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Diversifying Grapevines With Aromatic Plants Changes the Soil Habitat, Microbial Community Composition and Functions Toward More Efficient Substrate Use and Nutrient Allocation

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ABSTRACT

In vineyards facing soil degradation and biodiversity loss, crop diversification may improve sustainability, but its effects on the soil microbiome remain unclear. In a 3-year field study, we examined how diversifying the plant row under grapevine with aromatic plants affected topsoil properties (0–10 cm) in an organically farmed, steep-sloped vineyard. Specifically, we investigated the effects of diversification with oregano and thyme on microbial biomass, respiration, prokaryotic and fungal community compositions, enzyme activities, potential nitrification, and abiotic soil properties, including total and particulate organic carbon (TOC, POC), nutrient status, pH, and soil moisture. Grapevines alone with mechanical tillage served as control. The aromatic plants competed with grapevines by lowering soil nutrient contents and moisture. Aromatic plant litter had a small, mostly non-significant but consistent effect on POC contents, and POC stocks determined in the final year showed a slight increasing trend in the order control (10.9 ± 2.8 t POC ha⁻¹) < thyme (12.6 ± 3.1) < oregano (13.1 ± 4.1). Surprisingly, these changes coincided with a significant decrease in microbial biomass compared to control, indicating aromatic plant-microbe competition. Concomitant decreases in respiration and the activity of C-cycling enzymes but also the metabolic quotient, suggest lower carbon mineralisation but more efficient microbial carbon use. Multivariate statistics revealed that the prokaryotic community was primarily structured by abiotic soil properties, such as organic matter, nutrient and water availability. In contrast, the fungal community exhibited a stronger plant-specific response, with changes in composition likely driven by root-associated interactions, suggesting a more direct biotic influence. Especially, arbuscular mycorrhizal fungi and potential nitrification were promoted under both aromatic plants, which may benefit grapevine growth. Overall, we show that diversifying perennial agroecosystems such as vineyards with aromatic plants increases soil habitat heterogeneity with benefits for microbial diversity, carbon sequestration and nutrient cycling, demonstrating its positive impact on soil biodiversity and functioning.

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1 | Introduction

Crop diversification in perennial agroecosystems such as vineyards is a promising measure to reduce adverse impacts on soil functioning (Abad et al. 2021; Ochoa-Hueso et al. 2024; Rasmussen et al. 2024) and economic risks due to the provision of additional high-value plant materials with various marketing options (Zuazo et al. 2008; Rao 2015; Almagro et al. 2023). In viticulture, mainly grasses and legumes are nowadays used as additional plant covers (Ochoa-Hueso et al. 2024). In addition, aromatic plants such as oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) appear to be particularly suited for vineyard diversification because, unlike grasses and herbs, they can be cultivated in the grapevine (*Vitis vinifera*) row to provide soil coverage without compromising grapevine production (Dittrich et al. 2021). The few studies that have investigated the effects of crop diversification through the combined cultivation of aromatic plants and other permanent crops report consistently positive results (Zhang et al. 2021a; Almagro et al. 2023).

One of the important drivers of the positive effect of crop diversification is the increased input and belowground allocation of plant-derived carbon (C) sources (Zhang et al. 2021b; Wooliver et al. 2022). Furthermore, crop diversification improves pest and disease control, adaptability to arid climates and hence, a low water consumption, erosion control, and pollinator attraction (Zuazo et al. 2008; Rao 2015; Song and Han 2020; Zhang et al. 2021a; Almagro et al. 2023). On the other hand, there is the possibility of competition for soil water and nutrients. However, in the case of grapevines, this can have a positive effect on grape and must quality (Dittrich et al. 2021). Furthermore, because of the known positive influence of the soil organic matter (SOM) content on soil structure and water (Lal 2020) and nutrient holding capacity (Rice 2005), one can assume that competition between crops decreases with increasing SOM content. In this context, a recent global meta-analysis (Prairie et al. 2023) reported that particulate OM (POM; particulate organic residues mostly of plant origin; Witzgall et al. 2021) is particularly sensitive to the effects of crop diversity, tillage and duration of the cultivation.

The effects of crop diversification on abiotic soil properties also have an impact on soil microorganisms, which are fundamental for providing and maintaining soil functions (FAO 2020). Consequently, soil microorganisms have been shown to benefit from crop diversification with cover crops in permanent crops in general and in viticulture in particular (Steenwerth and Belina 2008; Di Bene et al. 2022). There is growing evidence from empirical studies and meta-analyses that increasing crop cover and plant diversity in agricultural systems enhance soil microbial abundance, respiration (McDaniel et al. 2014; Kim et al. 2020; Morugán-Coronado et al. 2022) and the activity of extracellular enzymes (Curtright and Tiemann 2021), and alter the microbial community composition (Wooliver et al. 2022; Yang et al. 2023). In comparison, less is known about the impact of aromatic plants on soil microorganisms, especially under field conditions. In an orchard production system, increases in microbial biomass and substantial changes in the microbial community were reported (Zhang et al. 2021a). Furthermore, aromatic plants synthesise essential oils, which consist of organic compounds with known antimicrobial

properties, e.g. volatile isoprenoids such as terpenes (Paster et al. 1995; Kumar et al. 2008; Sakkas and Papadopoulou 2017). Inhibiting effects on soil microbiota as well as stimulative effects on microbial abundance and activity have been observed (Vokou and Margaritis 1988; Paavolainen et al. 1998; Vokou and Liotiri 1999; Smolander et al. 2006; Adamczyk et al. 2015). Thus, a considerable impact of terpenes released by aromatic plants on vineyard soil microbiota can be expected (Thiele-Bruhn et al. 2024).

The knowledge presented shows that positive effects on soil microorganisms (e.g., through an increase in rooting and SOM) but also competitive or inhibitory effects (e.g., competition for water and nutrients; inhibiting terpenes) are possible through crop diversification with aromatic plants. However, field studies on the effects of growing aromatic plants on soil microbiota have not been published to our knowledge. Hence, this observational 3-year field study aimed to investigate the impact of crop diversification with aromatic plants on the soil microbial community. This study was carried out in an organically managed vineyard. Two diversification treatments, i.e. oregano (*Vitis vinifera* L. cv. 'Riesling' diversified with *Origanum vulgare*) and thyme (*Vitis vinifera* L. cv. 'Riesling' diversified with *Thymus vulgaris*) were compared with the control treatment, i.e. the sole cultivation of grapevines (*Vitis vinifera* L. cv. 'Riesling') combined with mechanical tillage (control). Samples were taken over 3 consecutive years and analysed for soil abiotic (TOC, POC, soil nutrient status, pH and soil water content) and microbiological parameters (microbial biomass, prokaryotic and fungal diversity and community composition, as well as enzyme activities, respiration, and nitrification). The specific objective was to identify the effects on the soil microbiota of two different aromatic plants used for crop diversification in comparison to conventional mechanical tillage underneath the grapevines. Thereby, this study focused on the effects on selected soil chemical properties, shaping the soil habitat for the microbiome, and on changes of the biomass, activity parameters and the diversity of the soil microbial community. We hypothesised that: (i) growing aromatic plants increase the SOM content and especially POM content both through increased litter fall and root biomass, (ii) which outweighs possible negative effects of nutrient and water competition, and that (iii) this results in an increased microbial biomass and activity (Bhattacharyya et al. 2022), which goes along with (iv) changes in the microbial community structural composition and diversity towards a more fungi-dominated community (Bartelt-Ryser et al. 2005). This should provide a better understanding of plant-soil-microbe interactions and their relationship to soil functioning in diversified agroecosystems.

