TACKLING THE SOIL MICROBIOME – CHALLENGES AND OPORTUNITIES

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Abstract

The health of the terrestrial ecosystems is directly dependent on the microbial composition that fulfills essential functions, such as sustaining plant growth, nutrient cycling and carbon sequestration. The study of the soil microbiome has gained popularity in the last decades due to its significant impact on the health of the environment and its inhabitants. This review explores the diversity and functions of soil microbial communities, with a particular focus on microbial dark matter, a subset of organisms that cannot be cultured through classical microbiological techniques. The evolution of DNA extraction methods and sequencing technologies coupled with the transition from amplicon sequencing to metagenome-assembled genomes (MAGs) and continuously developing bioinformatic pipelines has led to the discovery of novel microbial taxa, community networks, metabolic pathways and potentially useful molecules. Soil microbiome research is gaining momentum in Romania, as a big part of studies try to assess the impact of agricultural practices on the environment. Designing sustainable agricultural practices and implementing them with the goal of preserving the heterogeneity of the microbiome contributes significantly to the resilience of ecosystems, preserving the health of the environment, as well as the well-being of its residents.

Keywords: soil microbiome, microbial dark matter, metagenomics, DNA extraction, Next Generation Sequencing, amplicon sequencing, metagenome-assembled genomes

Introduction - The soil microbiome and us

In the past few decades, there has been growing recognition for the vital links between the ecosystem's health and that of plants, animals and humans. Themed under the umbrella of the term "OneHealth", the fitness of the aforementioned concepts reflects a worldwide objective driven by the concerning trends that the environment is subjected to, including climate change, emergent antimicrobial resistance, and diseases, as well as challenges related to ensuring food safety and security (Nadeu et al. 2023). Primarily, the soil acts as a nutrient storage and supplier, a fertile agricultural soil being able to sustain the production of qualitative food for animals and humans, all in a high yield. The nutrient content and its variations in the last two decades mirrors the global trend of the increasing need for higher quantities of feed along with the rise in population. The intensification of agriculture leading to a decline in the organic matter impairs the storage of the nutrients, their recycling into plant-available forms and their atmospheric and water distribution. The subsequent actions and decisions taken to attain the continuously-increasing food demand are the main reasons that lead to soil devaluation and successive deterioration of ecosystems (Brevik et al. 2020). The association between the ecosystem and their inhabitants is finely linked by the colonizing microbial communities. Within a high array

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of global ecosystems, microbes and especially prokaryotes dominate every habitat they inhabit through a high genetic and metabolic diversity, the soil harboring the most complex microbial communities out of all the environments (Nadeu et al. 2023). The terms "microbiome" and "microbiota" are intertwined, being used to describe microbial communities formed out of prokaryotes, fungi, viruses, algae, and protozoans that populate a specific habitat. The microbiome also includes the associations formed in these cooperative structures, within and outside the community, adding the encompassing environmental conditions (Marchesi and Ravel 2015). Microbiome research has rapidly grown in the last decades, especially following discoveries linking a dysregulated human gut microbiota to various gastrointestinal diseases (Fan and Pedersen 2021). The human microbiome is directly influenced by dietary habits. Consequently, the quality of the diet is linked to the agricultural practices, which are, in turn, dependent on multiple factors alongside the soil health. A healthy soil is described as a substrate capable of sustaining the productivity of plants and animals as well as with promoting their health. At the same time, this substrate has the capacity to manage the quality of the water and air, having a major contribution against climate change. And as everything is connected into the One Health concept, the health of the soil is largely sustained by the diverse accompanying microbiota (Banerjee and van der Heijden 2023). The soil microbiome is linked to numerous functions, aiding in bioremediation, discovery of antimicrobial substances, and sustainability and security of food systems, all of these having implications for the human health (Brevik et al. 2020).

Amidst the introduction of new molecular biology methods, the knowledge regarding various microbial communities has significantly increased in the last three decades. The advancement of -omics research encompassed the exploration of the complete genetic makeup of the microbiota in a culture independent fashion. This branch of study is also known as "metagenomics", term being oftentimes interchangeably and improperly used with "microbiome" (Marchesi and Ravel 2015). Comparative to the human microbiome research, the study of the soil bacterial communities hasn't been of much interest to the academic community (Stulberg et al. 2016). The interest for this ecological niche has surged over the last decade as there has been a dramatic rise in literature regarding the microbiome of the soil, the quality and health of soil being directly linked to the agricultural system and thus, all being dependent to the indigenous microbiota (Clarke et al. 2020; Hermans et al. 2023).

The composition of the microbiome and its functions

The composite microbes of the soil microbiota are represented primarily by bacteria and fungi, being followed by archaea, protists and viruses (Bar-On et al. 2018), distinguishing different habitats based on the diversity and distribution of species. Examples of such soil regions are the bulk soil and the rhizosphere (Xiong and Lu 2022). Among all known microbiomes, the soil microbiota is the most complex, with bacterial, fungal and archaeal species being the key players through their high metabolic diversity necessary to survive different environments (Fierer 2017). The study of the soil microbiome has been burdened by the limiting inability to culture most of the microorganisms. In consequence, culture-independent techniques emerged as a solution to explore the full extent of microbiota's diversity (He et al. 2008).

The microbial abundance of soil is high, being often reported that a single gram can contain billions of microorganisms representative of up to tens of thousands of species (Raynaud and Nunan 2014; Fierer 2017). Bacterial species are highly abundant in soil, comprising 70-90% of the total biomass, with fungi being subsequent, whereas the abundance of archaeal species insignificantly higher in extreme environments (X. Wang et al. 2024). In variable abundance, the bacterial representatives belong to the phyla *Pseudomonadota*, *Actinobacteria*, *Acidobacteriota*, *Verrucomicrobiota*, *Bacterioidota*, *Planctomycetes*, *Chloroflexi*, and *Firmicutes*.When it comes to fungi, the relative abundance picture is represented in big part by species from the phylum *Basidiomycota,* the rest being completed by *Ascomycota* and *Zygomycota* species (Fierer 2017; Delgado-Baquerizo et al. 2018; Labouyrie et al. 2023). The culturable bacterial fraction is diverse, being constituted of a significant number (over 88% out of the entire bacterial division) of *Pseudomonadota* species, with A*ctinobacteria, Firmicutes* and *Bacterioidetes* following (Nikolaki and Tsiamis 2013). Whitin a soil sample, the culturable fraction is dominated by the *Arthrobacter* genus (He et al. 2008). On the other hand, rare microbial species, although present in relatively small numbers, contribute to more than 65% of the diversity within the entire community (Xiong and Lu 2022). Scattered in between abundant microbial populations, with great dependence to the niche populated, there is an unculturable microbial fraction that plays a huge role in maintaining the balance of the entire community. Recognized as the "microbial dark matter", its role is well-known in the stability of the microbiome (Ma et al. 2023). The great heterogeneity and interactions between different taxa support the resilience and productivity of the ecosystem. This diversity is seen also on a functional level, with the majority of microbial strains performing important environmental functions, while a small fraction act as pathogens (Banerjee and van der Heijden 2023).

