

Beneath the apple trees - Exploring soil microbial properties under *Malus domestica* concerning various land management practices

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ABSTRACT

The presented study evaluated the impact of six land management practices on soil bacterial and fungal communities under apple trees: green field belts, forests, gardens with trees and ornamental plants, gardens with farm animals, and uncultivated and cultivated orchards. The study explored soil microbial properties including the enzymatic activity of dehydrogenases (tested by colorimetric reaction with 2,3,5-triphenyl-tetrazolium chloride), metabolic profile (using BiologTM ECO Plates), metataxonomy structure (Next Generation Sequencing using Illumina[®]), followed by physicochemical properties (pH, N, P, K, microelements concentrations, organic matter, and C_{org} content). The hypothesis was that different land management practices would influence soil microbial properties, with cultivated orchards expected to show significantly lower dehydrogenases activity, and higher substrate-based respiratory than biomass response, within substrate stress occurrence, when testing metabolic profiles, but also different composition and lower relative abundances of specific microbial taxa and different biomarker genera, compared to other treatments. We aimed to identify practices promoting diverse microbial substrate-based metabolic and taxa diversity responses, with a focus on *Bacillus* and *Trichoderma* abundances, which are potential biological agents against fungal pathogens. As expected the presented research revealed significant statistical variations in microbial communities among different land management practices in soil beneath apple trees. It was accordingly noted that cultivated orchards, but also green belts, clearly exhibited reduced microbial activity (3.59 and 4.76 TPF kg⁻¹ d⁻¹, respectively) compared to gardens and uncultivated orchards (12.08 and 9.89 TPF kg⁻¹ d⁻¹). Cultivated orchards notably showed higher respiration levels and substrate stress compared, especially to forests and other land management practices represented by a clear separation of observed according to Sneath's criteria in cluster analysis. Different land management practices induce unique stress responses in microbial communities: forests struggled with B-Methyl-D-Glucoside, gardens with Serine and Putrescine, cultivated orchards with D-Glucosaminic Acid and Cyclodextrin, and bounds with 2-Hydroxy-Benzoic Acid. Substantial differences were also observed in the relative abundance of the top ten bacterial and fungal orders, and biomarker genera representatives. In cultivated orchards, there was a significant decrease in the relative abundance of many bacterial taxa such as e.g. Rhizobiales, Burkholderiales, Vivinamibacterales, and fungal taxa including Eurotiales, and Saccharomycetales. Notably, no significant differences were noted for *Bacillus* abundance among tested management practices. Forests favored *Trichoderma* abundance the most among tested practices (relative abundance 0.05 %). In turn, *Trichoderma* representatives were revealed as biomarker genera in gardens with animals. *Williamsia* representatives, as found in uncultivated orchards were suggested to be a biomarker of less disturbance, resulting from area restoration. Overall, the study discussed how different land management practices influence soil microbial communities and their functional roles, emphasizing the potential impacts of use on soil health and biodiversity within its implications. The most important recommendation bullet points:

- using *Williamsia* representatives as a soil biomarker microorganism to indicate successful area restoration processes in apple orchards,
- using D-Glucosaminic Acid metabolic stress test to reveal early difficulties in controlling fungal pathogens in soil microbial communities,

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- promoting diverse plant covers and reasonably reduced agrochemical inputs in apple orchards for enhancing soil microbial resilience,
- preserving habitats of wild apple trees in forests or green belts, as these environments exhibit reduced metabolic stress and support the occurrence of fungi like *Trichoderma*, in the context of locations from which such isolates should be sought for further biocontrol use.

1. Introduction

The scale of apple production on a global level is substantial, with annual production exceeding 80 million metric tons in 2022 (Sottocornola et al., 2022). Thus, several countries play pivotal roles in the worldwide apple industry, with China, the United States, India, and Poland being among the major producers. The widespread appeal and usage of apples contribute to their significance in the global fruit industry. In the context of apple production, the European Union is committed to sustainability through two key initiatives: the European Green Deal and the Farm to Fork Strategy (Schebesta et al., 2020). These strategies aim to promote eco-friendly apple production while ensuring food security and emphasizing biodiversity preservation, intending to reduce pesticide use and associated risks by 50 % by 2030 (European Commission, 2020). It appears to be quite challenging since among others fungal diseases pose a significant threat to apple production, affecting both yield and fruit quality. Among the most common fungal pathogens impacting apple trees are *Neofabraea* spp., *Penicillium expansum*, *Botrytis cinerea*, *Botryosphaeria dothidea*, *Monilia* spp., and *Alternaria* spp. (Leng et al., 2023; Lee et al., 2023; Neri et al., 2023). Diseases manifest differently but all can lead to defoliation, reduced fruit quality, and economic losses if left unmanaged. Of course, eliminating fungicides in apple production can reduce the risk of fungal pathogens developing resistance and allow beneficial soil microorganisms to thrive, improving among others soil health and fertility (Pathak et al., 2022). However, apple production may become more susceptible to diseases, potentially resulting in lower yields and higher costs for producers who need alternative disease management practices (Holb, 2009). Sustainable apple production includes adopting integrated pest management practices (Mehla, 2023). Those alternatives include biological control and other sustainable orchard management practices (Fenta and Mekonnen, 2024).

It is known that balanced agriculture management practices are essential for fostering diverse and resilient soil microbial communities (Symochko and Pereira, 2024). This is important since microbial communities play a vital role in maintaining soil productivity, nutrient cycling, and overall ecosystem stability (Huang et al., 2024; Fraç et al., 2018; Fraç et al., 2022). The state of knowledge on soil microbes under apple trees highlights the significant impacts of different land management practices. Studies like Gupta et al. (2022), Shanmugam et al. (2011), Wang et al. (2022), Li et al. (2022), Xie et al. (2022), and Abdelfattah et al. (2016) show how practices such as mulching, intercropping, and rootstock types influence microbial communities, shaping the apple tree environment. All these investigations primarily focus on understanding the impact of different agricultural practices on soil microbial communities associated with apple trees in orchards for rational management. However, these studies do not compare apple orchards with natural or semi-natural environments. Even though, previous research, e.g. performed on the wild (found in the forest) and planted raspberries (Oszust and Fraç, 2021), led to broader conclusions and recommendations for its production. Identifying this as a research gap we proposed a study to compare soil microbial properties under apple trees in conventionally cultivated orchards, where agrochemicals and mineral fertilizers are used, with other land management practices resembling natural and semi-natural habitats for apple trees. These semi-natural habitats were previously utilized but are now reverting to natural conditions and becoming wild.

We offered an investigation comprising various techniques,

including dehydrogenase activity, metabolic profiles, metataxonomic analyses, and physicochemical properties of the soil background. The adoption of multiple methods to explore soil microbial properties was influenced by recent trends (Manda et al., 2023; Pylak et al., 2023). This multifaceted approach enhances the breadth, depth, reliability, and applicability of findings, thereby advancing our understanding of soil ecosystems and their role in broader ecological and agricultural contexts (Manda et al., 2023). The hypothesis was that various land management practices would impact soil microbial properties. It was anticipated that cultivated orchards would exhibit significantly lower dehydrogenase activity and higher substrate-based respiratory responses relative to biomass under substrate stress conditions when testing metabolic profiles. Additionally, these orchards were expected to have lower relative abundances of specific microbial taxa and show different biomarker genera compared to other practices. Particularly, the study aimed to identify the occurrence of microbial representatives that could indicate restoration processes in apple orchards. Notably, in the context of the observed development of trends related to integrated pest management practices (Ryalls et al., 2024), we seek land management practices that promote the abundance of microbial groups capable of serving as biological agents against fungal pathogens in apple orchards. Recent research has underscored the potential of diverse microorganisms as promising biocontrol agents targeting apple-related fungal pathogens (Oszust et al., 2023a). For instance, bacteria like *Bacillus* spp. and filamentous fungi such as *Trichoderma* spp. have shown considerable promise, exhibiting biocontrol efficacy through various mechanisms (Asad, 2022; Luo et al., 2022). Therefore, our investigation aimed to elucidate how specific land management strategies can enhance the prevalence of these beneficial microorganisms within communities. Overall, this assessment enhances the understanding of soil ecosystems beneath apple trees and their crucial role in broader ecological and agricultural contexts. It guides decision-making regarding site selection and ensures the effective exploration of valuable resources.

2. Materials and methods

2.1. Soil samples acquisition

Soil samples were obtained from 26 locations in Southeast Poland, including 37 apple trees, categorized according to land management practices as follows: belts (hedges) separating cultivated fields (B), forest areas (F), gardens with ornamental plants and fruit trees (G), courtyards/gardens where farm animals are kept (GA), orchards where the use of agrochemical treatments has been discontinued, uncultivated orchards (OU), and orchards cultivated in an integrated system (OC). The details on apple tree localization within the geographic coordinates of apple tree age and varieties (as declared by owners or land authorities) and management history are presented in Table 1. To differentiate OC and OU, it was assumed that OC was orchards in which different agrochemicals were used. Details of the agrochemicals used in OC and their quantities follow Table 2. The information includes the use of pesticides and fertilizers application for sites. The necessary permits from forestry authorities have been obtained for the collection of samples in forests as well and permissions have been obtained from private orchards and gardens owners.

The soil from adhering to apple tree roots (3.5 cm distance) was sampled in triplicate at a depth of 20–40 cm, following the removal of surrounding vegetation. Then, samples were placed into plastic bags and

Table 1

The factsheet on apple trees concerning various land management practices. Explanations: apple tree age and varieties as declared by land owners or authorities. Explanations: belts (hedges) separating cultivated fields (B), forest areas (F), gardens with ornamental plants and fruit trees (G), courtyards/gardens where farm animals are kept (GA), orchards where the use of agrochemical treatments has been discontinued, uncultivated orchards (OU), and orchards cultivated in an integrated system (OC); nd – no data available.

Method of land management	Localization	Apple tree variety	Apple tree age (years old)	Location (latitude) N	Location (longitude) E	Management history	
Bounds (B)	Lublin	nd	15	51.220202	22.627215	On the border of cropland and side of municipal asphalt paving, management continued >15 years	Limited chemical treatments or/and fertilization - sampling when rape cultivated - Sirius 250 EW: 200 l/ha, Kizeryt mineral nitrogen fertilizer: 200 kg/ha
	Chruślanki	nd	>50	51.012814	22.014071	On the border of cropland and local unpaved road, management continued >25 years	Limited chemical treatments or/and fertilization - sampling when wheat cultivated: fungicide - Delaro 325 S.C.: 0.5 l/ha, mineral nitrogen fertilizer - Polifoska 2 1 + S: 200 kg/ha, herbicide - Chwastox Trio: 2 l/ha
	Chruślina Kolonia	Kosztela	20	51.031289	22.007947	On the border of green wastelands and farm buildings, management continued >15 years	No chemical treatment or/and fertilization
	Baraki	Antonovka	>30	50.973528	23.300218	On the border of two green wastelands, management continued >25 years	No chemical treatment or/and fertilization
	Franciszków Stary	Gravenstein	70	51.100734	21.988053		
	Grądy	Biała Nalew	ca. 60	51.096165	22.220020		
	Grądy	Boiken	ca. 60	51.095843	22.219947		
	Przybysławice	Transparent de Croncels	ca. 90	51.367237	22.271997		
	Przybysławice	Antonovka	ca. 90	51.367170	22.272069		
	Forest (F)	Idalin	nd	>25	51.017247	21.991387	Forest, management continued >25 years
Marynopole		nd	nd	50.846510	22.039774		
Serniawy Kolonia		Antonovka	>70	51.334265	23.367624		
Serniawy Kolonia		White	>70	51.334265	23.367624		
Wólka		Transparent	nd	51.068611	21.884167		
Kolczyńska		nd	nd	51.068611	21.884167		
Garden (G)	Brzezice	Papirówka	>40	51.146808	22.894435	Garden with ornamental plants and fruit trees, management continued >25 years	No chemical treatment or/and fertilization
	Dys	Rennet	>30	51.299944	22.563278		
	Elizówka	White	>30	51.298222	22.575444		
	Kalinówka	Bohnapfel	90–100	50.860639	23.290472		
	Siedliska	Antonovka	>50	51.065272	22.936407		
	Pierwsze Świdnik	Jonathan	>15	51.200096	22.702692		
Zadworze	nd	>30	51.000889	22.255750	Limited chemical treatments or/and fertilization: Florovit, all-purpose fertilizer, 2–4 times a year		
Garden & Animals (GA)	Grabówka	Transparent	>70	50.946885	21.929213	Animals roam freely here: poultry, cattle, and/or goats are kept in this area, management continued >15 years	No chemical treatment or/and fertilization
	Ukazowa	de Croncels	>70	50.946947	21.929255		
	Grabówka	Bohnapfel	>70	50.946947	21.929255		
	Ukazowa	Bohnapfel	>70	50.946947	21.929255		
	Majdan Kozic Górnych	Roter Stettiner	>70	51.089213	22.731569		
	Szczebrzeszyn	Antonovka	>30	50.697945	22.968842		
Śmiary	nd	>70	52.044135	22.239975			
Wilków	Antonovka	>30	51.256439	21.875893			
Orchard Cultivated (OC)	Chruślina Kolonia	Gala	12	51.026361	22.004023	Usual practices of cultivation, pruning, or other care, management continued 10–12 years	Chemical treatment or/and fertilization, detailed information noted in Table 2
	Franciszków Stary	Calville Rouge d'hiver	10	51.095637	21.999804		
Orchard Uncultivated (OU)	Wilków	Champion	10	51.261055	21.884075	Usual practices of cultivation, pruning, or other care have been discontinued, leading to a state of neglect >20 years	No chemical treatment
	Chruślina Kolonia	Jonathan	>25	51.026967	22.003878		
	Franciszków Stary	McIntosh	>30	51.104271	21.989555		
	Góry	Ananas	ca. 100	51.351814	22.285082		
	Góry	White	ca. 90	51.351806	22.284977		
	Góry	Transparent	ca. 90	51.351806	22.284977		
	Grabówka	Close	>40	50.945576	21.918966		
	Baraki	Close	>40	50.945576	21.918966		
Leonin	Calville Blanc d'hiver	>30	51.104901	22.008197			
Rudka Kijańska	nd	80	51.437280	22.850237			

