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RESEARCH ARTICLE

REVISED Soil health and microbial diversity across land-use types: Evidence for agroecological management in peri-urban areas

[version 3; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background

Land-use change profoundly influences soil microbial communities, yet its impacts on richness and diversity remain incompletely resolved across taxa.

Methods

Here, we characterized fungal and bacterial communities in soils from four contrasting land-use types—including crop, reforested, agroforestry, and uncultivated land—located in the same pedoclimatic conditions, using high-throughput amplicon sequencing of Internal Transcribed Spacer (ITS) and 16S ribosomal RNA (rRNA) genes. We quantified species richness and Shannon diversity and examined their relationships with key physicochemical parameters.

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Any reports and responses or comments on the article can be found at the end of the article.

Results

Our results reveal that fungal and bacterial communities responded differently to land-use management. Fungal richness was highest in reforested soils, whereas bacterial richness was more uniformly distributed across land uses. Shannon diversity showed greater sensitivity than richness, suggesting that differences in the distribution of relative abundances among taxa contributed substantially to the observed patterns. Multivariate ordinations and correlation analyses further demonstrated that soil properties such as pH, total nitrogen, and cation exchange capacity were significant drivers of microbial community composition and diversity patterns.

Conclusions

Our study provides new empirical evidence of how land management shapes biodiversity and informs strategies for enhancing soil health and ecosystem potential resilience.

Plain language summary

Healthy soils support food production, clean water, and climate resilience. In this study, we examined how different land uses—crop fields, agroforestry, uncultivated areas, and reforestation—affect soil health in a peri-urban area of Milan. We analysed soil microbes, which are essential for nutrient cycling and ecosystem stability, along with key soil properties such as pH, nitrogen, and organic matter.

We found that land use strongly shapes microbial communities. Agroforestry soils hosted the highest number of unique microbial species, highlighting their ecological value, while reforested soils showed lower richness due to their early successional stage. Fungal communities were more sensitive to land management than bacterial ones. Soil chemical properties, especially cation-exchange capacity and nitrogen, played a major role in determining microbial patterns.

Overall, our results show that agroforestry can enhance soil biodiversity and resilience, supporting its role as a nature-based solution for sustainable land management in peri-urban landscapes.

Keywords

soil microbiome, agroecological management, soil health

This article is included in the Plant and Soil Health collection.

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REVISED **Amendments from Version 2**

The new version includes new Fig. 3 and Fig 8 small modifications to the abstract additional details on the methods and the results. Following the request of the referees we have removed the old Fig. 3 9 10 11 and 12. A typo in Eq 1 has been corrected.

Any further responses from the reviewers can be found at the end of the article

Introduction

For decades, conventional agricultural management has led to a gradual impoverishment and simplification of agricultural landscapes, compromising the overall health of the most productive areas.[1] Since the 1950s, agricultural landscapes have been progressively stripped of the ecological infrastructure that for centuries had supported their ecological, productive, and cultural functions.[2] This has led to the disruption of the multiple relationships and self-regulation mechanisms within the agroecosystem. As a result, the system has become vulnerable and unstable, highly dependent on external inputs to sustain its productive function,[3] unable to support other ecosystem functions across different scales – from soil domain, to the individual field, to the farming system, to the local landscape context and, more broadly, to the landscape unit.[4]

When considering the subset of peri-urban agricultural systems - ecotonal zones where the thinning of the urban fabric gradually gives way to natural, forestry, and pastoral land uses - these processes are further exacerbated. While urban systems confer numerous benefits to their inhabitants, they are also responsible for the profound alteration of the functioning of local and global ecosystems. This is achieved by the fragmentation and degradation of agricultural and natural habitats, leading to a reduction in biodiversity. Furthermore, urban systems disrupt hydrological systems, and modify energy flows and nutrient cycles. These dynamics, together with unsustainable land-use practices, affect agroecosystem functions and the provision of key ecosystem services (ES) on which the very livelihood of urban populations depends, e.g. supply of clean water, maintenance of soil fertility and health, food production, climate regulation and climate change mitigation, improvement of air quality.[5] Nonetheless, peri-urban fringe areas hold considerable potential for providing buffering and resilience functions for cities.[6–11]

Soil health - commonly defined as soil s ability to function as an ecosystem that sustains plants, animals, and human life - is increasingly recognized as a cornerstone of the broader concept of Global Health, as it underpins agricultural productivity, ecosystem services, human nutrition, and climate resilience. This is particularly relevant in peri-urban areas, where soils are expected to sustain food production while being exposed to intense pressures from urbanization and land-use change. This complexity requires the development of composite indicators that are rapid, sensitive, and reliable in capturing the multifunctionality of soils. Many recent papers explicitly call for harmonized methodologies able to link soil health assessment with food security, biodiversity, and climate policies.[12–15] In this perspective, soil health indicators should not only measure physical and chemical properties, but also integrate biological and functional dimensions, thereby providing a comprehensive and policy-relevant assessment tool.

To address these challenges, it is therefore necessary to rethink the territorial development of peri-urban fringes in order to maximize their delivering capacity of ES, enhancing their capacity to mitigate the negative externalities of urban systems. Recent advances in the literature highlight the potential of silvoarable agroforestry systems (SAFs) as a strategy to counteract soil degradation and biodiversity loss in Western Europe. According to De Clercq et al.,[16] SAFs contribute to soil biological health by fostering richer and more diverse soil communities and improving soil organic matter, litter-feeding macrofauna, and arbuscular mycorrhizal fungi, while also reducing soil bulk density. These effects are most pronounced in older stands and in proximity to tree rows, underscoring the role of temporal and spatial factors in shaping belowground processes. Despite these benefits, important research gaps remain—particularly concerning mesofauna, microbial activity, young stands, and deeper soil layers—indicating the need for more integrative indices that combine multiple biological parameters. Incorporating such findings into the broader context of sustainable land management underscores SAFs as a promising practice to enhance ecosystem services and soil resilience under climate and agricultural pressures.

In this context, in the present study we investigated soil health of different land uses representative of typical northern Italy peri-urban agricultural landscapes, i.e field annual crops, agroforestry, wooded areas, set-aside fields by integrating a variety of indicators such as (i) soil physicochemical properties linked to fertility and structure, (ii) microbial community composition and alpha-diversity derived from 16S ITS amplicon sequencing, (iii) multivariate analyses to relate community structure to environmental gradients (PCA/RDA), and (iv) prediction of functional potential of bacterial communities using PICRUSt2.

Results

Community composition and diversity patterns

To characterise differences in soil communities across land-use types, we analysed the taxonomic composition, richness, and diversity of fungal and bacterial communities, and quantified the overlap in species occurrence among land uses. Heatmap visualisation of the 25 most abundant fungal and bacterial genera revealed clear differences in taxonomic profiles among the four land-use types (Figure 1). Fungal communities showed stronger variation in dominant genera across land uses than bacterial communities, with certain genera significantly more abundant in specific samples and in specific land-use types; for instance, *Mortierella* and *Inocybe* are much more abundant in reforested than in any other soils. In contrast, the relative abundance in bacterial profiles displayed a higher homogeneity among samples, although clustering within the same land-use type is still visible to a certain degree. We note that *Gp6 Gemmatimonas*, and *Gaiella* are more abundant in crop and reforested soils, while *Gp1* and *Gp3* are more present in uncultivated soils. In general, fungal samples were dominated by genera such as *Mortierella Fusarium*, and *Trebouxia*, while bacterial communities showed high relative abundances of members of the *Acidobacteria* group, Gemmatimonas and *Gaiella*. It is worth noting that in both fungal and bacterial datasets, some of the most abundant features were not fully resolved to genus level, suggesting the presence of a substantial fraction of community members that remain taxonomically undercharacterised. These results underline the stronger sensitivity of fungal communities to land-use and land-use change, probably due to their symbiotic associations with plants and strong linkage with organic matter, namely lignin and complex organic molecules, in undisturbed conditions.[17] From an agroecological perspective, the observed land-use–associated differences in fungal diversity are consistent with the idea that management practices influencing soil organic matter and nutrient dynamics may contribute to shaping fungal communities.

Comparisons of diversity metrics at the species level showed that both fungal and bacterial richness and Shannon diversity varied noticeably across land-use types (Figure 2a). Uncultivated and reforested soils consistently exhibited lower richness than crop and agroforestry. Formal statistical testing (n = 3 per land-use type) detected significant differences only for richness: for bacteria, between crop and reforested soils ($p = 2.3 \times 10^{-2}$) and for fungi, between crop and reforested soils ($p = 4.0 \times 10^{-2}$) as well as between reforested and agroforestry soils ($p = 1.1 \times 10^{-2}$). No significant differences were detected for Shannon diversity among land-use types.

UpSet plots of species presence–absence (Figure 2b) showed that each land-use type contained unique fungal and bacterial taxa, but agroforestry soils had the highest number of exclusive taxa in both kingdoms, whereas reforested soils had the fewest. This suggests that agroforestry management is associated with increased compositional variety and may increase soil biodiversity relative to the other land uses examined. Significant overlap in community composition was observed, especially in bacteria, with over 700 species common to all the land-use types. In addition to species-level differences, significant shifts were also observed at higher taxonomic ranks. To evaluate compositional diversity across land-uses, we then computed Whittaker beta diversity within and between land-uses for fungal and bacterial communities. Within each land use (Figure 3), (W) = γ/α was consistently >1 for both kingdoms, indicating moderate taxonomic turnover among the three biological replicates. Turnover among replicates was generally higher for fungi than for bacteria. Pairwise comparisons between pooled land-use communities revealed moderate turnover across all land-use pairs, with the highest dissimilarity involving agroforestry and reforested soils. Together, these patterns suggest that land-use type is associated with compositional differences in both fungal and bacterial communities. Figure 4 and Figure 5 show fungal and bacterial phyla whose relative abundances varied significantly across land-use types, indicating that land-use effects are strong also at broader taxonomic groups. The strongest land-use contrasts for fungi were observed for *Chlorophyta* and *GS01*. For *Chlorophyta*, crop soils showed higher relative abundance than agroforestry (p = 5.101 10^{-3}) and uncultivated soils (p = 8.758 10^{-3}). For *GS01*, crop soils exceeded reforested (p = 5.199 10^{-3}) and uncultivated soils (p = 5.199 10^{-3}), and also differed from agroforestry (p = 2.470 10^{-2}). As for bacteria, the strongest land-use contrast was observed for candidate division *WPSUnclassified1*, between reforested and agroforestry soils, with markedly higher relative abundance under agroforestry (p = 2.454 10^{-4}). A similarly pronounced difference was detected between crop and agroforestry soils, again with higher values in agroforestry (p = 1.629 10^{-3}). For *Synergistetes*, reforested soils showed substantially higher relative abundance than agroforestry soils (p =4.227 10^{-4}), and also differed strongly from uncultivated soils (p = 8.348 10^{-4}). Notably, a larger number of bacterial phyla showed significant differences compared to fungi, likely reflecting the higher overall richness and taxonomic diversity of bacterial communities.

Correlation patterns among samples and abiotic parameters

To explore similarities among samples, we computed Pearson correlation matrices based on the bacterial, fungal and abiotic profiles of each sample; correlation matrices were represented as clustered heatmaps (Figure 6). Across all three data layers,

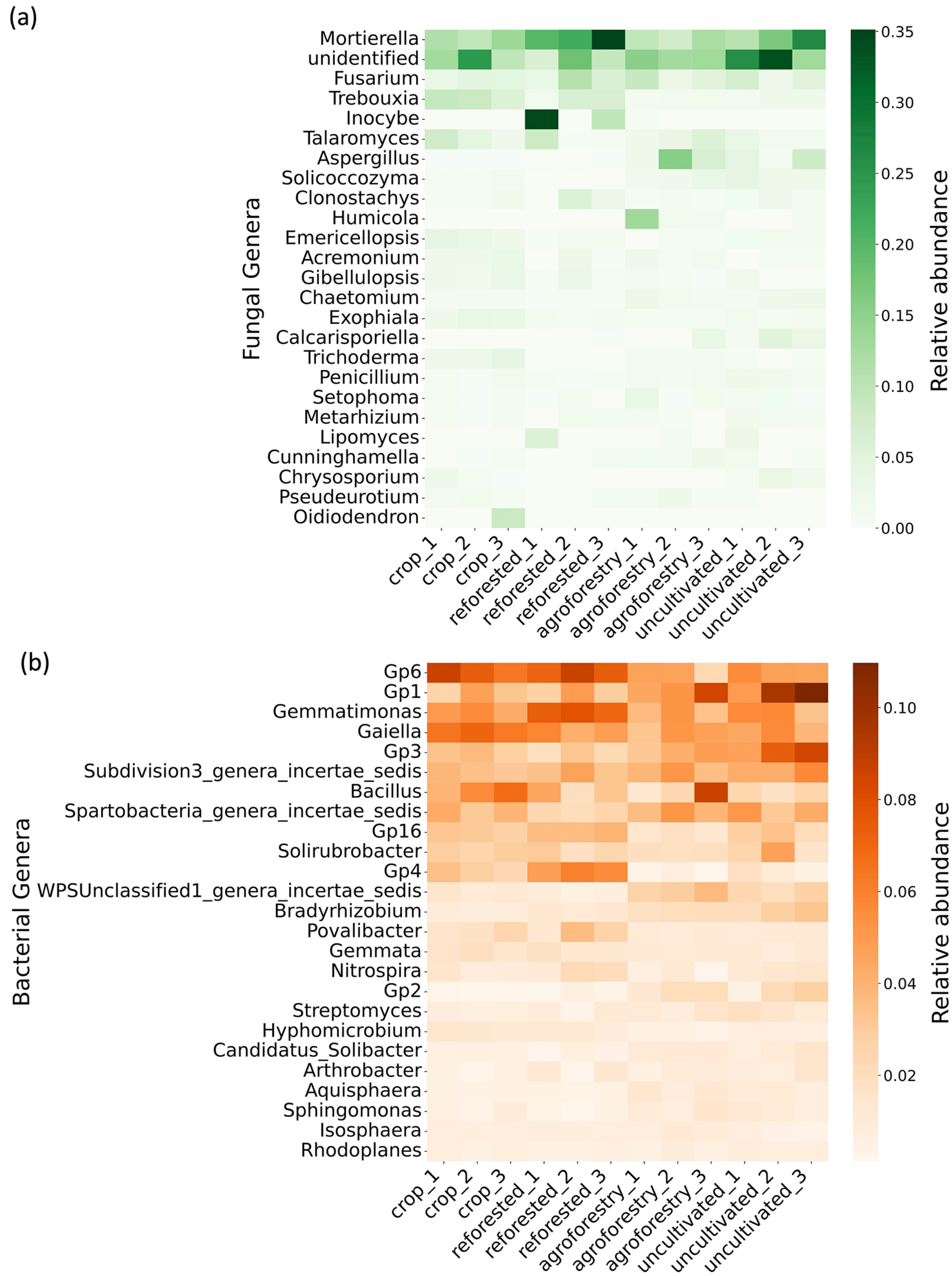

Figure 1. Heatmaps of a) the 25 most abundant fungal genera, and of b) the 25 most abundant bacterial genera across 12 soil samples representing four land-use types crop, reforested, agroforestry, and uncultivated). Genera are ordered by overall abundance. Color intensity reflects relative abundance within each sample.

samples generally clustered by land use, indicating consistent land-use–associated structuring of both microbial communities and abiotic conditions. Clustering was most distinct for bacterial communities, which separated into two main groups (crop–reforested vs uncultivated–agroforestry), whereas fungal communities showed weaker land-use clustering.