2 | Materials and Methods

2.1 | Study Site and Field Sampling Design

The study was conducted in a commercial, organically managed vineyard in the Mosel area of Germany, from May 2018–October 2020. The climate in the study area is temperate oceanic with mean annual temperature, precipitation and potential evapotranspiration, being 9.1°C, 722 and 687 mm, respectively. The experimental plot at 49°39'23.976"N, 6°33'27.936"E is

south-exposed, strongly inclined (~45%) and the vineyard soil was classified as Eutric Skeletic Regosol (Aric, Humic) (IUSS Working Group WRB 2022). Due to a large proportion (~50 wt.%) of coarse rock fragments (> 2 mm) and shallow (< 0.5 m) conditions, the soil is characterised by a warm and dry, continental pedoclimate. The fine earth has a sandy loamy texture and is composed of 60% sand, 25% silt, and 15% clay.

The soil in the differently treated rows of the vineyard site was repeatedly sampled twice a year over 3 consecutive years, starting from October 2018, following the establishment of the aromatic plants in May 2018. Coarse organic material and rock fragments were removed from the surface before sampling. To account for soil heterogeneity along the slope, a stratified sampling strategy was employed. Soil samples were collected at three distinct slope positions: upper, middle, and lower sections of the plot. Within each position, composite samples were taken with a hand shovel by combining 10 subsamples from 0 to 10 cm depth, close (< 10 cm) to the grapevine row, covering a distance of 15 m samples were taken to reduce microsite variability. This approach ensures that slope-induced differences in soil properties are captured in the analysis. A detailed schematic figure illustrating the layout of the experimental design is available in Dittrich et al. (2021). In total, nine (2018 and 2020 samplings), respectively, six (2019 samplings) replicate samples per sampling time and treatment were taken, stored in a cool container and transported to the laboratory immediately. Field-moist samples were sieved at < 2 mm and divided into two aliquots. For chemical analysis, the soil was air-dried, while aliquots for biological analysis were stored at -20°C. For DNA extraction (only for the three October samplings), five aliquots were selected based on the similarity of their OC content.

At the end of the field experiment, dropped grapevine leaves were collected from randomly chosen areas (six per treatment; 10 m row length and 0.3 m row width) to assess leaf dry mass and C and N contents in the differently treated rows. Dropped leaves were chosen because, during the experiment, aromatic plants acted as a physical barrier, retaining dropped grapevine leaves that would have otherwise been translocated. This approach was used to estimate carbon input to the soil via this retention mechanism. Moreover, soil bulk density was measured using the volume replacement method (Hartge and Horn 2009). Briefly, soil pits (six per treatment) of 20 × 20 × 20 (length × width × depth) were prepared with a hand shovel. Pit volumes (4–5 L) were assessed with commercially available sand, while the excavated soil material was air-dried and sieved at 2 mm for C and N analysis of SOM and POM (see section below). Stocks of total and particulate C and N were calculated as recommended in (FAO 2019): $stock_i (t C ha^{-1}) = C_i \times BD_{fine1_i} \times (1 - v_{G_i}) \times t_i \times 0.1$; where, $stock_i (t C ha^{-1})$ is the soil organic carbon stock of depth increment i ; $C_i (mg C g^{-1} \text{ fine earth})$ is the organic carbon content of the fine earth fraction (< 2 mm) in the depth increment i ; $BD_{fine1_i} (g \text{ fine earth } cm^{-3} \text{ fine earth})$ is the mass of fine earth per volume of fine earth = (dry soil mass [g] – coarse fragment mass [g]) / (soil sample volume [cm³] – coarse fragment volume [cm³]) in the depth increment i ; volume fraction fine earth ($cm^3 \text{ fine earth } cm^{-3} \text{ soil}$) = 1 – volume fraction coarse fragment [$cm^3 \text{ coarse fragment } cm^{-3} \text{ soil}$]; t_i is the thickness (depth, in cm) of the depth increment i ; 0.1 is a factor for converting $mg C cm^{-2}$ to $t C ha^{-1}$.

2.2 | Laboratory Analysis

2.2.1 | Abiotic Soil Properties

Total contents of C and N were determined on powdered (ball mill; Retsch MM400, Retsch GmbH, Haan, Germany) soil samples via high-temperature combustion using a vario EL cube (Elementar, Langensfeld, Germany). The samples were free of carbonates, therefore, the quantified total C content represents the total soil organic carbon (TOC). Concentrations of C and N in particulate organic carbon and nitrogen (PON) were determined on soil subjected to physical fractionation as described in Cambardella and Elliott (1992). To this end, 20 g of soil was dispersed by shaking overnight in 100 mL sodium hexametaphosphate ($Na_6[(PO_3)_6]$, 5 g L⁻¹). The mixture was poured on a 53 μm sieve and gently rinsed with deionised water. Subsequently, the sample material retained in the sieve was dried at 60°C before dry mass determination and grinding. Analysis of total C and N in POM was performed as described above for total C and N in bulk soil. Measurements of pH values were conducted in calcium chloride (CaCl₂, 0.01 M) soil suspensions [soil to solution ratio 1:2.5 (v:w)] with a 340i glass electrode (WTW GmbH, Weilheim, Germany). The same electrode was used to measure EC in a 1:2.5 (v:v) soil suspension with deionised water. Plant available phosphate (avP) and potassium (avK) were extracted using the calcium-acetate-lactate (C₅H₈CaO₅, CAL) method, NH₄-N and NO₃-N were extracted using potassium chloride (KCl, 2 M), and the gravimetric soil moisture was determined by the weight loss upon soil drying at 105°C for 24 h. These methods are described in more detail in Dittrich et al. (2021).

2.2.2 | Microbial Biomass, Respiration, Enzyme Activity and Potential Nitrification Rates

Before the analysis of microbial biomass, respiration, enzyme activities and potential nitrification, samples were thawed and biologically reactivated by pre-incubation according to ISO 18400-206, for example described in Álvaro-Fuentes et al. (2019).

Microbial biomass carbon (MBC) and nitrogen (MBN) were determined following chloroform (CHCl₃) fumigation extraction (Vance et al. 1987). Briefly, 25 g dry equivalent of the moist soil sample was exposed to a chloroform (CHCl₃) saturated atmosphere in a desiccator for 24 h. After subsequent careful evacuation of the CHCl₃, the microbial biomass was extracted with 100 mL CaCl₂ (0.01 M) solution; additionally, non-fumigated soil samples were used as control (Joergensen and Mueller 1996). For extraction, soil suspensions were agitated for 30 min using an orbital shaker (250 rpm) before filtration through a paper filter (595 ½, Whatman, Dassel, Germany). A TOC-TN analyser (Shimadzu TOC-V + TNN, Kyoto, Japan) was used to quantify total OC and N contents in the resulting extracts. MBC was calculated as EC/kEC, where EC is the OC extracted from the fumigated sample minus the OC extracted from non-fumigated aliquot, and the value of kEC is 0.45 (Joergensen and Mueller 1996). Likewise, MBN was calculated with the corresponding organic N contents and the correction factor kEN of 0.40 (Joergensen and Mueller 1996). The microbial C:N ratio (C:N_{MB}) was calculated by dividing MBC and MBN. The MBC:OC ratio was expressed as a percentage.