transported to the laboratory. Then soil was sieved, divided into two parts, and then kept in a cold room (+4 °C) and freezer (−20 °C) until analyses were performed to keep the soil fresh (not dried out). The collected soil samples underwent various analyses to assess their microbial biodiversity and physicochemical background. Microbial analyses included: dehydrogenase activity (DHA), which was measured to overall soil microbial activity; Biolog™ ECO plate analyses (ECO) were employed to examine soil microbial community metabolic diversity (on samples kept +4 °C) in and Illumina® Next-Generation Sequencing (NGS) was conducted using samples (on samples kept −20 °C) to assess the microbial community composition (metataxonomy). All the analyses were used, particularly in response to different land management practices.

2.2. Dehydrogenases activity

The overall microbial activity was evaluated within dehydrogenases activity (EC 1.1.1.1). The analyses were performed on fresh soil samples according to the colorimetric Thalmann's method, modified by Alef (1995), using 2,3,5-triphenyltetrazoliumchloride (TTC) (Merck, Darmstadt, Germany) as substrate.

2.3. Biolog™ ECO plates

The metabolic profiling of microbial communities followed Biolog™ ECO plate reads (at both 590 nm and 750 nm wavelengths) analyses as described in detail by Pylak et al. (2021) and according to previous research (Garland, 1997; Fraç et al., 2012). The results were presented as diversity indices such as Richness (R) and Shannon (H) (followed by data obtained at 590 nm and 750 nm wavelengths), average well color

development (AWCD) (based on 590 nm), average well density development (AWDD) (based on 750 nm), substrate stress index (SST), calculated as the ratio of 590 nm/750 nm values. The lower the SST value the higher the biomass production at lower substrate utilization, which corresponds to better growing conditions for the microbial community in given substrates (Pylak et al., 2023). For the SST representation (used in correlation analyses) substrates located at Biolog™ ECO plates were divided into five groups of carbon sources including carbohydrates, carboxylic and ketonic acids, amines and amides, amino acids, and polymers (Weber and Legge, 2009).

2.4. Next-Generation Sequencing

The metataxonomic analyses were conducted using Next-Generation Sequencing (NGS) technology, specifically Illumina® SBS (sequencing by synthesis) (San Diego, USA), with phylogenetic markers 16S for bacteria and ITS for fungi (eDNA metabarcoding) as summarized by Pylak et al., 2023 and Siegieda et al., 2023. Firstly, the eDNA from soil samples was extracted using the GeneMATRIX Soil DNA Purification Kit (Eutrx®, Gdańsk, Poland).

According to the adopted methodology to achieve high taxonomic resolution, the ITS1 fragment was taken under consideration (Wang et al., 2015). The ITS1 fragment of fungal DNA was amplified to identify the fungi present in each soil sample, with the primers as proposed by Bellemain et al. (2010) and a fungal metagenomic sequencing protocol by Illumina®. KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Cape Town, South Africa) was used for the amplification. We used the following conditions: 95 °C for 3 min of initial denaturation and 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s with the final step occurring at 72 °C for 5 min. The obtained amplicons were then

Table 2

The factsheet on agrochemicals applied in cultivated orchards as declared by landowners. Explanations are provided in Table 1.

Comprehensive plant treatment	Group of treatments	Chruślina Kolonia			Franciszków Stary			Wilków			
		Product name and dose (per hectare)									
Protective treatments	Fungicides and/or insecticides	Funguran OH: 0.75 kg – 2 times			Miedzian 50 WG: 1,5 kg			Miedzian 50 WP: 1 kg			
		Delan Pro: 2.5 l			Delan 700 WG: 0.5 kg – 4 times			Miedzian 50 WP + Delan 700 WG: 1 kg + 0.5 kg			
Plant nourishment	Foliar fertilization	Delan: 0.5 kg 1.9 kg – 2 times			Tepeki 50 WG: 0.14 kg			Delan 700 WG + Calypso 480 SC: 0.2 kg + 0.5 kg			
		Fontelis 200 SC: 0.75 l			Kendo 50 EW: 0.4 l			Aplosar 80 WG: 3 kg			
		Captan 80 WG + Chorus: 1.9 kg + 0.30 kg			Luna Experience 400 SC: 0.75 l			Delan 700 WG: 0.5 kg			
		Captan 80 WG + Sercadis: 1.9 kg + 0.3 l			Chorus 50 WG: 0.3 kg			Shavit Plus 80 WG: 2 kg			
		Captan 80 WG: 1.9 kg – 2 times			Sercadis: 0.25 l			Coragen 200 + Merpan 80: 175 ml + 1.9 kg			
		Captan 80 WG + Zato: 0.2 kg			Merpan 80 WDG: 1.9 kg – 5 times			Pennozeb 80 WP: 1.9 kg			
	Soil fertilization based on chemical analysis results		Captan 80 WG + Zato: 1.9 kg + 0.2 kg			Mospilan 20 SP: 0.125 kg			Sercadis 300 Se: 0.3 l		
			Captan 80 WG + Zato: 1.9 kg + 0.2 kg			Steward 30 WG: 0.2 kg			Merpan 80 + Steward 30: 1.9 kg + 0.2 kg		
			Captan 80 WG + Score 250 SC: 1.9 kg + 0.2 l			Coragen 200 SC: 0.175 l			Bellis 38 WG: 0.8 kg		
			Bellis 38 WG: 0.8 kg								
			Luna Experience 400 SC: 0.9 l								
			Teppeki: 0.14 kg								
Maintenance treatments	Pruning Irrigation Weed control	Coragen: 150 ml			Superba Forte: 1.5 kg			GreenAlga: 4 l			
		Movento: 2.25 l						GIBB Plus 11 SL: 0.5 l			
Specialized treatments	Thinning fruit set	Cynko-bor Interomag: 4 kg – 3 times			Magnesium sulfate heptahydrate: 6 kg – 4 times			YaraMila Complex: 180 kg			
		Magnesium lime: 300 kg/ha			Superba Forte: 1.5 kg + YaraIviva			Manure: 20 t			
		Yara Mila Complex 400 kg/ha			Calcinit: 100 kg						
		Ammonium nitrate 200 kg/ha			Eurofertil 29 N PR: 100 kg						
		Yara Unica Calcium 100 kg/ha									
		Yara liva Nitabor 100 kg/ha									
		Yes – Winter + Summer			Yes – Winter			Yes – Winter			
		Yes			No			No			
		Roundup 360 Plus + Chwastox Extra: 2 l + 1 l – 2 times			Agrosar 360 Sl: 0.5 l			Mechanical weeding			
		Colorado: 5 l			No			No			

purified using magnetic beads CleanNGS (CleanNA, Netherlands, Gouda). Each sample was indexed with a unique Illumina® sequencing adapter (Illumina Nextera set a, b, and c) using the following program: 95 °C for 3 min and 10 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s with a final step of 72 °C for 5 min. Samples were subsequently purified using magnetic beads and quantified using the Quantus fluorometer with QuantiFluor® ONE reagents (Promega, Madison, WI, USA). Library size was analyzed using D1000 DNA ScreenTape on TapeStation 4150 (Agilent Technologies Inc., Santa Clara, USA) and samples were diluted to the same concentration with PCR-grade water and pooled to a final concentration of 8 pM. The library was sequenced using the Illumina MiSeq platform (2 × 300 paired-end) with 15 % PhiX used as internal positive quality control (Illumina®, San Diego, CA, USA). The 16S fragment of bacterial DNA was amplified to identify the bacteria present in each soil sample. The stages of the analysis were analogous to the sequencing of the ITS1 fragment.

Raw sequence reads were submitted to the Sequence Read Archive (SRA) database within BioProjects PRJNA923149 for fungal and PRJNA923266 for bacterial communities. Quantitative positive controls were applied for the target microorganism groups (the MOCK community) (Sevim et al., 2019). The results were presented as diversity indices such as Chao1, ACE, Shannon, Simpson, InvSimpson, and Fischer.

The data was processed and analyzed using QIIME2 2020.11 (Bolyen et al., 2019). In QIIME2, ITSxpress (Rivers et al., 2018) was used for primer trimming, and DADA2 (Divisive Amplicon Denoising Algorithm v.2) (Callahan et al., 2016) was used for denoising, chimera removal (method: consensus and minimal fold parent overabundance parameter set to 12), merging and amplicon sequence variant (ASV) calling (Callahan et al., 2017). Taxonomy was assigned using the Scikit-learn (sklearn) classifier (Pedregosa et al., 2011) trained on a UNITE 8.3 database (ver. dynamic, 98.5 % similarity level) (Kõljalg et al., 2020; Nilsson et al., 2019).

2.5. Physicochemical analyses

Physicochemical analyses included the pH determination and P₂O₅, K₂O, Mg, microelements (B, Cu, Zn, Mn, Fe) content evaluation, the determination of organic matter and organic carbon (C_{org}), as well as the examination of mineral nitrogen including N-NO₃ and N-NH₄. The chemical analyses were performed at the Regional Chemical-Agricultural Station in Lublin according to the methodology summarized by Pylak et al., 2021.

2.6. Statistical analysis

The visualization and statistical evaluation of the NGS data was carried out using the R software v. 4.2.3 with the following packages: phyloseq (McMurdie and Holmes, 2013), DESeq2 v. 1.30.1 (Love et al., 2014), NetCoMi v. 1.0.2 (Peschel et al., 2021), ggplot2 (Wickham, 2016), vegan (Oksanen et al., 2007), tidyverse (Wickham, 2016), GUniFrac (Chen et al., 2021), file2meco (Liu et al., 2021), macodev (Liu et al., 2021) along with the microeco (Liu et al., 2021) package. Rarefaction curves (Fig. S1A and B, for bacterial and fungal analyses, respectively) show the number of ASV reads per total reads for each sample. The number of considered sequences was normalized to the smallest possible sample size (2000).

The network parameters explanation is as follows. Vertex refers to individual microorganisms represented by an amplicon sequence variant (ASV). Edge represents the connections or interactions between microbial individuals showing how they are linked together in the community. Average degree informs on average, how many other microbes each microbe interacts with, where a higher average degree means more interactions. Next average path length means finding the average distance between microbes in the community, in other words, how many interactions it takes between one microbe to another, on average. Network diameter conspires the longest distance between any

two microbes in the community. It shows how far apart the most distant microbes are in terms of interactions. The clustering coefficient measures how much microbes tend to form groups or clusters with their neighbors, a higher coefficient means microbes are more likely to interact with their nearby friends. Density tells how connected the microbial community is, where low density means many possible interactions are not happening, while a high density means most possible connections are being used. In turn, heterogeneity indicates how different the number of interactions is among microbes, where a low value means most microbes have a similar number of connections, while a high value means some have many more than others. Finally, centralization - shows if there are a few super-connected microbes that play a central role in the community, whereas low centralization means interactions are spread out more evenly among the microbes (Ding, 2023).

