). Oat, in turn, possesses a well-developed root system that improves soil structure, enhances aeration, and stimulates microbial activity (Lithourgidis et al., 2011). This synergy reduces the need for synthetic fertilisers and contributes to a sustainable, environmentally friendly agroecosystem (Kaut et al., 2008; Lithourgidis et al., 2006). However, the effect of intercropping on microbial communities depends on multiple factors, including crop ratio, soil type, climatic conditions, and plant growth stage (Liu et al., 2024). Soil microorganisms, an important component of the plant microbiome, is endophytes—microorganisms inhabiting internal plant tissues without causing visible disease symptoms. Endophytes establish complex symbiotic relationships with plants, enhancing their nutrition, growth, and tolerance to biotic and abiotic stressors (Hardoim et al., 2015; Santoyo et al., 2016). They produce growth-promoting substances, enzymes, and antimicrobial metabolites that suppress phytopathogens and contribute to plant resistance. Endophytes often originate from the rhizosphere microbial pool and represent a transitional group between soil and internal microbiota (Compant et al., 2010). Their universal distribution across nearly all plant species and agroecological environments highlights their significance as a key biological resource for sustainable agriculture.

Recent advances in metagenomic technologies have enabled a deeper exploration of these interactions. Through direct sequencing of soil and plant DNA, it is now possible to identify non-culturable microorganisms and determine their functional genes (Daniel, 2005; Handelsman, 2004). This approach allows assessment of taxonomic structure, alpha and beta diversity, and the functional

potential of microbial communities, as well as the relationships between these communities and environmental factors (Isali et al., 2024). Studies have shown that intercropping systems increase the abundance of bacterial genera, such as *Pseudomonas*, *Bacillus*, and *Rhizobium*, which are involved in nitrogen and phosphorus cycling, as well as fungal taxa associated with organic matter decomposition (Lian et al., 2019; Dang et al., 2021). Such microbial associations enhance nutrient availability, stimulate plant growth, and strengthen agroecosystem resilience (Ma et al., 2024).

Although the impact of intercropping on crop productivity has been well documented, its influence on soil and endophytic microbiota remains insufficiently studied, particularly under the temperate continental conditions of Bulgaria. The lack of data on the metagenomic structure of soil and endophytic communities in oat–vetch systems represents a significant research gap. Therefore, the present study aims to perform a comparative metagenomic analysis of soil and endophytic microbial communities in oat and vetch monocultures and their intercropping system. Using high-throughput sequencing and functional analyses, the study aims to assess the impact of various agroecosystems on the taxonomic diversity, biological activity, and ecological functions of microbial communities. The results are expected to provide new insights into the relationships between the structure and function of soil and endophytic microbiomes and to support the adoption of intercropping systems as an ecological alternative for sustainable soil fertility management.

MATERIALS AND METHODS

1. Experimental Design and Sampling

The field experiment was conducted during the vegetation season on an experimental plot characterised as typical leached smolnitsa soil with a neutral pH (6.8–7.0), moderate organic matter content (2.1%), and medium levels of available nitrogen and phosphorus. Three treatments were established: oat monoculture (Avena sativa L.), vetch monoculture (Vicia sativa L.), and an oat–vetch intercropping system at a 1:1 seed ratio. A randomised block design was employed, incorporating three independent replicates per treatment. Each plot measured 5 × 2 m, separated by 0.5 m buffer corridors to prevent the exchange of root exudates.

Soil samples were collected at the oat stem elongation and vetch budding stages from a depth of 0–20 cm using a sterile soil auger. Five subsamples per replicate were pooled into a composite sample (~500 g). A portion of the samples was used for physiological analyses, while the rest was stored at −20°C until DNA extraction.

2. Extraction of Total DNA

Endophytic microorganisms were isolated from fresh root tissues of oat and vetch collected from the experimental plots, as described by Costa et al. 2012. Plant surfaces were surface-sterilised sequentially with 70% ethanol (1 min), 2% sodium hypochlorite (3 min), and rinsed three times with sterile distilled water. The final rinse was plated on nutrient agar to verify sterility. Sterilised tissues were

homogenised in sterile phosphate-buffered saline (PBS, pH 7.0), and the resulting suspensions were used for microbial plating and DNA extraction. Total DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol optimised for root tissues rich in polysaccharides and polyphenols (Porebski et al., 1997). Approximately 100 mg of homogenised root tissue was incubated in 700 µL preheated CTAB buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% PVP-40) with 0.2% β-mercaptoethanol. After incubation at 65°C for 30 min, samples were extracted with chloroform: isoamyl alcohol (24:1), and DNA was precipitated from the aqueous phase with cold isopropanol. The pellet was washed with 70% ethanol, air-dried, and resuspended in 50 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Residual RNA was removed by RNase A treatment (10 µg/mL, 15 min, room temperature).

Total DNA from soil samples was extracted using the DNeasy PowerSoil Kit (Qiagen, Germany) according to the manufacturer's protocol, ensuring effective microbial lysis and removal of humic substances and PCR inhibitors. DNA quality and purity were evaluated using a Qubit 4.0 fluorometer (Thermo Scientific, USA) and confirmed by electrophoresis in a 1% agarose gel containing ethidium bromide. DNA concentration was standardised to 20 ng/µL before PCR amplification (Zhou, Bruns, & Tiedje, 1996).

3. Amplification, Library Preparation, and Sequencing

For analysis of endophytic bacterial communities in soil and plant samples, the V3–V4 region of the 16S rRNA gene was amplified using universal primers 799F (5′-AACMGGATTAGATACCCKG-3′) and 1193R (5′-ACGTCATCCCCACCTTCC-3′) (Chelius & Triplett, 2001). PCR reactions (25 µL) contained 12.5 µL of 2× KAPA HiFi HotStart ReadyMix, 1 µL of each primer (10 µM), 2 µL of DNA template, and nuclease-free water. Thermal cycling conditions included an initial denaturation at 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were visualised by electrophoresis in a 1.5% agarose gel and purified using AMPure XP Beads (Beckman Coulter). Purified amplicon concentrations were measured with a Qubit 4.0 fluorometer (Thermo Fisher Scientific).

3.1. Library Construction

Purified PCR products were used to construct dual-indexed amplicon libraries following the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Kozich et al., 2013). Nextera XT Index Kit (Illumina, USA) adapters and barcodes were added during a secondary PCR (8 cycles) to allow multiplexed sequencing. Libraries were purified again using AMPure XP Beads, size-verified (~550 bp for 16S and ~300–400 bp for ITS) on an Agilent 2100 Bioanalyzer, and quantified using the Qubit dsDNA HS Assay Kit. All libraries were normalised to 4 nM and denatured with 0.2 N NaOH.

3.2. Sequencing and Quality Control

Sequencing was performed on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (600 cycles, 2×300 bp, paired-end). Each run included an internal control (PhiX Control v3, 15%) to monitor sequencing accuracy.