Additionally, lipid extraction was applied following the protocol provided by Zelles and Bai (1993) from field-moist (10 g dry-mass equivalent) and frozen aliquots, using a phosphate (K_2HPO_4 , 0.05 M)-(CHCl₃)-methanol (CH₃OH) buffer. We focused on neutral lipids, which were separately eluted with CHCl₃ and quantified against methyl 11(Z)-hexadecenoate (CAS number: 822-05-9) obtained from Larodan (Solna, Sweden), to measure the neutral lipid 16:1 ω 5, a biomarker used to investigate arbuscular mycorrhizal fungi (AMF) abundance (Ngosong et al. 2012; Olsson and Lekberg 2022).

Respiration was measured according to Heinemeyer et al. (1989). Briefly, 200 mL min⁻¹ of carbon dioxide (CO₂)-free, humid air was channelled through tubes containing 30 g dry equivalent moist soil sample until steady-state conditions were reached, i.e. the initial CO₂ flush reached an equilibrium. An infrared gas analyser (ADC 225 MK3, The Analytical Development Company Ltd., Hoddesdon, UK) quantified the formed CO₂ and respiration was expressed as $\mu\text{g CO}_2\text{-C g}^{-1} \times \text{h}^{-1}$. The metabolic quotient (qCO₂) was calculated as the ratio of the microbial respiration and the MBC and expressed as $\mu\text{g CO}_2\text{-C} \times (\text{mg MBC})^{-1} \times \text{h}^{-1}$.

Enzyme activities were assessed based on colorimetric and fluorimetric approaches. The latter was adapted from Marx et al. (2001) and used for determination of β -D-glucosidase (BG; EC 3.2.1.21), N-acetyl-glucosaminidase (AG; EC 3.2.1.52), leucine-aminopeptidase (LA; EC 3.4.11.1), acid phosphomonoesterase (AP; EC 3.1.3.2) and aryl-sulfatase (AS; EC 3.1.6.1). Briefly, 1 g dry equivalent of moist soil sample was suspended in 100 mL deionised water and subjected to ultrasonic treatment at 50 J s⁻¹ for 2 min. Microplate-wells were used in which 50 μL of soil suspension was combined with 50 μL of buffer and 100 μL substrate solution (aminomethylcoumarin for LA and methylumbelliferyl for BG, AG, AP, AS). Multi-well plates were incubated for 4 h at 30°C, and fluorescence was measured in constant time intervals at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using the microplate reader Victor 1420 (Perkin Elmer, Waltham, USA).

The colorimetric approach was applied for the determination of the oxidoreductases dehydrogenase (DH; EC 1.1.1) and peroxidase (PO; EC 1.11.1.7; from April 2019). For dehydrogenase, iodotetrazolium chloride was used as substrate as originally described by Benefield et al. (1977) and modified by von Mersi and Schinner (1991). To this end, 2 g dry equivalent of moist soil sample was combined with 2 mL substrate solution, whereas control samples received 2 mL of buffer solution instead. All samples were incubated at 25°C for 4 h before the addition of 8 mL acetone and agitation on an orbital shaker (250 rpm) for 1 h. After centrifugation for 5 min at 2000g, the absorbance of the supernatants was quantified using a spectrophotometer (Shimadzu UV-1650 PC, Kyoto, Japan) at a wavelength of 485 nm.

For the determination of the peroxidase activity, the phenolic amino acid L-DOPA (C₉H₁₁NO₄) and hydrogen peroxide (H₂O₂) served as substrates. We applied the method described by Dick (2011). Peroxidase activity was determined by the difference in phenol oxidase activity and total oxidase activity by the same protocols, except that samples to determine the latter

additionally received H₂O₂. For that purpose, 0.5 g dry equivalent of moist soil sample was mixed with 3.2 mL (phenol oxidase) resp. A total of 3 mL (total oxidase) acetate buffer. For both activities, 2 mL of 10 mM L-DOPA were added, while a control sample for each activity received 2 mL acetate buffer. For total oxidase activity, 0.2 mL of 0.3% H₂O₂ were added. All samples were incubated at 25°C for 30 min. After centrifugation for 5 min at 2000g, the absorbance of the transferred supernatants was quantified on a spectrophotometer (Shimadzu UV-1650 PC, Kyoto, Japan) at a wavelength of 475 nm.

For the determination of the potential nitrification, we followed the method described in ISO 15685 (ISO 2012). Briefly, 25 g dry equivalent of moist soil sample was mixed with 100 mL substrate solution, containing diammonium sulphate (NH₄)₂SO₄ and sodium chlorate (NaClO₃) to inhibit biological nitrate formation. Samples were incubated for 6 h at 25°C on an orbital shaker at 175 rpm. After 2 and 6 h of incubation, aliquots of 2 mL were sampled from the soil slurry, and 2 mL of KCl were added to stop the ammonium (NH₄⁺) oxidation. Samples were centrifuged at 3000g for 2 min before nitrite quantification at 540 nm using the microplate reader $\mu\text{Quant ELx800}$ (Bio-Tek Instruments Inc., Winooski, USA).

The biomass-specific activities of enzymes and potential nitrification were calculated by normalisation to MBC and expressed as a percentage of the control soil.

2.2.3 | Soil DNA Extraction, Amplification and Sequencing

Soil DNA was extracted from 0.25 g dry equivalent of field-moist and frozen aliquots using the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Yield and quality of the obtained extracts were tested using NanoDrop and Qubit 2.0 device (Thermo Fisher Scientific, Waltham, USA).

For prokaryotic sequencing, the V4 region of the 16S rRNA gene was amplified with the 515 F/806 R primer set (Caporaso et al. 2011). PCR amplification was performed in a final volume of 25 μL containing 2 μL of DNA, 5 μL of 5X Q5 reaction buffer, 1.25 μL of each primer (10 μM), 0.25 μL of Q5 High-Fidelity DNA polymerase, 0.5 μL of 10 mM dNTPs and 14.75 μL of DNA/RNAase-free water (New England Biolabs, Germany). The thermal cycling conditions used for the PCR were: initial denaturation at 98°C for 30 s followed by 25 cycles of denaturation at 98°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 30 s, terminating with a final extension at 72°C for 10 min. Paired-end sequencing (2 \times 300 bp) for the 16S rRNA gene PCR amplicons was conducted on the Illumina MiSeq platform by LGC Genomic GmbH, Berlin, Germany. Before the analysis of sequencing data on the QIIME2 platform (Bolyen et al. 2019), raw sequence reads were initially reoriented into respective forward and reverse read files by using an in-house Python script (github.com/DamienFinn/MiSeq_read_reorientation) in order to address the blind ligation of Illumina adaptor and amplicon fragments. The Cutadapt paired-end method was employed for demultiplexing the reoriented sequence reads on the QIIME2 platform (Martin 2011). The

forward and reverse reads were then merged using the Vsearch join-pairs function (Rognes et al. 2016). Following the merging process, sequence reads were truncated at positions 280 and 40 utilising the DADA2 denoise-single function (Callahan et al. 2016). For 16S rRNA sequencing, taxonomy was assigned using the Silva 138 database (Quast et al. 2012; Yilmaz et al. 2014). Eukaryote-associated reads (mitochondria and chloroplasts) were subsequently filtered out.