Moreover, we computed the Pearson correlation matrices of the abiotic parameters to investigate relationships among chemical and physical soil properties. Analysis of the top 20 strongest pairwise correlations among abiotic variables

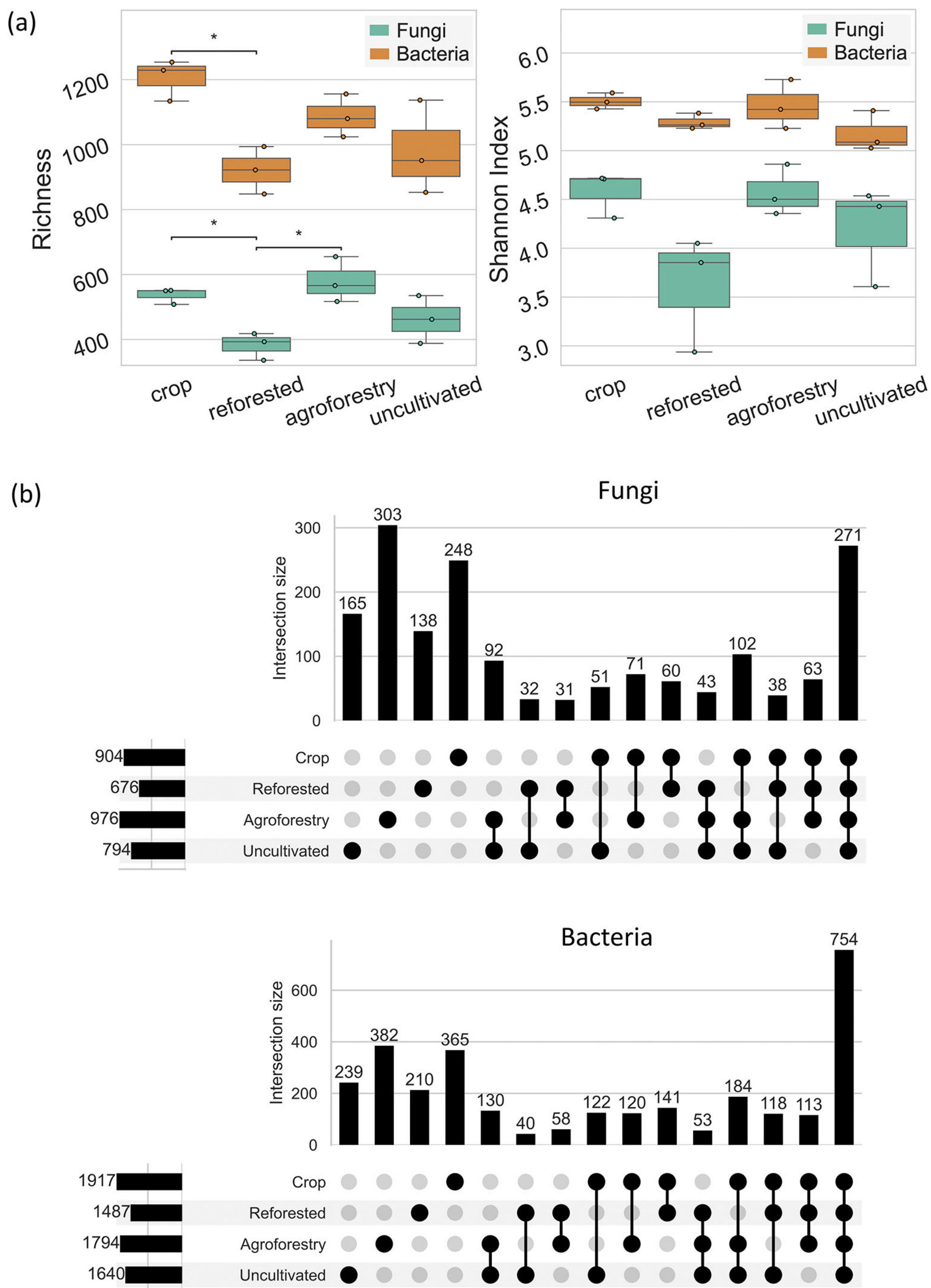

Figure 2. a) Boxplots of fungal and bacterial richness and Shannon s diversity index at species level across different land uses. Significant differences between land-uses, assessed with a Tukey HSD test on the mean values of each ecological index, are indicated with a bar over the couple of boxplots and a number of stars. Differences are considered statistically significant at p-value <0.05 and are reported with the following legend: $10^{-2} < p < 5 \cdot 10^{-2}$ (*), $10^{-3} < p < 10^{-2}$ (**), $10^{-4} < p < 10^{-3}$ (***), $p < 10^{-4}$ (****). (b) UpSet plots showing the intersection of bacterial and fungal species across four land use types. Each plot illustrates the number of species unique to or shared between different land uses. Horizontal bars represent the total number of species observed per land use, while the vertical bars show the size of species sets defined by each intersection.

(Figure 7) revealed different patterns across land-use types: crop and reforested soils showed more compact correlation clusters dominated by strong co-variation among fertility-related variables (e.g., organic matter, total nitrogen, and organic carbon), whereas agroforestry and uncultivated soils displayed more heterogeneous patterns with multiple smaller clusters. In these two land uses, several subgroups of variables emerged, leading to a higher structural complexity.

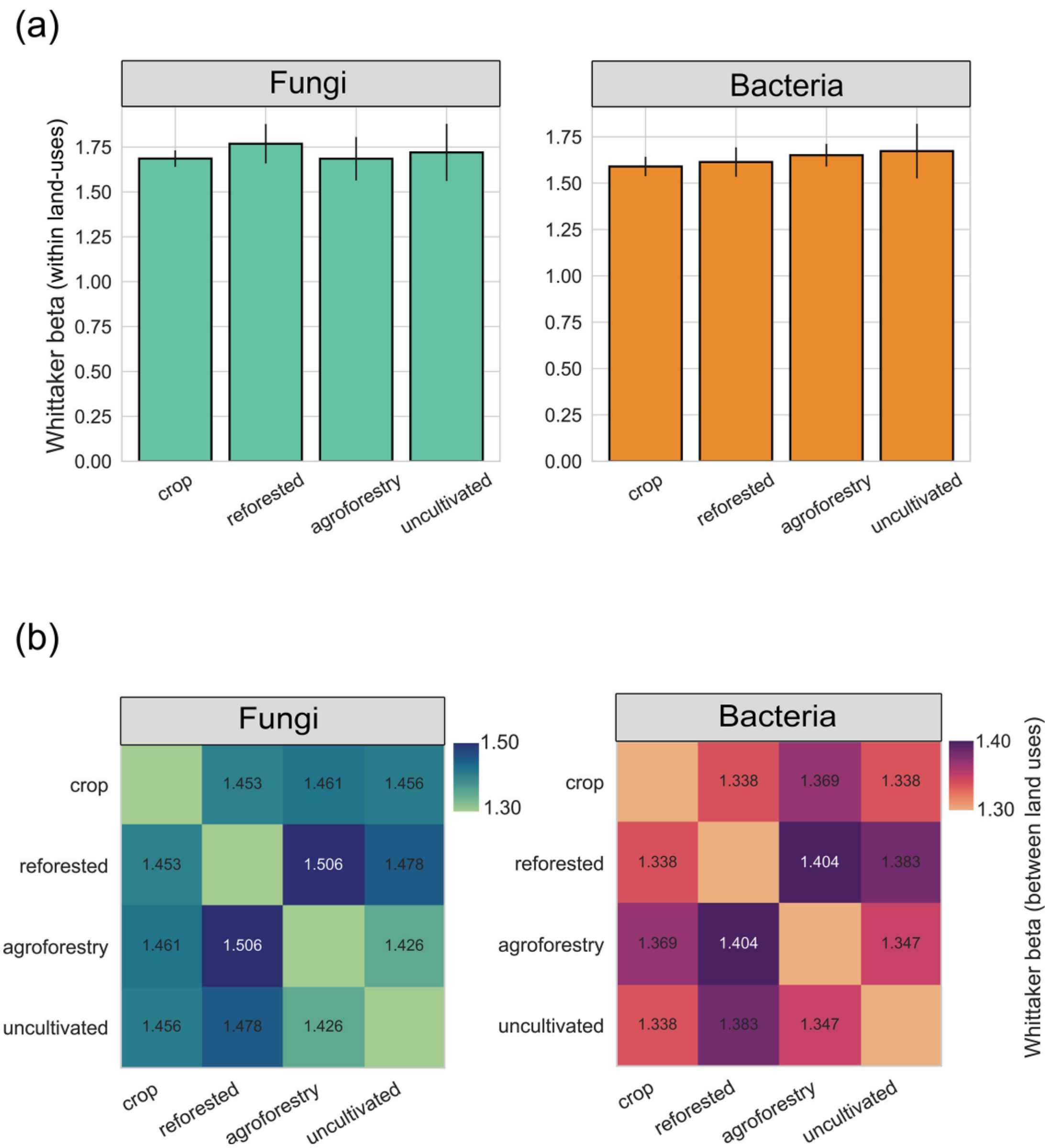

Figure 3. Ecological indices of fungal and bacterial communities across land-uses. (**a**) Barplots of fungal and bacterial Whittaker beta diversity at species level between replicates (within land-uses). The errorbars reflect the uncertainty on α diversity propagated into β. (**b**) Heatmaps of pairwise Whittaker beta diversity between land-use types for fungi and bacteria. Values were computed from pooled presence/absence profiles within each land use.

We also assessed significant differences for each abiotic parameter across land-use types; the parameters showing the strongest effects are reported in Figure 9. Land-use types differed most strongly in texture and fertility-related properties. Crop soils showed a higher silt fraction than agroforestry soils ($p = 8.379 \quad 10^{-4}$) and reforested soils ($p = 5.902 \quad 10^{-3}$). Reforested soils exhibited consistently higher values for several fertility-linked variables, including pH (reforested vs agroforestry: $p = 8.388 \quad 10^{-4}$), cation-exchange capacity (reforested vs agroforestry: $p = 7.186 \quad 10^{-4}$), and exchangeable base cations, with particularly pronounced differences in exchangeable Ca (reforested vs crop: $p = 1.195 \quad 10^{-5}$; reforested vs agroforestry: $p = 1.465 \quad 10^{-4}$) and exchangeable Mg (reforested vs agroforestry: $p = 1.618 \quad 10^{-5}$). Micronutrients also showed land-use–specific contrasts, including higher Mn in crop than reforested soils ($p = 2.209 \quad 10^{-3}$) and higher Cu in uncultivated than crop soils ($p = 1.710 \quad 10^{-3}$).

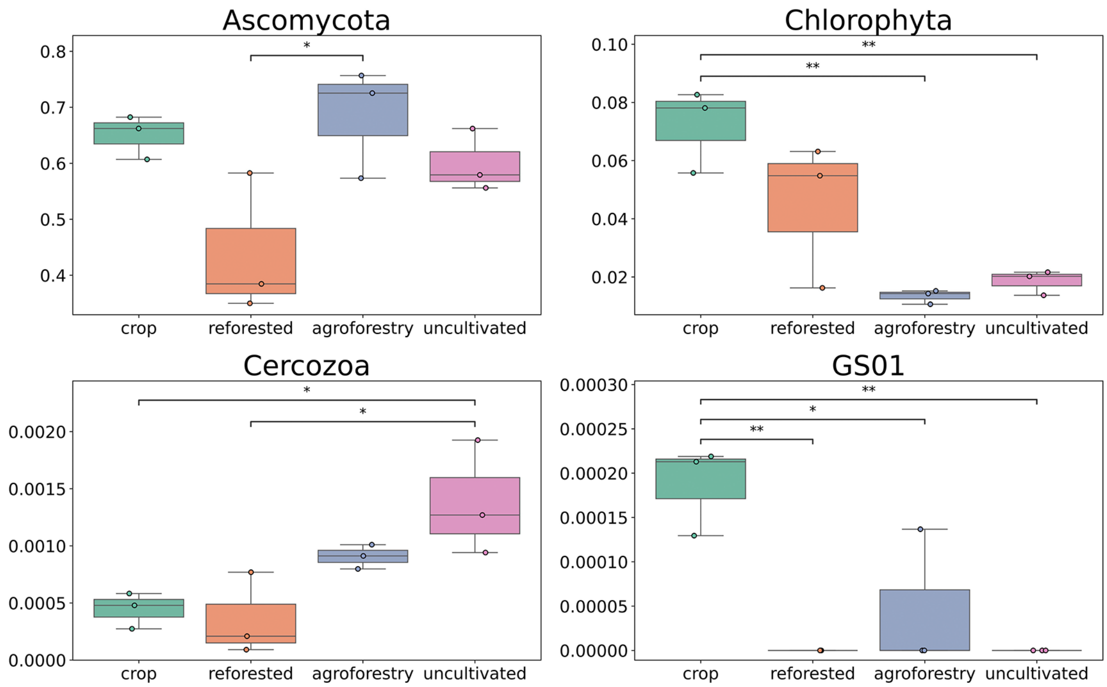

Figure 4. Boxplots of relative abundance of selected fungal genera across land-use types. Only taxa with statistically significant differences (assessed with Tukey HSD test) are shown. Differences are considered statistically significant at p-value <0.05 and are reported with the following legend: $10^{-2} < p < 5 \cdot 10^{-2}$ (*), $10^{-3} < p < 10^{-2}$ (**), $10^{-4} < p < 10^{-3}$ (***), $p < 10^{-4}$ (****).