4. Bioinformatic Processing and Data Analysis

Bioinformatic analyses were conducted using QIIME 2 (v.2023.2) and MG-RAST (Meyer et al., 2008). Raw reads were quality-filtered (Q > 30), merged, and chimaera-checked using USEARCH. Taxonomic classification was performed against the SILVA 138 database for bacteria and UNITE v 9.0 for fungi at a 97% similarity threshold. Alpha diversity indices (Shannon and Simpson; Magurran, 2004) and beta diversity (PCA based on Bray–Curtis dissimilarity) were computed. Taxonomic structure was assessed at the phylum, class, family, and genus levels, with relative abundance visualised as heatmaps and rank-abundance curves.

5. Statistical Analysis

Differences between treatments were analysed using ANOVA at $p < 0.05$. All statistical analyses were conducted in RStudio (v.2023.06).

6. Data Availability Statement

Sequencing data are available under the BioProject accession number PRJNA1345251, submission SUB15708592, at the NCBI database: https://www.ncbi.nlm.nih.gov/bioproject/1345251 (accessed on 14 October 2025).

RESULTS AND DISCUSSION

1. Sequencing Quality

Table 1 presents the main parameters obtained from high-throughput sequencing of four samples representing endophytic and soil microbial communities under oat–vetch intercropping and vetch monoculture. The parameters include the number of raw reads, the number of effective tags (after quality filtering), GC content (%GC), the proportion of nucleotide sequences with Q20 and Q30 quality scores, and the overall sequencing efficiency. A total of 1,245,360 raw reads were obtained from the Illumina MiSeq platform, of which 1,128,750 (90.6%) passed quality filtering (Q > 30). The average merged read length was 410 bp for the 16S rRNA region and 320 bp for the ITS region. After chimera removal, 26,584 bacterial and 18,913 fungal operational taxonomic units (OTUs) were identified at a similarity threshold of ≥97%. A comparable level of read depth and filtration efficiency has been reported in other metagenomic studies on soil microbiomes in agroecosystems (Chen et al., 2023), confirming the reliability of sequencing and subsequent analyses.

High-throughput sequencing of amplicon libraries derived from soil and endophytic samples of oat, vetch, and their intercropping system generated between 68,000 and 136,213 raw reads. After quality control (Q > 30) and the removal of chimeric and low-quality fragments, the number of effective tags ranged from 65,219 to 123,207, corresponding to an efficiency of 79–82%. This high efficiency indicates excellent sequencing quality and optimal library preparation. The GC content ranged between 54.3% and 56.9%, reflecting a stable taxonomic composition of the microbial communities and the presence of high-GC bacteria typical of the phylum *Actinobacteria* (Ventura et al., 2007). The Q20 (98%) and Q30 (93–94%) scores confirmed high sequencing accuracy and a minimal error rate (<0.1%), consistent with the standards required for metagenomic analyses (Illumina, 2022; Knight et al., 2018). The endophytic samples from vetch contained

nearly twice as many raw reads as those from oat–vetch intercropping, likely due to the higher microbial density within the tissues of the legume species. Soil microbiomes exhibited the highest GC% values and sequencing efficiency (>81%), suggesting the dominance of Gram-positive genera such as *Bacillus* and *Streptomyces*. The obtained metrics confirm that all samples meet international standards for sequencing quality and depth, providing a reliable basis for subsequent taxonomic and functional analyses (Kozich et al., 2013).

Table 1. Quality metrics of sequencing and filtering of nucleotide sequences from soil and endophytic samples in oat–vetch intercropping and vetch monoculture systems.

Sample name	Raw reads	Effective tags	GC (%)	Q20 (%)	Q30 (%)	Efficiency (%)
Root endophytes (oat–vetch)	68,000	65,219	54.35	98.06	93.79	79.05
Root endophytes (vetch)	128,350	123,207	54.37	98.13	93.97	80.67
Soil microbiome (intercropping, endophytic fraction)	136,213	73,85	56.78	98.06	93.77	81.75
Soil microbiome (vetch monoculture, endophytic fraction)	132,486	87,28	56.92	98.03	93.62	82.04

Legend: Raw reads represent the original sequence tags obtained after sequencing; effective tags are reads remaining after chimera and quality filtering, suitable for downstream analysis; Q20 and Q30 denote the percentage of bases with quality scores greater than 20 (error rate <1%) and 30 (error rate <0.1%), respectively; GC (%) represents the GC content of effective tags; efficiency (%) indicates the proportion of effective sequences among total raw reads.

2. Taxonomic Composition of the Bacterial Community

The taxonomic analysis of 16S rRNA nucleotide sequences revealed a clear dominance of several major bacterial phyla across all samples (Figure 1). The most abundant groups were Proteobacteria, Actinobacteriota, Acidobacteriota, and Bacteroidota, together accounting for over 80% of the total bacterial abundance. In group A (oat–vetch root endophytes), Actinobacteriota (≈50%) and Proteobacteria (≈30%) predominated. This composition is typical of plant endophytic niches, where actinobacteria are known to synthesise antimicrobial metabolites and plant growth-promoting compounds (Ventura et al., 2007). The high abundance of Proteobacteria reflects the active participation of genera such as Pseudomonas and Rhizobium in nitrogen fixation and plant protection (Philippot et al., 2013). In group B (vetch root endophytes), the ratio between the two dominant phyla shifted—Proteobacteria were more abundant (>50%), while Actinobacteriota decreased by approximately half compared with the mixed culture. This pattern

suggests that vetch tissues host active nitrogen-fixing bacteria typical of leguminous plants, consistent with findings by Peoples et al., (2009) and Tian et al., (2019). Soil microbiome samples (groups C and D) exhibited a more balanced taxonomic composition and higher phylum-level diversity. In the intercropping system (C), the relative abundance of Proteobacteria (≈40%) and Acidobacteriota (≈15%) was high, along with a moderate share of Actinobacteriota (≈20%). This indicates that mixed oat–vetch cultivation provides a favorable environment for microorganisms involved in the degradation of organic matter and nutrient mobilization (Luo et al., 2016; Chen et al., 2023). In the soil microbiome under vetch monoculture (group D), Proteobacteria remained dominant, but a slight increase in Actinobacteriota and Firmicutes was observed. This may be attributed to changes in vetch root exudates and the presence of symbiotic rhizobia, which alter microbial dynamics (Van Der Heijden et al., 2008; Seitz et al., 2023). Bacteroidota, Verrucomicrobiota, Myxococcota, and Chloroflexi occurred in lower but stable proportions (5–10%), reflecting their role in organic matter mineralisation and maintenance of soil health (Eichorst et al., 2018). The results confirm that the oat–vetch intercropping system promotes the development of a more functionally diverse bacterial community characterised by increased abundance of Proteobacteria and Acidobacteriota, which are key contributors to nitrogen and carbon biogeochemical cycling. The relative abundance analysis further confirmed that Proteobacteria, Actinobacteriota, and Acidobacteriota were the dominant taxa across all microbiomes, consistent with previous findings in agricultural soils exhibiting high biological activity (Philippot et al., 2013; Li et al., 2024).