As an essential component of soil composition, the vast diversity of microbial taxa mediates important and essential functions for the ecosystem, having either direct or indirect impact on the environment and its inhabitants. Specifically, the microbiota is capable of sequestering and storing carbon from the environment, , playing a big role in mitigating the greenhouse gasses and their effect (Dubey et al. 2019; Tao et al. 2023). The microbial diversity aids in the degradation of soil organic matter, an essential step in the cycle of nutrients in the environment, and by oxidizing organic residues left behind by plants and animals, nutrients are made available for the growing plants (Anthony et al. 2020). Fungal and bacterial species, primarily from the phylum *Actinomycetes* and the *Bacillus* genus target mostly proteins, making nitrogen available for other species (Bhatti et al. 2017; Nicolás et al. 2019; Gómez-Brandón et al. 2020; Rana Chhetri et al. 2022). The impact of bacteria on plant health and growth is significant as they interact with plant roots and aid the formation of beneficial relationships with growth promoting rhizobacteria, mycorrhizal fungi, and other microorganisms. Growth promoting microorganisms are represented by rhizobacterial species or mycorrhizal fungi found in the rhizosphere, root tissue or are integrated into the nodules of plants, that interact with the microbiome, either synergically or antagonistically, promoting plant growth through nutritional and hormonal balance regulation, aiding in nutrient eased solubilization and uptake along with providing resistance against pathogens. Under the influence of stress-inducing factors such as high salinity, heavy metal contamination, drought, and flooding, rhizhobacterial strains were seen to protect and promote the growth of the plants either alone or in synergy with mycorrhizal fungi. Mycorrhizal fungi facilitate water absorption and nutrient uptake, being estimated that around 80% of phosphorus is supplied to plants by them. Because of their localization and their potential in agriculture, research regarding the inoculation of growth-promoting microorganisms is of interest at the moment, as this approach could improve crop productivity and quality in a more sustainable way (Nadeem et al. 2014; Lopes et al. 2021). Nitrogen-fixing bacteria, such as species from genera *Achromobacter*, *Anabena*, *Azotobacter*, *Azospirillum*, *Rhizobium*, *Bradyrhizobium*, *Beijerinckia*, *Clostridium*, *Frankia*, *Klebsiella*, *and Nostoc*(Lopes et al. 2021) along with mycorrhizal fungi as *Funelliformes* sp., *Gigaspora* sp., and *Rhizophagus* sp. (formerly known as the genus *Glomus*) (Chalk et al. 2006) are featured as key players in maintaining soil fertility and sustaining terrestrial ecosystems, inoculi of one or more of these species being actively tested (Nadeem et al. 2014). All these microorganism associations highlight the intricate relationships between the soil microbiome and plants (Banerjee and van der Heijden 2023). Along with nitrogen-fixing bacteria, other species capable of fixing or producing derivatives out of phosphorus (*Arhtorbacter* sp., *Bacillus* sp., *Burkholderia* sp., *Penicillium* sp., *Pseudomonas* sp., *Serratia* sp., *Aspergillus* sp., *Achromobacter* sp., *Agrobacterium* sp., *Erwinia* sp., *Micrococcus* sp., *Rhizobium* sp.), sulfur (*Bacillus* sp.) and iron (*Azobacter* sp., *Bacillus* sp., *Fusarium sp.*, *Pseudomonas* sp., *Serratia* sp., *Streptomyces* sp., *Burkholderia* sp., *Enterobacter* sp., *Grimotella* sp.) (Lopes et al. 2021; Banerjee and van der Heijden 2023) have a direct role in the biogeochemical cycling of macro- and microelements. It is estimated that soil bacteria accounts for the bioavailability of 18 essential elements out of 29 elements necessary for maintaining plant health (Brevik et al. 2020; Banerjee and van der Heijden 2023).

Another function mediated by the soil microbiome involves conferring resistance to aboveground pests, a concept that is gaining interest in the agricultural field (Pineda et al. 2017; Pineda et al. 2020). Noteworthy to highlight, by aiding in the formation of soil aggregates, the microbiome maintains the soil structure, preventing its erosion and protecting the associations between the root system of the plants and the soil as a nutritive substrate (Bergmann et al. 2016; Angst et al. 2021).

In the last decade researchers have investigated the impact of heavy metal soil contamination, severe pollution, and the effect of climate change on the normal microbiota. Contamination with heavy metals negatively influences the structure of the microbiome, with descending relative abundance for species from phyla *Nitrospirae*, *Bacterioidia* and *Verrucomicrobia* (Li et al. 2020). Moreover, the relative abundance and species variability are impacted by elevated levels of aluminum, variable carbon-to-nitrogen ratios, available phosphorus, and pH levels (Hermans et al. 2017). Plastic pollution affects the soil microbiome's composition, abundance and functions by altering the water and carbon availability (Lear et al. 2021). Pesticide usage causes a decrease in the microbial population and diversity, and as a consequence, affects the nutrient cycling by the mycorrhizal fungi. Nonetheless, human actions affect the soil mainly through urbanization, unsustainable agricultural practices and intense cropping. A disrupted soil microbiome can affect the soil health and associated functions, with alterations in the microbiome potentially acting as a bioindicator of such conditions. Despite the significance and need of new pollution bioindicators, research is still in early stages (Banerjee and van der Heijden 2023). The various soil microbiome functions along with its disrupting factors are depicted in figure 1.

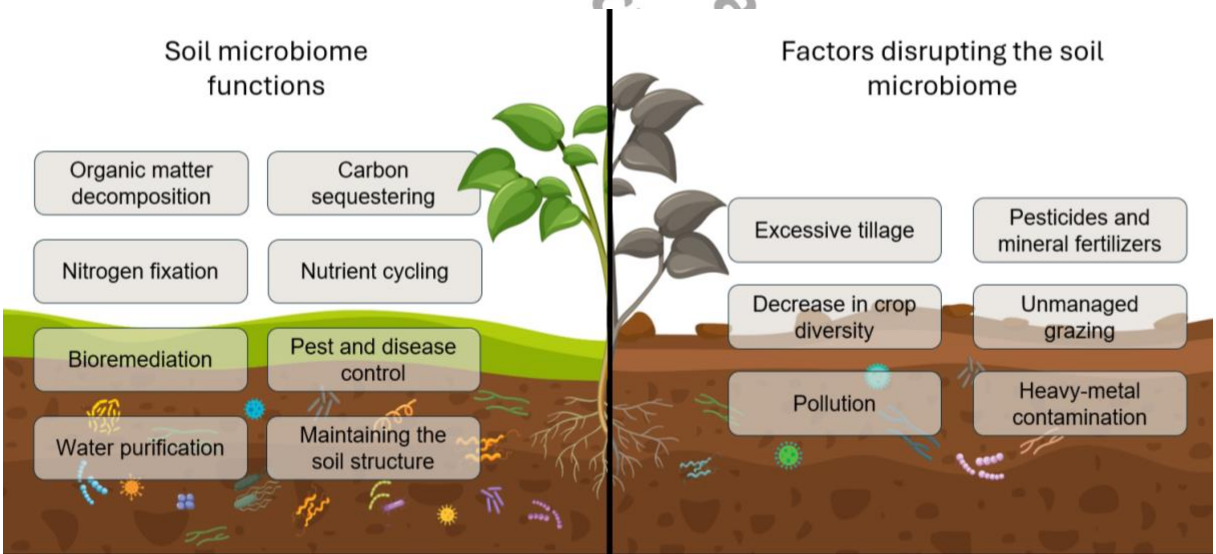

Figure 1 – Functions of the normal soil microbiota (left) and factors influencing negatively the soil microbiota diversity (right). The microbiome is a major contributor to the health of the soil, being associated with the normal growth and development of plants. (*Figure created using vectors from* www.vecteezy.com)

A recent study compared the soil microbial variations and microbial biomass from three sites used for urban leisure, traffic and urban agriculture. The researchers observed that the agricultural site had the lowest biodiversity of them all but high fungal richness, whereas the leisure site represented a stable setting for the development of specialized microbial communities and microbial plant symbionts (Christel et al. 2023).

Understanding the significance of a healthy soil and the interdependence of humans and microbial communities might lead us towards a cleaner environment that promotes sustainable agriculture and stable ecosystems.