The correlation matrix analysis was performed and results were visualized using the R software v.4.2.3 with the use of the following packages: corplot (Wei and Simko, 2021), ggcorrplot (Kassambara, 2022), Hmisc (Harrell Jr., 2023), fplot (Berge, 2020), and ggplot2 (Wickham, 2016). The cluster analysis (CA) and ANOVA evaluation were performed using Statistica 13.1 software (StatSoft®, Tulsa, Oklahoma, USA). The CA analysis, including the grouping of treatments (land management practices) and features (response to substrates located in Biolog™ ECO plates), was performed on the standardized data of the absorbance average values for readings obtained at 72 h of incubation, as there were the most representant data. A dendrogram representing the similarities of the carbon utilization patterns from substrates located in Biolog™ ECO plates was set on scaled axis bond distances (Ward's method, within Euclidean distance), with marked boundaries of Sneath's criteria (restrictive, 33 % and less restrictive, 66 %) (Sneath and Sokal, 1973; Oszust et al., 2019). Venn diagrams were constructed using InteractiVenn (Heberle et al., 2015).

All statistical analysis, except for the NGS data, was performed with the use of Statistica 13.1 software. Both data normality and homogeneity of variance were checked, and a one-way ANOVA with $p < 0.05$ was performed with the following Tukey tests to reveal statistically significant differences.

3. Results and discussion

This study examined the microbial communities present in the soil beneath apple trees grown in areas that are under different land management practices, including belts (hedges) separating cultivated fields (B), forest areas (F), gardens with ornamental plants and fruit trees (G), gardens (courtyards) where farm animals are kept (GA), orchards where the use of agrochemical treatments has been discontinued, uncultivated orchards (OU), and orchards cultivated in an integrated system (OC).

3.1. Overall microbial activity

The average level of dehydrogenase activity (DHA) characterizes the overall activity of microbial communities inhabiting the soil beneath apple trees concerning various land management practices is presented in Fig. 1. Significant differences in dehydrogenase activity were observed in certain practices. The highest level of dehydrogenase activity was noted in the soil beneath apple trees located in gardens (G) (12.08 ± 0.59 mg TPF kg⁻¹ d⁻¹) and uncultivated orchards (OU) (9.89 ± 0.42 mg TPF kg⁻¹ d⁻¹). DHA activity in forests (F) and in gardens with animals (GA) reached accordingly 6.36 ± 0.46 mg TPF kg⁻¹ d⁻¹ and 7.72 ± 0.29 mg TPF kg⁻¹ d⁻¹ (and also in this case, similarities are demonstrated). The lowest activity was found in the soil from cultivated orchards (OC), which reached 3.59 ± 0.15 mg TPF kg⁻¹ d⁻¹, and in green belts (B) (4.76 ± 0.14 mg TPF kg⁻¹ d⁻¹).

Many factors must be considered to comprehensively understand the mechanisms driving microbial activity in various land-management types. In gardens (G) and uncultivated orchards (OU), where DHA

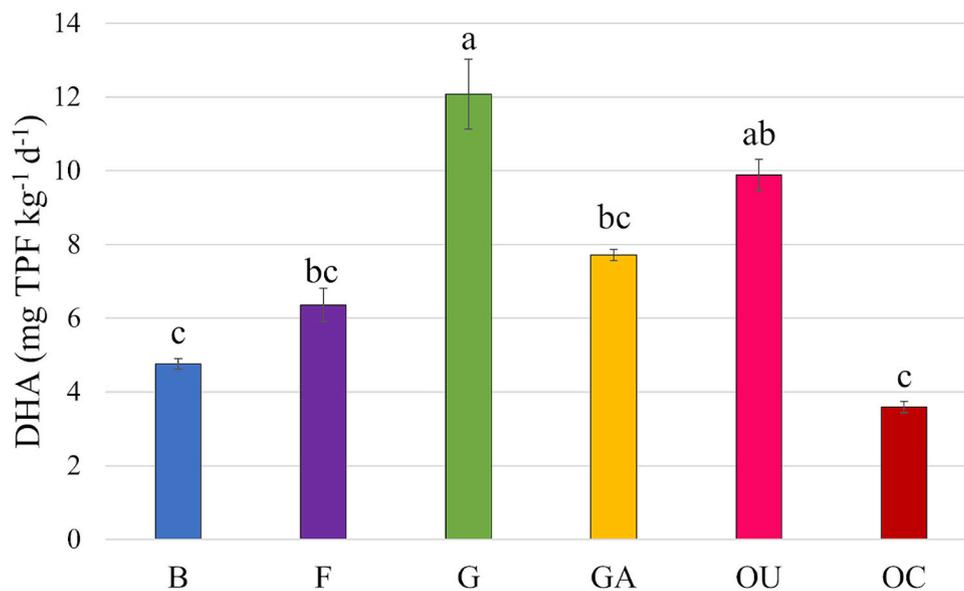


Fig. 1. Dehydrogenases (DHA) activity in the soil beneath the apple trees according to land management practices. Explanations: belts (hedges) separating cultivated fields (B), forest areas (F), gardens with ornamental plants and fruit trees (G), courtyards/gardens where farm animals are kept (GA), orchards where the use of agrochemical treatments has been discontinued, uncultivated orchards (OU), and orchards cultivated in an integrated system (OC); triphenylformazan (TPF); error bars indicate a standard deviation and different letters above the bars indicate significant differences (according to test ANOVA Tukey, $p < 0.05$).

levels were highest, the soil probably benefits from lower disturbance, diverse plant cover, and organic inputs (Ma et al., 2021). These conditions support a rich microbial community engaged in efficient organic matter decomposition and can be attributed to higher Net Primary Productivity (NPP), which generally leads to more organic material entering the soil, that supports a more active microbial community and thus higher dehydrogenase activity (Thies and Grossman, 2023). Thus, lower diversity resulted from fewer types of root exudates and organic compounds entering the soil, which can limit the diversity and activity of microbial communities. Diverse plant species release a wider range of organic compounds through root exudates, which serve as substrates for soil microbes. In monoculture orchards, the limited variety of root exudates can diminish microbial activity (Furmanczyk et al., 2024). Forests (F) and gardens with animals (GA) exhibited moderate DHA levels, reflecting their semi-natural environments that sustain relatively stable microbial communities Settineri (2017).

Conversely, cultivated orchards (OC) and green belts (B) showed the lowest DHA levels. Intensive agricultural practices such as compaction and applications of agrochemicals, and monoculture in cultivated orchards can disrupt soil structure and deplete organic matter, reducing microbial habitats and enzymatic activity. This normally leads to diminished microbial diversity and metabolic function. Agricultural machinery can compact the soil, reducing pore space and oxygen availability (Das, 2024). This compaction limits the movement of air and water, making conditions less favorable for microbial activity (Dugan et al., 2024). What is more, cultivated orchards that receive agrochemicals can create imbalances in soil nutrient levels (Lepp et al., 2024). High levels of nitrogen and phosphorus from fertilizers can inhibit certain microbial processes and alter microbial community composition, leading to reduced dehydrogenase activity. Cultivation probably leads to the depletion of soil organic matter, which is a crucial source of nutrients and energy for microbes. Reduced organic matter inputs mean less substrate for microbial growth and enzyme production (Dincă et al., 2022). What is more agricultural practices can temporarily increase or decrease soil pH (Kai and Adhikari, 2021), influencing microbial activity. Soil pH and C/N ratio determine variations in soil microbial communities and enzymatic activities of the agricultural ecosystems (Xu et al., 2020).

Testing DHA in soil beneath apple trees among various management

practices has revealed that the most overall active microbial communities are found in the soil beneath apple trees in gardens and uncultivated orchards. This finding underscores the significance of mostly (semi)-natural environments with diverse plants and provides valuable insights for isolating microbial strains with potential applications in biotechnology, agriculture, and environmental management and agrees e.g. with Fikri et al. (2018). However, it needs to be highlighted that excessively high soil dehydrogenase activity may indicate nutrient imbalances or environmental stressors that could potentially harm apple trees over time (Rutkowski et al., 2022). While DHA serves as the very first indicator of active microbial communities, its extremely elevated levels could suggest disruptions in nutrient cycling or soil health that may not support optimal tree growth. Monitoring soil conditions and overall ecosystem health alongside DHA levels is crucial to accurately assess potential impacts on tree health and long-term sustainability (Daunoras et al., 2024).

3.2. Metabolic profiles of microbial communities – the significance of testing responses at different levels

Traditionally, researchers have described microbial diversity using a Biolog™ system based on either substrate catabolism (respiration) or biomass production separately (Frąc et al., 2012; Oszust et al., 2014). However, this study combined these parameters and also considered substrate stress conditions. This holistic approach provides a more comprehensive understanding of microbial communities and their activities in the soil concerning comparing only respiratory activity or toxicity (Oszust et al., 2023b). When microbes have a high respiratory response but low biomass production, it suggests that they are expending a lot of energy (respiration) but not growing much. This condition can be considered stressful for the microbes, as they are not efficiently converting substrate into biomass (Pinzari et al., 2017). Thus, the substrate stress index (SST index) is a critical parameter as it reflects the stress conditions that the microbial community may be experiencing (Pylak et al., 2023).

3.2.1. General differences in metabolic profiling

The metabolic profiles of microbial communities inhabiting the soil beneath apple trees were assessed according to land management

practices using all three mentioned above responses to BiolTM ECO plate substrate. Therefore, dendrograms were generated for respirometry activity (Fig. 2A), for biomass production (Fig. 2B), and for the substrate stress index (Fig. 2C) to test general substrate-based differences, while heat maps were constructed correspondingly for respirometric activity (Fig. 2D), biomass production (Fig. 2E), and substrate stress Index (Fig. 2F) to explore substrate-specific based differences between communities beneath apple trees. When considering the functional diversity of microbial communities based on the restrictive Sneath's criterion (33 %) presented in dendrograms toward tested substrates, a clear separation was observed among all the examined soils beneath apple trees considering land management practices in terms of respiration level (resulting from substrate catabolism), biomass production, as well as the occurrence of substrate stress conditions (SST index). Differences between the soil of cultivated orchards (OC) and forests (F) became distinctly evident when examining respiration levels and substrate stress in comparison to the other land management practices. A similar situation occurred when a less restrictive Sneath's criterion (66 %) was applied. In the case of respiration, forests (F) and cultivated orchards (OC) communities clustered together, while in terms of biomass production, the OC was separated from the other. When assessing the stress situation, both the OC and the F separated. Some similarity was observed between pairs gardens (G) and belts (B), as well as uncultivated orchards (OU) and gardens with animals (GA), which clustered together.

In other words, the study found that different land management practices had a significant impact on the metabolic profiles of microbial communities. Notably, cultivated orchards (OC) and forests (F) showed distinct patterns in respiration, biomass production, and substrate stress when compared to other practices. This suggests that the way land is managed, such as through agriculture or forestry, can strongly influence the microbial communities in soil. When a less restrictive criterion (66 %) was applied, some clustering patterns changed. For instance, in terms

of respiration, forests and cultivated orchards clustered together, indicating some functional similarity in terms of microbial respiration. However, for biomass production and substrate stress, cultivated orchards and forest communities still showed differences. Gardens and green belts, as well as uncultivated orchards and gardens with animals, exhibited some similarities, implying that certain land management practices might have overlapping effects on microbial communities. It is important to note that for more accurate results regarding the similarity of grouped objects, clustering at a level of 33 % should be used.