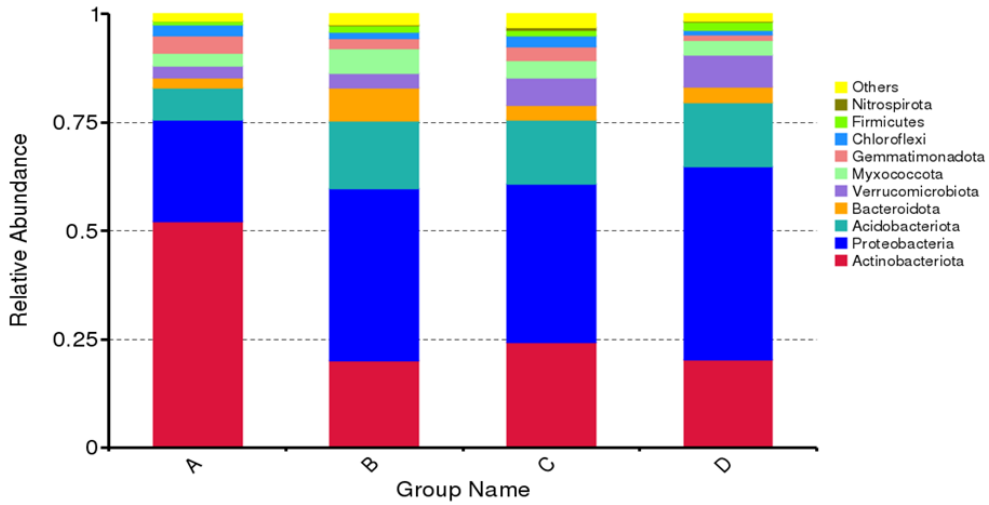

Figure 1. Relative abundance of dominant endophytic bacterial phyla in soil and root samples under different agroecological conditions (A–D). A – Root endophytes (oat–vetch); B – Root endophytes (vetch); C – Soil microbiome (oat–vetch field); D – Soil microbiome (vetch field).

3. Biodiversity Curves

3.1. Rarefaction Curves

The rarefaction curves illustrate the saturation of detected operational taxonomic units (OTUs) as the number of sequenced reads increases. In all four samples, the curves reached a plateau after approximately 60,000 sequences, confirming that the sequencing depth was sufficient for reliable assessment of bacterial diversity (Knight et al., 2018). The highest number of OTUs was observed in the soil microbiome from the intercropping system (C) and in the vetch endophyte samples (B), exceeding 2,200 OTUs, indicating richer microbial communities in these samples. This can be attributed to the combined effects of the legume component on nitrogen fixation and the diversity of root exudates that stimulate microbial colonisation (Tian et al., 2019). In contrast, the oat–vetch endophyte samples (A) and the soil microbiome from vetch monoculture (D) showed lower OTU counts (approximately 2,000 and 1,900, respectively), likely due to the selective nature of the endophytic niche and a less complex microbial network in monocultures. The overall saturation pattern and the close alignment of the curves among samples confirm comparable sequencing depth, enabling reliable cross-group comparisons of α- and β-diversity (Chen et al., 2023).

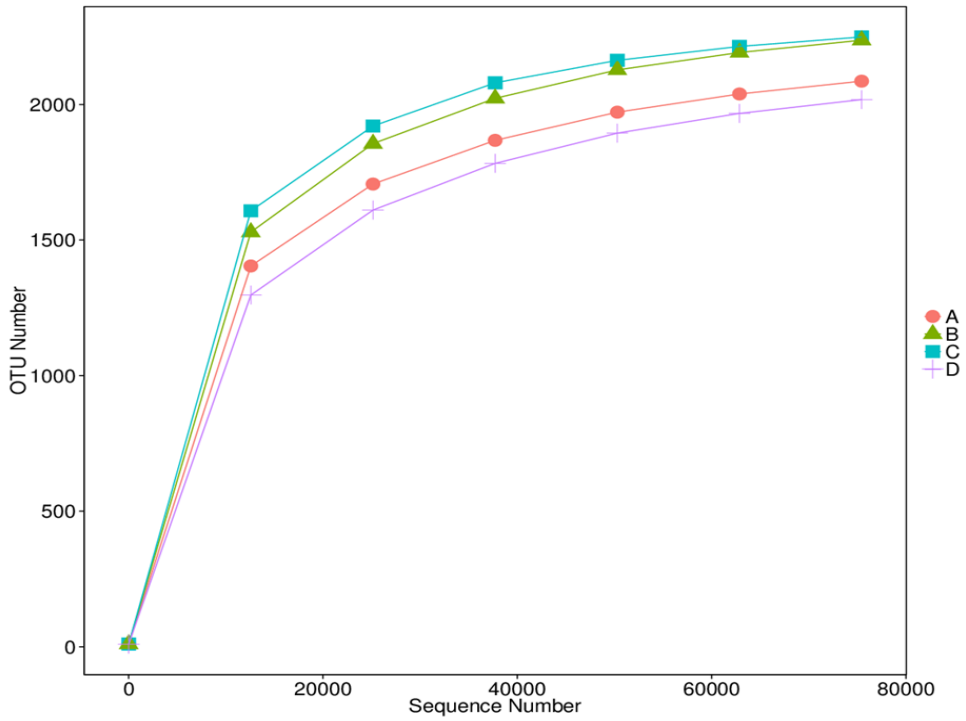

Figure 2. Rarefaction curves showing the saturation of OTUs with increasing numbers of sequenced reads for root endophytic and rhizosphere microbiomes. (A) Root endophytes (oat–vetch); (B) Root endophytes (vetch); (C) Rhizosphere microbiome (oat–vetch); (D) Rhizosphere microbiome (vetch).

3.2. Abundance Curves

The abundance curves illustrate the distribution of relative abundances among bacterial taxa. All four groups exhibited a characteristic long-tailed pattern, typical of microbial communities composed of many rare taxa and a few dominant species. Samples C (rhizosphere microbiome of oat–vetch) and B (root endophytes of vetch) showed flatter curves, indicating greater evenness in taxon distribution and a more balanced community structure. This suggests that intercropping and the legume component promote microbial diversity by reducing the dominance of single phyla such as Proteobacteria. In contrast, samples A (root endophytes of oat–vetch) and D (rhizosphere microbiome of vetch) displayed steeper abundance curves, reflecting lower evenness and the prevalence of several dominant taxa. Such patterns likely result from host plant selectivity toward endophytic microbiota and lower ecological plasticity within monoculture-associated communities (Philippot et al., 2013). The combined results from the diversity curves (Figures 2 and 3) indicate that intercropping (C) supports the highest taxonomic richness and evenness, confirming its beneficial impact on soil microbial diversity. The legume crop (vetch) further enhances microbial community complexity through symbiotic nitrogen fixation and increased exudation of organic acids. Root endophytes were less diverse than soil microbiomes, reflecting the selective colonisation processes within plant tissues Zhu and Morel (2019). These findings are consistent with the observations of Zhang et al. (2019), emphasising that mixed cropping systems foster more stable and functionally enriched microbial communities, contributing to improved biological activity and resilience of agroecosystems.