If it's too small, it doesn't mean it's not powerful: the microbial dark matter

The study of microorganisms found in the environmental niches primarily focused on isolating and characterizing them from a pure culture. The DNA sequencing methods described in 1977 changed the whole perspective on taxonomical classification of bacteria, transitioning from the field of microbiology to that of molecular biology. Phylogenetical studies based on sequencing followed shortly as the gene encoding the small subunit of the ribosome was described as a feasible taxonomic marker (Woese and Fox 1977; Woese et al. 1990; Nikolaki and Tsiamis 2013). Although the first bacterial genome was successfully sequenced in 1995, it didn't take long for researchers to try to characterize a bacterial community (Land et al. 2015). Other marker genes taken into consideration in metagenomic studies are the internal transcribed spacer (ITS) region for distinguishing fungal species and the 18S and 23S rRNA for other eukaryotes that compose the microbiota (Pérez-Cobas et al. 2020; Nam et al. 2023). It was a great surprise to find out that the already described soil microbiome through culture techniques accounted for 1% of the total microbiota found in the environment. The great unculturable microbial fraction, recognized as the "microbial dark matter" is being represented in big part by archaea and bacteria (Solden et al. 2016; Jiao et al. 2021; Ma et al. 2023). This reservoir of newly identified species was described later as a new clade, appointed the name of Candidate Phyla Radiation (Hug et al. 2016; Jiao et al. 2021). The identification of previously uncharacterized microbes presents a potential resolution to emerging medical and biotechnological challenges. Given that the majority of antimicrobial substances discovered in the "Golden Age" were of microbial origin, the diverse and numerous species present within microbial dark matter became an exciting subject to pursuit in context of combating the antimicrobial resistance phenotypes (Ma et al. 2023). Other noteworthy potential applications are represented by the bioremediation capacity from soil and water, generation of biofuels and agricultural fertilizers as well as the synthesis of disease markers (Nikolaki and Tsiamis 2013). The considerate complexity and heterogeneity of the soil microbial dark matter presents numerous challenges in the investigation of this ecological community. A number equal to one million is estimated to represent the unknown species (Zha et al. 2022). Studying these novel organisms require considerable computational resources along with bioinformatic tools capable to mine through the data, a significant obstacle being the absence of reference genomes in databases.

How is soil microbiota affected by current agricultural practices

Because the health of the soil relies on the constituent microbiota, external factors that have a negative impact on the microbial communities interfere with the soil's ability to sustain the well-being of plants, animals and humans while also contributing to a cleaner environment. As pollution and environmental changes are taking their chance to hinder the soil's microbiome functions, the conventional agricultural practices pose a harmful influence on the long term sustainability of food production (Food and Agriculture Organization United Nations 2022; Hermans et al. 2023; Nadeu et al. 2023). Practices such as excessive tillage, usage of antimicrobial substances, synthetic fertilizers and pesticides with excessive grazing lead to loss of biodiversity and homogenizes the microbial community of the soil (as seen in figure 1).

Consequently, this leads to soil erosion and compaction as well as pesticide contamination, all with a bad prognostic for the future of food (Banerjee and van der Heijden 2023; Hermans et al. 2023). Trying to preserve the health of the soil and the high yield of crops, regenerative agriculture approaches have been described in recent years taking into consideration the need of enhancing crop resilience to environmental stresses. In opposition to traditional agriculture, the sustainable agriculture movement is represented by practices such as reduced tillage with low or no usage of mineral fertilizers and pesticides. For protecting the biodiversity, the recommendations follow that there should be crop rotation practices between fields with diversified plants cultivated as well as managing the grazing of livestock towards quick recoveries of skimmed soil patches (Hermans et al. 2023).

Multiple projects have been amended in the last decades in the hope of saving the environment, and by this, the soil microbiota that contributes greatly to the agricultural sector. Understanding and tackling the potential that the soil microbiome holds are essential for optimizing agricultural practices and enhancing crop resistance to environmental stresses in a sustainable manner (Nadeu et al. 2023).

The soil microbiota of Romania – What we know up until now

Research on soil microbial diversity is currently gaining momentum in Romania. A multitude of studies have set the stage for uncovering the microbial complexity of the soil, with a primary focus on its implications for sustainable agriculture and the preservation of environmental diversity. A great part of research conducted on the Romanian soil microbiome take culturing or metataxonomic approaches, bacterial strains being the primary focus of these studies. Numerous studies tried to describe the extremophile species from Romania, from either soil, sediments, karst or water from habitats defined by severe conditions that don't allow the survival of most organisms (Andrei et al. 2017; Sarbu et al. 2018; Chiciudean et al. 2022; Bogdan et al. 2023; Szekeres et al. 2023). A map displaying the geographical coordinates associated to the soil microbial studies conducted in Romania is represented in figure 2.

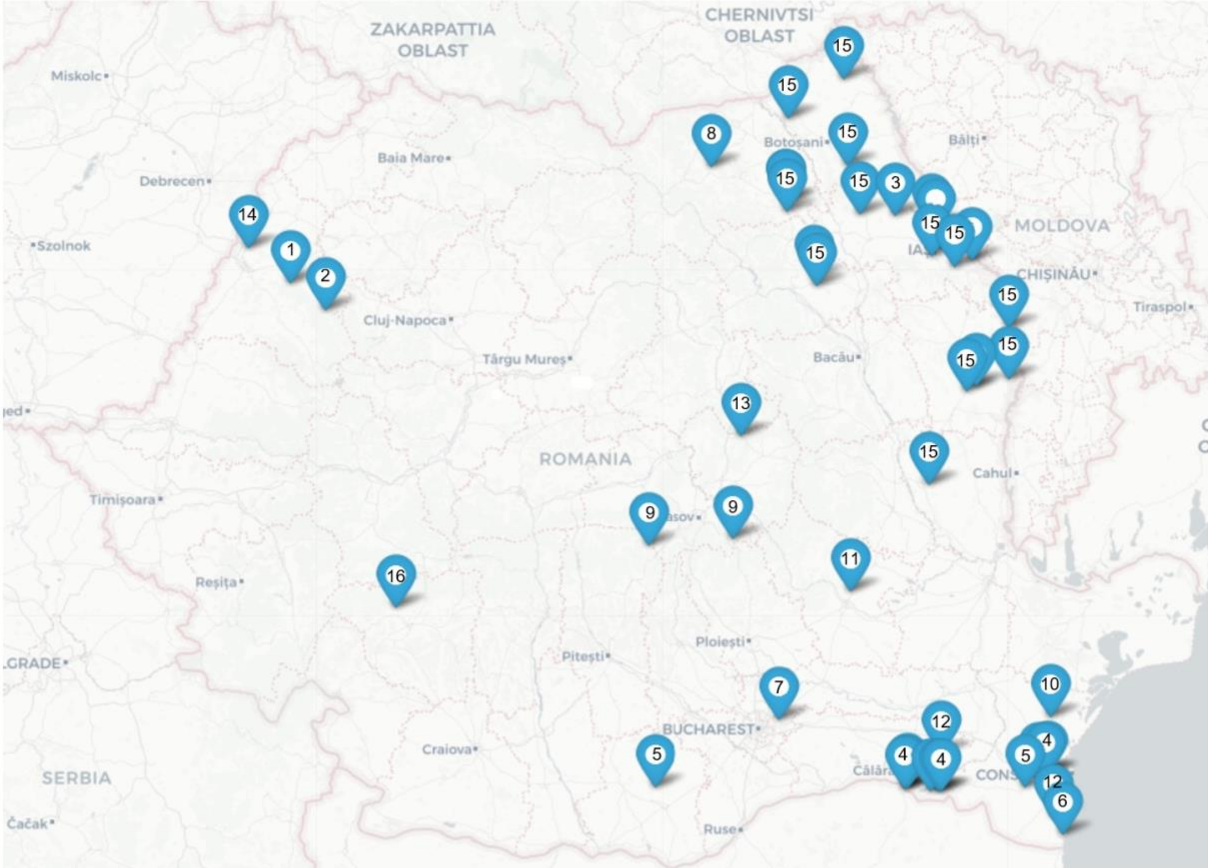

Figure 2 – Soil microbial studies conducted in Romania between the years 2005-2024. Legend: **1 -** Onet et al. 2024**; 2** - Bogdan et al. 2023; **3 -** Gafencu et al. 2023; **4 -** Steiner et al. 2023**; 5 -** Ghiță et al. 2022; **6 -** Chiciudean et al. 2022**; 7 -** Dușa et al. 2022; **8 -** Choma et al. 2021**; 9 -** Dinca et al. 2021**; 10** - Matei et al. 2020; **11 -** Toader et al. 2019; **12** - Ditu et al. 2018; **13** - Sarbu et al. 2018; **14 -** Onet et al. 2019**; 15 -** Ulea et al. 2017; **16** - Gornoavă et al. 2005

For instance, the samples examined from the Sulfur Cave were characterized by the presence of *Mycobacteria sp., Ferroplasmaceae sp., Acidithiobacillus sp.,* and *Metallibacterium sp.* with the first taxon being the most abundant (Sarbu et al. 2018). The diverisity of taxons from the soil samples collected in the Leșu cave is represented by taxons primarily from the phyla *Pseudomponadota, Verrucomicrobio, Actinomycetota, Acidobacteriota. Patescibacteria, Nitrospirota*. The central difference between the different collection sites was the abundance. Even though all the samples contained species from the mentioned phyla, their abundance was different throughout all the collection sites (Bogdan et al. 2023).