Multivariate analysis of soil communities and abiotic parameters

In order to assess how abiotic parameters shape microbial community structure, we performed ordination analyses. Principal Component Analysis (PCA) of abiotic variables (Figure 8a) explained a substantial fraction of variance in the first two components (> 60) and showed clear separation of samples according to land use. Top abiotic contributors to the first principal component were Cation-Exchange Capacity, Total Nitrogen and Boron. These soil features are closely linked to the management of organic matter and organic carbon, indicating that land-use differences align with a gradient in nutrient status and related soil properties. Agronomic practices aimed at increasing organic inputs and enhancing specific nutrient cycles are crucial for positively shaping soil microbiomes.

Redundancy Analysis (RDA) of fungal and bacterial taxonomical profiles, where abiotic parameters were used as explanatory variables, confirmed significant associations between abiotic factors and soil community structure. The first 2 components of the RDA explained more than 30 of the variance, and both fungal and bacterial species were found to cluster in this 2-dimensional space, although this result is much more evident for bacteria. Vectors of the explanatory variables (CEC, B, exchangeable Ca and Mg, and OM) define the main constrained gradient. In the fungal RDA, the reforested samples project in the same direction as the vectors for CEC and exchangeable base cations (Ca, Mg), indicating that fungal community differences among land uses are associated with these fertility-related parameters. This interpretation is consistent with the abiotic boxplots, where reforested soils show higher CEC, exchangeable Ca, and exchangeable Mg, compared with the other land uses. In the bacterial RDA, the most prominent feature is the alignment of agroforestry points with exchangeable Ca and Mg vectors.

Functional analysis of bacterial communities

To investigate whether land-use type influences microbial functional potential, we used PICRUSt2 to predict bacterial functional profiles; in particular, we computed Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) terms, Enzyme Commission (EC) numbers, and MetaCyc metabolic pathways for each land-use. Principal component analysis of these profiles consistently separated the different land-uses (Figure 10a–c), although uncultivated and agroforestry EC profiles partially overlap in the 2-dimensional space defined by the first 2 components of the PCA. We also created stacked bar plots of the 15 most abundant functions (Figure 10d–f) for each functional profile across the different land-uses. These stacked plots represent about 10 of the total predicted functional profile, indicating that the vast majority of detected functions occur at lower relative abundances. Moreover, the most prevalent functions displayed

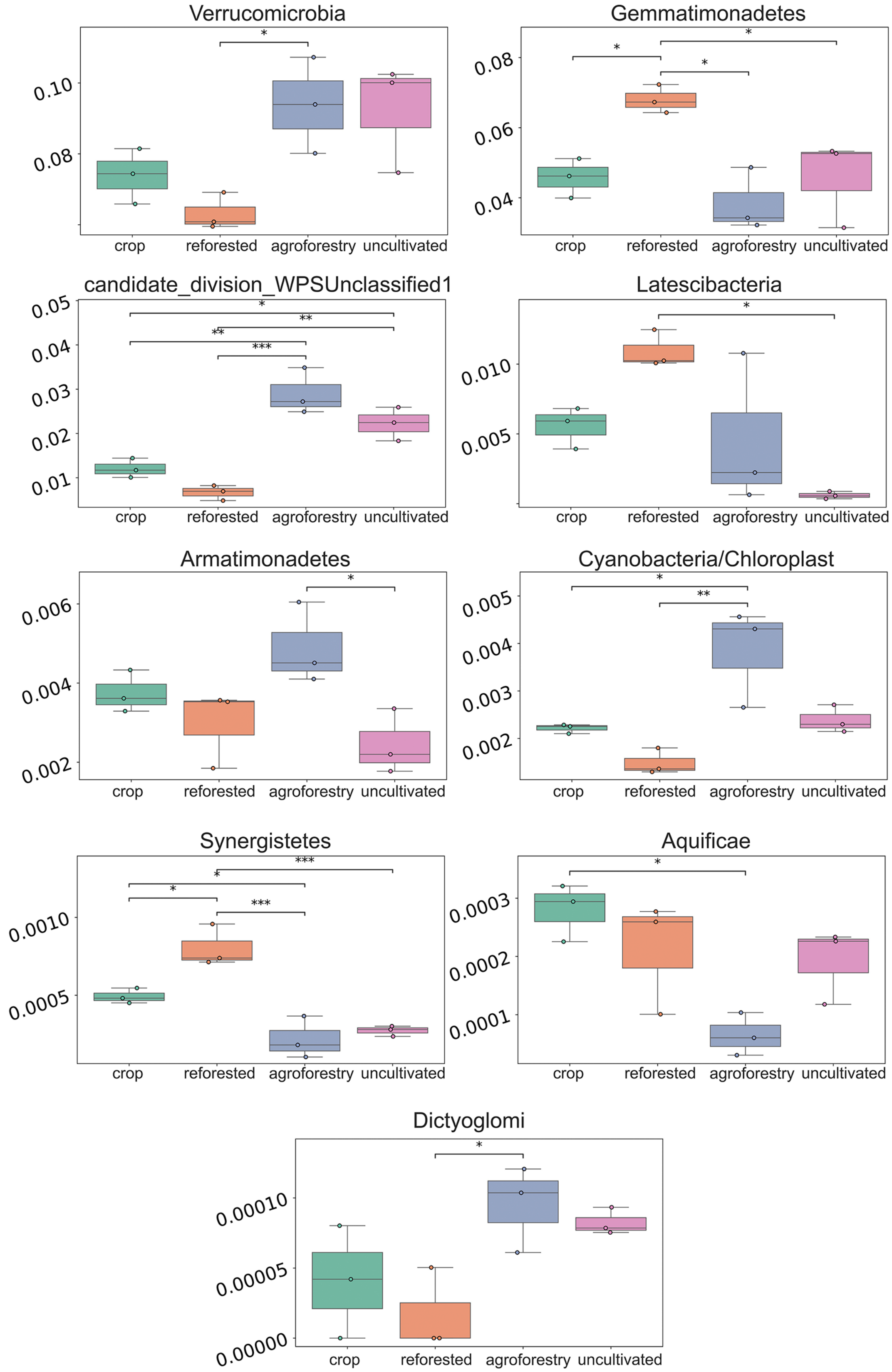

Figure 5. Boxplots of relative abundance of selected bacterial phyla across land-use types. Only taxa with statistically significant differences (assessed with Tukey HSD test) are shown. Differences are considered statistically significant at p-value <0.05 and are reported with the following legend: $10^{-2} < p < 5 \cdot 10^{-2}$ (*), $10^{-3} < p < 10^{-2}$ (**), $10^{-4} < p < 10^{-3}$ (***), $p < 10^{-4}$ (****).

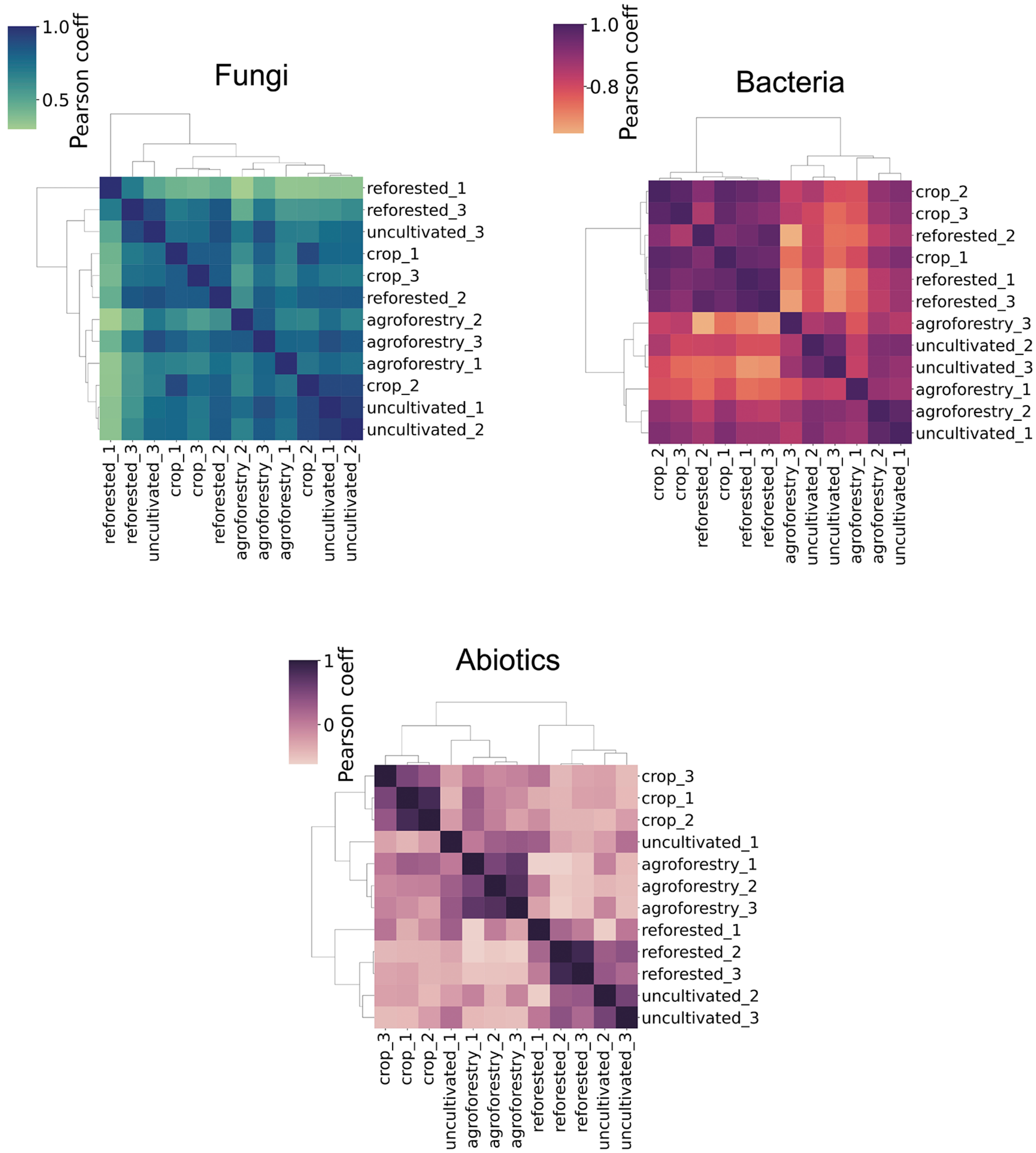

Figure 6. Heatmaps of pairwise Pearson correlations among samples based on fungal, bacterial, and abiotic profiles. Each panel displays hierarchical clustering of the 12 soil samples based on their correlations. Darker colors indicate stronger positive correlations.

broadly similar relative abundances across land-use types, suggesting that a common set of core metabolic capacities dominates bacterial communities regardless of management regime; land-use–associated differences emerge primarily in the multivariate structure of the predicted functional profiles and in the long tail of lower-abundance functions. In particular, at the pathway level, reforested soils showed a comparatively higher contribution of fatty-acid -oxidation II (plant peroxisome), whereas uncultivated soils displayed a slightly greater representation of pathways related to central metabolism and biosynthesis (e.g., pentose phosphate pathway and L-methionine biosynthesis IV). In contrast, cropland samples tended to show marginally higher contributions for adenosine nucleotides degradation II. At the KEGG Ortholog level, differences were similarly small, but uncultivated (and to a lesser extent agroforestry) samples showed slightly higher relative contributions of membrane-associated/transport and signaling-related functions (e.g., TonB/periplasmic proteins). At the EC level, the predicted enzyme composition also appeared largely conserved across land uses, with only minor shifts—for example, uncultivated and agroforestry samples tended to exhibit marginally higher contributions of core replication/translation-associated enzymes (e.g., DNA-directed polymerases/helicases).

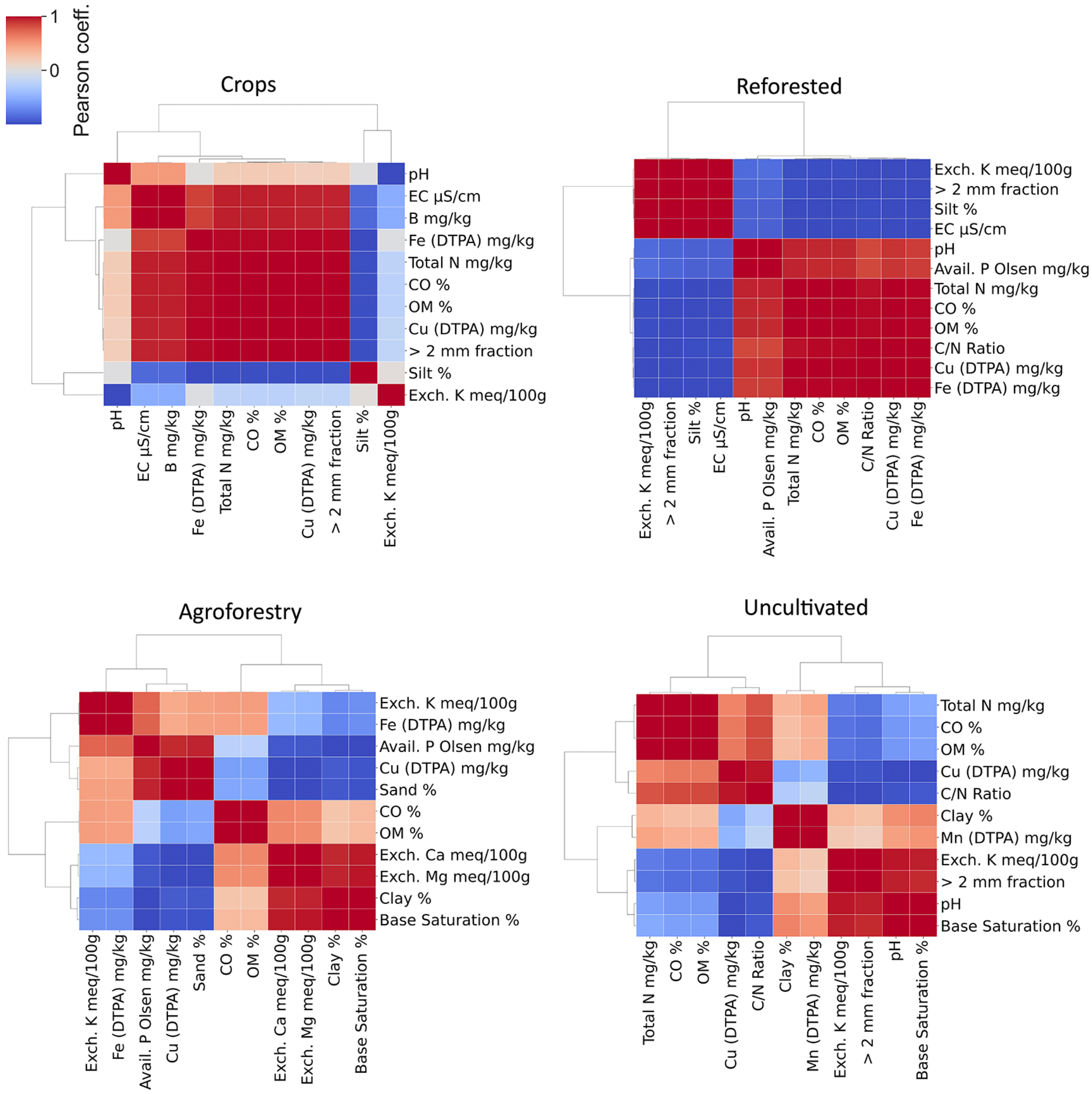

Figure 7. The figure displays a clustered heatmap of the top 20 strongest pairwise Pearson correlations by absolute value) among abiotic variables. Only variables involved in at least one of these top correlations were retained in the plot. The color scale represents the Pearson correlation coefficient.