3.3. Heatmap of the Taxonomic Structure of Bacterial Communities

The heatmap (Figure 4) visualises the relative abundance of dominant bacterial genera across the analysed samples. The colour gradient from blue to red represents changes from low to high abundance, with individual phyla indicated by distinct colour codes. The samples were predominantly composed of members of Proteobacteria, Actinobacteriota, and Acidobacteriota, consistent with the results obtained at the phylum level. In the oat–vetch endophytic samples (A), an increased abundance of the genera Sphingomonas, *Rhodanobacter*, and *Burkholderia–Caballeronia–Paraburkholderia* (phylum Proteobacteria) was observed. These genera are characteristic of endophytic microorganisms involved in plant growth promotion, phytohormone production, and the degradation of phenolic compounds (Compant et al., 2010). In the vetch endophytes (B), the genera *Bradyrhizobium* and *Acidibacter* dominated. These taxa are associated with nitrogen fixation and phosphate solubilization. *Bradyrhizobium* is a well-known symbiont of legumes and likely contributes to the enrichment of the endophytic microbiota with functionally active taxa (Delamuta et al., 2013). The soil samples exhibited greater diversity and evenness in the distribution of bacterial genera. In the oat–vetch rhizosphere (C), *Granulicella, Bryobacter*, and *Pseudonocardia*—belonging to Acidobacteriota and Actinobacteriota—were prevalent. These microorganisms play key roles in the degradation of complex organic matter and contribute to soil structure stabilisation (Ward et al., 2009).

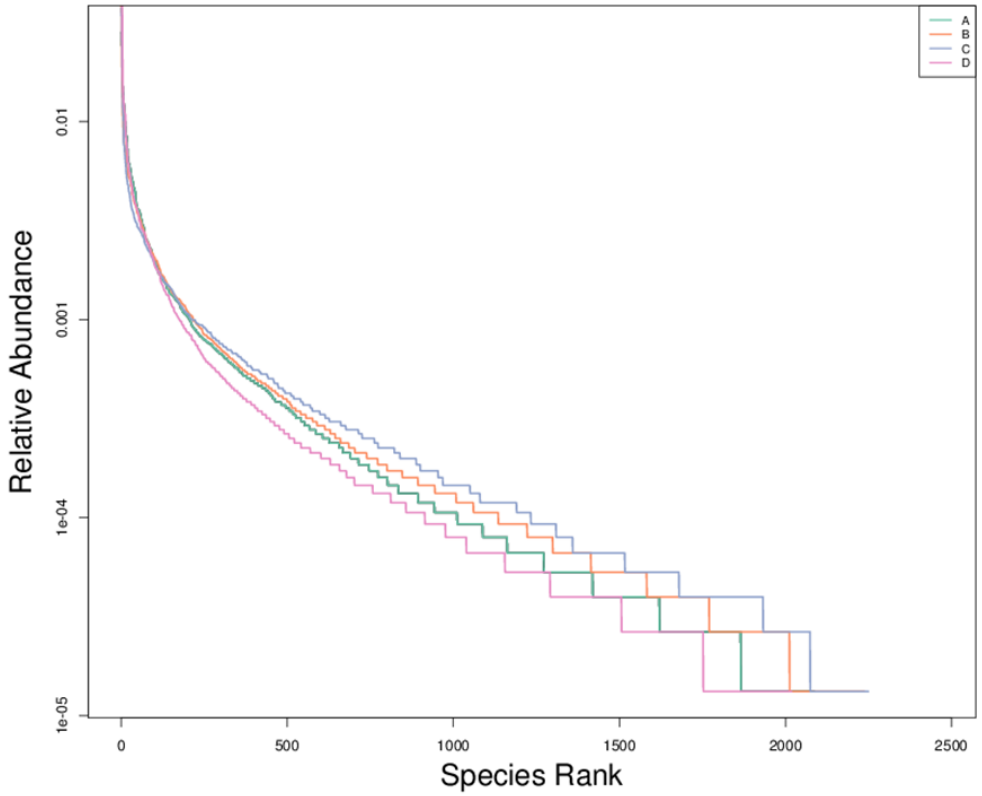

Figure 3. Abundance curves illustrating the distribution of relative abundance and evenness of bacterial taxa across the four analyzed groups. (A) Root endophytes (oat–vetch); (B) Root endophytes (vetch); (C) Rhizosphere microbiome (oat–vetch); (D) Rhizosphere microbiome (vetch).

In the vetch rhizosphere (D), *Streptomyces*, *Solirubacter*, and *Nocardioides* predominated—typical actinobacteria known for their ability to produce antibiotics and extracellular enzymes that regulate microbial interactions in the rhizosphere (Ventura et al., 2007). *Mycobacterium* and *Acidothermus* were detected in both C and D, suggesting the presence of microorganisms adapted to recalcitrant organic substrates and higher microbial activity in the intercropping system (Sisodia et al., 2025). Groups A and B (root endophytes) clustered closely together, indicating a similar composition of internal bacterial microbiota shaped primarily by the host plant species. In contrast, soil endophytic microbiome in C and D formed a separate cluster characterised by higher complexity and a more balanced taxonomic distribution. This distinct separation highlights the differences between internal (endophytes in plants) and external (endophytes in soil) microbial niches and demonstrates the influence of intercropping on the ecological structure of soil bacterial communities.