The rhizosphere bacterial communities of five rare plant species (*Adonis vernalis, Opopanax chironium, Asphodeline lutea. Paeonia tenuifolia, Potentilla emilii-popii*) were investigated using a mass spectrometry approach. With a focus on the cultivable fraction of the rhizosphere microbiota, the findings indicate that the genera variation among samples was not high. Species from genera such as *Bacillus, Pantoea, Serratia, Pseudomonas* were present in almost all of the samples analysed, these microorganisms having a function in mediating the plant growth. The outlook of the research states that the discovery of beneficial strains along with microbial indicators showcasing the health of the plant might be useful in conservation approaches (Ditu et al. 2018).

Soil pollution is majorly affecting the health of the crops along with their yield. Ulea et al. (2017) studied the impact of the agricultural practices and the seasonal variability on different soil types from Moldavia region. They took into consideration the abundance and composition of bacterial strain as indicators for the health of the soil. Compared on a temporal scale from

May to September, the highest bacterial abundance was registered in spring, whilst the lowest was registered in autumn. The agricultural practices directly influenced the microbial community abundance and dynamics as an undisturbed forest soil presented the highest bacterial count, whereas a vineyard soil which was subjected to a set of conventional agricultural practices presented the lowest bacterial count. Concluding, the authors state that the dynamics and changes in the structure of the soil bacterial population contribute to a better management of the agricultural habits, leaning towards a healthier future for the environment and promoting sustainable food production (Ulea et al. 2017).

The majority of studies were based on cultural methods for the identification of bacterial species. Consequently, the full picture of the whole studied soil microbiome hasn't been painted yet, as the unculturable fraction accounts for 99% of the whole microbiome. The study of soil microbial communities could contribute to developing more sustainable agricultural practices, leading to a healthier environment for the future generations. Food obtained through green and eco-friendly practices not only reduces the impact of agriculture but also improves the nutritional quality, promoting a long-term sustainable system for the food production.

DNA extraction – the essence of a metagenomics protocol

Studying the complex microbial communities present in a specific niche has opened doors to new insights into their ecological interactions, metabolic capacities, and evolutionary processes. The conventional culturing methods can't give an answer to all the questions that arise from a microbial network as they can't entirely portray its composition. With the advent of molecular biology methods, the study of taxa by amplicon sequencing and the field of metagenomics emerged answering a considerate number of questions. Metagenomic studies generate large quantities of data and even larger challenges to take into consideration, all in exchange for creating an almost perfect microbial picture on the canvas of the ecological niche and environmental changes.

A basic metagenomics protocol is described by the acquisition of the sample from the environment, extraction of the nucleic acids and their processing, sequencing, and analysis of the obtained data. The central step of the pre-sequencing stage consists of the nucleic acid isolation, step influencing both the quality and quantity of DNA for successive analysis. A lot of attention has been invested in soil DNA extraction methods, primarily due to the particularities of each technique and the varying outcomes in dependence to each environmental sample taken into analysis. Characteristics of a DNA extraction protocol from soil samples have been extensively reviewed by Wydro (2022). The DNA extraction from soil samples can be done through indirect or direct approaches. The indirect isolation of nucleic acids involves the separation of the cells from the soil sample, followed by their lysis. As eukaryotic cells are excluded, the separated organisms are represented by bacteria and archaea. Even though high amounts of DNA are extracted, this becomes a disadvantage for downstream analyses. Another impediment of this approach is the inability to study eukaryotic sequences and their interactions with prokaryotes. Direct isolation of DNA from soil implies the processing of the whole sample, the cells present in the soil matrix being lysed. This approach is beneficial for obtaining high yields and allowing the analysis of a high number of microorganisms (Wydro 2022).

A sum of factors that may influence the quantity and quality of the extracted DNA from soil samples include the organic content and type of the soil, the lysis method, the samples size, its transport and storage until downstream processing (Wydro 2022). The outcomes of a metagenomic protocol may also be influenced by the batch effect or the limited number of replicates taken into analysis (Child et al. 2024 Preprint). Soil contains a high number of impurities. Of interest are the humic acids that can co-precipitate and inhibit the DNA extraction process, consequently resulting in the failure of the PCR reaction. The physical, chemical and enzymatic lysis techniques employed in extracting the DNA from environmental samples are key determinants of the microbial diversity recovered (Wydro 2022). For example, metagenomic studies encounter challenges with the sample preparation process, as it could impact the number of lysed cells, mostly affected being the fungal species (Child et al. 2024 Preprint). Papers comparing different protocols, commercial kits or laboratory developed methods have emerged, each test being ran on different types of soil samples, trying to assess the best kits in regard of DNA yield, purity and impact on downstream analysis (Plassart et al. 2012; Santos et al. 2015; Tanase et al. 2015; Child et al. 2024 Preprint; Jensen et al. 2024). Table 1 provides a summary of DNA extraction methods and kits reported and compared in the literature from the last two decades with emphasis to the soil types taken into analysis.

Table 1 – Comparison of commercial DNA extraction kits and laboratory-developed methods used for soil microbial community analysis, as reported in the literature over the past two decades. It summarizes the DNA yields and purity ratios obtained, with reference to the specific soil types tested.

Commercial kit / method	DNA yield	A_{260}/A_{280} A_{260}/A_{230}	Soils tested on	Reference
DNeasy® PowerSoil® Pro Kit (Qiagen)	60 ± 21 ng/mg	N/A N/A	Arable, pasture, woodland, healthy soil	Child et al. 2024 Preprint
	0.5 - 68-8 ng/µl	0.75 - 5.31 0.01 - 0.4	Martian soil, mars stimulant soil	Wang et al. 2024
DNeasy® 96 PowerSoil® Pro QIAcube® HT Kit (Qiagen)	0.16 - 4.20 µg	1.79 - 2.88 0.95 - 2.15	Beach sand, clay, organic, sand, sand-clay	Jensen et al. 2024
QIAamp DNA Stool Mini KitTM (Qiagen)	4.7 - 54.7 ng/µl	N/A N/A	Compost, soil, mangrove sediment, decaying coffee pulp	Guillén-Navarro et al. 2015
ExtroSpin® Soil Kit (Lvjia Agro-tech Co., Ltd)	0.3 - 0.5 ¼g/g soil	1.69-1.82 0.08-0.19	Paddy soil, clayey soil	Li et al. 2014
FastDNA™ SPIN Kit for Soil (MP BioMedicals)	32 ± 17 ng/mg soil	N/A N/A	Arable, pasture, woodland, healthy soil	Child et al. 2024 Preprint
	1914.6-20333.33 ng	1.26-1.87 0.06-0.35	Woodland	Bollmann-Giolai et al. 2020
	2.1 ug/g soil	1.9 ± 0.2 N/A	Permafrost	Vishnivetskaya et al. 2014
	3.51 ± 0.03 µg/g soil	1.50 - 1.62 N/A	Garden soil, sewage sludge, lake soil, compost	Devi et al. 2015
	8.39 - 9.33 ng/µl	2.47 - 2.7 0.001	Martian soil, mars stimulant soil	Wang et al. 2024
	1.45-2.26 ¼ g/g soil	1.74-1.84 1.23-1.52	Paddy soil, clayey soil	Li et al. 2014