Methods

Study area

The study is applied to an agricultural area on the southeastern edge of the city of Milan (Figure 11), which has been the focus of recent strategic transformation plans aimed at establishing an agroecological experimentation laboratory,[18] integrated into the Living Lab Milano Porta Verde system:[19] an open-air laboratory dedicated to the socio-environmental regeneration of the area. The study area covers approximately 100 ha, part of the South Milan Agricultural Park - Vettabbia Valley system, an ancient strategic axis linking city and countryside.[20] The area is predominantly composed of agricultural lands (arable crops, horticulture, permanent and fallow grasslands) and semi-natural areas (young reforestation). Since 2019, the implementation of 2 ha under productive agroforestry management - a multilayered agro-silvo-pastoral system inspired by regenerative practices - has begun (Figure 1). The study area belongs to an alluvial context (Po Plain) dominated by Cambisols (WRB Classification System[21]), and Gleysols strips along water courses. A loamy-sandy texture predominates: loose and relatively permeable soils, easily workable, typical of alluvial contexts. Soil samples were collected 16–17 April 2023 for DNA analysis while samples for soil abiotic analysis were collected 27 June 2023. Four land use types were investigated as treatments: crop field (crop), uncultivated area (uncultivated), wooded area (reforested), agroforestry system (agroforestry) (a summary of management history (current management, previous land use, products) is included in Table 1). Their selection was based on:

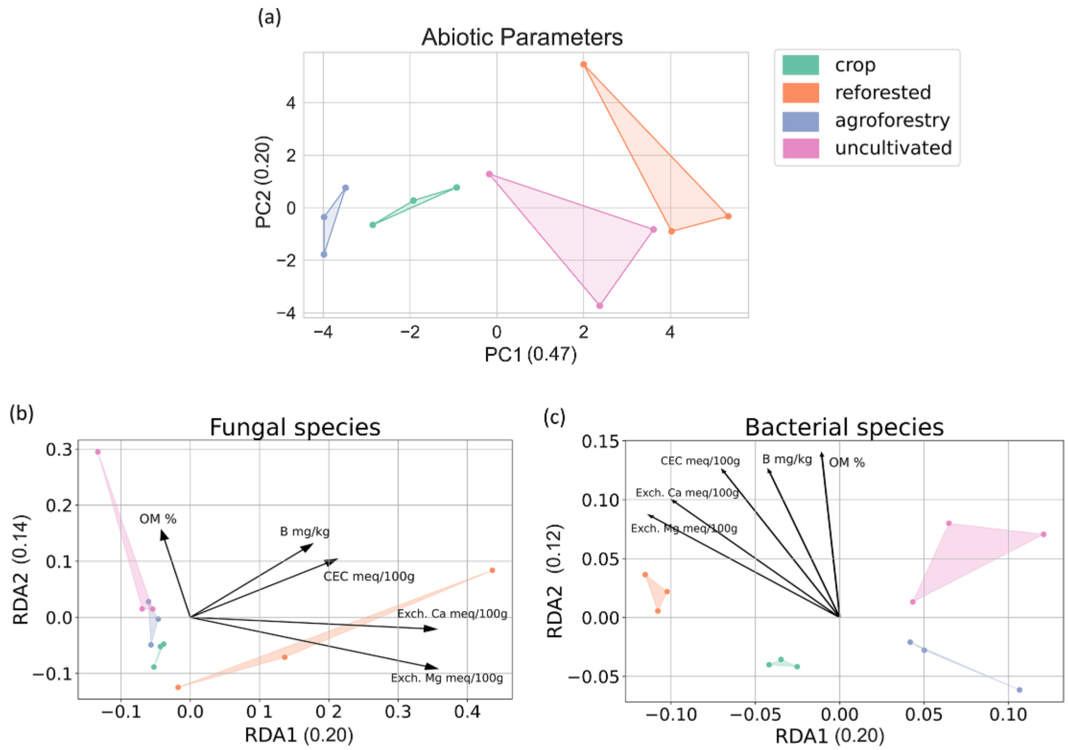

Figure 8. Ordination plots of abiotic and species data. (**a**) Principal Component Analysis (PCA) of abiotic parameters across samples. (**b**) Redundancy Analysis (RDA) of fungal species constrained by selected abiotic variables. (**c**) Redundancy Analysis (RDA) of bacterial species constrained by selected abiotic variables. Convex hulls connect replicates within each land use group to visually highlight the clusters. The values between parentheses report the variance explained by each component. The arrows represent vectors of the explanatory abiotics.

- the intention to compare the agroforestry system with different local land uses, yet still of agro-silvo-pastoral or semi-natural type;
- the opportunity to compare sites characterized by the same pedological substrate (same cartographic Unit[22]).

In each site, sampling was conducted at selected nodes of an 8 × 8 grid with a 20 m mesh (Figure 11): we sampled randomly in each grid, for a total of 3 soil sampling points for each site, avoiding marginal points, which often coincided with the edges of the site (roads, ditches, tree rows) and were more likely to be subject to disturbances that could influence the analytical results. Points that coincided with specific sources of heterogeneity within the site were also excluded, such as those located very close (< 2 m) to tree rows crossing the uncultivated area. The sampling was carried out by removing litter and picking soil slices through a spade at a depth of 0–30 cm with fixed thickness. Table 2 reports the soil parameters investigated. Abiotic parameters were measured at Labs & Technological Services AGQ, S.L. (https://agqlabs.com/en/).

Metagenomic sequencing library preparation, sequencing and analysis

The next generation sequencing experiments, which included quality control and the initial bioinformatics analysis, were executed by Genomix4life S.R.L. (Baronissi, Salerno, Italy). DNA quality and quantity were checked with the NanoDropOne spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the Qubit Fluorometer 4.0 (Invitrogen Co., Carlsbad, CA, USA). 16S amplification was performed using primers targeting the hypervariable V3 and V4 region of the 16S rRNA gene 314F(5 -CCTACGGGNGGCWGCAG-3) and 806R (5 -GACTACHVGGGTATCTAATCC-3),[23] while ITS amplification used the ITS3 – ITS4 primers (ITS3f 5 - GCATCGATGAAGAACGCAGC -3 ITS4r: 5 -TCCTCCGCTTATTGATATGC -3). PCR amplification and library preparation followed the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA). Briefly, a two-step PCR protocol was used: the first PCR amplified the V3–V4 region of the bacterial 16S rRNA gene using locus-specific primers containing Illumina overhang adapters, while the second PCR attached dual indices and sequencing adapters using the Nextera XT Index Kit (Illumina). Amplifications were performed in duplicate for each sample to reduce PCR bias and the resulting

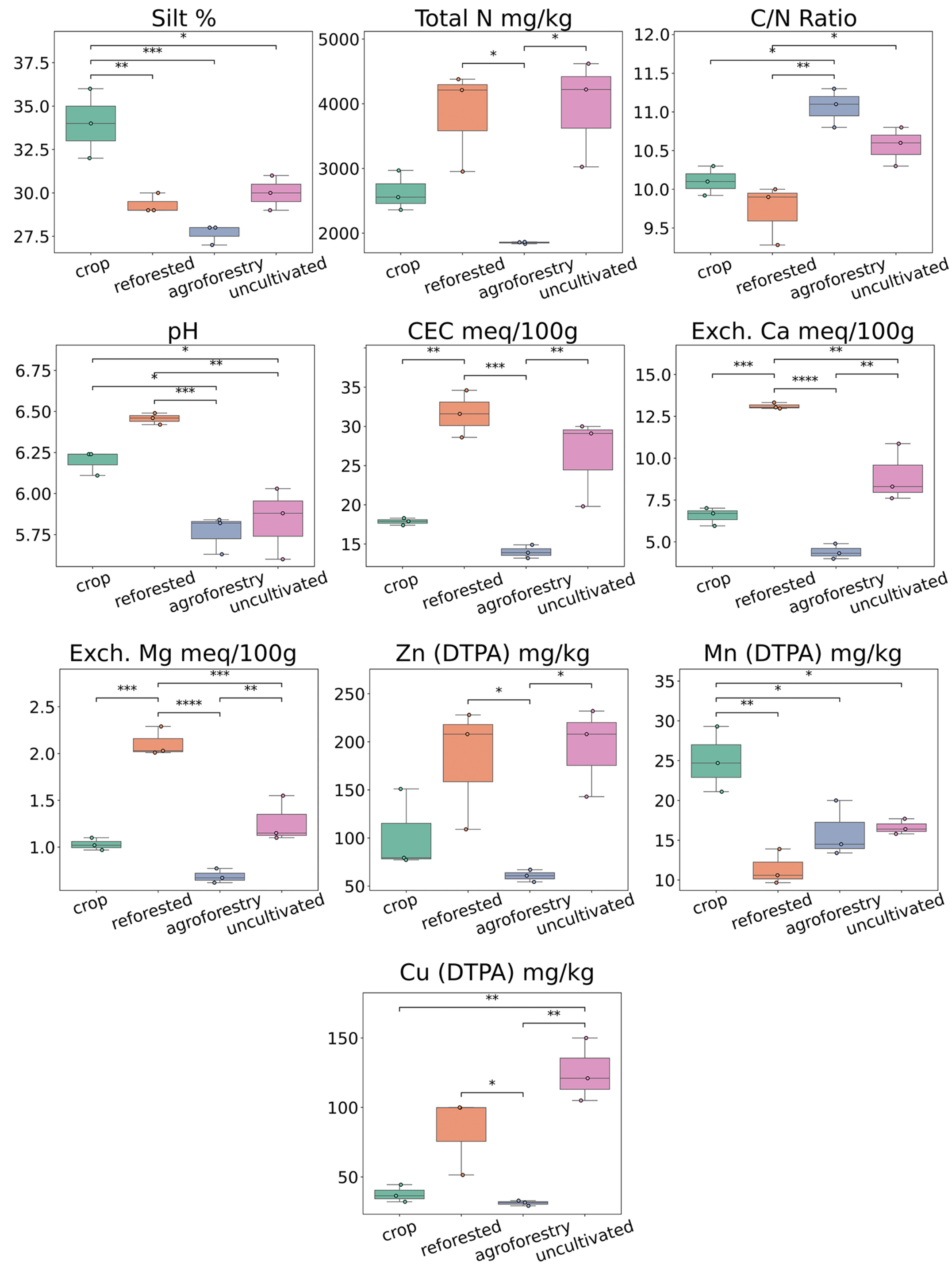

Figure 9. Boxplots of abiotic parameters across land-use types. Only parameters with statistically significant differences (assessed with Tukey HSD test) are shown. Differences are considered statistically significant at p-value <0.05 and are reported with the following legend: $10^{-2} < p < 5 \cdot 10^{-2}$ (*), $10^{-3} < p < 10^{-2}$ (**), $10^{-4} < p < 10^{-3}$ (***), $p < 10^{-4}$ (****). Abbreviation keys are reported in Table 2).

products were pooled prior to library preparation. A negative control, including all reagents used in amplification and library preparation but excluding DNA template, was included to monitor potential contamination. The negative control included during library preparation did not generate a detectable library profile. During the sequencing run no reads were obtained from the negative control sample. Therefore, no sequences from the negative control were present or required filtering in the

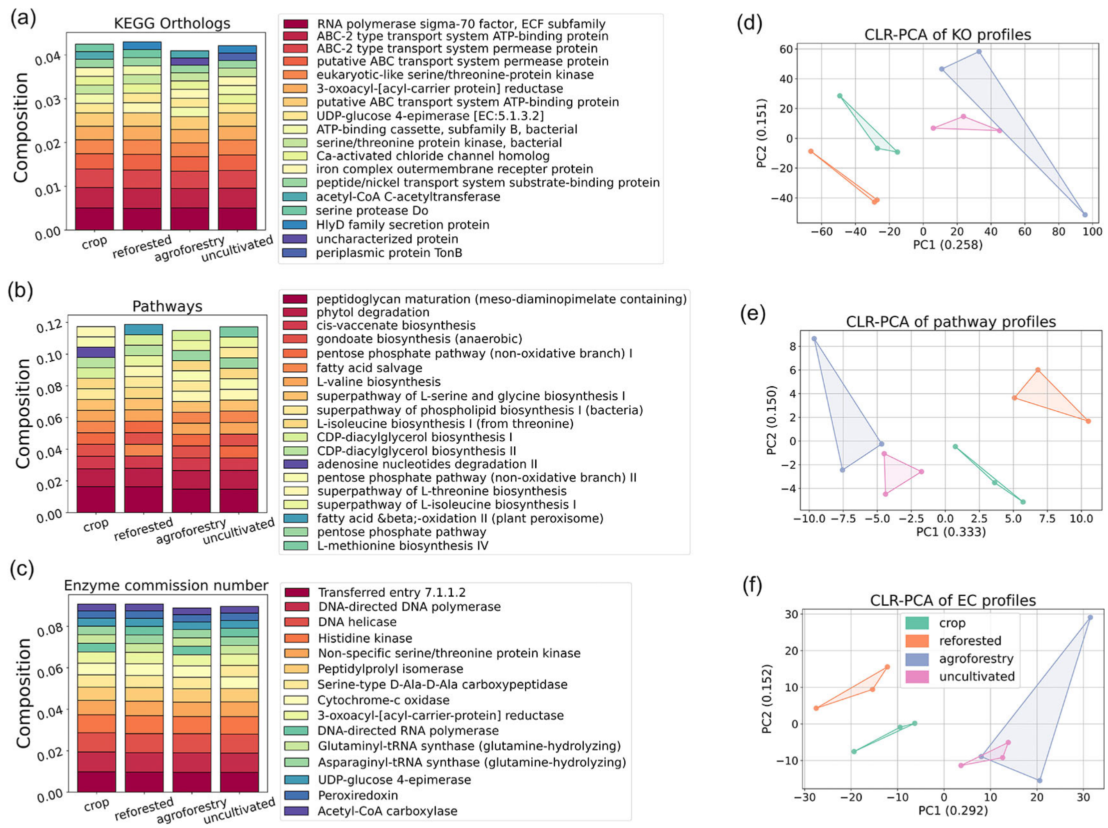

Figure 10. a) b) c) Stacked plots of the 15 most represented functions as expressed by KEGG, EC and MetaCyc metabolic pathways across land-use type. (d) (e) (f) Principal Component Analysis (PCA) of bacterial KEGG, EC and MetaCyc metabolic pathways functional profiles across land-use type, as obtained from PICRUSt2 analysis. Convex hulls connect replicates within each land use group to visually highlight the clusters. The values between parentheses report the variance explained by each component.