3.2.2. Common substrate-specific trends

Common substrate trends were revealed, which means that regardless of the specific land management practices employed, certain substrates (Pyruvic Acid Methyl Ester, D-Mannitol, L-Arginine, Hydroxybutyric Acid, L-Asparagine, and L-Serine) consistently led to robust microbial respiration (Fig. 2D). These substrates served as easily metabolizable carbon sources, providing both energy and electron donors for microorganisms. Microbial communities efficiently harnessed these substrates to produce adenosine triphosphate (ATP), reflecting their proficiency in utilizing these compounds for energy production and metabolic activities. On the other hand, substrates such as i-Erythritol, Glycogen, α -Ketobutyric Acid, α -Cyclodextrin, and 2-Hydroxy Benzoic Acid did not induced significant microbial respiration (Fig. 2E). These compounds were likely not favorable sources of energy or electron donors for the microbial community, resulting in limited or negligible stimulation of respiration. This suggests that the mentioned substrates had a minimal role in the energy metabolism of the microbial community. In terms of biomass production variation (Fig. 2E), D-Mannitol significantly increased microbial biomass. Conversely, several substrates (D-Glucosaminic Acid, Glycyl-L-Glutamic Acid, Itaconic Acid, L-Phenylalanine, L-Threonine, D-Galactonic Acid Lactone, i-Erythritol, D-Malic Acid, DL- α -Glycerol Phosphate, Glycogen, α -Cyclodextrin, and α -Ketobutyric Acid) resulted in notably low biomass production. This

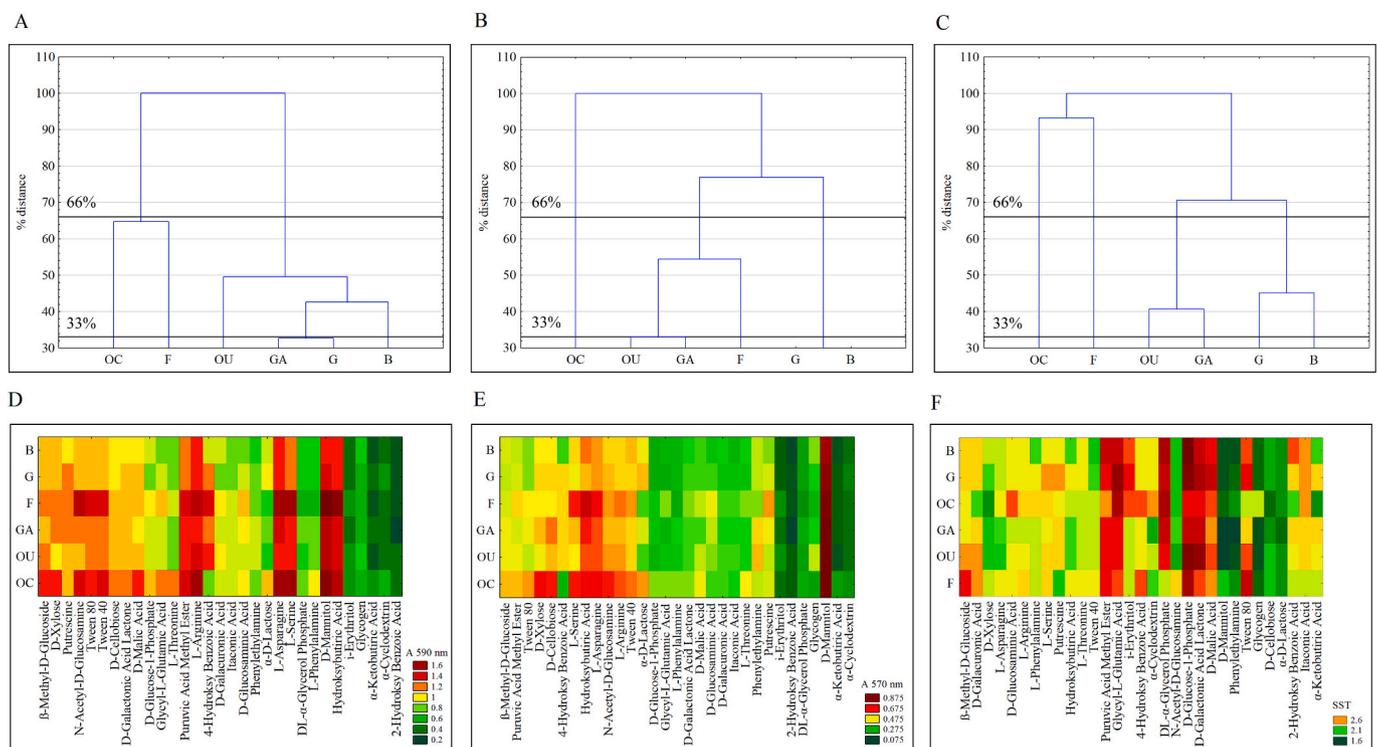


Fig. 2. Metabolic profiles of microbial communities inhabiting the soil beneath the apple trees according to land management practices following BiolTM ECO plates analyses. Dendrograms based on: A) respirometric activity (A 590 nm), B) biomass production (A 750 nm), C) substrate stress index (SST); and heat maps dendrograms based on: D) respirometric activity (A 590 nm), E) biomass production (A 750 nm), F) substrate stress index (SST). Explanations are provided in Fig. 1. Statistical analyses included Ward's Agglomerative Hierarchical Clustering Method within Sneath's criteria (strict, 33 % and less restrictive, 66 %) was applied.

indicates that D-Mannitol was a favorable substrate for microbial growth, while others did not support substantial biomass increase.

It is a common phenomenon that microbial communities exhibit substrate-specific trends, resulting in positive, negative, or neutral responses in terms of respiratory activity and biomass production, regardless of the specific conditions under which they function, as we demonstrate. Understanding the drivers of microbial community stability, including resistance (insensitivity to disturbance) and resilience (the rate of recovery after disturbance) is important for predicting community response to disturbance (Shade et al., 2012). The authors explained this manifestation using both individual and population properties. Individual properties include plasticity, stress tolerance, and dormancy. Population properties encompass adaptation, growth rate, stochastic expression, dispersal, diversity, turnover, and emergent properties. They also provided an outlook, viewing communities as systems of genes and their functions. In our case study, each management practice significantly influenced microbial communities, as demonstrated by both analytical approaches: biomass measurement and respiratory investigation. Notably, the combined approach, which focuses on substrate stress division (Fig. 2F), most clearly highlights these distinctions. Thus, it appears to be the optimal method for profiling the metabolic capabilities of microbial communities.

3.2.3. Substrate-specific based differences

The stress conditions within microbial communities exhibited a sophisticated substrate pattern with distinctive features that most strongly differentiated each method of land management practice. In forest (F) soils, microbial communities experienced significant stress when exposed to B-Methyl-D-Glucoside, indicating challenges in breaking down this glycoside. In garden (G) soils, stress responses were most pronounced with Serine and Putrescine, suggesting difficulties in amino acid and polyamine metabolism. Cultivated orchard (OC) soils showed a unique pattern, with stress responses to D-Glucosaminic Acid and Cyclodextrin, but not to Tween 80. This indicates challenges in utilizing amino sugars and cyclic oligosaccharides but not surfactants. In the bounds (B), microbial communities experienced the most stress with 2-Hydroxy-Benzoic Acid, highlighting difficulties in breaking down complex aromatic acids. These substrates were the most distinctive in differentiating the stress responses associated with each land management practice. The way land is managed has a pronounced impact on the response of microbial communities to different substrates in terms of stress.

The findings highlighted in OC are particularly profound. D-Glucosaminic acid originates from the oxidation of glucosamine, an amino sugar present in chitin, and various polysaccharides. Stress observed in cultivated orchard soils suggests that microbial communities may encounter challenges in chitin degradation, potentially impacting their ability to combat soil-borne pathogens. Antagonistic microorganisms producing chitinolytic enzymes play a pivotal role in chitin breakdown, crucial for the biological control of plant pathogens and biological recycling processes (Unuofin et al., 2024). Metabolic pathways can become blocked due to the accumulation of intermediates (D-Glucosaminic acid) if subsequent enzymes are not sufficiently active to process them further (Fuhrmann, 2021). The emergence of metabolic stress conditions can be seen as an early biomarker of disrupted chitin metabolism within microbial communities. When confirmed, this highlights potential challenges in combating fungal pathogens by the community.

3.3. Metataxonomical composition of microbial communities

Understanding the metataxonomical composition of microbial communities is crucial for unraveling the intricate web of interactions that govern microbial dynamics (Paudel, 2023). One essential aspect of microbial communities lies in the abundance of dominant taxa (Liu et al., 2024). As the Authors mentioned, identifying and quantifying the top ten dominant taxa offers an overlook of the community's taxonomic

hierarchy, highlighting the key players shaping the ecosystem. Within the taxonomy overview, the relative abundance of representatives carries practical implications (Oszust et al., 2021).

3.3.1. Insights from multidimensional scaling analysis

The first step in describing the composition of microbial communities (Fig. 3), namely bacteria (Fig. 3A) and fungi (Fig. 3B) inhabiting the soil of apple trees conspired with multidimensional scaling analysis (MDS). This analysis was applied to compare different localizations (as presented in Tables 1 and 2) including different land management methods and helped visualize and understand the similarities and dissimilarities among these locations based on the tested variable (meta-taxonomy) investigated. The MDS analysis uncovered the quite close spatial relationships between different particular land management practices. This finding should be generally regarded as a normal phenomenon since there were communities tested associated with the same plant (Hout et al., 2013). However, it can be noted that orchards-cultivated localizations (OC) were the most tightly clustered group both for bacterial (Fig. 3A) and fungal (Fig. 3B) communities. This results from more similar and relatively restrictive agrochemical and agrotechnical treatments conducted in every localization of the OC, compared to more natural processes occurring in green belts (hedges) (B), forests (F), gardens (G), gardens with animals (GA), orchards, not modified by, e.g., with mineral fertilizers or pesticides application. The most diverse group among tested land management practices (as shown by outlining localizations) was noted to be the B, the F, and the OC. Outlier sites that deviate significantly from the general trends in grouping within land management practices observed. These outliers indicate unique conditions or anomalies occurring according to those land management practices. The obtained differences show a positive aspect in the context of considering the communities of microorganisms inhabiting given areas.

3.3.2. Relative abundance of the ten dominant taxa

The relative abundance of the ten dominant bacterial orders residing in the soil beneath apple trees, as influenced by various land management practices, is depicted in Fig. 4A. Those are the taxa that usually are responsible for creating the core microbiome, and they might be responsible for having the most significant impact on the environment or biological processes under study (Sun et al., 2024). The relative abundance refers to the proportional representation or prevalence of the ten most dominant microbial taxonomic orders within a community. This information provides insights into the composition of the bacterial population for understanding which microbial groups are the most prevalent or abundant in a given environment.

A noticeable statistical trend was the lower relative abundance of many of these microbial groups in cultivated orchards compared to other land management practices. The Rhizobiales (Rhi) order, known for their nitrogen-fixing abilities (Delgado et al., 2007), was found to be less abundant in cultivated orchards (OC) compared to other land management practices. This decrease may be attributed to the use of synthetic fertilizers in orchards, reducing the need for nitrogen fixation. The Rhizobiales order typically forms symbiotic relationships with leguminous plants to facilitate nitrogen acquisition. In the OC, these bacteria may find less opportunity to thrive due to altered nutrient dynamics. The Burkholderiales (Bur) order, versatile bacteria involved in various biogeochemical cycles (Voronina et al., 2015), also displayed lower relative abundance in the OC. This could be due to the potential disruption of their ecological niche caused by agrochemicals, which can affect their role in nutrient cycling and organic matter decomposition. The Vivinamibacterales (Viv) order, a less-studied order, showed lower abundance in the OC as well. The reasons for this decrease warrant further investigation, but it may be linked to changes in soil chemistry and microbiota composition induced by agrochemical treatments. The Chthoniobacterales (Cht) and Pirellulales (Pir) orders, known for their contributions to nutrient cycling and organic matter degradation