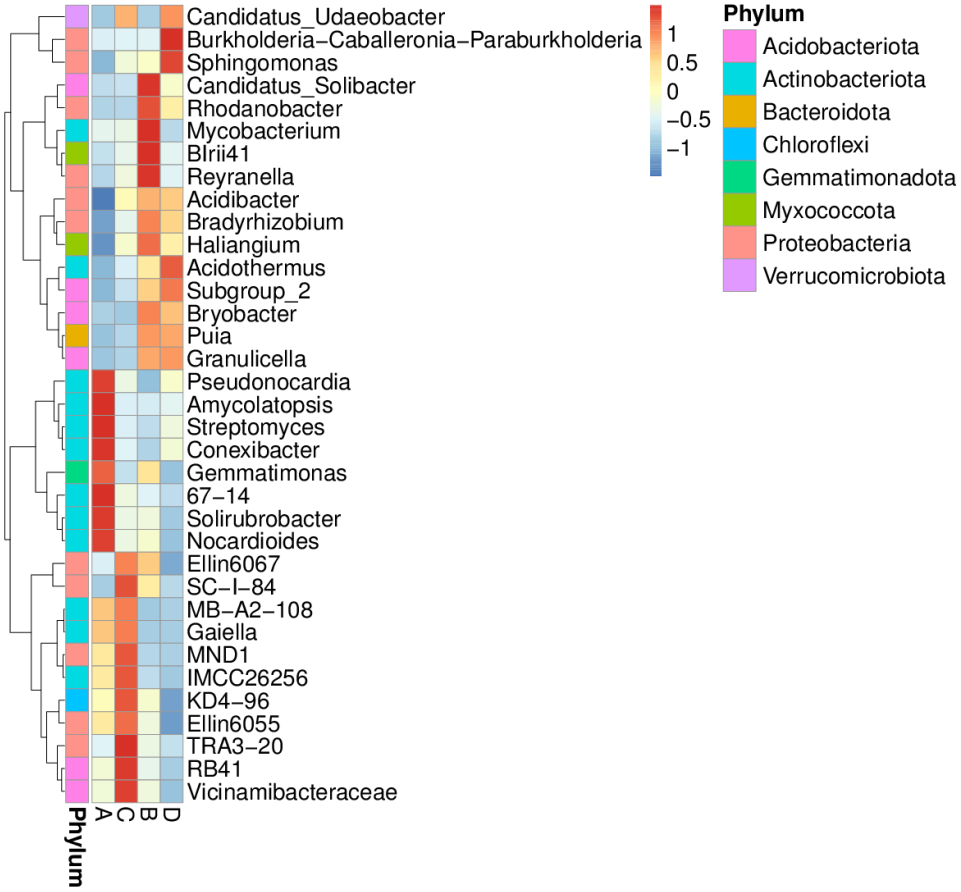

Figure 4. Heatmap of the relative abundance of dominant bacterial genera in root endophytic and rhizosphere samples.(A) Root endophytes (oat–vetch); (B) Root endophytes (vetch); (C) Rhizosphere microbiome (oat–vetch, intercropping system); (D) Rhizosphere microbiome (vetch). The color scale (ranging from –1 to +1) represents normalized abundance values, with red tones indicating higher abundance. The legend on the right denotes the phylum-level affiliation of the respective genera.

4. α-Diversity Analysis

4.1. Shannon Index

The Shannon index (Figure 5) is a widely used measure of α-diversity, integrating both species richness and evenness within a microbial community (Shannon & Weaver, 1949). In this study, values ranged from 8.3 to 9.4, indicating high microbial diversity across all samples. The highest index was observed in the rhizosphere microbiome of the oat–vetch intercropping system (C, 9.35), followed by vetch endophytes (B, 9.1) and oat–vetch endophytes (A, 8.9). The lowest diversity was recorded in the vetch soil microbiome (D, 8.3). These results suggest

that intercropping significantly enhances microbial diversity, particularly in soil samples. The increased diversity likely results from the broader spectrum of root exudates and distinct root architectures of oat and vetch, which create a more heterogeneous and resource-rich environment (Tian et al., 2019). The higher index in vetch endophytes (B) compared with oat–vetch endophytes (A) may reflect the presence of symbiotic bacteria such as *Bradyrhizobium*, *Burkholderia*, and *Sphingomonas*, known for their diverse physiological roles and stress tolerance (Compant et al., 2010; Delamuta et al., 2013). Conversely, the lower Shannon index in vetch soil microbiomes (D) could be linked to the dominance of a few Actinobacterial genera (*Streptomyces*, *Nocardioides*), a pattern typical for monocultures with reduced plant diversity and microbial evenness (Petkova et al., 2025).

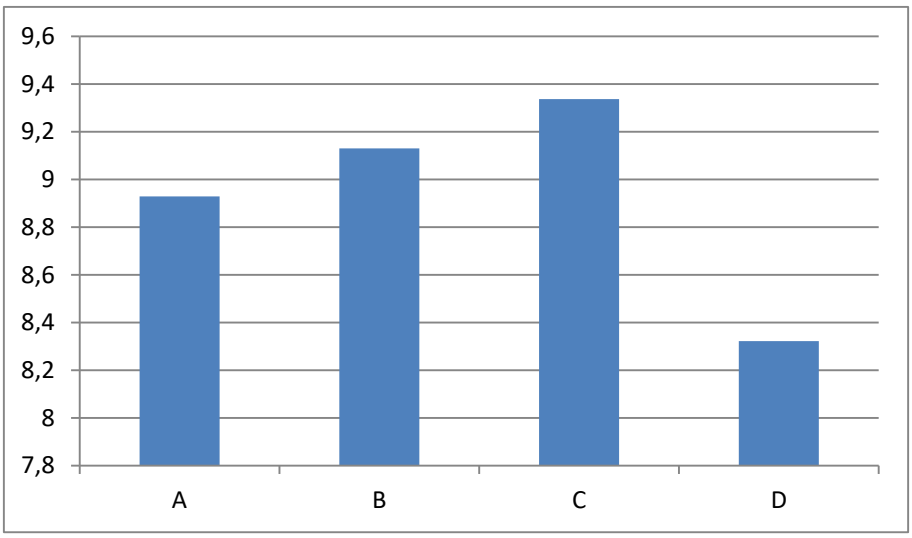

Figure 5. Shannon diversity index for endophytic and soil microbial communities. A – Root endophytes (oat–vetch); B – Root endophytes (vetch); C – Rhizosphere microbiome (oat–vetch); D – Rhizosphere microbiome (vetch).

4.2. Simpson Index

The Simpson index (Figure 6) measures the evenness and dominance structure of microbial communities, with values closer to 1 indicating greater diversity and lower dominance (Simpson, 1949). Across all samples, the index ranged narrowly between 0.988 and 0.995, confirming confirm rich and evenly distributed microbial assemblages. The highest diversity was detected in A (oat–vetch endophytes) and C (oat–vetch soil microbiome) (0.995 each), while the lowest occurred in D (vetch soil microbiome) (0.988). These findings corroborate the Shannon index pattern, demonstrating that oat–vetch intercropping fosters more balanced and functionally stable microbial communities in both rhizosphere and plant tissues. This can be attributed to the synergistic effect of diverse root exudates from both crops, which expand ecological niches and sustain higher microbial evenness (Li et al., 2024). Slightly lower Simpson values for vetch

endophytes (B, 0.994) suggest moderate dominance by a few symbiotic taxa, such as *Bradyrhizobium* and *Burkholderia*. The lowest index in vetch monoculture soils (D) indicates reduced evenness and the predominance of limited taxa (e.g., *Streptomyces*), consistent with monocultural systems (Liu et al., 2022). The combined assessment of Shannon and Simpson indices confirms that intercropping enhances both taxonomic richness and structural evenness, contributing to greater stability and functional resilience of agroecosystems (Philippot et al., 2013).