downstream analyses. Libraries were quantified using a Qubit fluorometer (Invitrogen Co., Carlsbad, CA, USA) and pooled to obtain an equimolar concentration of 4 nM for each index-tagged sample, with the addition of the PhiX Control Library. The pooled libraries underwent cluster generation and sequencing on the MiSeq platform (Illumina) using a 2X250 bp paired-end format. The amplicon sequencing data were analyzed using the 16S Metagenomics application available in the Illumina BaseSpace Sequence Hub. This pipeline performs read processing and taxonomic classification using a k-mer–based Naïve Bayesian classifier derived from the Ribosomal Database Project (RDP) algorithm.[24] The RefSeq RDP 16S v3 database was used for taxonomic assignment of 16S rRNA sequences. For the ITS datasets, the same analytical framework was used; however, taxonomic assignment was performed using the UNITE reference database, which is specifically curated for fungal ITS sequences.[25] Therefore, the pipeline itself was not fundamentally different, but the reference database used for taxonomic classification was adapted to account for the differences between bacterial 16S and fungal ITS markers. The UNITE Fungal ITS Database v7.2 is based on FASTA from https://doi.org/10.15156/BIO/587475 Includes singletons set as RefS (in dynamic files).

Statistical analysis

Raw taxa abundances were first processed by normalizing the values for each sample to relative abundances (ranging from 0 to 1). To visualise the composition of the most common taxa at genus-level, we generated heatmaps showing the relative abundance of the 25 most abundant fungal and bacterial genera across the 12 soil samples.

To quantitatively assess the composition of soil communities, we calculated two alpha-diversity metrics: observed richness (number of unique species) and Shannon diversity index.

These metrics were computed separately for fungal and bacterial taxa, based on species-level metagenomic assignments from each of the 12 soil samples.

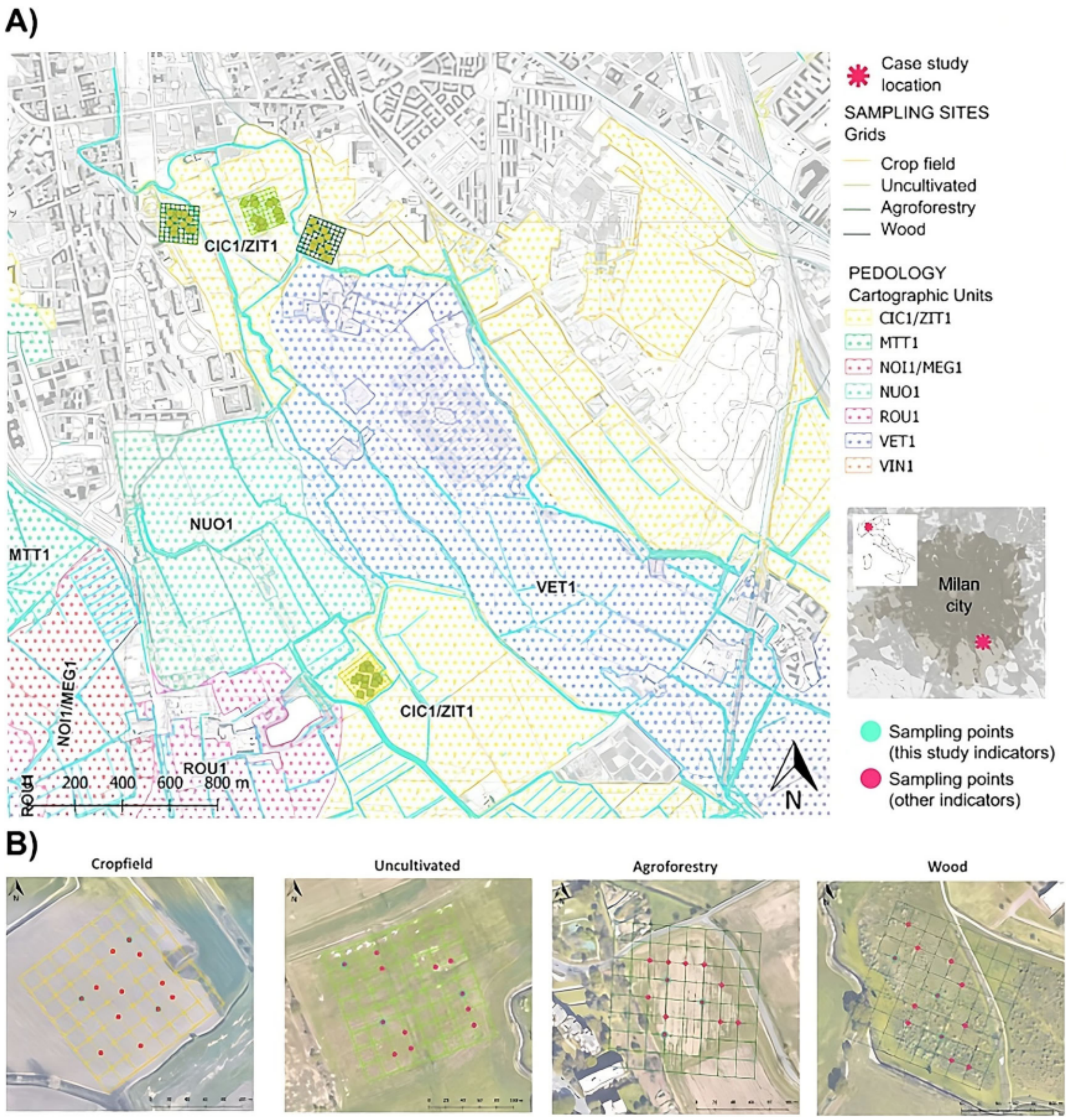

Figure 11. a) The study area location in Milan metropolitan area and sampling sites location in CIC1/ZIT1 Soil Cartographic Unit - Cambisols; b) sampling sites location and sampling protocol.

Richness was defined as the count of distinct taxa detected per sample. The Shannon diversity index was calculated as:

$$= \sum_{i=1}^{N} P_i \log P_i \tag{1}$$

where N is the number of observed taxa in a sample and p_i is the relative abundance of the ith taxon. To visualise the ecological indices, we created barplots of richness and Shannon s index across all 12 samples and boxplots across the different land-uses (crop, reforested, agroforestry, uncultivated), for both fungal and bacterial species. The ecological indices were computed using custom Python (v3.12.4) code, while the plots were created using the seaborn (v0.13.2) and matplotlib (v3.8.4) libraries.

To complement diversity and abundance-based metrics, we also assessed the overlap of microbial taxa across land-use types using UpSet plots.[26] These diagrams summarize taxa overlap among land uses: horizontal bars indicate the total taxa per land use, and vertical bars indicate the number of taxa in each intersection defined by the filled-dot combinations. Single-dot intersections represent land-use–unique taxa, whereas multi-dot intersections represent shared taxa. A taxon

Table 1. Sampling sites main features.

Land use type	Abbr.	Current management	Current management start date	Previous land use	Products
Annual crop field	Crop	Conservation agriculture	About 25 years ago	Conventional crop field	Corn (2–3 yrs) in rotation with winter wheat (1 yr) and soyabean (1 yr)
Wooded area	Reforested	Semi-natural	2018 (planting)	Uncultivated area	No products
Agroforestry system	Agroforestry	Regenerative agriculture	2019 (planting)	Conventional crop field	Fruits, berries, eggs
Uncultivated area	Uncultivated	Mowings	Stable for decades	Crop field	Hay

was considered present in a specific land-use type if it was found in one or more replicates. These plots visualize the intersections of species sets and were created using the UpSetPlot (v0.9.0) package in Python.

To assess how soil communities and environmental parameters vary across different land-use types, we performed comparative analyses focusing on diversity metrics, taxonomic composition, and abiotic factors. The 12 soil samples were grouped according to their land-use classification: crop, reforested, agroforestry, and uncultivated (3 replicates per group).

Richness and Shannon diversity values and relative abundances of fungal and bacterial species were compared across land-use types using boxplots. Statistical differences were assessed via one-way ANOVA followed by Tukey s Honest Significant Difference (HSD) post hoc test.[27] Significance was defined at $p < 0.05$. Given the large number of species detected, only those exhibiting statistically significant differences between land-use types were visualized. Assumptions for parametric testing were assessed by applying the Shapiro–Wilk test for normality and Levene s test for homogeneity of variance across land-use types. We note that, given the small number of biological replicates (n = 3 per group), these diagnostics must be interpreted cautiously. To evaluate compositional turnover among samples and between land-use types, we computed beta diversity using the Whittaker approach. Starting from species-level relative abundance tables, we converted abundances to presence/absence by considering a taxon as present when its relative abundance exceeded a fixed threshold ($p > p_0$). For each land-use type g, we computed the mean within-sample richness (alpha diversity) as

$$\bar{}_g = \frac{1}{n_g} \, {}_{k=1}^{n_g} \; {}_{gk} \tag{2}$$

where ${}_{gk}$ is the number of taxa present in replicate k of land use g and $n_g = 3$ is the number of replicates. We then computed the pooled richness (gamma diversity) within each land use as

$$\gamma_g = | \, {}_{k=1}^{n_g} S_{gk} | \tag{3}$$

where S_{gk} denotes the set of taxa present in replicate k. Whittaker beta diversity within each land use was defined as

$$\beta_g^{(\mathrm{W})} = \frac{\gamma_g}{\bar{}_g} \tag{4}$$

with larger values indicating greater turnover (i.e., lower overlap) among replicates within the same land-use type. To summarize turnover between land-use types g and h, we first defined the pooled sets $S_g = {}_k S_{gk}$ and $S_h = {}_k S_{hk}$, then computed ${}_g = |S_g|$, ${}_h = |S_h|$, and $\gamma_{gh} = |S_g \cup S_h|$. Pairwise beta diversity between land uses was calculated as

Table 2. Abiotic parameters: complete names and abbreviations used in figures.

Abiotic parameter	Abbreviation
Sand	Sand
Clay	Clay
Silt	Silt
Total Nitrogen mg/kg	Total N mg/kg
C/N Ratio	C/N Ratio
Organic Matter	OM
OC	OC
Available Phosphorus Olsen mg/kg	Avail. P Olsen mk/kg
pH	pH
Electrical Conductivity (S cm $^{-1}$, 20 °C)	EC S/cm
Cation-Exchange Capacity meq 100 g $^{-1}$	CEC meq 100 g $^{-1}$
Base Saturation	Base Saturation
Exchangeable Calcium meq/100 g	Exch. Ca meq/100 g
Exchangeable Potassium meq/100 g	Exch. K meq/100 g
Exchangeable Magnesium meq/100 g	Exch. Mg meq/100 g
Zinc (DTPA) mg/kg	Zn (DTPA) mg/kg
Manganese (DPTA) mg/kg	Mn (DTPA) mg/kg
Iron (DTPA) mg/kg	Fe (DTPA) mg/kg
Boron (mg/kg)	B mg/kg
Copper (DTPA) mg/kg	Cu (DTPA) mg/kg
> 2 mm fraction	> 2 mm fraction

$$\beta_{gh}^{(\mathrm{W})} = \frac{\gamma_{gh}}{\frac{{}_{g} + {}_{h}}{2}} \tag{5}$$

where higher values indicate greater compositional turnover between pooled land-use communities.

Abiotic soil parameters were compared across land-use types using the same procedure. Electrical conductivity (EC) and boron (B) included left-censored observations (reported as $<0.7\ \mu$S/cm and < 0.5 mg/kg, respectively). These values were set at one-half the respective detection limit for the analysis. Again, we represented only the parameters where significant differences were found. All visualizations and tests were implemented using custom Python scripts with seaborn (v0.13.2), scipy (1.12.0), and statannotations (v0.7.1).

First, we assessed samples similarity computing pairwise Pearson correlation matrices among the 12 soil samples using three different variable sets: bacterial species abundances, fungal species abundances, and abiotic parameters. These correlation matrices were visualized as heatmaps paired with hierarchical clustering.

Moreover, to identify and visualize the strongest associations among abiotic soil parameters, we computed pairwise Pearson correlation coefficients using the full set of measured variables for each one of the four land-uses types. To reduce visual clutter, we implemented a filtering strategy to retain only the most strongly correlated variable pairs.

Specifically, we first computed the Pearson correlation matrix across all abiotic parameters, using the absolute values of the coefficients. We then ranked all unique variable pairs by the magnitude of their correlation coefficients and selected the top 20 strongest associations (excluding self-correlations). The set of variables involved in these top correlations was extracted, and a reduced correlation matrix was constructed using only this subset. This filtered matrix was visualized as a heatmap with hierarchical clustering.

This analysis was carried out using custom Python (v3.12.4) scripts, while the heatmaps were created using seaborn (v0.13.2) package.

Before performing Multivariate Analysis, all variables were scaled to zero mean and unit variance using the StandardScaler function from the sklearn (v1.4.2) package. To reduce the dimensionality of the abiotic dataset and identify environmental gradients, we applied Principal Component Analysis (PCA) to the standardized abiotic parameters using the PCA function from sklearn (v1.4.2). The first two principal components were used to visualize the samples in reduced space, with samples colored by land-use type. Convex hulls were drawn around groups to highlight clustering by land use using the ConvexHulls function from scipy (v1.12.0).