For fungal sequencing, libraries were prepared using a modified version of the method proposed by Smith and Peay (2014). Sequencing libraries were generated by PCR amplification using locus-specific primers (ITS1f-ITS2) tailed with the Illumina adapters. The reverse primers were barcoded to allow multiplexing using the 12-base Golay barcodes (Caporaso et al. 2012). PCR amplification was performed in a final volume of 30 μ L containing 3 μ L of buffer 10X, 0.7 μ L of each primers (10 mM), 0.9 μ L of 50 mM MgSO₄, 0.6 μ L of 10 mM dNTP and 0.12 μ L of Invitrogen Platinum Taq DNA polymerase High Fidelity (Cat No.: 11304-011, Thermo Fisher Scientific). The thermal cycling conditions used for the PCR were: initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 1 min and extension at 72°C for 1 min, terminating with a final extension at 72°C for 10 min. Before sequencing, amplicon libraries were mixed together with 10% PhiX control library to increase the sequences diversity. Libraries were sequenced on the Illumina MiSeq (Illumina, San Diego, USA) platform, generating 300 bp paired-end reads.

2.2.4 | Bioinformatics Analysis

Raw reads were filtered using Trimmomatic (Bolger et al. 2014) to remove adapters and low-quality reads using a quality cutoff of 20 in a 20 bp sliding windows. Paired-end reads were merged using Pear (Zhang et al. 2014). Amplicon sequence variants (ASVs) were generated using the QIIME2 DADA2 plugin (Callahan et al. 2016). ASVs were clustered into OTUs based on a 97% sequence similarity using QIIME2 vsearch cluster-features-open-reference tool (Rognes et al. 2016). Taxonomy was assigned using the UNITE database version 8.2, dynamic (Abarenkov et al. 2024). Functional profiles of the identified prokaryotic and fungal OTUs were depicted from FAPROTAX (Louca et al. 2016) and FunGuild (Nguyen et al. 2016). The 16S rRNA sequence reads have been deposited in the European Nucleotide Archive database (Project Accession number PRJEB75200). The ITS sequence reads have been stored in the NCBI database (<https://www.ncbi.nlm.nih.gov/>) under the project number PRJNA1174674.

2.3 | Statistical Analysis

All analyses were performed using R Statistical Software (R Core Team 2023) and visualised using the ggplot2 package (Wickham et al. 2023). To cope with the hierarchical design of soil sampling, we applied generalised mixed effects modelling (GLMM), to compare the effects of the diversification treatments with the control management over time on soil edaphic and microbiological properties. Unless otherwise specified, statistical

significance was analysed using the functions lmer from lme4 package (Bates et al. 2023) and cftest from the multcomp package (Hothorn et al. 2023). To identify the numeric edaphic variables explaining variation of microbial properties, a stepwise backward elimination of non-significant variables was performed using the update function from the stats package (R Core Team 2023). The plotQunif function from the DHARMA package (Hartig and Lohse 2022) was applied to produce quantile-quantile plots to visually inspect the uniformity of distributions. Microbial alpha diversity indices were calculated using the diversity function from the vegan package (Oksanen et al. 2022). Microbial beta-diversity (community composition) was analysed using principal coordinates analysis (PCoA) of the Bray-Curtis species dissimilarity using the cmdscale function from the stats package. The anosim function from the vegan package was used to test beta-diversity divergence between the treatments. Finally, the bioenv function from the vegan package was used to identify the subset of edaphic variables with maximum rank correlation with community dissimilarity.

3 | Results

3.1 | Effects of Diversification on Edaphic Properties

Data of the analysed abiotic soil properties are summarised in relation to the sampling time and treatment (Table 1). Over the course of the field experiment, TOC and POC concentrations ranged from 2.87 ± 0.42 to 3.67 ± 0.60 wt.%, and from 1.14 ± 0.33 to 1.85 ± 0.61 wt.%, respectively. Despite high variations in OM due to the stone-rich soil, diversification with oregano tended to increase TOC, on average by ~3%, relative to the control management. Diversification with thyme had variable and inconsistent effects on TOC concentrations. By contrast, oregano and thyme uniformly elevated POC concentrations to varying extents (by 1.7%–31.6%), being significant at $p < 0.05$ for oregano in October 2020 (Table 1). At the end of the experiment, bulk density and carbon stock estimations showed a slight trend towards lower bulk density and higher carbon stocks under both diversification treatments. Specifically, TOC and POC stocks followed an increasing trend from control (13.0 ± 2.3 resp. 10.9 ± 2.8 t TOC/POC ha⁻¹) to thyme (14.3 ± 2.5 resp. 12.6 ± 3.1 t TOC/POC ha⁻¹) to oregano (15.1 ± 3.9 resp. 13.1 ± 4.1 t TOC/POC ha⁻¹), though these differences were not statistically significant (Table 1). Likewise, the estimated potential C and N inputs via grapevine leave retention followed the same order, being control (169 ± 14 kg C resp. 4.2 ± 0.2 kg N) < thyme (306 ± 52 kg C resp. 7.7 ± 1.3 kg N) < oregano (327 ± 71 kg C resp. 8.3 ± 1.9 kg N) (Supporting Information S1: Table S4). At the plant level, oregano showed significantly higher total biomass ($p < 0.05$) and root biomass ($p < 0.001$) compared to thyme (Supporting Information S1: Table S3). Leaf C:N ratios and stem carbon content were significantly higher in thyme than in oregano ($p < 0.05$; Supporting Information S1: Table S3). Although not statistically significant, stem and root C:N-ratio also tended to be higher in thyme, with a more pronounced tendency for root C:N (Supporting Information S1: Table S3).

In contrast to the effects on SOM and POM increasing with time, all other soil abiotic properties tended to be or were even significantly reduced in the diversified treatments. In most

TABLE 1 | Edaphic properties per sampling time and treatment.