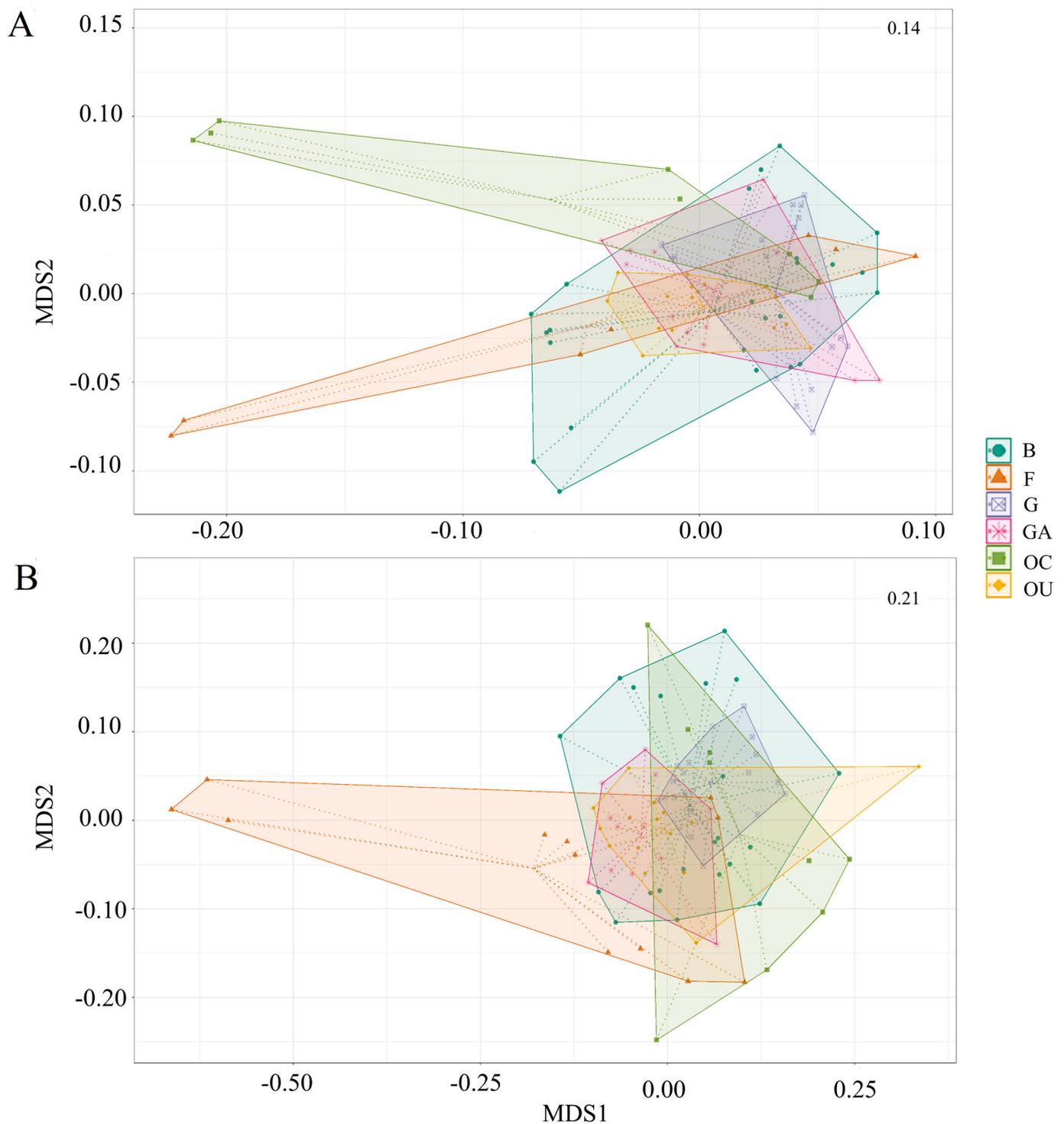


Fig. 3. Multidimensional scaling (MDS) analysis of A) bacterial and B) fungal communities in the soil beneath apple trees under different land management practices using Bray-Curtis dissimilarity. Explanations are provided in Fig. 1.

(Köberl et al., 2020; Dedysh, 2021), were also less abundant in cultivated orchards. The impact of agrochemicals on these bacteria may be hindering their beneficial roles in soil health. The Polyangiales (Pol) order exhibited lower relative abundance in the OC. This order is involved in various ecological functions, including the decomposition of complex organic materials (Saggu et al., 2023). The disruption of their population in cultivated orchards may affect soil organic matter turnover.

The relative abundance of the ten top fungal orders is presented in

Fig. 4B. The Mortierellales (Mor) and Eurotiales (Eur) orders, key players in organic matter decomposition (Wagner et al., 2013), displayed lower relative abundance in the OC compared to other types of land management practices. The use of agrochemicals might be affecting these fungi's roles in breaking down organic materials and nutrient cycling. The Saccharomycetales (Sac) and Rhizophydiales (Rhp) orders, which include yeasts and chytrids (syn. Chytridiomycota, are a group of fungi belonging to the phylum Chytridiomycota) (Khomich et al., 2017), respectively, exhibited reduced abundance in the OC. Their roles in

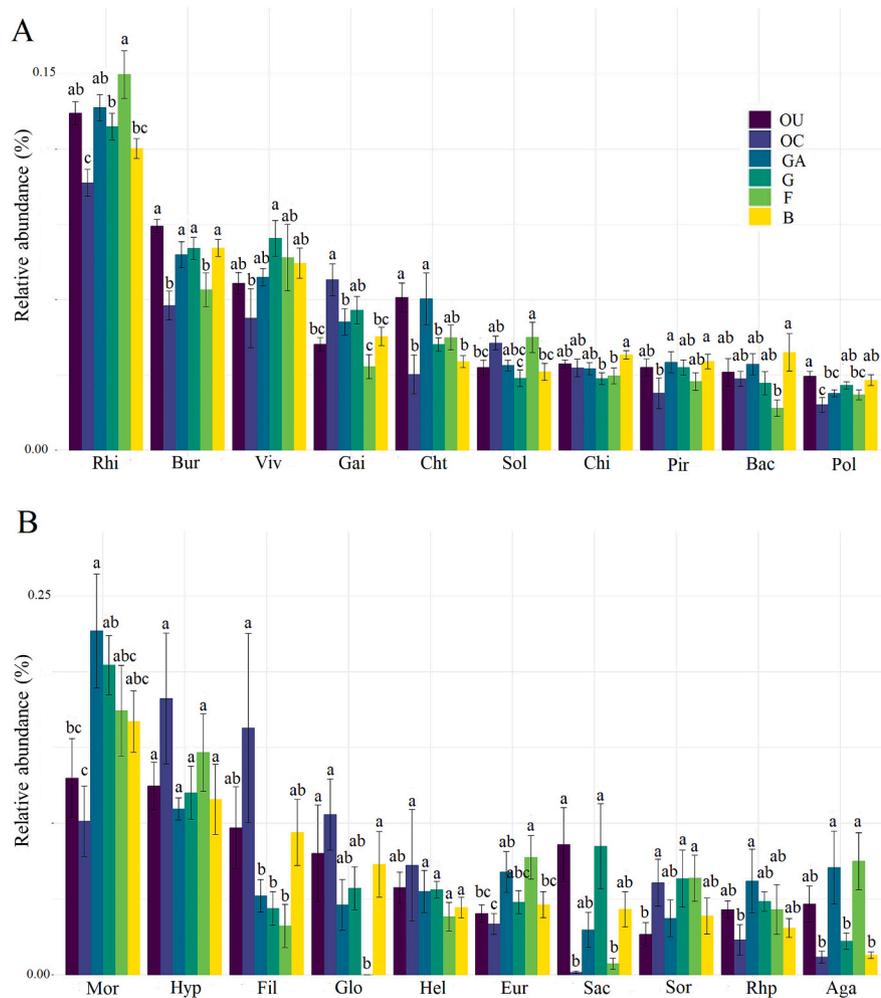


Fig. 4. Relative abundance (%) A) bacteria and B) fungi inhabiting the soil beneath the apple trees according to land management practices. Ten top microbial groups according to orders taxonomy: bacteria - Rhizobiales (Rhi), Burkholderiales (Bur), Vivinamibacterales (Viv), Gaiellales (Gai), Chthoniobacterales (Cht), Solirubobacterales (Sol), Chitinophagales (Chi), Pirellulales (Pir), Bacillales (Bac), Polyangiales (Pol), fungi: Mortierellales (Mor), Hypecreales (Hyp), Filobasidiales (Fil), Glomerellales (Glo), Helotiales (Hel), Eurotiales (Eur), Saccharomycetales (Sac), Sordariales (Sor), Rhizophydiales (Rhp), Agaricales (Aga). Other explanations are provided in Fig. 1. Error bars indicate a standard deviation and different letters above the bars indicate significant differences among land management practices inside each order group (according to test ANOVA Tukey, $p < 0.05$).

nutrient cycling and interactions with plant roots may be impacted by agrochemical treatments. This study reveals intriguing findings regarding the increased relative abundance of specific fungal orders in cultivated orchards compared to alternative land management practices, shedding light on the impact of cultivation practices on these fungal communities. The Eurotiales (Eur) order, which includes fungi like *Penicillium* and *Aspergillus*, exhibited a reduced relative abundance in the OC. These fungi are renowned for their pivotal roles in organic matter decomposition and nutrient cycling (Houbraken et al., 2014). Including manure with animal excrement employed in the GA, including the introduction of organic matter through agricultural activities, creates conditions conducive to the growth of Eurotiales which contributes to their increased relative abundance in these locations. Their ability to break down organic materials enhances nutrient availability in the rhizosphere, which can potentially benefit soil health and productivity. Saccharomycetales (Sac), encompassing yeasts, also showed a lower relative abundance in the OC. Yeasts are versatile microorganisms actively engaged in numerous nutrient-cycling processes (Vero et al., 2019). In cultivated orchards, factors such as increased availability of fermentable organic matter or alterations in soil conditions may favor the proliferation of yeasts. Their presence contributes to nutrient cycling and may influence the intricate interactions between plants and microorganisms in the rhizosphere, with potential implications for orchard

health and crop quality. The Rhizophydiales (Rhp) order, a group of chytrid fungi, also showed reduced relative abundance in the OC. Chytrids are recognized for their interactions with plant roots, potentially playing a role in nutrient acquisition (Morgan et al., 2005). The cultivated environment of the OC not offer conditions conducive to the growth of chytrids, possibly due to heightened root exudate release or alterations in soil chemistry.

The relative abundance of microbial taxa in the soil of apple trees varies significantly based on land management practices. All in all, disturbance level, organic inputs, net primary productivity (NPP), root exudates and organic compounds, agrochemical applications, soil compaction, soil nutrient imbalances, soil pH alterations, and depletion of soil organic matter are the main drivers of observed differences, as described for dehydrogenase activity.

3.3.3. Microbial biomarkers of management practices by linear discriminant analysis

While presenting results from the top ten most abundant taxa is beneficial, it's also important to acknowledge that less abundant taxa can also be ecologically or biologically significant when regarded as biomarkers (Segata et al., 2011). To reveal the microbial biomarkers with significantly different relative abundance on genus level among different land management practices, linear discriminant analysis (LDA)

with effect size (LEfSe) was employed (Fig. 5). Within this, Fig. 5A stands for bacterial, and Fig. 5B fungal community. Specific taxa that are significantly different between the groups were identified, with longer bars indicating higher discriminative power.

Our results revealed distinct bacterial biomarkers indicative of

specific management practices beneath apple trees. In cultivated orchards (OC), genera such as *Pseudolabrys*, *Conexibacter*, and *Blastococcus* were significantly enriched, suggesting a potential association with agricultural management inputs. Conversely, uncultivated orchards (OU) harbored unique bacterial genera such as *Williamsia*, likely

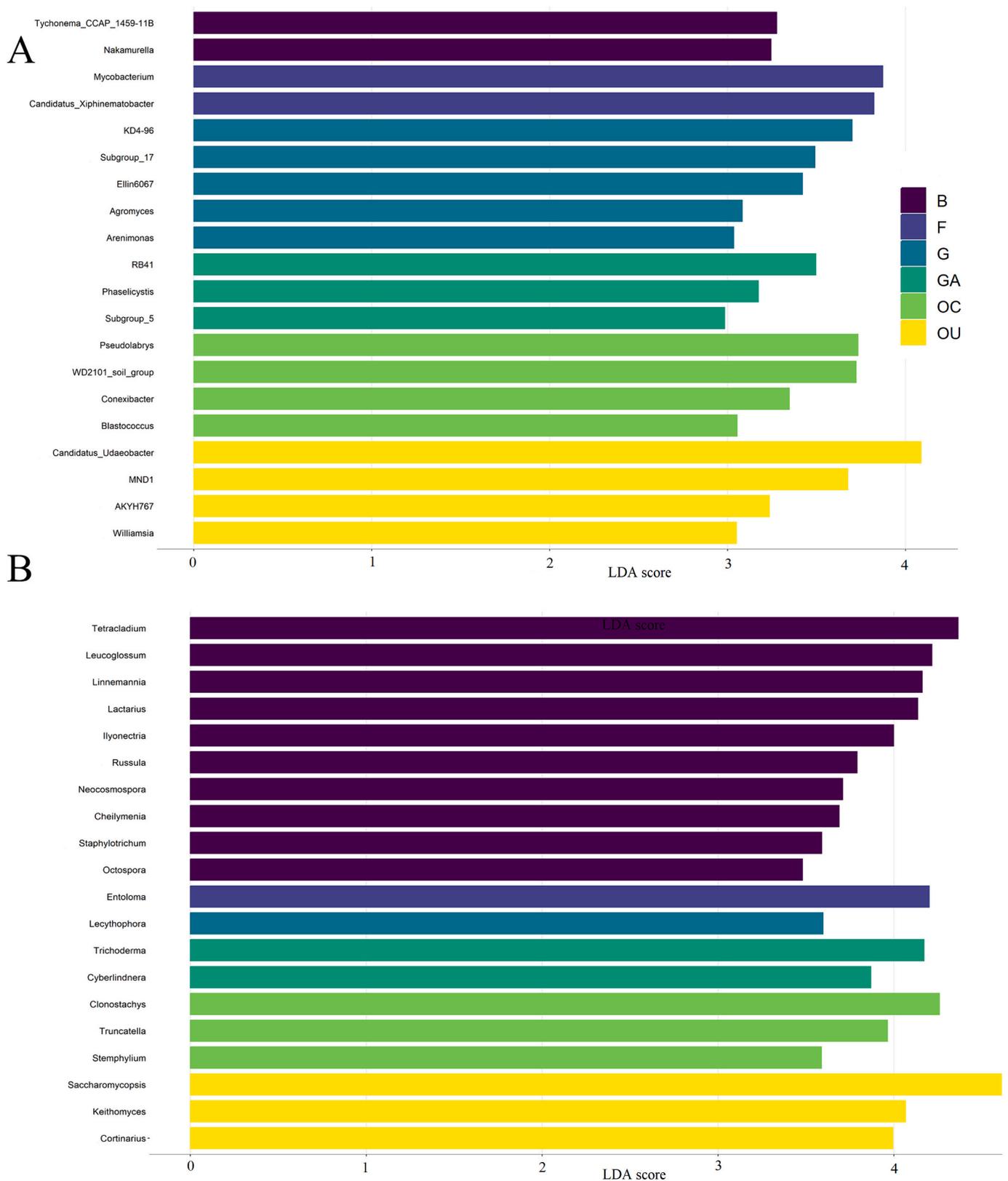


Fig. 5. Microbial biomarkers of management practices according to linear discriminant analysis (LDA) with effect size (LEfSe). Explanations are provided in Fig. 1.