To investigate the relationships between soil community composition and abiotic variables, we performed Redundancy Analysis (RDA) using species-level abundance data for fungi and bacteria as response matrices and a selected subset of abiotic parameters as explanatory variables. Because the number of abiotic variables exceeded the number of samples, we retained as explanatory variables only a selection of abiotic parameters. We first selected candidate predictors based on their contribution to the main abiotic gradient captured by PCA, retaining variables with an absolute loading >0.25 on the first principal component (PC1). To limit redundancy among predictors, we then assessed pairwise correlations among the selected abiotic variables and removed highly collinear variables using a correlation threshold of $|r| = 0.9$. The final set of abiotic predictors used in the RDA was: Cation Exchange Capacity (CEC), Boron (mg/kg), Exchangeable Calcium, Organic Matter, and Exchangeable Magnesium (meq/100 g).

RDA was performed using the rda function from the scikit-bio (v0.6.3) package. We visualised the results with a scatter plot in the 2-dimensional space defined by the main components of the RDA using matplotlib (v3.8.4).

Raw paired-end FASTQ files were reprocessed in QIIME 2[28] (qiime2-amplicon-2025.7 version) to generate a PICRUSt2-compatible amplicon sequence variant (ASV) table together with the corresponding representative-sequence FASTA. Denoising, was performed with q2-dada2 (v2024.10.0) using denoise-paired. The resulting amplicon sequence variant (ASV) feature table and representative sequences were used to predict functional profiles with PICRUSt2[29] (v2.4.1). In particular, we predicted Kyoto Encyclopedia of Genes and Genomes (KEGG).

Orthology (KO) terms, Enzyme Commission (EC) numbers, and MetaCyc metabolic pathways for each sample. These functional profiles were aggregated by land-use type; for each functional profile, we generated stacked bar plots showing the relative abundance of the 15 most prevalent functions across land uses. In addition, centered log-ratio (CLR) transformation was applied to each table (after compositional normalization), and principal component analysis (PCA) was performed to visualise patterns of functional differentiation among the four land-use categories. These exploratory analyses were conducted separately for KO, EC, and pathway profiles to compare the degree and structure of functional variation associated with land use. We note that the ASV table resulting from this reprocessing step may differ from the feature table produced by the Illumina pipeline. This approach was adopted because representative sequences were not available from the Illumina pipeline outputs, preventing direct use of that table in PICRUSt2. We note that functional profiles obtained with PICRUSt2 are inferred by reference-genome mapping and phylogenetic placement rather than by direct measurement of functional genes; for this reason, these results should be interpreted cautiously as predictive, also in light of the fact that PICRUSt2 used the QIIME 2-derived ASV table, which may differ from the Illumina pipeline feature table used for other statistical analyses.

Discussion

In the present study we investigated, in typical northern Italy peri-urban agricultural landscapes, how land use is able to influence soil microbial communities, abiotic soil parameters, and functional potentials. This kind of study is important to identify the best agroecological practice in peri-urban area able to preserve a healthy soil. In fact, it is known that the expanding urbanization affects biodiversity and agrobiodiversity, for example in a recent paper was discussed the complex relationship between these aspects also focusing to urban and peri-urban land for the best use in terms of biodiversity.[30] Overall, our study highlights the need for composite soil health indicators that integrate microbial, chemical, and physical parameters, as recommended by international frameworks such as the FAO s Global Soil Partnership and the EU Soil Strategy for 2030. Despite the robustness of our findings, the limited number of replicates (n=3 per land-use type) constrains the statistical power of some comparisons, particularly for fungal communities where natural variability can be high.

Agroecological practices, included within the framework of Nature-Based Farming Solutions (e.g., landscape features, agroforestry), have the potential to activate and implement processes of diversification, ecological regeneration and multifunctional re-functionalization of rural areas.[31] In particular, agroforestry management, understood broadly as the management of productive and non-productive tree and shrub components, both within fields and along boundaries, such as hedgerows, tree lines, buffer strips, and small wooded patches,[32] is distinctive in its ability to interact with different levels of agroecosystem organization.[33]

Our results collectively suggest that agroforestry systems compared to uncultivated, reforested and crop land use, can play a pivotal role in restoring soil health and enhancing EC, reinforcing their importance as nature-based solutions for sustainable land management. Across the four peri-urban land uses, we observed consistent land-use–associated differences in both abiotic soil properties and microbial community structure, supporting the view that land management is a key determinant of soil health indicators in this landscape. In fact, the observed differences in fungal and bacterial community composition across land-use types confirm that land management has a measurable impact on soil microbiota. Agroforestry soils were characterized by the highest number of unique taxa, whereas reforested soils showed the lowest richness and diversity, aligning with previous studies reporting that microbial communities in early-stage reforested soils are typically less diverse and more compositionally simplified than in later successional stages.[34–36]

In a recent meta-analysis where was investigated the effects of agroforestry on ES provision in Europe,[37] was reported that, compared to conventional land uses such as pastures, arable crops, or forests, agroforestry supports higher levels of biodiversity and ecosystem goods and services. In this connection, the study by Beillouin *et al*.[38] confirmed the capacity of agroforestry to significantly influence multiple ES, including biodiversity, production, water regulation and quality, pest and disease control, and soil quality.

Furthermore, we found that fungal communities had a stronger variation in dominant genera across land uses in comparison to bacterial communities. The recurrent dominance of genera such as Fusarium and Trebouxia in the fungal dataset, together with the prevalence of Acidobacteria and Gemmatimonadetes in the bacterial dataset, suggests that these groups may respond to management-driven gradients in soil resources and disturbance. The high abundance of Mortierella is of particular interest, as some studies have indicated that Mortierella is associated with disease-suppressive soils, which goes with the accentuated complexity of undisturbed and diversified systems such as Silvoarable Agroforestry Systems (SAF).[39–41] These symbioses are crucial for the nutrient cycle in forests and can play an important role in ecological succession processes. Considering the richness, we found the highest levels for crops and agroforestry for both bacteria and fungi. All together these analysis pointed out the deep impact of land use on the soil microbiome.

We then investigated how abiotic parameters such as for example cation-exchange capacity (CEC), total nitrogen, and boron, affect microbial communities. Multivariate analyses (PCA and RDA) revealed that abiotic parameters were key determinants of microbial community structure. This finding supports the hypothesis that soil fertility parameters not only reflect the chemical status of soils but also shape microbial niches and resource availability. The different correlation structures among abiotic parameters across land uses may reflect differences in how soil properties vary under distinct management contexts. Crop and reforested soils showed tighter clustering among fertility-related variables (e.g., organic matter/organic carbon and total nitrogen), suggesting that a smaller set of coupled drivers may dominate these systems. In contrast, agroforestry and uncultivated soils exhibited more heterogeneous clustering patterns of abiotic variables, suggesting a higher degree of spatial complexity and potentially greater resilience to disturbances. Similar work has linked increased soil diversity and network complexity to enhanced ecosystem multifunctionality and resilience,[42] although interpretation of correlation structure as "stability" should be treated cautiously and validated with time-series or perturbation data. Functional predictions using PICRUSt2 indicated that while a core set of metabolic functions was shared across all land uses, agroforestry soils exhibited a broader functional repertoire. This functional diversification is consistent with the idea that agroforestry systems enhance ecological functions beyond productivity by providing microhabitats and diversified root exudates, as well as creating microhabitats that support a wider range of microbial guilds, thereby improving nutrient cycling and soil multifunctionality.[43,44] Our results provide evidence that management practices can influence not only who is present in the soil microbiome but also what functions they are capable of performing. This is aligned with the growing body of literature advocating agroforestry as a key tool in the ecological refunctionalization of rural and peri-urban landscapes.[45,46] By promoting biodiversity, improving soil structure, and enhancing carbon sequestration, agroforestry systems directly contribute to several Sustainable Development Goals (SDGs), including Zero Hunger (SDG 2), Climate Action (SDG 13), and Life on Land (SDG 15). The ES provided by agroforestry-managed soils include: soil conservation; soil health; carbon sequestration; nutrient cycling and enrichment; and the sequestration, transformation, and detoxification of chemicals into non-toxic forms. These benefits are difficult to measure independently, as they are interconnected. For example, increased carbon sequestration enhances soil nutrient status by improving cation exchange capacity, soil stability, and soil quality, while also reducing soil erosion.[47]

Conclusion

Our findings show that land-use type has a significant impact on soil microbial communities, with agroforestry emerging as a promising agroecological practice. By increasing microbial diversity, supporting unique taxa, and broadening functional profiles, agroforestry can enhance ecosystem services, including nutrient cycling, carbon sequestration, and resilience to environmental stress. These findings support the incorporation of agroforestry into peri-urban agricultural landscapes as a strategy to balance food production with biodiversity conservation and climate regulation.

From a policy standpoint, this study emphasizes the importance of soil health indicators that incorporate biological aspects, as recommended by the FAO and the EU Soil Strategy 2030. Agroecological methods should be prioritized in land management frameworks to enhance soil ecosystem services and global health.

Ethics and consent

Ethical approval and consent were not required.

Data availability

UNIMI Dataverse. Replication Data for: Soil Health and Microbial Diversity Across Land-Use Types: Evidence for Agroecological Management in Peri-Urban Areas. https://dataverse.unimi.it/dataset.xhtml?persistentId=doi:10.13130/RD_UNIMI/ZGZY4Q[48]

This project contains the following underlying data:

16 s Genus Level Aggregate Counts 2.csv: ITS OTU table at genus level ITS Species Level Aggregate Counts.csv: ITS OTU table at species level 16 s Species Level Aggregate Counts.csv: 16 s OTU table at species level ITS Genus Level Aggregate Counts.csv: 16 s OTU table at genus level Samples.xlsx: metadata specifying the type of soil for each sample.

Data is available under the terms of the CC BY 4.0 licence.

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Publisher Full Text

45. Castle SE, Miller DC, Merten N, *et al.*: **Evidence for the impacts of agroforestry on ecosystem services and human well-being in high-income countries: a systematic map.** *Environ Evid.* 2022; **11**(1): 10.
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46. Jose S: **Agroforestry for ecosystem services and environmental benefits: an overview.** *Agroforest Syst.* 2009; **76**(1): 1–10.
Publisher Full Text

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Publisher Full Text

48. Replication Data For: Soil Health and Microbial Diversity Across Land-Use Types: Evidence for Agroecological Management in Peri-Urban Areas.
Reference Source

Open Peer Review

Current Peer Review Status: ✔ ?

Version 1

Reviewer Report 25 February 2026

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Carolyn Cornell
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In the manuscript "Soil health and microbial diversity across land-use types: Evidence of agroecological management in peri-urban areas", the authors aimed to determine how land-use type, and therefore the associated management, impacted soil health using "different indicators" across peri-urban landscapes typical to northern Italy, including a crop field, uncultivated area, reforested land, and agroforestry site. Three soil samples were collected per site during a single collection to examine abiotic soil properties, and bacterial and fungal communities using amplicon sequencing. Based on the study, the authors concluded that land-use type significantly impacts soil microbial communities and that agroforestry may help increase or preserve diversity in agricultural land. The authors also said that the study provides mechanistic insight into how land management shapes biodiversity and informs strategies for improving overall soil health.

While the authors conducted an interesting study, it was difficult to assess the robustness due to insufficient detail in the materials and methods, especially regarding sequencing depth and data processing. The sample size also limits the study's power. There were also several conclusions drawn on the impact of abiotic properties and soil microbial communities, with few actual correlations done between the two datasets. The number of figures could also be reduced, with many showing very similar information. Additionally, the introduction set the manuscript up to focus on soil health, yet the results did not necessarily follow that theme. Pointing out more on the differences between sites that would support that a certain land-use type may be "healthier" because you found, for example, more beneficial soil taxa or increased carbon/OM compared to more managed lands, would help better frame the story.

Comments by section:
Abstract

- P. 1: Suggest changing "—such as" in the methods part of the abstract to "including" since that is the full list of land-use types used, not just several examples.

- P. 2: Evenness is mentioned in the results section of the abstract, while the conclusion drawn makes sense, it would be better to actually calculate evenness as one of your alpha-diversity metrics.
- P. 2: In the results section of the abstract, while it was found that the abiotic factors pH, nitrogen, and CEC differed with land-use type, no correlations were made to the microbial communities. It was almost done with the RDA, but the vectors showing the abiotic factors were not included in the plot.
- P. 2: I would change the word "mechanistic" as the study did not show a mechanism but an association or correlation.
- P. 2: Suggest changing "resilience" to "potential resilience" as this was not examined in this study.

Introduction:

- P. 4: The last sentence of the first paragraph in the introduction is too long, making it hard to follow. Break it up into two sentences.
- P. 4: In this sentence, "When considering the subset of peri-urban agricultural systems, these processes are further exacerbated." I suggest moving the peri-urban fringe definition to the beginning of the paragraph, closer to where this term is introduced.
- P. 4: Need to define "soil health" as the definition is not always consistent. It also quickly moves on to the importance of soil health as a whole, but it would help to mention its particular relevance to peri-urban areas, which was the topic of the previous paragraph.
- P. 4: "According to 16,.." Should be changed to "author's name and colleagues" followed by the citation.
- P. 4: "contest" needs to be changed to "context" in the last paragraph of the introduction.
- P. 4: The description of the study could be improved in the last paragraph. These "different indicators" need to be explained more, especially if the authors are suggesting they can be used in other studies as indicators of soil health.