Sampling Treatment <i>n</i>	October 2018						April 2019						October 2019						April 2020						October 2020					
	Ctrl		Ore		Thy		Ctrl		Ore		Thy		Ctrl		Ore		Thy		Ctrl		Ore		Thy		Ctrl		Ore		Thy	
	9	9	9	9	9	9	6	6	6	6	6	6	6	6	6	6	6	6	6	9	9	9	9	9	9	9	9	9	9	
TOC [%]	3.67 ± 0.60	3.38 ± 0.66	3.44 ± 0.58	3.44 ± 0.58	2.95 ± 0.48	3.07 ± 0.45	2.92 ± 0.39	2.90 ± 0.56	2.92 ± 0.52	2.94 ± 0.44	2.87 ± 0.42	2.87 ± 0.44	2.87 ± 0.42	2.94 ± 0.44	2.87 ± 0.42	2.94 ± 0.44	2.76 ± 0.44	2.76 ± 0.44	2.87 ± 0.42	2.94 ± 0.44	3.07 ± 0.48	3.07 ± 0.48	3.07 ± 0.48	3.07 ± 0.48	3.21 ± 0.70	3.21 ± 0.70	2.96 ± 0.45	2.96 ± 0.45		
TN [%]	0.34 ± 0.04	0.32 ± 0.05	0.33 ± 0.05	0.33 ± 0.05	0.28 ± 0.03	0.30 ± 0.05	0.28 ± 0.03	0.28 ± 0.04	0.27 ± 0.04	0.28 ± 0.04	0.28 ± 0.03	0.28 ± 0.03	0.28 ± 0.03	0.28 ± 0.04	0.28 ± 0.03	0.28 ± 0.04	0.27 ± 0.03	0.27 ± 0.03	0.28 ± 0.03	0.28 ± 0.03	0.31 ± 0.04	0.31 ± 0.04	0.31 ± 0.04	0.32 ± 0.05	0.32 ± 0.05	0.30 ± 0.04	0.30 ± 0.04			
C:N _{SOM}	10.8 ± 0.6	10.6 ± 0.4	10.4 ± 1.7	10.4 ± 1.7	10.5 ± 0.6	10.2 ± 0.7	10.4 ± 0.4	10.4 ± 0.5	10.8 ± 0.4	10.5 ± 0.4	10.3 ± 0.4	10.5 ± 0.4	10.3 ± 0.4	10.5 ± 0.4	10.3 ± 0.4	10.5 ± 0.4	10.2 ± 0.6	10.2 ± 0.6	10.3 ± 0.4	10.5 ± 0.4	9.9 ± 0.5	9.9 ± 0.5	9.9 ± 0.5	10.0 ± 0.5	10.0 ± 0.5	9.9 ± 0.5	9.9 ± 0.5			
POC [%]	1.70 ± 0.47	1.44 ± 0.33	1.57 ± 0.39	1.57 ± 0.39	1.77 ± 0.47	1.85 ± 0.61	1.80 ± 0.35	1.14 ± 0.33	1.40 ± 0.29	1.50 ± 0.34	1.68 ± 0.31	1.50 ± 0.34	1.68 ± 0.31	1.72 ± 0.33	1.72 ± 0.33	1.75 ± 0.36	1.75 ± 0.36	1.75 ± 0.36	1.75 ± 0.36	1.51 ± 0.38	1.51 ± 0.38	1.51 ± 0.38	1.51 ± 0.38	1.85 ± 0.57*	1.85 ± 0.57*	1.59 ± 0.36	1.59 ± 0.36			
PON [%]	0.14 ± 0.03	0.13 ± 0.02	0.13 ± 0.03	0.13 ± 0.03	0.16 ± 0.03	0.16 ± 0.05	0.16 ± 0.03	0.11 ± 0.03	0.12 ± 0.02	0.14 ± 0.02	0.18 ± 0.02	0.14 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.17 ± 0.03	0.18 ± 0.03	0.18 ± 0.03	0.18 ± 0.03	0.14 ± 0.03	0.14 ± 0.03	0.17 ± 0.05*	0.17 ± 0.05*	0.17 ± 0.05*	0.17 ± 0.05*	0.15 ± 0.03	0.15 ± 0.03			
C:N _{POM}	12.1 ± 1.1	11.1 ± 0.9	12.1 ± 0.9	12.1 ± 0.9	11.1 ± 0.7	11.6 ± 0.6	11.3 ± 0.5	10.4 ± 1.0	11.7 ± 1.1**	10.7 ± 0.9	9.3 ± 0.6	10.7 ± 0.9	9.3 ± 0.6	10.1 ± 0.3	10.1 ± 0.3	9.7 ± 0.7	9.7 ± 0.7	9.7 ± 0.7	9.7 ± 0.7	10.8 ± 0.7	10.8 ± 0.7	10.9 ± 1.0	10.9 ± 1.0	10.9 ± 1.0	10.9 ± 1.0	10.6 ± 0.7	10.6 ± 0.7			
pH [CaCl ₂]	6.56 ± 0.09	6.51 ± 0.08	6.53 ± 0.06	6.53 ± 0.06	6.71 ± 0.07	6.67 ± 0.08	6.69 ± 0.06	6.61 ± 0.11	6.57 ± 0.11	6.53 ± 0.13*	6.64 ± 0.08	6.53 ± 0.13*	6.64 ± 0.08	6.61 ± 0.05	6.61 ± 0.05	6.60 ± 0.07	6.60 ± 0.07	6.60 ± 0.07	6.63 ± 0.05	6.63 ± 0.05	6.63 ± 0.08	6.63 ± 0.08	6.63 ± 0.08	6.63 ± 0.08	6.63 ± 0.08	6.63 ± 0.08	6.63 ± 0.08			
EC [μ S cm ⁻¹]	126 ± 12	92 ± 15***	105 ± 15*	105 ± 15*	165 ± 15	135 ± 17**	149 ± 13	198 ± 30	178 ± 13	190 ± 27	134 ± 11	190 ± 27	134 ± 11	118 ± 11	118 ± 11	118 ± 12	118 ± 12	118 ± 12	218 ± 32	218 ± 32	210 ± 24	210 ± 24	210 ± 24	210 ± 24	168 ± 25***	168 ± 25***				
NH ₄ -N [mg kg ⁻¹]	5.0 ± 1.8	4.0 ± 1.7*	4.6 ± 2.2	4.6 ± 2.2	2.0 ± 0.9	0.8 ± 0.3*	1.0 ± 0.4	0.8 ± 0.8	0.3 ± 0.3	0.7 ± 0.5	2.0 ± 0.4	0.7 ± 0.5	2.0 ± 0.4	1.9 ± 0.9	1.9 ± 0.9	1.8 ± 0.3	1.8 ± 0.3	1.8 ± 0.3	2.9 ± 0.8	2.9 ± 0.8	1.8 ± 0.6*	1.8 ± 0.6*	1.8 ± 0.6*	2.2 ± 0.6	2.2 ± 0.6					
NO ₃ -N [mg kg ⁻¹]	9.6 ± 3.7	4.7 ± 2.3***	6.3 ± 2.9***	6.3 ± 2.9***	nd	nd	nd	1.1 ± 0.4	2.3 ± 1.3	1.7 ± 0.7	0.4 ± 0.5	1.7 ± 0.7	0.4 ± 0.5	0.7 ± 0.8	0.7 ± 0.8	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	7.1 ± 4.0	7.1 ± 4.0	8.3 ± 3.9	8.3 ± 3.9	8.3 ± 3.9	5.4 ± 2.6	5.4 ± 2.6					
avP [mg kg ⁻¹]	212 ± 23.1	179 ± 24.7***	194 ± 15.9*	194 ± 15.9*	200 ± 12.5	180 ± 40.7	173 ± 50.1*	147 ± 7.2	135 ± 7.2	142 ± 9.7	156 ± 15.6	142 ± 9.7	156 ± 15.6	149 ± 16.7	149 ± 16.7	148 ± 13.2	148 ± 13.2	148 ± 13.2	152 ± 12.2	152 ± 12.2	140 ± 26.2	140 ± 26.2	140 ± 26.2	137 ± 15.9	137 ± 15.9					
avK [mg kg ⁻¹]	689 ± 56.9	710 ± 160.4	669 ± 128.5	669 ± 128.5	275 ± 18.6	222 ± 65.6	212 ± 53.5	293 ± 26.1	225 ± 20.8	215 ± 18.5*	225 ± 27.6	215 ± 18.5*	225 ± 27.6	186 ± 8.7	186 ± 8.7	189 ± 23.3	189 ± 23.3	189 ± 23.3	298 ± 26.6	298 ± 26.6	287 ± 33.3	287 ± 33.3	287 ± 33.3	249 ± 26.4	249 ± 26.4					
Moisture [wt.%]	10.0 ± 1.1	8.6 ± 1.4	8.5 ± 1.2*	8.5 ± 1.2*	18.8 ± 1.3	16.4 ± 2.1*	16.8 ± 1.5*	21.5 ± 1.4	20.2 ± 1.1	19.2 ± 1.0*	12.8 ± 1.8	19.2 ± 1.0*	12.8 ± 1.8	11.4 ± 1.5	11.4 ± 1.5	11.4 ± 1.1	11.4 ± 1.1	11.4 ± 1.1	28.8 ± 1.7	28.8 ± 1.7	25.4 ± 3.1***	25.4 ± 3.1***	25.4 ± 3.1***	24.0 ± 2.1***	24.0 ± 2.1***					
Bulk density [g cm ⁻³]																														
TOC [t ha ⁻¹]																														
TN [t ha ⁻¹]																														
POC [t ha ⁻¹]																														
PON [t ha ⁻¹]																														
PON [t ha ⁻¹]																														