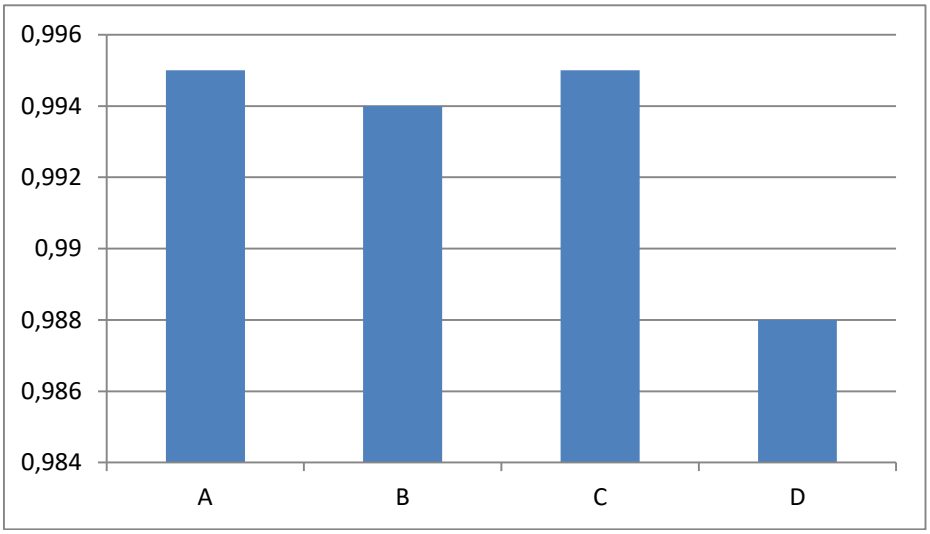

Figure 6. Simpson diversity index for endophytic and soil microbial communities. A – Root endophytes (oat–vetch); B – Root endophytes (vetch); C – Rhizosphere microbiome (oat–vetch); D – Rhizosphere microbiome (vetch).

5. Beta Diversity Analysis of Microbial Communities

Beta diversity describes the degree of dissimilarity in the taxonomic composition among samples, reflecting the spatial heterogeneity of microbial communities (Anderson et al., 2011). The beta diversity indices shown in Figure 7 reveal clear distinctions between endophytic and soil microbiomes. The highest variability was observed in the root endophytes of oat–vetch (A) and vetch (B), indicating greater within-group heterogeneity and a strong influence of host plant species and tissue specificity on microbial composition. This supports the hypothesis that endophytic environments are highly selective, allowing colonisation by only a limited number of taxa adapted to plant physiological conditions (Compant et al., 2010; Hardoim et al., 2015). In contrast, the soil microbiomes from oat–vetch intercropping (C) and vetch monoculture (D) exhibited lower beta diversity values, suggesting a more homogeneous taxonomic composition among replicates. This pattern likely reflects the stabilising effect of the soil environment and the relatively uniform physicochemical conditions in the rhizosphere, which reduce large fluctuations in community structure (Fierer, 2017). Comparative analysis between systems indicates that intercropping (C) decreases within-group

variability and promotes a more stable and resilient microbial structure compared to vetch monoculture (D). This finding confirms that mixed cropping enhances ecological consolidation of the microbiome through diversified root exudates and complementary nutrient utilisation (Chen et al., 2023; Li et al., 2024).

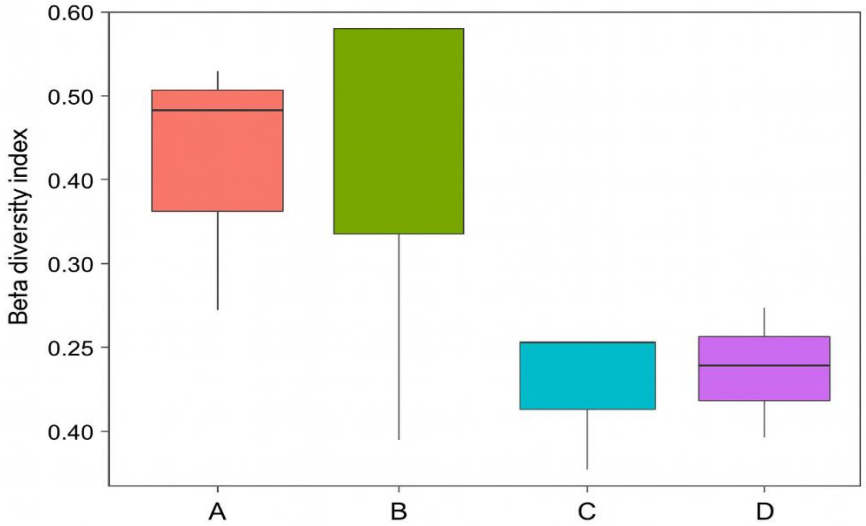

Figure 7. Beta diversity indices of endophytic and soil microbial communities in oat–vetch intercropping and vetch monoculture systems.

6. Principal Component Analysis (PCA)

PCA is a multivariate statistical method used to visualise structural differences among microbial communities and to assess taxonomic similarity based on metagenomic data (Jolliffe & Cadima, 2016). This approach reduces data dimensionality by extracting principal components (PC1, PC2, and PC3) that explain the greatest proportion of variance within the dataset. As shown in Figure 8, the first three components accounted for approximately 88% of the total variance (PC1 – 65%, PC2 – 17.25%, PC3 – 5.8%), indicating high explanatory power of the model. The spatial distribution of data points revealed a clear separation between root endophytic (A and B) and soil (C and D) samples, confirming that both plant species and isolation environment strongly influence microbial community composition (Philippot et al., 2013). Samples from the oat–vetch intercropping system (A and C) clustered closer together, reflecting similar microbial profiles across both endophytic and soil communities. This pattern corresponds to the higher α-diversity indices observed earlier and supports the idea that intercropping promotes the establishment of specialised and functionally interconnected microbial assemblages (Zhu & Morel, 2019; Sisodia et al., 2025). In contrast, vetch monoculture samples (B and D) were positioned further apart along PC1, indicating a more distinct and specialised community structure, likely resulting from selective root exudate effects typical of single-species cultivation (Brooker et al., 2015). PCA revealed distinct clustering patterns according to both the cultivation system (monoculture vs. intercropping) and isolation environment (root endophytes vs. soil

endophytes). These findings confirm that oat–vetch intercropping fosters more balanced and functionally coherent microbial structures, highlighting its potential as an effective agroecological strategy for maintaining soil health and microbial diversity.

Figure 8. Principal Component Analysis (PCA) of microbial communities based on taxonomic composition (genus level). Axes PC1 and PC2 explain 65% and 17.25% of the variance, respectively. A – Root endophytes (oat–vetch; orange); B – Root endophytes (vetch; gray); C – Rhizosphere microbiome (oat–vetch; purple); D – Rhizosphere microbiome (vetch; brown).