Methods:

- The overall organization of the data analysis of the methods is a bit confusing. It makes more sense to break your methods into sequence analysis and statistical analysis, rather than a section for each test.
- I do not understand the reasoning behind using the "Illumina 16S Metagenomics pipeline" in one section, then, in another section, saying the data was processed again using "QIIME 2." Only one should be used as they will produce different OTU/ASV tables. No link or reference to the Illumina pipeline was provided, so I was unable to see how the methods compared.
- Why not perform beta-diversity analysis? This would be a better comparison of diversity differences across sites as it takes into account the community structure/taxa at each site.
- The methods need to be checked for tense usage. Often using the present tense when needing to use the past tense when describing tests and sampling.
- P. 11: Add a sentence making it clear that the management history is presented in Table 1. I was looking for it in the text because it wasn't clear that I could find it in the table.
- P. 11: Confused on why four quadrants were established and one sampling point was established in each, but only 3 samples were taken.
- P. 11: When were the samples collected? Include the year and the month at least. This is important since it is said some land uses were established in 2019, so it is helpful to know how long since the establishment of the site where samples were taken.
- P. 14: There is no information given on how soil parameters were measured. Either cite other papers that have this information, or, if they were sent somewhere, include the

location and the link so readers can find the methods there.
- P. 14: Change “DNA quality control checks” to “DNA quality and quantity were checked” since the Nanodrop is generally for quality and Qubit for quantity.
- P. 14: Add the specific 16S primers (e.g., 314F and 806R).
- P. 14: Add a link or citation where the specific PCR protocol can be found. I also recommended including a short summary, such as whether it was done in replicates, in two steps, etc.
- P. 14: Add a link or citation for the “16S Metagenomic pipeline”. Was there a different pipeline for the ITS, or was it adapted at all for differences between ITS and 16S reads?
- P. 14: The database versions used are dated. Ideally, should use more recent releases of both databases.
- P. 14: It is mentioned that negative controls were included. Were any sequences found in the negative controls, and if so, include how they were accounted for in the data.
- P. 14: While using relative abundance normalization for the data is fine, information should be included on the average sequencing depth for each dataset, and if there was a cutoff on the minimum number of sequences considered usable for a sample. Given soil diversity, sequencing depth can be important.
- P. 15: It should be mentioned that the metrics used are both alpha-diversity metrics.
- P. 15: Does the “16S Metagenomic pipeline” produce OTUs or ASVs? It is generally just referred to as species-level assignments in the methods.
- P. 16: For the UpSet plots, was a taxa considered to be present in both land uses if it was present in just one of the replicates or more?
- P. 16: Was the data checked for normality and homogeneity of variance before determining that a parametric test (ANOVA) was the right choice? If so, include that in the methods.
- P. 18: Was there a correlation coefficient cutoff value to be considered “strongly” correlated?
- P. 19: Did you consider the correlation between the abiotic factors when doing your RDA? Also, need to add the vectors of the abiotic factors to the RDA so readers can see which factors were associated with each land-use type.
- P. 19: Please add a statement about the use of PICRUSt, such as, “however, these predictions are based on reference genomes and do not represent direct measurements of functional genes. Therefore, results should be interpreted cautiously,” considering its limitations.

Results

- For the results, my main comments would be to reduce the number of figures to make the message more concise. For example, the alpha-diversity metrics are shown by field with box plots (Fig. 2), then by replicate (Fig. 3). Unless a main point is within site variation, then Figure 3 is not needed. The same for showing heatmaps of the top 20 correlations between abiotic factors, then showing heatmaps with all variables.
- In the results text, there needs to be a stronger focus on the differences between land uses to convey that converting to certain land-use types may improve “soil health,” as seen by increases in diversity, richness, OM, etc., to match the story set out in the introduction.
- P. 4: When saying “significantly” in terms of results, it can be helpful to put the p-value in parentheses after it, especially since the method is after the results, where significance is defined. I recommended this for the whole results section.
- P. 4: “certain genera significantly more abundant in specific samples and in specific land-use types”, which ones especially on a land-use base comparisons?
- P. 4: The genera “Mortierella, Fusarium, and Trebouxia” need to be italicized.

- P. 4: I do not think this conclusion is supported because no differences in relative abundance were explained between sites in the text and no differences in organic inputs have been presented yet, “From an agroecological point of view, this results clearly demonstrate the role of agronomical management that enhance soil organic inputs (e.g., manure, green mulch, compost application, agroforestry litterfall) in sustaining fungal diversity.”
- P. 4-5: For the differences in alpha-diversity metrics, state if they were significant, not just noticeable. The statement about replicating following it makes it sound like you would have found significant values if you had more replicates, which is not necessarily true. Consider rewording or removing that statement.
- P. 5: Fungi is a kingdom, not a domain.
- P. 5-6: “Such diversification contributes to ecosystem resilience and multifunctionality, which are essential for effective biological pest control.” A statement like this is better for the discussion, so you can show that other studies have shown this. Otherwise, it appears to be an unsupported claim in the results.
- P. 6: Again, explain the differences in what taxa you saw that were significantly different between land uses. The figure captions also says genera instead of phyla. It would also make more sense to talk about the differences in phyla (Fig. 4/5) before talking about the genera (Fig. 1).
- P. 6: What is meant by “bacterial and fungal profiles”? Do you mean the special-level abundance tables? This information on what went into the tables needs to be included in the Figure 6 caption. Based on the coefficient gradient for your heatmap, I also cannot tell if it includes negative correlations. I number at the bottom of the gradient needs to be included.
- P. 7: The conclusions on soil stability based on abiotic variable correlation need to be moved to the discussion, so examples that support this can be cited. It would also be a good place to discuss how the management specific to the crop and reforested sites can lead to shared drivers of these properties.
- P. 8: Redundant/highly correlated variables can impact a PCA and RDA. It would be useful to include details in the figures' captions and in the methods section on how this was handled, since strong correlations between factors were shown in Figure 7.
- P. 8: Discussing the differences in abiotic factors between land uses (Fig. 13), which help show what factors shift under the different management and may help support the PCA results below, showing that there are differences in soil factors by land use.
- P. 8: I believe I mentioned this before, but all the abiotic vectors are in the RDA, so you can discuss which factors were associated with different land uses.
- P. 9: For the functional differences, again explain the important differences between land use. Did you see a loss or gain of a certain function with land use?

Discussion

- Overall, the discussion needs to focus more on how the results presented were caused by differences in land use and how the management practices associated with those land uses may have shaped them. This then needs to be brought back to the idea of soil health. Currently, it is not focusing enough on the differences between land uses, and again, it just says there were broad differences between land uses.

Altogether, the authors present an interesting study on how land use type may influence soil microbial diversity and overall soil health in a peri-urban landscape. However, significant revisions are needed to improve the clarity, robustness, and alignment between the stated objectives and the results. The authors should provide more detail in the Materials and Methods section on sequencing data and management practices for each land-use type. The number of figures should be reduced, and the order of figures reconsidered to group similar topics (e.g., keep all relative abundance differences together). The results and discussion should focus more on differences between land-use types, mentioning specific differences and how management practices influence soil properties and microbial communities to support claims about soil health. Lastly, moderate claims about mechanisms and resilience to better align with the data presented.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Soil microbial ecology, nitrogen cycling, and agricultural systems

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 25 Mar 2026

Caterina La Porta

REVIEWER #2
Reviewer: In the manuscript “Soil health and microbial diversity across land-use types: Evidence of agroecological management in peri-urban areas”, the authors aimed to determine how land-use type, and therefore the associated management, impacted soil health using “different indicators” across peri-urban landscapes typical to northern Italy, including a crop field, uncultivated area, reforested land, and agroforestry site. Three soil samples were collected per site during a single collection to examine abiotic soil properties, and bacterial and fungal communities using amplicon sequencing. Based on the study, the authors concluded that land-use type significantly impacts soil microbial communities and

that agroforestry may help increase or preserve diversity in agricultural land. The authors also said that the study provides mechanistic insight into how land management shapes biodiversity and informs strategies for improving overall soil health.

While the authors conducted an interesting study, it was difficult to assess the robustness due to insufficient detail in the materials and methods, especially regarding sequencing depth and data processing. The sample size also limits the study's power. There were also several conclusions drawn on the impact of abiotic properties and soil microbial communities, with few actual correlations done between the two datasets. The number of figures could also be reduced, with many showing very similar information. Additionally, the introduction set the manuscript up to focus on soil health, yet the results did not necessarily follow that theme. Pointing out more on the differences between sites that would support that a certain land-use type may be "healthier" because you found, for example, more beneficial soil taxa or increased carbon/OM compared to more managed lands, would help better frame the story.
Answer: We thank the review for her comments. We have now revised the manuscript accordingly.
Comments by section:

Reviewer: Abstract P. 1: Suggest changing "—such as" in the methods part of the abstract to "including" since that is the full list of land-use types used, not just several examples.
Answer: Done

----- Reviewer: Abstract P. 2: Evenness is mentioned in the results section of the abstract, while the conclusion drawn makes sense, it would be better to actually calculate evenness as one of your alpha-diversity metrics.
Answer: We changed accordingly

----- **Reviewer:** Abstract P. 2: In the results section of the abstract, while it was found that the abiotic factors pH, nitrogen, and CEC differed with land-use type, no correlations were made to the microbial communities. It was almost done with the RDA, but the vectors showing the abiotic factors were not included in the plot.
Answer: We agree with the referee. We revised the RDA analysis and the plots (Fig. 6) updated to include the abiotic vectors, so the associations between land-use clustering and the main abiotic gradients are now explicit.

--------- Reviewer: Abstract P. 2: I would change the word "mechanistic" as the study did not show a mechanism but an association or correlation.
Answer: Changed accordingly.

---------- **Reviewer** Abstract P. 2: Suggest changing "resilience" to "potential resilience" as this was not examined in this study.
Answer: Done

--------------- **Reviewer:** Introduction**:** P. 4: The last sentence of the first paragraph in the introduction is too long, making it hard to follow. Break it up into two sentences.

Answer: Done

---------------- **Reviewer**: Introduction P. 4: In this sentence, “When considering the subset of peri-urban agricultural systems, these processes are further exacerbated.” I suggest moving the peri-urban fringe definition to the beginning of the paragraph, closer to where this term is introduced.
Answer: Done -

--------------- **Reviewer:** Introduction P. 4: Need to define “soil health” as the definition is not always consistent. It also quickly moves on to the importance of soil health as a whole, but it would help to mention its particular relevance to peri-urban areas, which was the topic of the previous paragraph.
Answer: We revised the paragraph by adding a definition of soil health and a sentence that directly mentions its importance to peri-urban areas (page 3).

----------------- **Reviewer:** P. 4: “According to 16,..” Should be changed to “author’s name and colleagues” followed by the citation.
Answer: Done

------------------- **Reviewer:** P. 4: “contest” needs to be changed to “context” in the last paragraph of the introduction.
Answer: Done

Reviewer: P. 4: The description of the study could be improved in the last paragraph. These “different indicators” need to be explained more, especially if the authors are suggesting they can be used in other studies as indicators of soil health.
Answer: We revised the manuscript as suggested by the referee.

------------------- **Reviewer: Methods:** The overall organization of the data analysis of the methods is a bit confusing. It makes more sense to break your methods into sequence analysis and statistical analysis, rather than a section for each test.
Answer: We modified and renamed the sections as suggested by the referee.

----------------------------- **Reviewer: Methods:** I do not understand the reasoning behind using the “Illumina 16S Metagenomics pipeline” in one section, then, in another section, saying the data was processed again using “QIIME 2.” Only one should be used as they will produce different OTU/ASV tables. No link or reference to the Illumina pipeline was provided, so I was unable to see how the methods compared.
Answer: PICRUSt2 requires not only a species abundance table but also the corresponding FASTA file of representative sequences. The output we received from the Illumina 16S Metagenomics workflow consisted of taxonomic abundance summaries and raw FASTQ files, but it did not include a representative-sequence FASTA associated with the OTU/feature identifiers. For this reason, we reprocessed the raw FASTQ files in QIIME 2 to infer ASVs and the corresponding representative sequences FASTA. These QIIME 2 outputs were the inputs used for PICRUSt2. We have revised the Methods to clarify this rationale.

---------------------------------- **Reviewer: Methods:** Why not perform beta-diversity analysis? This would be a better comparison of diversity differences across sites as it takes into account the community structure/taxa at each site.
Answer: We thank the referee for the suggestion. We have now included a new figure with beta-diversity analysis, a description in the Method section, the results and discussion sections.

------------------------ **Reviewer: Methods** The methods need to be checked for tense usage. Often using the present tense when needing to use the past tense when describing tests and sampling.
Answer: Done

----------------------- **Reviewer: Methods** P. 11: Add a sentence making it clear that the management history is presented in Table 1. I was looking for it in the text because it wasn't clear that I could find it in the table.
Answer: Done

------------------------ **Reviewer: Methods** P. 11: Confused on why four quadrants were established and one sampling point was established in each, but only 3 samples were taken.
Answer: The referee is right, the method was not clear. We analysed three samples randomly for each point. We corrected this point now at page 8.

----------------------------------- **Reviewer: Methods** P. 11: When were the samples collected? Include the year and the month at least. This is important since it is said some land uses were established in 2019, so it is helpful to know how long since the establishment of the site where samples were taken.
Answer: We have now added this information in Study Area of Methods section (page 8).

------------------------------- **Reviewer:** Methods P. 14: There is no information given on how soil parameters were measured. Either cite other papers that have this information, or, if they were sent somewhere, include the location and the link so readers can find the methods there.
Answer: We have now included this information in the Methods' section (page 8).

------------------------- **Reviewer:** Methods P. 14: Change "DNA quality control checks" to "DNA quality and quantity were checked" since the Nanodrop is generally for quality and Qubit for quantity.
Answer: Done

-------------------- **Reviewer:** Methods P. 14: Add the specific 16S primers (e.g., 314F and 806R).
Answer: Done.

----------------- **Reviewer:** Methods P. 14: Add a link or citation where the specific PCR protocol can be found. I also recommended including a short summary, such as whether it was done in replicates, in two steps, etc.

Answer: Done

------------------- **Reviewer:** Methods P. 14: Add a link or citation for the “16S Metagenomic pipeline”. Was there a different pipeline for the ITS, or was it adapted at all for differences between ITS and 16S reads?
Answer: Done -

------------------- **Reviewer:** Method P. 14: The database versions used are dated. Ideally, should use more recent releases of both databases.
Answer: The amplicon sequencing data were analyzed using the 16S Metagenomics application available in the Illumina BaseSpace Sequence Hub. This pipeline performs read processing and taxonomic classification using a k-mer–based Naïve Bayesian classifier derived from the Ribosomal Database Project (RDP) algorithm (Wang et al., 2007). The taxonomic assignment for 16S rRNA sequences was performed using the RefSeq RDP 16S v3 database. For the ITS datasets, the same analytical framework was used; however, taxonomic assignment was performed using the UNITE reference database, which is specifically curated for fungal ITS sequences (Kõljalg et al., 2013). Therefore, the pipeline itself was not fundamentally different, but the reference database used for taxonomic classification was adapted to account for the differences between bacterial 16S and fungal ITS markers. We now added this part in the Methods section.