influenced by reduced anthropogenic disturbance and natural succession processes. Forest (F) areas exhibited a different profile, characterized by the presence of representatives of *Mycobacterium* sp. and *Xiphinematobacter* sp. Gardens, particularly those with animals (GA), showed a higher abundance of *Agromyces* and *Arenimonas*.

Among the microorganisms mentioned, *Williamsia* spp. stands out in terms of its function. In uncultivated orchards or less managed environments, *Williamsia* representatives may be regarded as a bioindicator of soil health by showing how well the soil maintains its organic content and supports microbial diversity crucial for nutrient cycling and soil stability. *Williamsia*, a genus of Actinobacteria, is known for its ability to degrade complex organic compounds. Its presence probably indicates adaptation to less disturbed environments with potentially lower nutrient availability compared to cultivated orchards (Yassin et al., 2007). The other genera mentioned, such as specially *Agromyces*, *Pseudolabrys*, etc., also play roles in nutrient cycling and decomposition but are often associated with more nutrient-rich or disturbed soils (Wongkiew et al., 2022).

Distinct fungal communities were observed: *Clonostachys*, *Truncatella*, and *Stemphylium* in cultivated orchards (OC), *Saccharomyces*,

Keithomyces, and *Cortinari* in uncultivated orchards (OU), *Entoloma* in forests (F), *Lecytophora* in gardens (G), *Trichoderma* and *Cyberlindnera* in gardens with animals (GA). Each of these fungal genera represents a distinct ecological niche or function within its respective habitat, making them potential biomarkers that reflect unique environmental conditions or management practices.

3.3.4. Relative abundance of *Trichoderma* and *Bacillus* representatives

Trichoderma and *Bacillus* representatives play essential roles in soil ecosystems, contributing to nutrient cycling, organic matter decomposition, and overall soil health (Singh et al., 2024; Thepbandit and Athinuwat, 2024). Although Bacillales including *Bacillus* and Hypocreales, including *Trichoderma* representatives, were not the dominant taxa in the studied soils (Fig. 4), as is a normal phenomenon in most soils, their biotechnological potential prompted us to perform an analysis of their relative abundance in apple tree soils under various land management practices (Fig. 6). Although, the study did not reveal significant differences in the relative abundance of *Bacillus* individuals (Fig. 6A), the data indicated that forest soils (F) (Fig. 6B) provide the most favorable niche for the *Trichoderma* genus representatives (0.05 %

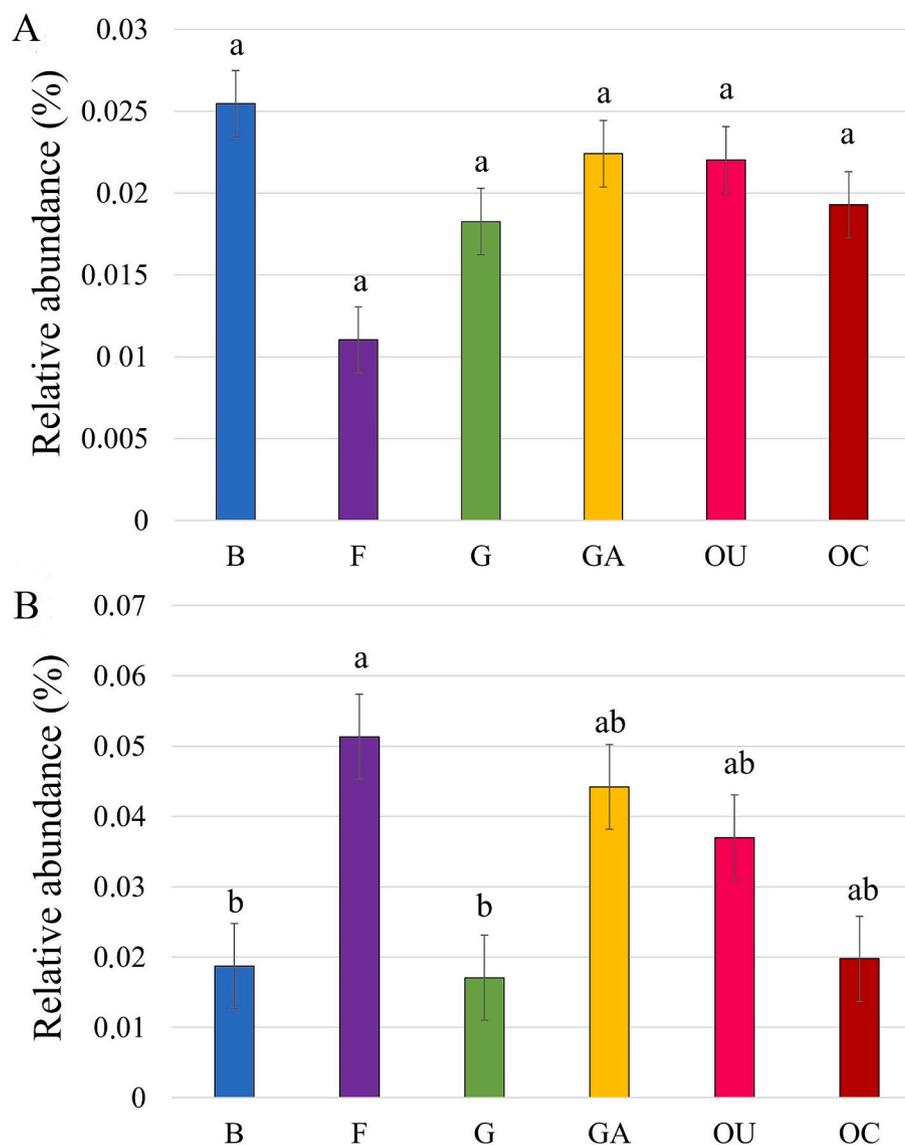


Fig. 6. Relative abundance (%) of A) *Bacillus* and B) *Trichoderma* representatives, inhabiting the soil beneath the apple trees according to land management practices. Explanations are provided in Fig. 1. Error bars indicate a standard deviation and different letters above the bars indicate significant differences (according to test ANOVA Tukey, $p < 0.05$).

of relative abundance) compared to other tested management practices, such as green belts (B) and gardens (G). This finding suggests that forest conditions are conducive to the growth and proliferation of *Trichoderma* fungi among tested management practices. However, microbial biomarker testing, as indicated by LDA analysis (Fig. 5B), demonstrated that the genus *Trichoderma* exhibits the highest discriminative power for gardens with animals (GA).

It is important to note that the *Trichoderma* genus is known for its beneficial properties, such as being a biocontrol agent against plant pathogens and promoting plant growth (Marchuk Larrea et al., 2024). Therefore, forests might be of particular interest to those looking to harness the potential benefits of *Trichoderma* in agriculture or ecological systems. The higher relative abundance of *Trichoderma* species in forests compared to gardens and green belts is likely due to a combination of natural ecological factors and human-induced alterations in soil and environmental conditions. Our findings are in agreement with our previous research. Shortly, the fungal isolates belonging to the *Trichoderma* genus were obtained from the rhizosphere of the wild raspberry plants from forests and were subsequently used to develop a biopreparation with antagonistic properties against fungal pathogens (Oszust et al., 2021).

To the best of our knowledge, no prior studies have directly compared the relative abundance of both genera within specific apple tree habitats under different land management practices. However, similar research was conducted by Huang et al. (2018) across consecutive cropping fields of greenhouse strawberries. This approach streamlines the process for further investigations for isolations of strains. In other words, the presented strategy demonstrates that tools such as the NGS community sequencing can be harnessed to reduce the number of environment types, locations, or ecological niches, where traditional methods are used to search for specific groups of isolates concerning known potential practical applications.

3.3.5. Overlap and uniqueness of microbial representatives - Venn diagrams recognition

Going forward, the Venn diagrams were used to visually represent the overlap and uniqueness of microbial communities in different land management practices. The percentages in Fig. 7A represent the proportion of bacteria representatives that are unique to each land management practice, indicating the diversity and distinctiveness of the bacterial communities in each management practice. Taxa represented by unique ASV are shown in Table S1.

The green belts demarcating cultivated fields (B) exhibited the most noteworthy percentage of unique bacteria, standing at 7.5 %. This suggests the presence of a distinct bacterial community within this environment. Forest areas (F) and gardens with farm animals (GA) showed lower but still considerable percentages of unique bacteria, at 5.1 % and 5.3 %, respectively. Following closely, orchards cultivated (OC) displayed a substantial percentage of unique bacteria, reaching 4.4 %. Gardens featuring ornamental plants and fruit trees (G) presented a noteworthy 3.7 % of unique bacteria. Orchards left uncultivated (OU) and orchards cultivated (OC) displayed relatively lower percentages of unique bacteria, measuring at 1 % and 4.4 %, respectively.

The percentages in Fig. 7B represent the proportion of fungal amplicon sequence variants that are unique to each land management practice, indicating the diversity and distinctiveness of the fungal communities in each setting. The highest percentage of unique fungi (15.7 %) was found in gardens (G), while forest areas (F) also had a notable percentage of unique fungi (15.7 % and 10.7 % respectively). Green belts separating cultivated fields (B) also showed significant fungal diversity with unique fungal representatives (9.7 %). In contrast, uncultivated orchards (OU) and gardens with farm animals (GA) had relatively lower percentages of unique fungal representatives. Orchards cultivated in an integrated system (OC) had a moderate percentage of unique representatives (6.6 %).