Note: Values represent means ± standard deviation. Asterisks indicate significant differences at $p \leq 0.05 = *$, $p \leq 0.01 = **$ and $p \leq 0.001 = ***$ between the diversification treatments and the control. Ctrl = control (grapevine monoculture); Ore = grapevine diversified with oregano and Thy = grapevine diversified with thyme. Uppercase letters indicate significant differences among treatments at the final sampling ($p \leq 0.05$), as determined by the Kruskal-Wallis test followed by Dunn's post hoc test with Bonferroni correction for multiple comparisons. This applies to selected parameters only.

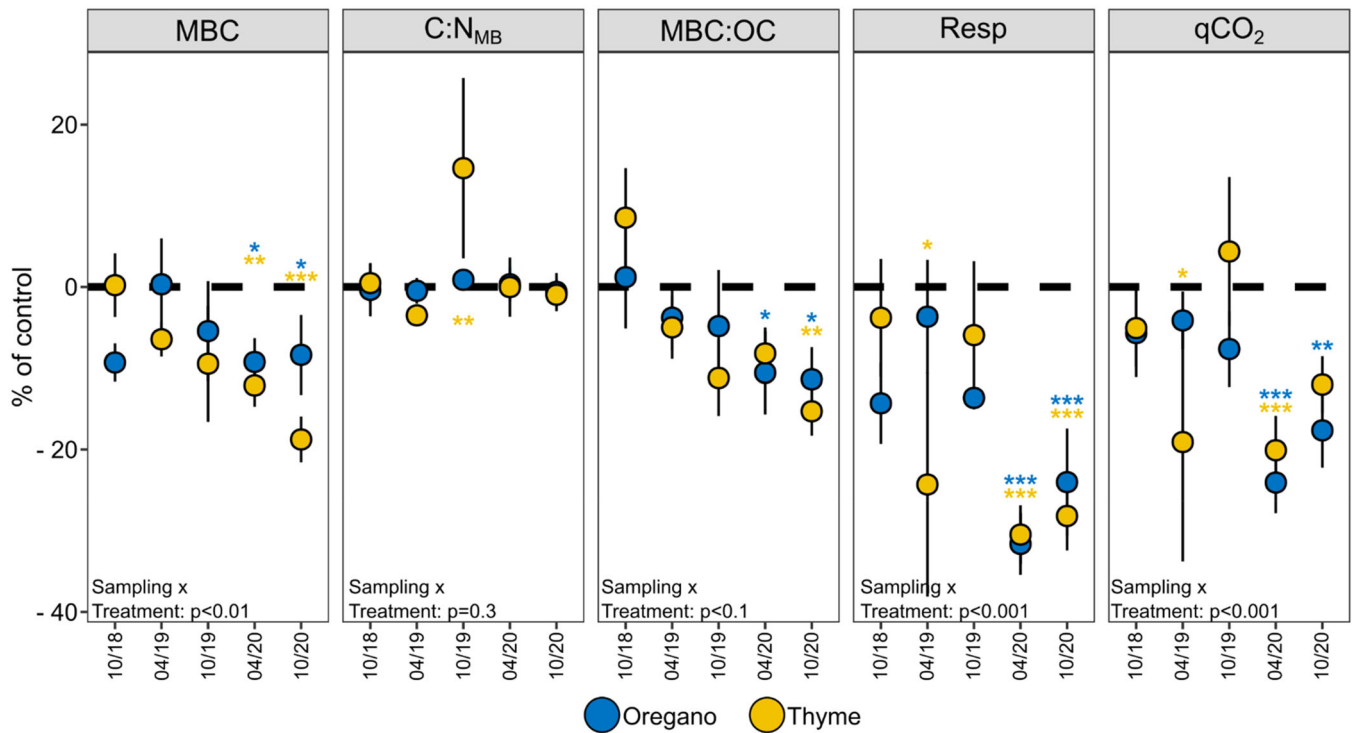


FIGURE 1 | Effect of vineyard diversification with oregano (blue) or thyme (yellow) on the relative change (% of control) of soil microbiological indices per sampling time. MBC = microbial biomass carbon, C:N_{MB} = C:N ratio of microbial biomass, MBC:OC = ratio of microbial biomass carbon to total organic carbon, Resp = respiration, qCO₂ = respiration per unit MBC (metabolic quotient). Data points represent mean values and vertical lines the standard deviation. Asterisk represent significance levels at $p \leq 0.05 = *$, $p \leq 0.01 = **$ and $p \leq 0.001 = ***$.

cases, pH values did not significantly differ between the treatments. However, in four out of five samplings, pH under the diversification treatments showed a non-significant trend toward being slightly lower than in the control, except for thyme in October 2019, where a significantly lower pH was observed. The electrical conductivity was consistently decreased under both diversification treatments. While the strength of reduction decreased over time in oregano, the strongest reduction for thyme occurred at the end of the experiment (October 2020: 168 ± 25 vs. $218 \pm 32 \mu\text{S cm}^{-1}$ in the control treatment; $p < 0.001$). Levels of NH₄-N were lower under both diversification treatments compared to the control, with stronger and significant ($p < 0.05$) reductions in three out of five samplings under oregano. By contrast, NO₃-N concentrations were significantly ($p < 0.001$) reduced only in October 2018 under both diversification treatments. During later stages, minor differences or even increased NO₃-N levels were found in most diversified samples compared to the control. Both diversifications resulted mostly in a consistent reduction of avP and avK. Gravimetric soil moisture was consistently lower under oregano and thyme throughout all samplings, with the most significant ($p < 0.001$) reduction in October 2020 (control: 28.8 ± 1.7 ; oregano: 25.4 ± 3.1 and thyme: 24.0 ± 2.1 wt.%; Table 1).