Commercial kit / method	DNA yield	A_{260}/A_{280} A_{260}/A_{230}	Soils tested on	Reference
FastDNA™-96 Soil Microbe DNA extraction Kit (MP BioMedicals)	0.02 - 2.91 µg	2.08 - 3.17 0.18 - 1.95	Beach sand, clay, organic, sand, sand-clay	Jensen et al. 2024
HiPurA soil DNA isolation kit (Himedia)	3.52 µg/ g soil	N/A N/A	Agricultural fields	Tanveer et al. 2016
Modified HiPurA soil DNA isolation kit	7.35 µg/ g soil	N/A N/A	Agricultural fields	Tanveer et al. 2016
ISOIL for Beads Beating kit (Nippon Gene)	1.02 - 2.15 ¼g/g soil	1.77-1.92 1.17-1.32	Paddy soil, clayey soil	Li et al. 2014
MagBeads FastDNA™ Kit for Soil (MP BioMedicals)	38 ± 20 ng/mg	N/A N/A	Arable, pasture, woodland, healthy soil	Child et al. 2024 Preprint
Meta-G-NomeTM DNA Isolation Kit (Epicentre Biotechnologies)	0.06 µg/g soil	1.7 ± 0.02 N/A	Permafrost	Vishnivetskaya et al. 2014
Power Lyzer™ PowerSoil® DNA Isolation Kit (Qiagen, formerly MOBIO)	8.7–47.5 µg/ g soil	1.8-1.9 1.5-2.1	Grassland, arable	Santos et al. 2015
	0-1203.33 ng	2.02-2.12 0.82-1.77	Woodland	Bollmann-Giolai et al. 2020
	0.9 µg/g soil	> 2.00 N/A	Permafrost	Vishnivetskaya et al. 2014
	2.5-3.5 ng/µl	N/A N/A	Beach sand	Gallard-Gongora et al. 2022
	2.47–6.96 ± 1.56 µg/g soil	1.13–1.64 1.28–1.58	Agricultural yellow loess soil	Kathiravan et al. 2015
PowerMax Soil™ (Qiagen)	0.8-0.9 ng/µl	N/A N/A	Beach sand	Gallard-Gongora et al. 2022
SPINeasy® DNA Pro Kit for Soil (MP BioMedicals)	40 ± 12 ng/mg	N/A N/A	Arable, pasture, woodland, healthy soil	Child et al. 2024 Preprint
Soil DNA Isolation Kit (NorgenBiotech)	1.08± 0.18 µg/ g soil	2.31± 0.17 0.29± 0.12	Rich humic acid and clay content soil polluted with kerosene	Tanase et al. 2015
Soil DNA extraction kit (MACHEREY-NAGEL)	14 µg/µl	2.2 0.86	Loam	Basim et al. 2020

Commercial kit / method	DNA yield	A_{260}/A_{280} A_{260}/A_{230}	Soils tested on	Reference
Soil Master DNA extraction kit (Epicentre)	0.79 μg/ml	1.32 1.21	Rhizospheric soil	Fatima et al. 2014
Zymo Research Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research)	12 ± 16 ng/mg	N/A N/A	Arable, pasture, woodland, healthy soil	Child et al. 2024 Preprint
ZymoBIOMICS1 96 MagBead DNA Kit (Zymo Research)	0.03 - 1.08 μg	1.38 - 1.68 0.03 - 0.07	Beach sand, clay, organic, sand, sand-clay	Jensen et al. 2024
ZR Soil Microbe DNA Miniprep™ (Zymo Research)	11.5 - 62.5 ng/μl	N/A N/A	Compost, soil, mangrove sediment, decaying coffee pulp	Guillén-Navarro et al. 2015
ISO-11063 Standard Method	3.87± 0.23 μg / g soil	N/A N/A	Crop soil, forest soil, grassland	Plassart et al. 2012
ISOm	19.03± 2.22 μg/g soil	N/A N/A	Crop soil, forest soil, grassland	Plassart et al. 2012
	21.5–43.4 μg/ g soil	1.5± 0.010 1.6-1.8	Grassland, arable	Santos et al. 2015
GnS-GII	26.26±2.20 μg/ g soil	N/A N/A	Crop soil, forest soil, grassland	Plassart et al. 2012
	8.2–49.7 μg/ g soil	1.6-1.7 1.5-1.6	Grassland, arable	Santos et al. 2015
Tanase et al. 2015 modified GnS-GII	40±6.16 μg/ g soil	1.55±0.05 0.56±0.05	Rich humic acid and clay content soil polluted with kerosene	Tanase et al. 2015
S	49.38±9,8 μg/ g soil	1.52± 0.02 0.69± 0.02		
SP	75.70±9.4 μg/ g soil	0.74± 0.02 0.38± 0.08		
S-CTAB	25.58±8.62 μg/ g soil	1.56± 0.02 0.62± 0.02		
SDE	468-2913.33 ng	1.29-1.45 0.60 - 0.87	Woodland	Bollmann-Giolai et al. 2020
PEG/NaCl method	0.73 μg/ml	1.26 1.12	Rhizospheric soil	Fatima et al. 2014
Mannitol-PBS-PEG/NaCl method	2.2 μg/ml	1.81 1.84	Rhizospheric soil	Fatima et al. 2014
Mannitol-PBS-PEG method	2.36 μg/ml	1.84 1.93		
Mannitol-PBS-CTAB	2.67 μg/ml	1.85 2.07		
Phenol-chloroform	7.5 - 125.0 ng/μl	N/A N/A	Compost, soil, mangrove	Guillén-Navarro et al. 2015

Commercial kit / method	DNA yield	A_{260}/A_{280} A_{260}/A_{230}	Soils tested on	Reference
Enzymatic lysis	7.5 - 75 ng/µl	N/A N/A	sediment, decaying coffee pulp	
Lysozyme method	12.5 - 100 ng/µl	N/A N/A		
Modified enzymatic lysis	0 - 100 ng/µl	N/A N/A		
Protocol A	10 µg/µl	1.9 2.4	Loam	Basim et al. 2020 (Basim et al. 2020)
Protocol B	14 µg/µl	1.6 0.65		
Protocol D	135 µg/µl	2 2.2		
Manual method	232.42 µg/g soil	N/A N/A	Agricultural fields	Tanveer et al. 2016
Slurry method	8.6-8.7 ng/µl	N/A N/A	Beach sand	Gallard-Gongora et al. 2022
Tsai and Olson 1991 method	3.38 ± 0.05 µg/ g soil	1.33 - 1.48 N/A	Garden soil, sewage sludge, lake soil, compost	Devi et al. 2015
	7.55 ± 0.73 µg/g soil	1.18 ± 0.015 0.82 ± 0.035	Garden soil, domestic and cellulose waste dumping sites, sewage contaminated site	Verma et al. 2017
Yeates et al. 1998 method	3.42 ± 0.04 µg /g soil	1.40 - 1.56 N/A	Garden soil, sewage sludge, lake soil, compost	Devi et al. 2015
Modified Yeates et al. 1998 method	5.87 ± 0.04 µg/g soil	1.72 - 1.82 N/A		
Modified Yeates et al. 1998 method	23.62± 4.65 µg/g soil	1.23± 0.06 0.92± 0.04	Agricultural yellow loess soil	Kathiravan et al. 2015
Zhou et al. 1996 method	1.29 ± 0.02 µg/ g soil	1.14 - 1.29 N/A	Garden soil, sewage sludge, lake soil, compost	Devi et al. 2015
	19.1±1.74 µg/g soil	1.25±0.03 0.94±0.04	Garden soil, domestic and cellulose waste dumping sites, sewage contaminated site	Verma et al. 2017
Siddhapura et al. 2010 method	8.51 ± 0.93 µg/g soil	1.34±0.03 1.25±0.03	Garden soil, domestic and cellulose waste dumping sites, sewage contaminated site	Verma et al. 2017
Singh et al. 2014 method	1.33 ± 0.16 µg/ g soil	1.02±0.01 1.00±0.01		
Verma et al. 2017 method	15.55±0.80 µg/ g soil	1.74±0.03 1.70±0.02		