Overall, the findings suggest that the diversity and distinctiveness of

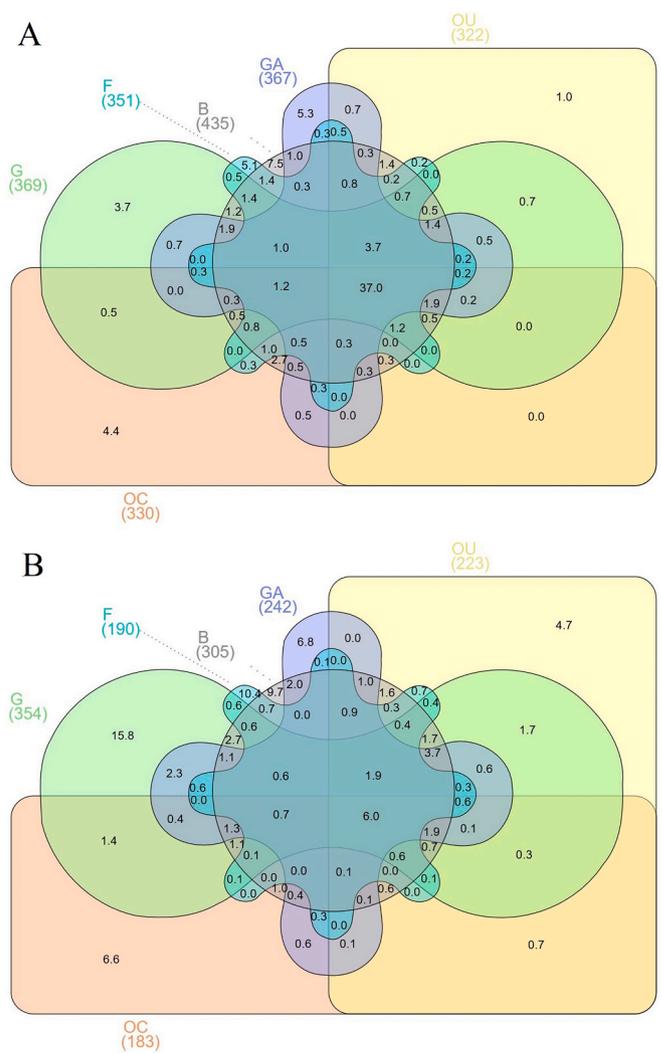


Fig. 7. Venn diagrams of microorganism distribution (%) in soil beneath apple trees according to land management practices in A) bacterial and B) fungal communities. Explanations are provided in Fig. 1. The number of amplicon sequence variants (ASVs) noted in each land management practice is indicated in parentheses. Refer to Table S1 for the list of shared microorganisms among these land management practices.

bacterial and fungal communities vary among the different land management practices, with gardens (G) and green belts separating cultivated fields (B) showing significant uniqueness in both bacterial and fungal communities. Forest areas (F) also exhibit distinct fungal communities, while other practices have varying levels of uniqueness in their microbial communities. To offer a comprehensive overview of the microbial communities beneath apple trees, disregarding the influence of land management practices, a microbial interaction network was constructed. In describing a microbial community network the following aspects are normally examined: the number of microbes, their interconnections, the typical distance between them, their tendency to cluster, the level of activity within the community, the distribution of interactions, and the presence of highly connected “hub” microbes. These details provide valuable insights into how microbes collaborate and function within the community (Fischbach and Segre, 2016).

3.3.6. Distribution of interactions – microbial network comprehension

Microbial interaction network microbial communities regardless of land management practice, inhabiting the soil beneath the apple trees are shown in Fig. 8A for bacteria and Fig. 8B for fungi with the detailed network statistics as shown in Table 3. The fungal community

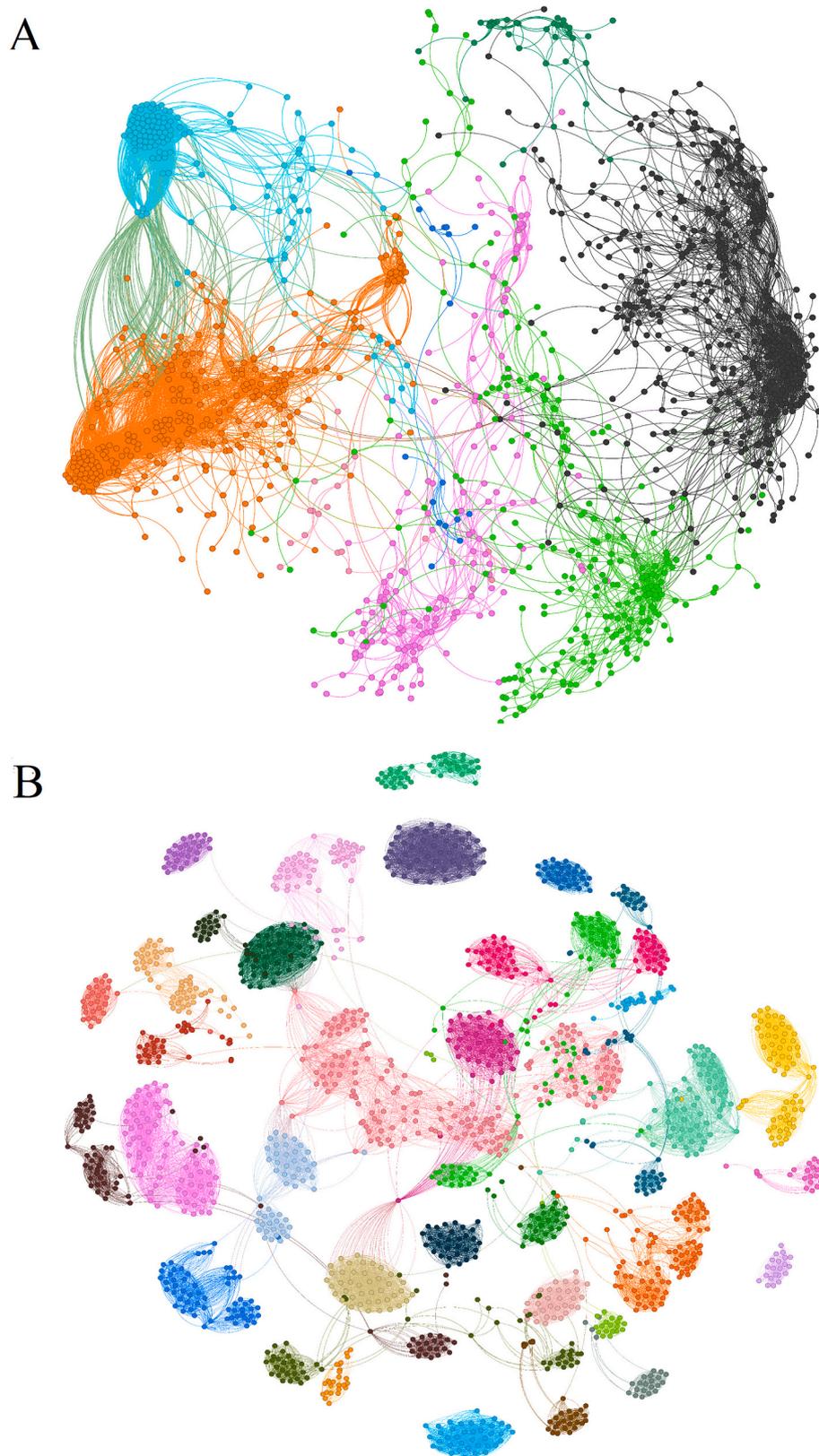


Fig. 8. Microbial interaction network of A) bacterial and B) fungal communities, inhabiting the soil beneath the apple trees. For network statistics see [Table 3](#).

was found to be larger, comprising 1955 taxa representatives (as noted within different amplicon sequence variants (ASVs)), with 21,512 connections, while the bacterial community consisted of 1505 representatives with 11,365 connections. This indicates that the fungal community was more extensive and interconnected. Fungal representatives in the

community, on average, had a higher degree (22.01) than bacterial representatives (15.1). This means fungi tend to have more connections, fostering a richer web of interactions ([Matchado et al., 2021](#)). The bacterial community offers easier navigation with a shorter average path length (5.02) and smaller network diameter (15). In contrast, the fungal

Table 3

Microbial interaction network statistics of bacterial and fungal communities, inhabiting the soil beneath the apple trees. For graphics please see Fig. 8.

Network parameter	Bacteria	Fungi
Vertex	1505	1955
Edge	11,365	21,512
Average degree	15.10	22.01
Average path length	5.02	7.41
Network diameter	15.00	22.00
Clustering coefficient	0.69	0.92
Density	0.01	0.01
Heterogeneity	1.40	0.55
Centralization	0.04	0.03

community has longer average paths and larger diameters (7.41 and 22, respectively), suggesting more extended interaction pathways (Faust and Raes, 2012). Fungal representatives exhibited a notably higher clustering coefficient (0.92) compared to bacterial (0.69). This highlights that fungi tend to form densely interconnected clusters, while bacteria are less clustered. Both communities share the same network density (0.01), indicating that interactions are relatively sparse in both communities. This implies that only a small fraction of potential connections were realized. Bacteria displayed a higher degree of heterogeneity (1.4) in degree distribution, meaning that some have significantly more connections than others. In contrast, the fungal community showed a more even degree distribution with lower heterogeneity (0.55). Both communities maintained low centralization values (0.04 for bacteria and 0.03 for fungi), revealing decentralized structures where connections are distributed broadly among representatives.

The bacterial community differed from the fungal community in several key ways. The fungal community was characterized by its larger size and a higher level of interrelation, indicating a greater diversity of fungal representatives and a complex network of interactions among them. Additionally, fungal representatives had a higher average degree of interaction, implying that they were more engaged with other representatives. However, these interactions in the fungal community tended to take longer paths, suggesting a more convoluted web of relationships. In contrast, the bacterial community was smaller, less densely connected, and offered shorter interaction pathways, indicating a lower diversity of bacterial representatives and a simpler network of interactions among bacteria. Furthermore, the bacterial community displayed more heterogeneous connectivity, with some representatives having many connections while others had fewer. It also exhibited slightly lower clustering, implying a less pronounced formation of tightly-knit groups. Both the fungal and bacterial communities maintained decentralized structures with low centralization levels, meaning that power and influence were distributed across multiple representatives rather than concentrated in a few central representatives. These structural and functional distinctions provided valuable insights into the differences between fungal and bacterial communities beneath apple trees in terms of their ecological roles and dynamics.

3.4. Connections among activity, metabolic, taxonomic microbial properties, and physicochemical features

The data provided in Table S2 gives insights into the physicochemical properties of soils under different management practices across various locations. Surprisingly, not cultivated orchards (OC), but gardens (G) and garden with animals (GA) management practices generally have the highest values for most parameters. However, noticeable location-dependent variations were obvious. Forest (F) consistently shows the lowest values, reflecting the minimal agricultural intervention and natural nutrient cycling processes typical of forest ecosystems. However, for better exploration correlation matrix was proposed. Namely, a correlation matrix was employed to evaluate the interrelationships among microbial functions, taxonomic features, and

physicochemical properties within the soil. Fig. 9 represents findings and explanations for the observed correlations. Robust and significant positive correlations were observed among the tested fungal and bacterial α -diversity indices calculated from metataxonomy data. These indices included Fisher, Chao1, ACE, Inverse Simpson, Simpson, and Shannon indices. These results suggest that an increase in both fungal and bacterial diversity is associated with higher richness and evenness within their communities. Significant positive correlations were found between various nutrient content parameters, such as potassium oxide (K_2O), mineral nitrogen (N_{min}), nitrate nitrogen ($N-NO_3$), and ammonium nitrogen ($N-NH_4$). These findings indicate that these nutrient factors are interrelated, with changes in one nutrient potentially affecting the others. Strong positive correlations were observed among α -diversity indices calculated from Biolog™ ECO plates data. This indicates that there is a positive correlation between the functional diversity metrics and the diversity of the microbial community as measured by Biolog™ analysis, but still significant were certain nutrient elements content, such as zinc (Zn), magnesium (Mg), copper (Cu), iron (Fe), and boron (B). This suggests that these elements may have weaker or more intricate relationships with each other. Less significant correlations were identified between zinc (Zn), copper (Cu), and phosphorus (P_2O_5), as well as between the DHA and these elements. These results imply that the relationships between these elements and dehydrogenases activity may be more complex and warrant further investigation. A weaker correlation was also found between fungal α -diversity indices calculated from metataxonomy data and DHA, indicating a less straightforward relationship between fungal diversity and DHA content. Less significant correlations were observed between the substrate stress index (SST) based on amino acids and other SST substrate groups, as well as between magnesium (Mg) and α -diversity indices calculated from Biolog™ data. These findings suggest that these relationships are not as strong as others in the dataset. Last, but not least, it was noted that pH was positively correlated to DHA, C_{org} , and organic matter content. This correlates with the findings of Kumar et al. (2018) in apple orchards.

Negative correlations were discovered between bacterial α -diversity indices calculated from metataxonomy data and mineral nitrogen (N_{min}), nitrate nitrogen ($N-NO_3$), and ammonium nitrogen ($N-NH_4$). This implies that higher bacterial diversity may be associated with lower levels of these nitrogen compounds, possibly due to competition or niche differentiation among bacteria. Negative correlations were found between the average well color development index (AWCD) and the average well density development (AWDD), as well as between organic carbon (C_{org}) and organic matter. These negative associations suggest that increased microbial activity (AWCD and AWDD) may be linked to lower organic carbon content. Negative correlations were identified between potassium oxide (K_2O) and the AWCD, as well as between K_2O and the AWDD. This implies that higher potassium levels may be associated with reduced microbial activity. Negative correlations were also observed between the Simpson index for fungi and manganese (Mn) and zinc (Zn). This suggests that higher fungal diversity may be associated with lower concentrations of these trace elements, possibly due to fungal nutrient uptake or competition with other microorganisms.