3.2 | Effects of Diversification on Microbial Biomass, Respiration and Related Quotients

Microbial properties in relation to sampling time and treatment are shown in Figure 1 (percentage change compared to control) and Supporting Information S1: Table S1 (absolute values). MBC

varied between $365.7 \pm 70.5 \mu\text{g g}^{-1}$ and $521.5 \pm 78.6 \mu\text{g g}^{-1}$. Over time, we observed a significant decrease in MBC (sampling \times treatment: $p < 0.01$), in the microbial biomass normalised to the SOM content MBC:OC (sampling \times treatment: $p < 0.1$), microbial respiration (sampling \times treatment: $p < 0.001$) and qCO₂ (sampling \times treatment: $p < 0.001$) due to diversification. This was particularly evident in the final year of the field study. The reducing effect of diversification on MBC was relatively constant under oregano (ranging between -5% and -9% compared to control), while under thyme, the reduction in MBC continuously increased over time (reaching -19% compared to control in October 2020; total: 423.7 ± 44.1 vs. 521.5 ± 78.6 in the control; $p < 0.001$). However, the microbial biomass' C:N_{MB} ratio was largely unaffected by diversification. With regard to respiration, both diversification treatments had a similar reducing impact compared to the control, ranging from -3.7% to -31.6% for oregano and from -3.8% to -30.5% for thyme.

The applied GLMM identified TN, POC, PON, EC, avK, avP and gravimetric soil moisture as most important for explaining MBC variation (Supporting Information S1: Table S5). Respiration was best explained by MBC, C:N_{POM}, NH₄-N, avK and avP.

3.3 | Effects of Diversification on Prokaryotic and Fungal Diversity, Community Structure and Composition

Prokaryotic species richness ranged from 685 ± 307 to 1018 ± 873 , while prokaryotic alpha diversity varied from 5.7 ± 0.4 to 5.9 ± 0.4 (Shannon), 162.4 ± 30.8 to 198.7 ± 58.7

(inverse Simpson), and 298.6 ± 84.3 to 387.3 ± 197.0 (Hill number) (Table 2). No statistically significant differences in prokaryotic species richness or alpha diversity were detected among treatments. Fungal species richness ranged from 34 ± 3 to 80 ± 6 , and fungal alpha diversity from 1.9 ± 0.2 to 2.6 ± 0.3 (Shannon), 4.1 ± 1.3 to 6.4 ± 2.2 (inverse Simpson), and 6.8 ± 1.3 to 13.4 ± 4.6 (Hill number) (Table 2). In some cases, the treatments showed lower fungal alpha diversity compared to the control, yet these differences were not statistically significant, except for a reduction in fungal Shannon index and Hill number ($q = 1$) between the control and oregano in October 2020 ($p < 0.05$).

The prokaryotic community (Figure 2A) was mainly composed of the phyla *Proteobacteria* (22%–26%), *Actinobacteriota* (18%–26%), *Acidobacteriota* (15%–20%), *Bacteroidota* (7%–10%), *Verrucomicrobiota* (5%–7%), *Firmicutes* (4%–5%), *Chloroflexi* (3%–4%), *Crenarchaeota* (3%–4%) and *Planctomycetota* (3%–4%). The relative abundance of *Acidobacteriota*, *Crenarchaeota* and *Verrucomicrobiota* consistently increased in both diversification treatments in 2019 and 2020 compared to the control (Figure 2A and Supporting Information S1: Table S2). The fungal community (Figure 2B) was mainly composed of the phyla *Ascomycota* (27%–54%), *Basidiomycota* (6%–11%) and *Mortierellomycota* (14%–27%). The *Ascomycota* phylum consistently showed a higher relative abundance under both diversification treatments throughout the field study. In contrast to oregano, where the relative abundance of *Ascomycota* decreased again in the final year, steady increases were observed for thyme (+4%, +46.4%, +83.3%; Figure 2B and Supporting Information S1: Table S2).

Functionality assignment of prokaryotic OTUs via FA-PROXTAX revealed an increased relative abundance of taxa involved in aerobic ammonia oxidation and nitrification under both diversification treatments in October 2019 and 2020 (Figure 2C). For the fungal OTUs, an elevated relative abundance of fungal taxa assigned to AMF was depicted for oregano and thyme in October 2019 and 2020 by FUNGuild (Figure 2D). Accordingly, the AMF biomarker 16:1 ω 5 was increased under both diversifications (Supporting Information S1: Figure S1).

Multivariate ordination analysis of the Bray-Curtis dissimilarity was applied to analyse the impact of diversification on prokaryotic (Figure 3A) and fungal (Figure 3B) community structure. A clear separation in prokaryotic community dissimilarity was only observed in 2020, when the microbial community composition under both diversifications uniformly differed from the control treatment. The differentiation of the fungal community dissimilarity appeared already in 2019, ending up in three distinct clusters corresponding to the three treatments in 2020. The differentiation in prokaryotic and fungal community dissimilarity was also confirmed by the applied ANOSIM approach. The bioenv procedure was applied to identify the subset of soil variables with maximum rank correlation with prokaryotic and fungal community dissimilarities. For the prokaryotic community, the soil variables with the closest correlation included C:N_{SOM} and avP (October 2018), C:N_{SOM}, pH and POC (October 2019), and MBC and pH (October 2020). The fungal community most strongly correlated with EC, pH and avP (October 2018), TN, PON and avK (October 2019),

TABLE 2 | Microbiological alpha diversity indices per sampling time and treatment.

Diversity index	October 2018			October 2019			October 2020		
	Ctrl	Ore	Thy	Ctrl	Ore	Thy	Ctrl	Ore	Thy
Prokaryotes (16S)									
Richness	822 ± 260 ^A	857 ± 649 ^A	737 ± 552 ^A	1018 ± 873 ^A	848 ± 686 ^A	685 ± 307 ^A	1015 ± 609 ^A	817 ± 518 ^A	981 ± 517 ^A
Shannon	5.8 ± 0.2 ^A	5.8 ± 0.4 ^A	5.7 ± 0.4 ^A	5.9 ± 0.4 ^A	5.7 ± 0.4 ^A	5.7 ± 0.3 ^A	5.8 ± 0.3 ^A	5.7 ± 0.3 ^A	5.9 ± 0.3 ^A
invSimpson	178.6 ± 46.4 ^A	197.9 ± 60.5 ^A	180.9 ± 40.8 ^A	198.7 ± 58.7 ^A	162.4 ± 30.8 ^A	164.1 ± 26.3 ^A	163.3 ± 28.8 ^A	174.2 ± 35.5 ^A	177.6 ± 20.3 ^A
Hill; $q = 1$	338.5 ± 64.1 ^A	357.8 ± 170.2 ^A	320.0 ± 137.4 ^A	387.3 ± 197.0 ^A	320.7 ± 141.2 ^A	298.6 ± 84.3 ^A	353.0 ± 122.3 ^A	327.6 ± 122.8 ^A	362.2 ± 106.6 ^A
Fungi (ITS)									
Richness	80 ± 6 ^A	78 ± 11 ^A	71 ± 9 ^A	45 ± 14 ^A	48 ± 4 ^A	34 ± 3 ^A	53 ± 3 ^A	50 ± 3 ^A	55 ± 5 ^A
Shannon	2.5 ± 0.1 ^A	2.6 ± 0.3 ^A	2.4 ± 0.2 ^A	2.1 ± 0.1 ^A	2.0 ± 0.2 ^A	1.9 ± 0.2 ^A	2.4 ± 0.2 ^A	2.0 ± 0.2 ^B	2.2 ± 0.1 ^{AB}
invSimpson	5.9 ± 0.3 ^A	6.4 ± 2.2 ^A	5.5 ± 1.0 ^A	5.0 ± 0.7 ^A	4.3 ± 1.2 ^A	4.1 ± 1.3 ^A	5.5 ± 1.1 ^A	4.3 ± 0.8 ^A	5.5 ± 0.3 ^A
Hill; $q = 1$	12.2 ± 0.9 ^A	13.4 ± 4.6 ^A	11.3 ± 2.0 ^A	8.6 ± 1.3 ^A	7.8 ± 1.6 ^A	6.8 ± 1.3 ^A	10.8 ± 1.8 ^A	7.9 ± 1.4 ^B	9.4 ± 0.8 ^{AB}