Commercial kit / method	DNA yield	A_{260}/A_{280} A_{260}/A_{230}	Soils tested on	Reference
Verma and Satyanarayana 2011 method	11.23 ± 1.04 µg/ g soil	1.48 ± 0.030 1.32 ± 0.055		
Volossiouk et al. 1995 method	9.36 ± 0.60 µg/ g soil	1.11±0.02 0.85±0.05		
Bürgmann et al. 2001 method	33.8 ± 2.71 µg/g soil	1.27± 0.03 0.86± 0.02	Agricultural yellow loess soil	Kathiravan et al. 2015
Kathiravan et al. 2015 method	42.48± 5.59 µg/g soil	1.24–1.43 0.52-0.96	Agricultural yellow loess soil	Kathiravan et al. 2015
Porteous et al. 1994 method	9.31 - 15.89 ± 1.34 µg/g soil	1.04± 0.02 0.80± 0.01		

In 2012, a standardized method for extracting microbial DNA was published under the name "ISO-11063: Soil quality - Methods to directly extract DNA from soil". Although this method could be used to isolate bacterial DNA from soil samples, the other microbial species from the soil such as archaea and fungi were overlooked. Thus, diverse approaches were explored with much greater success in describing all the constituents of the soil microbiota (Plassart et al. 2012; Terrat et al. 2012; Terrat et al. 2015). By testing different protocols to discover the best ones when it comes to capture a snapshot of the soil microbiome, different standard-derived, developed in laboratory methods emerged. Two methods that became popular because of the results obtained were GnS-GII and ISOm. The ISOm standard is a method derived from the last-mentioned international standard that implies the usage of FastPrep® bead-beating (MP BioMedicals, USA). Compared with the GnS-GII method that involves the use of the same mechanical lysis step and being time consuming, it is more lightweight, meaning it could be routinely applied when working with a big batch of samples. The DNA obtained from using each method varies in quantity and quality, being much greater than using the standard ISO protocol. The authors concluded that the ISOm methods was the best option to use in extracting DNA for metagenomic studies, as the GnS-GII method introduced heterogeneity in the bacterial composition (Plassart et al. 2012; Terrat et al. 2015). The soil homogenization process was described as the most significant step to have an impact on the procedure efficiency (Plassart et al. 2012). Despite that applying the FastPrep® bead-beating in the last-mentioned protocols provided a higher DNA yield than the standard method, the results differed in between methods, with the greatest variations between soil types being registered when working with the GnS-GII protocol. This method had the highest distinguishing capacity between the soil types, being able to assess the heterogeneity of the microbial community accurately even though the yield was not the expected one (Terrat et al. 2012).

As the years passed, the methodology was advancing as the soil microbiome field was gaining popularity. Commercially available kits assess a variety of isolation methods to achieve high DNA yields, purity and integrity of nucleic acids while maintaining a high throughput and reproducibility. Various studies have compared different DNA extraction protocols for metagenomics analysis, across diverse soil sample types, such as agricultural, polluted, forest, and many more. These comparisons have highlighted the importance of selecting an optimal DNA extraction method to ensure accurate microbial community profiling and functional information retrieved (Tanase et al. 2015; Child et al. 2024 Preprint). GnS-GII was compared with other modified methods, such as S and S-CTAB. Interestingly, when tested on humic and kerosene-polluted soil samples, the S and S-CTAB exhibited superior performance or the results were equal to the ones obtained by applying the GnS-GII method, the DNA yield and purity being suitable for consecutive analyses. The highest DNA yield was obtained through the SP method, being almost two-fold higher than the yield obtained from the GnS-GII, although the purity was the lowest. Taking into consideration the higher DNA yield and proportionately equal purity when compared with the GnS-GII method, the authors concluded that the S method could be a great alternative when studying humic and clay soils (Tanase et al. 2015). The GnS-GII and ISOm methods were compared with the Power Lyzer™ PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, California) to assess their capacity to extract the protist DNA from grassland and arable soil samples. Although the GnS-GII and the ISOm had good yields of extracted DNA, the MoBio isolation kit had the best yield and purity, with reasonable cell-breaking capability and great abundance recovery ability, aspects important for describing the small fraction of soil protists, an important component of the microbiome (Santos et al. 2015).

In a very recent study, researchers compared the extraction capacity of five different kits for isolating DNA from soil samples taken from a pasture, an arable field, a dry healthy soil, and one collected from woodland. Some of the main differences between the samples was the pH of the environment along with the organic composition from the substrate. The authors compared the kits based on the characteristics of the extracted DNA: yield, purity, integrity, the impact on the read length based on the contrast between DNA length and read length, taxonomic classification rates based on DIAMOND aligned reads, and the effect of soil composition on the last-mentioned aspects. The analysis of the tested kits is summarized in table 2.

Table 2 – Comparison between different soil DNA extraction kits. The analyzed samples were representative of a pasture and arable field (neutral soil), dry healthy soil and woodland soil (acidic soil). Comparison between these kits could be interpreted from the graphical descriptors: ↑ - the best results, ● – average results, ↓ - the least favorable results (Child et al. 2024 Preprint)

Kit name	DNA yield	DNA purity	DNA integrity	Average DNA length	Average read length	Decrease in average read length	Impact on taxonomic classif.
FastDNA™ SPIN Kit for Soil	●	↓	↑	↑	↓	High decrease	●
SPINeasy® DNA Pro Kit for Soil	●	●	↑	↑	↑	High decrease	↑
MagBeads FastDNA™ Kit for Soil	●	↓	↑	↑	↓	High decrease	●
DNeasy® PowerSoil® Pro Kit	↑	↑	↑	↓	↑	Low decrease	↑
Zymo Research Quick-DNA Fecal/Soil Microbe MiniPrepTM Kit	↓	●	●	↓	●	Low decrease	↑

Based on their assessment, the authors determined that the optimal DNA extraction kit for soil samples is the DNeasy® PowerSoil® Pro Kit (Qiagen, UK), given its superior DNA yield, purity, and integrity. The decrease in read length that seems to normalize the performance of other kits with relatively average scores in the mentioned aspects is low for the DNA extracted using this kit. When it comes to the fungal communities, the Zymo Research Quick-DNA Fecal/Soil Microbe MiniPrepTM Kit (Cambridge Bioscience, UK) showed the lowest percentage of reads whereas the other kits closely followed with higher number of reads, with FastDNA™ SPIN Kit (MP BioMedicals, UK) for Soil, and MagBeads FastDNA™ Kit for Soil (MP BioMedicals, UK) leading the ranking. Average results could be seen for the SPINeasy® DNA Pro Kit for Soil (MP BioMedicals, UK), with an average yield and a high decrease in read length (Child et al. 2024 Preprint).

Sequencing technologies and their impact in revealing the soil microbial dark matter

The concept of microbial ecology is described by the relationship that forms inside a microbial community and outside of it, in regard to the interaction of the microbiota with the environment. Revealing the phylogenetic diversity of a sample can be tackled through metataxonomic or metabarcoding approaches, and uncovering the complex associations from soil samples has witnessed remarkable progress with the advent of metagenomics. By directly studying the genetic material of a microbial community with the aid of cutting-edge next generation sequencing (NGS) technologies and continuously evolving bioinformatic pipelines, the field of metagenomics has seen a great development in the last decade. The great advantage that metagenomics offers in uncovering the complexity of the microbiome resides in the ability to study the unculturable fraction of the microbial population, the soil' microbial dark matter.