The metagenomic analysis revealed clear differences in microbial community structure and diversity between intercropping and monoculture systems. Oat–vetch intercropping supported higher bacterial diversity in both root and rhizosphere compartments, as indicated by elevated alpha diversity indices. These findings suggest that plant diversity enhances microbial richness and evenness, likely due to varied root exudates and expanded ecological niches. Taxonomic analysis showed that Actinobacteria and Proteobacteria were dominant across all treatments, consistent with their known roles in nutrient cycling and plant growth promotion. Notably, intercropped samples exhibited greater relative abundance of genera such as *Rhizobium* and *Pseudomonas*, which are associated with nitrogen fixation, phosphate solubilization, and biocontrol functions. The presence of these taxa underscores the potential of intercropping to recruit beneficial microbes that support plant productivity. Beta diversity analysis further demonstrated distinct microbial assemblages across treatments, with intercropped samples forming separate clusters. This suggests that intercropping not only increases diversity but also shapes community composition in a manner distinct from monocultures. These shifts may be attributed to root–root interactions and complementary nutrient use, which create a unique rhizospheric environment. Functionally, microbial communities in intercropping systems showed enrichment in genes related to nitrogen metabolism, organic matter degradation, and stress tolerance. Such traits are essential for maintaining soil health and reducing

dependence on synthetic fertilisers. The observed microbial dynamics align with previous studies, indicating that diversified cropping systems can enhance ecosystem services through microbial mediation. The results highlight the capacity of oat–vetch intercropping to foster complex, functionally active microbial communities in both root and rhizosphere environments. These findings support the integration of intercropping as a sustainable strategy to improve microbial biodiversity and soil function in agricultural systems.

CONCLUSIONS

In conclusion, the results highlight the capacity of oat–vetch intercropping to foster complex, functionally active microbial communities in both root and rhizosphere environments. These findings support the integration of intercropping as a sustainable strategy to improve microbial biodiversity and soil function in agricultural systems.

ACKNOWLEDGEMENTS

This research was funded by the Bulgarian National "Science Fund", grant number KP-06-DO 02/5. The authors acknowledge the financial support through the partners of the Joint Call of the Cofund ERA-Nets SusCrop (Grant N° 771134), FACCE ERA-GAS (Grant N° 696356), ICT-AGRI-FOOD (Grant N° 862665) and SusAn (Grant N° 696231).

REFERENCES

Anderson, M. J., Crist, T. O., Chase, J. M., Vellend, M., Inouye, B. D., Freestone, A. L., & Vázquez, D. P. (2011). Navigating the multiple meanings of β diversity: A roadmap for the practicing ecologist. *Ecology Letters, 14*(1), 19–28. https://doi.org/10.1111/j.1461-0248.2010.01552.x

Brooker, R. W., Bennett, A. E., Cong, W. F., Daniell, T. J., George, T. S., Hallett, P. D., ... & White, P. J. (2015). Improving intercropping: A synthesis of research in agronomy, plant physiology and ecology. *New Phytologist, 206*(1), 107–117. https://doi.org/10.1111/nph.13132

Chelius, M. K., & Triplett, E. W. (2001). The Diversity of Archaea and Bacteria in Association with the Roots of *Zea mays* L. *Microbial ecology*, 252-263. https://doi: 10.1007/s002480000087.

Chen, X., Chen, J., & Cao, J. (2023). Intercropping increases soil N-targeting enzyme activities: A meta-analysis. *Rhizosphere*, *26*, 100686. https://doi.org/10.1016/j.rhisph.2023.100686

Compant, S., Clément, C., & Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology & Biochemistry, 42*(5), 669–678. https://doi.org/10.1016/j.soilbio.2009.11.024

Costa, L. E. D. O., Queiroz, M. V. D., Borges, A. C., Moraes, C. A. D., & Araújo, E. F. D. (2012). Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (Phaseolus vulgaris). *Brazilian Journal of Microbiology*, *43*, 1562-1575. https://doi: 10.1590/S1517-838220120004000041.

Dang, K., Gong, X., Zhao, G., Wang, H., Ivanistau, A., & Feng, B. (2020). Intercropping alters the soil microbial diversity and community to facilitate nitrogen assimilation: a potential mechanism for increasing proso millet grain yield. *Frontiers in Microbiology*, *11*, 601054. https://doi.org/10.3389/fmicb.2020.601054

Daniel, R. (2005). The metagenomics of soil. *Nature Reviews Microbiology, 3*(6), 470–478. https://doi.org/10.1038/nrmicro1160

Delamuta, J. R. M., Ribeiro, R. A., Ormeño-Orrillo, E., Melo, I. S., Martínez-Romero, E., & Hungria, M. (2013). Polyphasic evidence supporting reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 63*(Pt 9), 3342–3351. https://doi.org/10.1099/ijs.0.049130-0

Eichorst, S. A., Trojan, D., Roux, S., Herbold, C., Rattei, T., & Woebken, D. (2018). Genomic insights into the Acidobacteria reveal strategies for their success in terrestrial environments. *Environmental Microbiology, 20*(3), 1041–1063. https://doi.org/10.1111/1462-2920.14043

Fierer, N. (2017). Embracing the unknown: Disentangling the complexities of the soil microbiome. *Nature Reviews Microbiology, 15*(10), 579–590. https://doi: 10.1038/nrmicro.2017.87

Hardoim, P. R., van Overbeek, L. S., & van Elsas, J. D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology, 16(10): 463-71*. https://doi: 10.1016/j.tim.2008.07.008.

Isali, I., Wong, T. R., & Tian, S. (2024). Best practice guidelines for collecting microbiome

Jolliffe, I. T., & Cadima, J. (2016). Principal component analysis: A review and recent developments. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences, 374*(2065), 20150202. https://doi.org/10.1098/rsta.2015.0202

Kaut, A. H. E. E., Mason, H. E., Navabi, A., O'Donovan, J. T., & Spaner, D. (2008). Organic and conventional management of mixtures of wheat and spring cereals. *Agronomy for Sustainable Development*, *28*(3), 363-371. https://doi: 10.1051/arpo:2008017

Knight, R., Vrbanac, A., Taylor, B. C., et al., (2018). Best practices for analysing microbiomes. *Nature Reviews Microbiology, 16*(7), 410–422. https:// doi: 10.1038/s41579-018-0029-9.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina platform. *Applied and Environmental Microbiology, 79*(17), 5112–5120. https://doi.org/10.1128/AEM.01043-13

Li, C., Chen, X., Jia, Z., Zhai, L., Zhang, B., Grüters, U., & Müller, C. (2024). Meta-analysis reveals the effects of microbial inoculants on the biomass and diversity of soil microbial communities. *Nature Ecology & Evolution*, *8*(7), 1270-1284. https://doi: 10.1038/s41559-024-02437-1

Lian, T., Mu, Y., Jin, J., Ma, Q., Cheng, Y., Cai, Z., & Nian, H. (2019). Impact of intercropping on the coupling between soil microbial community structure, activity, and nutrient-use efficiencies. *PeerJ*, *7*, e6412. https:// doi: 10.7717/peerj.6412.