The correlation matrix provided insights into the relationships between microbial diversity, nutrient availability, physicochemical properties, and elemental composition in the soil of apple trees. These findings show the complex interplay between microbial communities and their environment beneath the apple trees and are consistent with the findings of Jiao et al. (2023) and Xie et al. (2022).

4. Conclusions

The study evaluated the impact of six different land management practices on soil bacterial and fungal communities beneath apple trees. These practices included green field belts, forests, gardens with trees and ornamental plants, gardens with farm animals, and both uncultivated

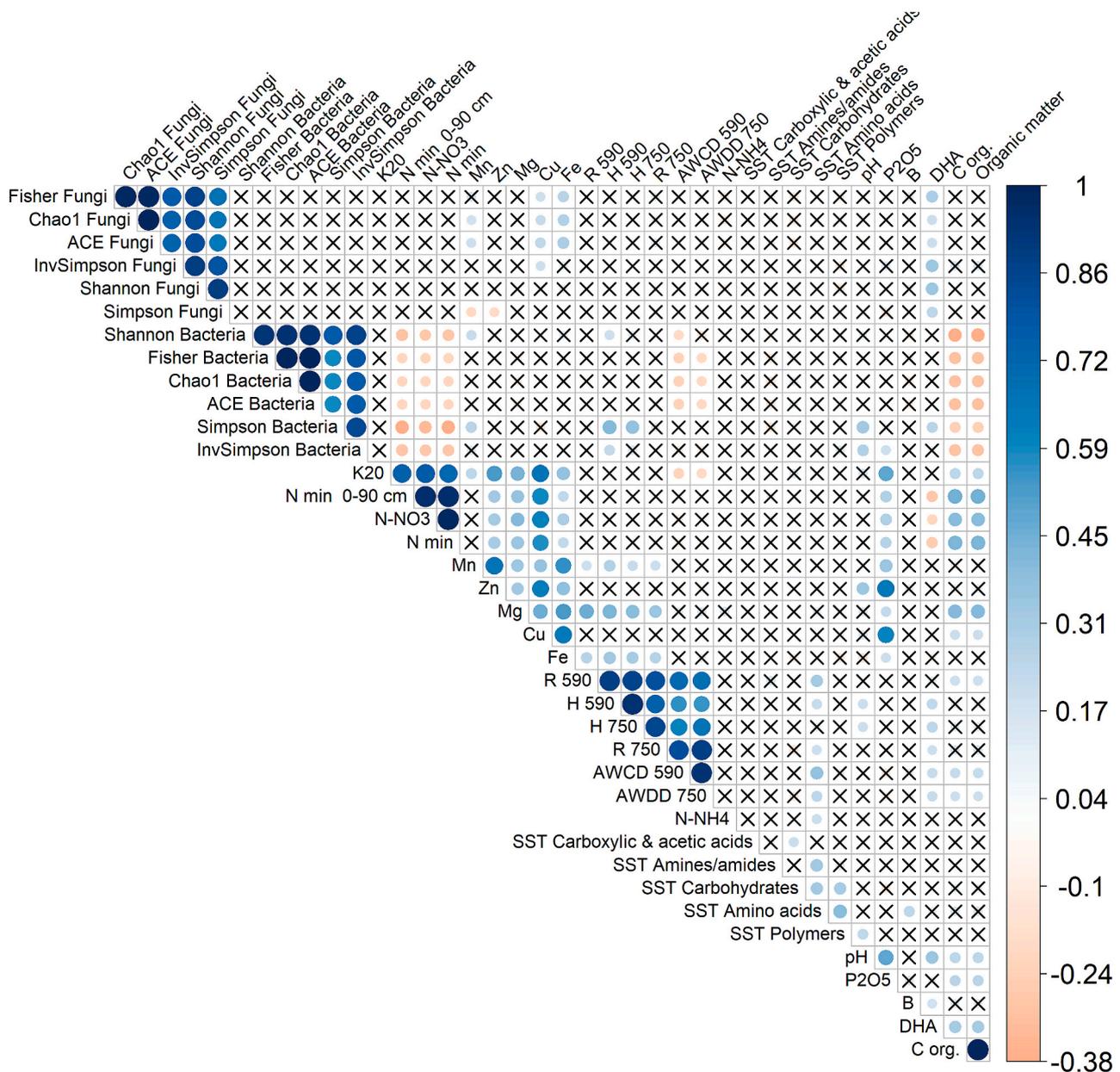


Fig. 9. Correlation matrix between microbial functional and taxonomical, and physicochemical parameters in the soil beneath the apple trees. Explanations: Shannon index (H), Richness index (R), average well color development index (AWCD), average well density development (AWCD), substrate stress index (SST), dehydrogenases activity (DHA), organic carbon (C_{org}), potassium oxide (K_2O), phosphorus pentoxide (P_2O_5), mineral nitrogen (N_{min}), nitrate nitrogen ($N-NO_3$), ammonium nitrogen ($N-NH_4$), manganese (Mn), zinc (Zn), magnesium (Mg), copper (Cu), iron (Fe), boron (B), $\lambda = 590$ nm (590), $\lambda = 750$ nm (750), pH; not significant (X); the larger and darker the circle, the higher the correlation value.

and cultivated orchards. The research focused on several soil microbial properties: overall activity of dehydrogenases (measured through colorimetric reaction with 2,3,5-triphenyl-tetrazolium chloride as substrate), metabolic profile (assessed using Biolog™ ECO Plates), metataxonomy structure (analyzed via Next Generation Sequencing using Illumina®), and physicochemical properties (such as pH, nitrogen, phosphorus, potassium, microelements concentrations, organic matter, and carbon content). The hypothesis was that different land management practices would influence soil microbial properties, with cultivated orchards expected to exhibit significantly lower dehydrogenase activity, higher substrate-based respiration, and different microbial taxa composition compared to other treatments. The applicable goal was to identify practices that promote diverse microbial metabolic and taxonomic composition responses, with particular attention to *Bacillus* and *Trichoderma*, which are potential biological agents against fungal

pathogens.

The results confirmed notable variations in soil microbial properties beneath apple trees across different land management practices. Cultivated orchards and green belts showed reduced overall microbial activity compared to gardens and uncultivated orchards. The significance of testing metabolic responses at different levels was highlighted. Within this, cultivated orchards demonstrated higher respiration levels and substrate stress, particularly when compared to forests, as evidenced by clear separation in cluster analysis. Unique substrate-based patterns were observed among the different management practices. Different land management practices induce unique stress responses in microbial communities: forests struggle with B-Methyl-D-Glucoside, gardens with Serine and Putrescine, cultivated orchards with D-Glucosaminic Acid and Cyclodextrin, and bounds with 2-Hydroxy-Benzoic Acid. The emergence of metabolic stress conditions with D-Glucosaminic Acid was

mentioned as an early biomarker of disrupted chitin metabolism within soil microbial communities, underscoring potential challenges in combating fungal pathogens in these communities. Certain substrates, like Pyruvic Acid Methyl Ester and D-Mannitol, promoted robust respiration and biomass production, while others, like i-Erythritol and Glycogen D-Glucosaminic Acid, were less effective, regardless of management practice. Significant differences were found in the relative abundance of the top ten bacterial and fungal orders, regarded as core microbiome but also biomarker genera. Following this cultivated orchards showed a significant decrease in the relative abundance of bacterial taxa such as Rhizobiales, Burkholderiales, and Vivinamibacterales, and fungal taxa including Eurotiales and Saccharomycetales. As for identified microbial biomarkers under apple trees linked to specific land management practices cultivated orchards had enriched genera like *Pseudolabrys*, *Conexibacter*, and *Blastococcus*, indicating agricultural inputs. Uncultivated orchards featured *Williamsia* sp., suggesting this microorganism to be a biomarker of less disturbance, resulting from area restoration. Forests were characterized by e.g. *Xiphinematobacter* sp., while gardens with animals had more *Agromyces* and *Arenimonas* representatives connected to more natural management practice. What is more, we demonstrated that forests favored *Trichoderma* abundance the most among tested practices, implying that soil in forest habitats of apple trees is a promising source for finding isolates of this genus for further screening of their biotechnological features. Although, we did not obtain a definitive answer in the context of *Bacillus*, presented results highlight the potential of employing advanced techniques as a tool in a more sensible approach to screening locations suitable for searching specific groups of microorganisms. All in all, the study examined the connections among microbial activity, metabolic and taxonomic properties, and physicochemical features of soils under various land management practices.

The factors contributing to the observed differences were discussed. These included such attributes as diverse plant cover vs monoculture, where contrasting impacts of root exudates and organic inputs enter the soil, but also organic matter decomposition, net primary productivity, nutrient input, and other agricultural practices. Crucially, the interplay between microbial activity, physicochemical properties of soil, metabolic profiles, and taxonomic features paints a comprehensive picture of microbial community differences. Understanding these interconnections is pivotal for ecosystem function description.

5. Recommendations

The proposed management practices closely emulate natural habitats, semi-natural habitats (formerly utilized but now reverting to natural conditions and becoming wild), and conventionally managed apple orchard monocultures with relatively extensive agrochemical treatments. The implications of our findings lead to broader conclusions, recommendations, and future directions.

Demonstrating the benefits of mimicking natural conditions in apple orchards through diverse plant covers and reduced agrochemical inputs can guide apple producers in enhancing soil microbial health, promoting biodiversity, and fostering sustainable horticultural practices. By advocating less intensive management practices conserving plant biodiversity, and indicating *Williamsia* genus representatives as a biomarker microorganism to indicate less disturbance and successful area restoration, this study supports broader ecological goals and aligns with global sustainability initiatives. Regular monitoring of soil microbial communities to adjust management dynamically would aid in the early detection of potential issues and allow timely interventions by apple producers to maintain soil health.

Integrating microbial metabolic profiling, which combines respiration and biomass production with substrate stress, significantly enhances the accuracy of soil microbial assessments during monitoring. This holistic approach yields practical insights and holds promise for future scientific investigations and monitoring efforts. This

methodology's capability to capture microbial responses is crucial for evaluating the impacts of diverse factors on ecosystem health. When complemented by other microbial community analysis methods such as molecular techniques and community composition assessments, it forms a robust framework for comprehensive ecological evaluations. Using the D-Glucosaminic Acid metabolic stress test to identify early challenges in controlling fungal pathogens within soil microbial communities is recommended based on the findings of the study.

Identifying beneficial microbial taxa such as *Trichoderma* under specific management practices, simultaneously referring to potential applications in biocontrol and biotechnology, contributes to sustainable integrated pest management strategies. Research and policy efforts should support the development and application of microbial inoculants derived from less disturbed environments. Therefore, policy should prioritize preserving habitats of wild apple trees in forests or green belts, as these environments exhibit reduced stress and support beneficial fungi like *Trichoderma*.

Overall, these findings address challenges in sustainable horticulture and advocate for pathways toward resilient, environmentally friendly apple production systems. While the research yields positive outcomes, it's essential to acknowledge potential limitations in practical implementation. These include fluctuating agricultural policies, evolving trends in plant protection products, and economic constraints. The use of biopreparations in agriculture can be encouraged by incorporating these practices into eco-schemes and providing subsidies or financial incentives to farmers who adopt them. For instance, regular monitoring of microbial properties in orchards may pose financial challenges. Additionally, reduced reliance on fungicides could increase susceptibility to diseases, potentially leading to lower yields and higher costs for producers. However, the focus should not solely be on eliminating fungicides but on targeted strategies to manage fungal pathogens effectively and enhance biodiversity.

Further research is needed to explore the actual specific functions of microbial taxa in the context of not only soil but the entire plant under different management practices. Treating the apple tree as a holobiont and analyzing the microbiome of its various compartments, including the soil beneath it, as well as the fruit, leaves, roots, and other parts would be worthwhile. Equally important are the physiological aspects of the plant itself, which should be compared to obtain a comprehensive understanding of the complex relationship between plants and their associated microorganisms. Encourage interdisciplinary collaboration among microbiologists, agronomists, ecologists, and soil scientists to explore the complex interactions between microbes, plants, and management practices. This holistic approach can lead to more effective strategies for sustainable apple orchard management.

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Ethics approval and consent to participate

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Raw sequence reads were submitted to the Sequence Read Archive (SRA) database within BioProjects PRJNA923149 for fungal and PRJNA923266 for bacterial communities.

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CRediT authorship contribution statement

Klaudia Zawadzka: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Karolina Oszust:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Michał Pylak:** Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis. **Jacek Panek:** Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Agata Gryta:** Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Magdalena Frąc:** Writing – review & editing, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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