The history of microbiome studies encompasses multiple time-stamps, all overlapping on the evolution of sequencing technologies. In the early days, the pioneering technology used to describe microbial communities was Sanger sequencing. At the time, newly described phylogenetic markers, mainly ribosomal genes, were sequenced, making possible the discovery of microbial diversity from different samples. This approach has later been termed as metataxonomics. Sanger sequencing technology implies the use of terminator nucleotides, yielding a maximum of 96 reads averaging 650 base pairs per run. The emergence of the high throughput, parallel sample sequencing technologies of the second generation achieved greater sample yields at lower costs than the first sequencing generation. Four technologies contoured this period, with Illumina sequencing passing the test of time. The first technology employed was the 454-sequencing platform. This determined the nucleotide sequence through the detection of a signal obtained in the DNA polymerization reaction. The luminous signal was determined by the released pyrophosphate. Compared with Sanger sequencing, the advantage of this technology was represented by higher yields at lower prices, but with shorter reads averaging 250 nucleotides. Reads determined with the G5 FLX Pyrosequencer could be used to assemble small genomes, such as bacterial and viral ones. This is mainly due to the quality and the contiguity of genomic data (Nikolaki and Tsiamis 2013). The primary drawbacks identified in the quality of the sequences obtained were the inaccurate insertions and deletions determined by long homopolymeric regions. Acquired by Roche in 2007, the pyrosequencing technology can't be used anymore as the related reagents and platforms were discontinued less than a decade ago (Escobar-Zepeda et al. 2015). Formerly known as Solexa, the Illumina platforms were the second to emerge. Employing dye-labelled reversible terminators in DNA polymerization though bridge-PCR on a glass surface, this technology is feasible for shotgun metagenomics for the high throughput and high quality. Even though the small read lengths (<150 nucleotides) seem to constitute a drawback, the error rates less than 1% and the small running costs along with the advanced bioinformatic tools developed to process the reads conquered the field (Quince et al. 2017). Another short-read sequence technologies that have been developed in the last two decades are represented by the SOLiD platform that uses the ligation of fluorescently labelled di-base probes and the Ion Torrent platform that detects the signal emitted by the protons released during DNA polymerization. The error rates of these two technologies range from <0.06% to <1.78% for the Ion Torrent platform. The output yields and

the running costs are not comparable to the Illumina platforms, this being two of the reasons Illumina gained popularity (Nikolaki and Tsiamis 2013; Escobar-Zepeda et al. 2015).
The principal challenge associated with the fragment sequences obtained through short-read sequencing is represented by the accurate assembly of genomes, as the coverage of the sequence fails to accurately represent the genome. The third generation of sequencing technologies highlights the significance of the long reads, which enables the sequencing of whole genomes. Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) developed sequencing platforms employing new sequencing procedures. PacBio platforms are characterized by the single molecule real time (SMRT) sequencing technology. Recent advancements employ a circularized DNA strand with hairpin adapters that act as primers for a polymerase. Upon binding to the polymerase, the DNA is loaded in a chamber termed zero-mode waveguide. As the polymerase incorporates fluorescently labelled nucleotides, a distinct signal is detected, allowing to differentiation of the nucleotide sequence. ONT platforms employ a fixed nanopore that allows a single strand of DNA or RNA to pass through it. As the nucleotide strand translocates the nanopore, the ionic flow is altered, with variations in the recorded charges translating into the nucleotide sequence. These new principles output sequences averaging in lengths over 10 kilobases. This represents the first advantage of long read sequencing over short read sequencing: the ability to generate fewer reads with wider coverage. Another advantage is the identification of structural variants along with assessing epigenetic modifications. Comparable to sequencing short DNA fragments, the sequencing of lengthy DNA strands represents a challenge for the third generation of sequencers concerning error rates. Recent advancements within these technologies has seen great progress with increasingly higher sequencing accuracies (Kim et al. 2024).
As sequencing technologies evolved, a series of advantages and drawbacks have emerged when uncovering the soil microbiome. NGS technologies facilitate a thorough evaluation of the microbial diversity, advancements in these technologies allowing the identification of novel microbial taxa, as well as uncovering the unculturable fraction of the microbiome through deep sequencing of environmental samples. By directly sequencing the microbial community from an environmental sample, NGS bypasses the need for culturing microorganisms such as bacteria and fungi. Through the application of whole genome sequencing, previously unidentified genes and biochemical pathways have been uncovered. Sequencing technologies such as ONT make gene expression analysis possible, as this method is feasible for RNA sequencing. As NGS platforms continue to evolve, tackling the microbiome becomes more affordable, accompanied by a reduction in sequencing time. On the other hand, due to the high yields and huge volumes of data generated, costly computational resources are required to analyze the metagenomic data, as well as bioinformatic pipelines, and basic programing expertise. Sequence quality might be subjected to artifacts associated with error rates of sequencing platforms or the extraction methods used. When multiple samples are multiplexed, a metagenomic study might be influenced by the batch effect which can negatively impact the analytical outcomes. The discovery of new microorganism may also be impacted by the lack of reference genomes in public databases (Garg et al. 2024). Two frequently used sequencing platforms in metagenomics, namely Illumina and ONT, although they facilitate the comprehensive characterization of microbial communities, they exhibit differences in accuracy and taxonomic resolution. More specifically, Illumina MiSeq provides superior accuracy as the MinION sequencer offers longer reads at a lower initial cost (Stevens et al. 2023).
Recent studies have introduced innovative approaches like culturomics-based metagenomics, aimed to enhance the recovery of both taxonomic and functional diversity in desert soils, capturing previously missed diversity and enabling the identification of novel bacterial candidates. The culturomics-based metagenomics approach combines the cultivation of the samples under multiple culture conditions, followed by 16S amplicon sequencing and shotgun

sequencing. This approach resulted in an increase in the number of amplicon sequence variants (ASVs) and qualitatively metagenome-assembled genomes (MAGs). Despite this, the relative abundance and the functional pathways present in the *in situ* environment have not been properly represented. The integration of multi-omics approaches in metagenomic studies represents a promising future approach in recovering the untapped microbial dark matter. (Li et al. 2023).

Altogether, metagenomic analyses managed to uncover a big piece of the soil microbiome puzzle, but the whole picture is not even half complete. Even though databases were populated with sequences of new taxa obtained at reasonable costs, advances in the field are still sought as the interaction network of these microorganisms could describe the applicative potential of the microbiome. A metagenomic analysis cannot describe by itself all the particularities of the functionally active community as the sequenced DNA is composed out of relic DNA of dead or metabolically inactive species or by DNA trapped in biofilms. In dependence to external factors, such as climatic conditions following season's change, varying nutrient quantities and even the spatial separation in soil aggregates, metagenomics miss on the metabolic versatility of the microbiome dependent on the exterior and the microbiome's interactions within- and outside of it. By cumulating and contextualizing the genomic data with those obtained from metatranscriptomic, metaproteomic, metabolomic analyses, as well as the effect of the external-conditioning factors, obtaining a more comprehensive description of the microbiome's potential could be attained. Metatranscriptomics describes the activity of the microbial community and their adaptations while metaproteomics includes the post-translational modifications of proteins which could aid in the discovery of novel species when compared to genome bins. However, modelling detailed interaction networks of the soil microorganisms in regard to their active metabolic pathways which include signaling metabolites or synergic/agonistic interactions between the members of the community is what the future hopes to hold in its approaches (Jansson and Hofmockel 2018). Specifically, culturomics approaches could aid in describing species interactions with a higher resolution but are hindered by the lack of growth particularities (Liu et al. 2022).