Lithourgidis, A. S., Dordas, C. A., Damalas, C. A., & Vlachostergios, D. N. (2011). Annual intercrops: An alternative pathway for sustainable agriculture. *Australian Journal of Crop Science,* *5*(4), 396–410. https://search.informit.org/doi/10.3316/informit.281409060336481

Lithourgidis, A. S., Vasilakoglou, I. B., Dhima, K. V., Dordas, C. A., & Yiakoulaki, M. D. (2006). Forage yield and quality of common vetch mixtures with oat and triticale in two seeding ratios. *Field Crops Research*, *99*(2-3), 106-113. https://doi.org/10.1016/j.fcr.2006.03.008

Liu, J., Zhang, W., Teng, C., Pang, Z., Peng, Y., Qiu, J.,& Ding, C. (2024). Intercropping changed the soil microbial community composition but no significant effect on alpha diversity. Frontiers in Microbiology, 15, 1370996. https://doi.org/10.3389/fmicb.2024.1370996

Liu, Z., Li, S., Liu, N., Huang, G., & Zhou, Q. (2022). Soil microbial community driven by soil moisture and nitrogen in milk vetch (Astragalus sinicus L.)–rapeseed (Brassica napus L.) intercropping. *Agriculture*, *12*(10), 1538. https://doi.org/10.3390/agriculture12101538

Luo, S., Yu, L., Liu, Y., Zhang, Y., & Ding, W. (2016). Soil microbial community and functional diversity in intercropping systems. *Agriculture, Ecosystems & Environment, 230*, 142–150. https://doi.org/10.1016/j.agee.2016.05.036

Ma, H., Zhou, J., Ge, J., Zamanian, K., Wang, X., Yang, Y.,... & Zang, H. (2024). Oat/soybean intercropping reshape the soil bacterial community for enhanced nutrient cycling. Land Degradation & Development, 35(17), 5200-5209. https://doi.org/10.1002/ldr.5290

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E. M., Kubal, M., ... & Edwards, R. (2008). The metagenomics RAST server–a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC bioinformatics*, *9*(1), 386. https://doi.org/10.1186/1471-2105-9-386

Muthukumar, A., Udhayakumar, R., & Naveenkumar, R. (2017). Role of bacterial endophytes in plant disease control. In *Endophytes: Crop Productivity and Protection: Volume 2* (pp. 133-161). Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-66544-3_7

Peoples, M. Á., Brockwell, J., Herridge, D. F., Rochester, I. J., Alves, B. J. R., Urquiaga, S., & Jensen, E. S. (2009). The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis*, *48*(1), 1-17. https://doi.org/10.1007/BF03179980

Petkova, M., Shilev, S., Popova, V., Neykova, I., & Minev, N. (2025). Intercropping of Oats with Vetch Conducts to Improve Soil Bacteriome Diversity and Structure. *Microorganisms*, *13*(5), 977. https://doi.org/10.3390/microorganisms13050977

Handelsman, J. (2004). Metagenomics: Application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews, 68*(4), 669–685. https://doi.org/10.1128/MMBR.68.4.669-685.2004

Philippot, L., Raaijmakers, J. M., Lemanceau, P., & van der Putten, W. H. (2013). Going back to the roots: The microbial ecology of the rhizosphere. *Nature Reviews Microbiology, 11*(11), 789–799. https://doi.org/10.1038/nrmicro3109

Porebski, S., Bailey, L. G., & Baum, B. R. (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant molecular biology reporter*, *15*(1), 8-15. https://doi.org/10.1007/BF02772108

Ray, S., Maitra, S., Sairam, M., Sameer, S., Sagar, L., Divya, B., & Gitari, H. I. (2025). The nexus between intercropping systems, ecosystem services and sustainable agriculture: A review. *Research on Crops*, *26*(1). https://doi.org/10.31830/2348-7542.2025.ROC-1166

Santoyo, G., Moreno-Hagelsieb, G., del Carmen Orozco-Mosqueda, M., & Glick, B. R. (2016). Plant growth-promoting bacterial endophytes. *Microbiological research*, *183*, 92-99. https://doi.org/10.1016/j.micres.2015.11.008

Shannon, C. E., & Weaver, W. (1949). *The Mathematical Theory of Communication.* Urbana, IL: University of Illinois Press.

Seitz, V. A., Chaparro, J. M., Schipanski, M. E., Wrighton, K. C., & Prenni, J. E. (2023). Cover crop cultivar, species, and functional diversity is reflected in variable root exudation composition. *Journal of Agricultural and Food Chemistry*, *71*(30), 11373-11385. https:// doi: 10.1021/acs.jafc.3c02912.

Simpson, E. H. (1949). Measurement of diversity. *Nature, 163*(4148), 688. https://doi.org/10.1038/163688a0

Sisodia, P., Gryta, A., Pathan, S. I., Pietramellara, G., & Frąc, M. (2025). Roots to Riches: Unearthing the Synergy of Intercropping, Microbial Interactions, and Symbiotic Systems for Sustainable Agriculture: A Review. *Agronomy*, *15*(9), 2243. https://doi.org/10.3390/agronomy15092243

Tian, X. L., Wang, C. B., Bao, X. G., Wang, P., Li, X. F., Yang, S. C.,... & Li, L. (2019). Crop diversity facilitates soil aggregation in relation to soil microbial community composition driven by intercropping. Plant and Soil, 436(1), 173-192. https://10.1007/s11104-018-03924-8

Tuna, C., and Orak, A. (2007). The role of intercropping on yield potential of common vetch (*Vicia sativa* L.)/oat (*Avena sativa* L.) cultivated in pure stand and mixtures. *Journal of Agricultural and Biological Science*, *2*(2), 14-19.

Van Der Heijden, M. G. A., Bardgett, R. D., & van Straalen, N. M. (2008). The unseen majority: Soil microbes as drivers of plant diversity and productivity. *Ecology Letters, 11*(3), 296–310. https:// doi: 10.1111/j.1461-0248.2007.01139.x.

Wang, G., Bei, S., Li, J., Bao, X., Zhang, J., Schultz, P. A.,... & Zhang, J. (2021). Soil microbial legacy drives crop diversity advantage: Linking ecological plant–soil feedback with agricultural intercropping. Journal of Applied Ecology, 58(3), 496-506. https://doi.org/10.1111/1365-2664.13802

Zhu, S., & Morel, J. B. (2019). Molecular mechanisms underlying microbial disease control in intercropping. *Molecular Plant-Microbe Interactions*, *32*(1), 20-24. https:// doi: 10.1094