--------------------- **Reviewer:** Methods P. 14: It is mentioned that negative controls were included. Were any sequences found in the negative controls, and if so, include how they were accounted for in the data.
Answer: The negative control included during library preparation did not generate a detectable library profile. During the sequencing run no reads were obtained from the negative control sample. Therefore, no sequences from the negative control were present or required filtering in the downstream analyses. We now added this part in the Methods section.

-------------------- **Reviewer:** Methods P. 14: While using relative abundance normalization for the data is fine, information should be included on the average sequencing depth for each dataset, and if there was a cutoff on the minimum number of sequences considered usable for a sample. Given soil diversity, sequencing depth can be important.
Answer: The average sequencing depth across samples was approximately 40,000 reads per sample. For quality control purposes, we considered ~10,000 reads per sample as the minimum threshold required for a sample to be included in the downstream analyses. This threshold was selected to ensure sufficient sequencing depth for reliable microbial community profiling, while minimizing potential biases associated with low-coverage samples. Similar thresholds have been widely used in microbiome studies to ensure adequate representation of microbial diversity and to improve the robustness of comparative analyses.

-------------------- **Reviewer:** Methods P. 15: It should be mentioned that the metrics used are both alpha-diversity metrics.
Answer: Done

-------------------- **Reviewer:** P. 15: Does the “16S Metagenomic pipeline” produce OTUs or ASVs? It is generally just referred to as species-level assignments in the methods.
Answer: The Illumina BaseSpace 16S Metagenomics pipeline does not generate OTUs or ASVs. Instead, it performs direct taxonomic classification of reads using a k-mer–based Naïve Bayesian classifier derived from the Ribosomal Database Project (RDP) algorithm. Each read is assigned directly to the most probable taxonomic level based on the reference database, and the output is therefore reported as taxonomic profiles rather than OTU or ASV tables.

------------------- **Reviewer:** Methods P. 16: For the UpSet plots, was a taxa considered to be present in both land uses if it was present in just one of the replicates or more?
Answer: We agree with the referee and we have now clarified this point in the manuscript in the Methods section (Statistical Analysis on page 10).

----------------------- **Reviewer:** Methods P. 16: Was the data checked for normality and homogeneity of variance before determining that a parametric test (ANOVA) was the right choice? If so, include that in the methods.
Answer: We checked normality across land-uses applying the Shapiro–Wilk test and homogeneity of variance using Levene’s test. However, due to the small number of replicates, we acknowledge that the results of these tests should be interpreted cautiously. We included these details in the Methods section.

----------------------- **Reviewer:** Methods P. 18: Was there a correlation coefficient cutoff value to be considered “strongly” correlated?
Answer: We did not apply a correlation coefficient threshold. “Strongest” correlations were defined operationally for visualization purposes as the **top 20 variable pairs ranked by absolute Pearson’s r** within each land-use type. This filtering was used to reduce visual clutter and highlight the most important associations in the heatmap, rather than to assert a clear definition of “strong” correlation.

--------------------- **Reviewer:** Methods P. 19: Did you consider the correlation between the abiotic factors when doing your RDA? Also, need to add the vectors of the abiotic factors to the RDA so readers can see which factors were associated with each land-use type.
Answer: We have now assessed collinearity among the abiotic predictors and repeated the RDA after removing redundant variables using a pairwise-correlation threshold ($|r| = 0.9$), retaining only predictors below this threshold. The resulting ordination was highly similar to the original analysis, indicating that our conclusions are robust to predictor multicollinearity. We have now added this procedure to the Methods section. We also added vectors of the retained abiotic parameters to the RDA plots.

--------------------------- **Reviewer:** Methods P. 19: Please add a statement about the use of PICRUSt, such as, “however, these predictions are based on reference genomes and do not

represent direct measurements of functional genes. Therefore, results should be interpreted cautiously," considering its limitations.
Answer: Done

-------------------- **Reviewer:** Results For the results, my main comments would be to reduce the number of figures to make the message more concise. For example, the alpha-diversity metrics are shown by field with box plots (Fig. 2), then by replicate (Fig. 3). Unless a main point is within site variation, then Figure 3 is not needed. The same for showing heatmaps of the top 20 correlations between abiotic factors, then showing heatmaps with all variables.
Answer: We agree with the referee and we have now moved Fig.3 in the supplementary together with Fig.9, 10, 11 and 12**.**

--------------------- **Reviewer:** Results In the results text, there needs to be a stronger focus on the differences between land uses to convey that converting to certain land-use types may improve "soil health," as seen by increases in diversity, richness, OM, etc., to match the story set out in the introduction.
Answer: We revised the Results section as suggested by the referee.

---------------------- **Reviewer:** Results P. 4: When saying "significantly" in terms of results, it can be helpful to put the p-value in parentheses after it, especially since the method is after the results, where significance is defined. I recommended this for the whole results section.
Answer: Done

----------------------- **Reviewer:** Results P. 4: "certain genera significantly more abundant in specific samples and in specific land-use types", which ones especially on a land-use base comparisons?
Answer: We expanded those sentences by clearly specifying some of the observed patterns.

--------------------- **Reviewer:** Results P. 4: The genera "Mortierella, Fusarium, and Trebouxia" need to be italicized.
Answer: Done -

---------------------- **Reviewer:** Results P. 4: I do not think this conclusion is supported because no differences in relative abundance were explained between sites in the text and no differences in organic inputs have been presented yet, "From an agroecological point of view, this results clearly demonstrate the role of agronomical management that enhance soil organic inputs (e.g., manure, green mulch, compost application, agroforestry litterfall) in sustaining fungal diversity."
Answer: We have revised this statement to avoid implying a mechanistic explanation and we moderate our statement by substituting the expression 'this results clearly demonstrate...' with 'the results are consistent with the idea that...'

------------------------------ **Reviewer:** Results P. 4-5: For the differences in alpha-diversity metrics, state if they were significant, not just noticeable. The statement about replicating following it makes it sound like you would have found significant values if you had more replicates, which is not necessarily true. Consider rewording or removing that statement.
Answer: We agree. We have revised the text to explicitly state which alpha-diversity comparisons were statistically significant and removed wording regarding replication-size. We now describe the observed patterns descriptively and report significance only when supported by the statistical tests.

--------------------------- **Reviewer:** Results P. 5: Fungi is a kingdom, not a domain.
Answer: Fixed

----------------------- **Reviewer:** Results P. 5-6: “Such diversification contributes to ecosystem resilience and multifunctionality, which are essential for effective biological pest control.” A statement like this is better for the discussion, so you can show that other studies have shown this. Otherwise, it appears to be an unsupported claim in the results.
Answer: This sentence has been removed.

-------------------------- **Reviewer:** Results P. 6: Again, explain the differences in what taxa you saw that were significantly different between land uses. The figure captions also say genera instead of phyla. It would also make more sense to talk about the differences in phyla (Fig. 4/5) before talking about the genera (Fig. 1).
Answer: We corrected accordingly

----------------------------- **Reviewer:** Results P. 6: What is meant by “bacterial and fungal profiles”? Do you mean the special-level abundance tables? This information on what went into the tables needs to be included in the Figure 6 caption. Based on the coefficient gradient for your heatmap, I also cannot tell if it includes negative correlations. I number at the bottom of the gradient needs to be included.
Answer: We corrected accordingly.

------------------------- **Reviewer:** Results P. 7: The conclusions on soil stability based on abiotic variable correlation need to be moved to the discussion, so examples that support this can be cited. It would also be a good place to discuss how the management specific to the crop and reforested sites can lead to shared drivers of these properties.
Answer: We corrected accordingly.

------------------ **Reviewer:** Results P. 8: Redundant/highly correlated variables can impact a PCA and RDA. It would be useful to include details in the figures' captions and in the methods section on how this was handled, since strong correlations between factors were shown in Figure 7.
Answer: We corrected accordingly

---------------------- **Reviewer:** Results P. 8: Discussing the differences in abiotic factors between land uses (Fig. 13), which help show what factors shift under the different management and may help support the PCA results below, showing that there are differences in soil factors by land use.
Answer: We corrected accordingly.

----------------------- **Reviewer:** Results P. 8: I believe I mentioned this before, but all the abiotic vectors are in the RDA, so you can discuss which factors were associated with different land uses.
Answer: We have now described the associations between vectors of explanatory abiotics and different land-uses in the Results section where RDA is discussed.

-------------------- **Reviewer:** Results P. 9: For the functional differences, again explain the important differences between land use. Did you see a loss or gain of a certain function with land use?
Answer: We added a paragraph describing some examples of functionalities that were comparatively more present in a land-use compared to the others, for all three categories of functions.

------------------- **Review** Discussion Overall, the discussion needs to focus more on how the results presented were caused by differences in land use and how the management practices associated with those land uses may have shaped them. This then needs to be brought back to the idea of soil health. Currently, it is not focusing enough on the differences between land uses, and again, it just says there were broad differences between land uses.
Answer: We have substantially revised the Discussion to highlight land-use contrasts and to interpret observed patterns in terms of land-use–associated management contexts. Moreover, we moved interpretive statements (e.g., implications of abiotic correlation structure and diversification benefits) from Results to Discussion, as suggested. Relevant citations have been added in support of interpretations regarding diversification, ecosystem multifunctionality, and pest-suppressive agroecosystems.

---------------------- Review Discussion Altogether, the authors present an interesting study on how land use type may influence soil microbial diversity and overall soil health in a peri-urban landscape. However, significant revisions are needed to improve the clarity, robustness, and alignment between the stated objectives and the results. The authors should provide more detail in the Materials and Methods section on sequencing data and management practices for each land-use type. The number of figures should be reduced, and the order of figures reconsidered to group similar topics (e.g., keep all relative abundance differences together). The results and discussion should focus more on differences between land-use types, mentioning specific differences and how management practices influence soil properties and microbial communities to support claims about soil

health. Lastly, moderate claims about mechanisms and resilience to better align with the data presented.
Answer: We thank the reviewer for the very useful and detailed comments. We have now extensively revised the manuscript incorporating all the suggestions.

Competing Interests: No competing interests were disclosed.

Reviewer Report 24 February 2026

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Shefali Azad
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This study is conceptually strong and analytically rich and tackles a timely ecological issue. That is, the relationships between microbial diversity, soil health, and land use, which ultimately informs the ecological trade-offs of land management.
The methods section was very detailed and allows for replication by other parties. Is it a decision of the journal to have results before methods? I was confused by this layout, but it did create an interesting reading/interpreting experience. The relatively low #replicates does complicate the interpretation of statistical results, given that soil microbial communities are patchy on a finer scale - I was glad to see that this was addressed at the end of the discussion, but perhaps it could be shifted to earlier in the discussion to add context. In general - what are the results saying about beta diversity of the land use types, considering sample size?
I appreciated the variety of statistical analysis and visualizations. In particular I think the UpSet diagram could be better explained - I had not encountered this before and it revealed a lot of information, but it took external searching to understand it.
Minor note, this line seemed incomplete: "Agroforestry soils were characterized by the highest number of unique taxa, whereas reforested soils showed the lowest richness and diversity, aligning with previous studies that highlight the early successional stage of reforestation systems" - aligning with previous studies that highlight what about the early successional stage?
This line: "The predominance of Mortierella, Fusarium, and Trebouxia in fungal communities, as well as the dominance of Acidobacteria and Gemmatimonadetes in bacterial communities, underscores the potential of these taxa as bioindicators of soil health and management intensity." - is not necessarily a leap, but perhaps a functional extrapolation? Does presence signal relative soil health if widespread across land-use types, and through what mechanisms? (Mortierella unpacking excluded).

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and does the work have academic merit?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Agroecology, biotechnology, data management, statistical analysis (wildlife biology focus).

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 25 Mar 2026

Caterina La Porta

REVIEWER #1

Reviewer: This study is conceptually strong and analytically rich and tackles a timely ecological issue. That is, the relationships between microbial diversity, soil health, and land use, which ultimately informs the ecological trade-offs of land management.
Answer: We sincerely thank the reviewer for the careful and thoughtful evaluation of our manuscript and for recognizing its conceptual strength and analytical richness. We address each comment below and describe the revisions implemented in the updated manuscript. --

Reviewer: The methods section was very detailed and allows for replication by other parties. Is it a decision of the journal to have results before methods? I was confused by this layout, but it did create an interesting reading/interpreting experience.
Answer: We thank the reviewer for highlighting this point. The structure follows the Open Research Europe article template, which allows flexibility in manuscript organization. In our case, we adopted a Results-first structure to foreground the empirical findings before detailing methodological procedures.

-- Reviewer: The relatively low #replicates does complicate the interpretation of statistical

results, given that soil microbial communities are patchy on a finer scale - I was glad to see that this was addressed at the end of the discussion, but perhaps it could be shifted to earlier in the discussion to add context.
Answer: We fully agree that soil microbial communities exhibit fine-scale spatial heterogeneity, and the limited number of biological replicates constrains statistical power. In the revised manuscript, we have moved the discussion of replication limits earlier in the Discussion section on page 19, accordingly.

-- Reviewer #1: In general - what are the results saying about beta diversity of the land use types, considering sample size?
Answer: We thank the referee for the suggestion. We have now included beta-analysis and added the description in the Methods, Results and Discussion sections

---- **Reviewer**: I appreciated the variety of statistical analysis and visualizations. In particular I think the UpSet diagram could be better explained - I had not encountered this before and it revealed a lot of information, but it took external searching to understand it.
Answer: We have added a brief explanation to the Methods section of the manuscript describing how to interpret the UpSet diagram (set sizes, intersection sizes, and how intersections correspond to shared vs. land-use–specific taxa) on page 9.

--- **Reviewer:** Minor note, this line seemed incomplete: "Agroforestry soils were characterized by the highest number of unique taxa, whereas reforested soils showed the lowest richness and diversity, aligning with previous studies that highlight the early successional stage of reforestation systems" - aligning with previous studies that highlight what about the early successional stage?
Answer: Our intent was to indicate that the comparatively lower richness and diversity in reforested soils is consistent with previous studies reporting that early successional reforestation systems often host simpler, less diverse microbial communities. We have revised the sentence accordingly on page 13.

---- Reviewer #1: This line: "The predominance of Mortierella, Fusarium, and Trebouxia in fungal communities, as well as the dominance of Acidobacteria and Gemmatimonadetes in bacterial communities, underscores the potential of these taxa as bioindicators of soil health and management intensity." - is not necessarily a leap, but perhaps a functional extrapolation? Does presence signal relative soil health if widespread across land-use types, and through what mechanisms? (Mortierella unpacking excluded).
Answer: We agree with the referee and we have now changed the sentence accordingly on page 13.

Competing Interests: No competing interests were disclosed.