This way, multiple approaches are being taken at the moment to characterize the interaction network of the soil microbiome. Research on the mangrove sediments to assess the microbial community assembly using a genome scale metabolic modelling-based approach and network analysis from MAGs and metatranscriptomic data concluded that over half of the assembled species had a high potential of metabolic interactions. Still, from the entire community taken into study, over 98% of the microorganism pairs were not seen to interact with one another through sharing metabolites. However, five small groups of microorganisms were seen to interact divergently into successfully carrying out metabolic functions (Du et al. 2022). Adaptation to drought stress response on the rhizosphere microbiome was studied using MAGs and metatranscriptomic data for an agricultural site. Researchers observed that in drought conditions, the microbiome is enriched in bacterial groups such as *Actinobacteria*, possessing traits for carbohydrate metabolism and iron transport. When disrupting the iron homeostasis, the drought adapted microbes were affected, and in turn, the plant's ability to withstand the stress as well (Xu et al. 2021).

From amplicon sequencing to metagenome-assembled genomes (MAGs)

Studying the genetic material from environmental samples has taken different approaches along the way, as briefly described in the last section. The terminology used in the field tries to differentiate the genome microbiome studies, taking into consideration both the sequencing material and intended outcome. In this context, the concepts of metataxonomics (which involves the sequencing of phylogenetic markers) and metagenomics (which involves the sequencing of the whole genetic material of a sample) describe two independent approaches to take in studying environmental samples. Owing to their highly conserved and hypervariable regions, phylogenetic marker-based taxonomy became the easiest way to classify organisms, achieved by amplifying shorter regions of these genes, and subsequently sequencing these amplicons. This approach has been known in the field as amplicon sequencing. On the other hand, shotgun or long-read sequencing of an environmental sample describe an authentic metagenomics approach, the whole DNA content of a sample being taken into consideration. The main advantages of sequencing the whole genome lie in its capacity to uncover new functional genes, metabolisms and obtaining draft genomes of uncultured organisms, which encompass members of the microbial dark matter - element that cannot be achieved at the same scale by sequencing taxonomic markers. Additionally, another great advantage of sequencing the whole genome is avoiding the PCR biases that might appear when amplifying marker genes (Pérez-Cobas et al. 2020; Nam et al. 2023). Metagenomics data is processed into metagenome-assembled genomes (MAGs), a further refinement of metagenomic approaches. The reconstruction of MAGs has aided for the uncovering of bacterial diversity, especially discovering the microbial dark matter (Quince et al. 2017).

Amplicon sequencing takes into consideration the sequences of targeted amplified phylogenetic markers. 16S rRNA is specific for the identification of prokaryotes and identifying eukaryotes has resided in the sequencing of 18S, 26S or ITS. These marker genes are characterized by hypervariable regions that allow the classification of taxons down to species level (Pérez-Cobas et al. 2020; Nam et al. 2023).

NGS made available the evolution of metagenomics by making possible the description of the full diversity of complex microbial communities through deep-sequencing. This transition provides a more comprehensive understanding of the microbiome. Metagenomic data is processed in the scope of constructing a representative picture through the MAGs. A basic bioinformatic pipeline for the construction and analysis of MAGs consists of quality control of sequenced reads, genome reconstruction through assembly and binning, high-resolution taxonomic and functional prediction, and data visualization. A vast array of bioinformatic tools and databases currently used in genomic reconstruction and following analysis have been reviewed by Wajid et al. (2022). Even though the computational resources needed to conduct such analyses are considerably pricey, they provide greater insight into the complete picture of a microbiome (Nam et al. 2023).

Large-scale excavation efforts have reconstructed metagenome-assembled genome bins, revealing a vast number of unknown species-level genome bins that significantly expand the microbial diversity and functional landscape of the soil. Ma and colleagues (2023) tackled the soil's microbial dark matter from 3304 metagenome data. After reconstructing over 40,000 metagenome-assembled genome bins, they identified 21,077 species-level genome bins, out of which, almost 80% were unidentified species-level genome bins. The authors identified many unknown genes that need further analysis, as well as a great number of potential biosynthetic gene clusters that might code for useful secondary metabolites. Associations between viruses and hosts was described by a numerous range of viruses that infect different bacterial hosts, with prophages taken as the best predictor of these associations. Last but not least, they analyzed the "immune system" of the microbial community, discovering over 8500 CRISPR-Cas genes, the soil microbiome portraying a large resource of Cas proteins (Ma et al. 2023).

In another study, Singh et al. (2023) constructed MAGs from the International Space Station, their analysis revealing insights into microbial metabolic and antimicrobial potential, as well as the network interactions within the community. By undertaking a metagenome-to-phenome approach, two bacterial and one fungal novel species were also discovered. The authors conclude that the reconstructed genomes contribute to our understanding of microbial life in microgravity and low-dose irradiation when compared to the microorganism's evolution on Earth (Singh et al. 2023).

Conclusions

The soil microbiome is a central component of the environment, maintaining ecosystem functions and supporting agricultural productivity. Composed in big part by bacteria and fungi, the varying abundance and diversity of species characterizes different soil types and supports numerous soil functions such as carbon sequestration, organic matter decomposition, nutrient cycling, bioremediation, aggregate formation, pest and disease control along with many others. The soil microbiome's composition and functions are dependent to numerous factors, with agricultural practices, such as excessive tillage, use of pesticides and mineral fertilizers, significantly influencing and potentially disrupting these microbial communities. Observing this interdependence, sustainable agricultural practices are created and started being implemented in the field, aiming to preserve the health of the soil and quality of food, as a final objective. The development that the field of metagenomics has seen in the last years shows promising future approaches for describing the complexity of the microbiome, as well as identifying novel microbial species and new metabolic pathways with application in medicine and biotechnology. Soil microbial dark matter presents as a huge reservoir of such pathways that have not been described and new metabolites that might have bioactive potential (Ma et al. 2023). Rise in antimicrobial resistance genes determined by wastewater containing antibiotics or animal waste is due to increase because of the global demand for food production and pollution. Although the ecological stress leads to formation in soil bacteria of compounds similar to antimicrobials, their discovery is still much slower than the emergence of resistance phenotypes (Brevik et al. 2020). At the same time, discovery of novel antimicrobials, probiotics, biocontrol agents is hampered by the incapacity to cultivate many of these microorganisms (Fierer 2017; Liu et al. 2022). To understand the functional potential of interesting but yet-uncultured microorganisms, developing cultivating methods to isolate these species is a priority now. -omics data helped in broadening the knowledge about new microorganisms and their theoretic potential, but they cannot confirm that what is gene-encoded also functions as hypothesized. Thus, culturing microorganisms overpowers metagenomic analysis as it facilitates the study of biochemical and physiological traits under different, but controlled growth conditions. Advancements in using metagenomic data are made for selective isolation and cultivation; where possible, growth traits are deduced. However, metagenomic data quality still relies on DNA extraction methods, element that can represent a drawback in deducting such traits (Liu et al. 2022).

Efficient DNA extraction methods remain decisive for accurate downstream microbial analysis by sequencing. Comparison between different nucleic acid extraction methods, either applied from commercially available kits or lab developed has been an intriguing subject of discussion. Soil contains many inorganic and organic substances, along with PCR-inhibitors that can affect the extraction process and downstream metagenomic analyses. Thus, because of soil's nature, extracted DNA yield and quality vary, even when using the same method. Even though the metagenomics of the soil becomes more and more of interest in the actual context of trying to conserve biodiversity and acquire food security, integrating a cost-effective method is even harder. Lab-developed protocols appear to achieve the results of the DNA extraction kits but their efficacy and bias has not been properly described in the majority of cases by using an extraction control made out of a known quantity of bacterial cells.

Combined short- and long- read sequencing approaches or culturomics based metagenomics are just some of the latest procedures used to obtain metagenome-assembled genomes of a high quality. Along with the continuously updating bioinformatic pipelines and databases which hope to facilitate new discoveries through data mining, the field of soil metagenomics promises notable discoveries in agriculture, pharmacology, ecosystem preservation – being valuable for the OneHealth concept.

Code availability: The Python (v3.12.4) script used for mapping the coordinates on the Romania map can be accessed at the following address: https://github.com/AndaMM/Map_coordinates_in_Ro.

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