Note: Values represent means ± standard deviation. Uppercase letters indicate significant differences at $p \leq 0.05$ among the treatments. Ctrl = control (grapevine monoculture); Ore = grapevine diversified with oregano and Thy = grapevine diversified with thyme.

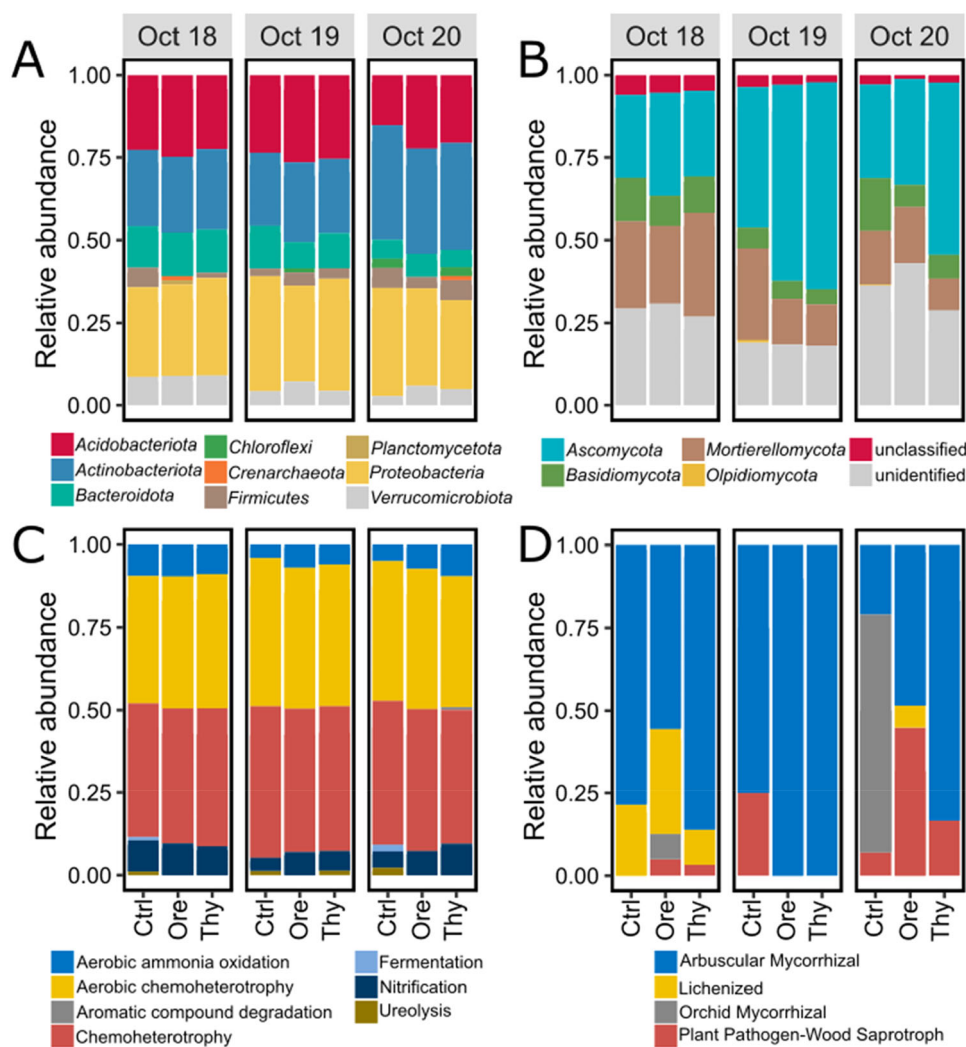


FIGURE 2 | Bar plot showing relative abundances of main phyla for prokaryotic (A, only phyla with abundance > 5%) and fungal (B, only phyla with abundance > 1%) communities, as well as the corresponding annotation of (C) ecologically relevant functions performed by prokaryotes via FAPROTAX and (D) functional guilds of fungal OTUs via FunGuild per sampling time and treatment. Ctrl = control (grapevine monoculture); Ore = grapevine diversified with oregano and Thy = grapevine diversified with thyme.

and TN, MBC, C:N_{POM}, avK, NO₃-N and gravimetric soil moisture (October 2020) (Figure 3A,B).

3.4 | Effects of Diversification on Enzyme Activity and Potential Nitrification

Results on the enzyme activities, the enzyme activities normalised to the MBC (biomass-specific enzyme activities) and potential nitrification in relation to sampling time and treatment are shown in Figure 4. The activities of enzymes involved in organic C and N cycling (BG, AG, LA and PO) generally decreased over time, resulting in most cases in lower activity in the diversified treatments compared to the control. Although less pronounced, this trend was also evident for the biomass-specific activity of BG, AG and PO. The activities of DH, AS, AP and PN showed an opposite trend, and mostly increased in the diversified treatments compared to the control, with the largest differences in the last year of the experiment. This effect was even more pronounced for the corresponding biomass-specific enzyme activities (Figure 4B).

4 | Discussion

4.1 | Aromatic Plants Alter the Soil Habitat

Aromatic plant development contributed to organic matter input into the soil, with POC contents and stocks showing a tendency to increase over time for both diversification treatments (Table 1). While overall differences among treatments were not statistically significant, POC content in October 2020 was significantly higher under oregano compared to the control ($p < 0.05$). This occurred mainly through the root biomass produced, whereas the above-ground biomass was largely harvested, and through the increased retention of grapevine litter in and around the diversified rows. Consequently, this demonstrates that increasing crop cover and diversity through the additional cultivation of aromatic plants can effectively contribute to C sequestration in vineyards. The stronger increase in carbon stocks and contents observed under oregano (compared to thyme) can be explained by the significantly higher primary productivity, i.e., higher above- and belowground biomass production of oregano. In agricultural systems, it is well known

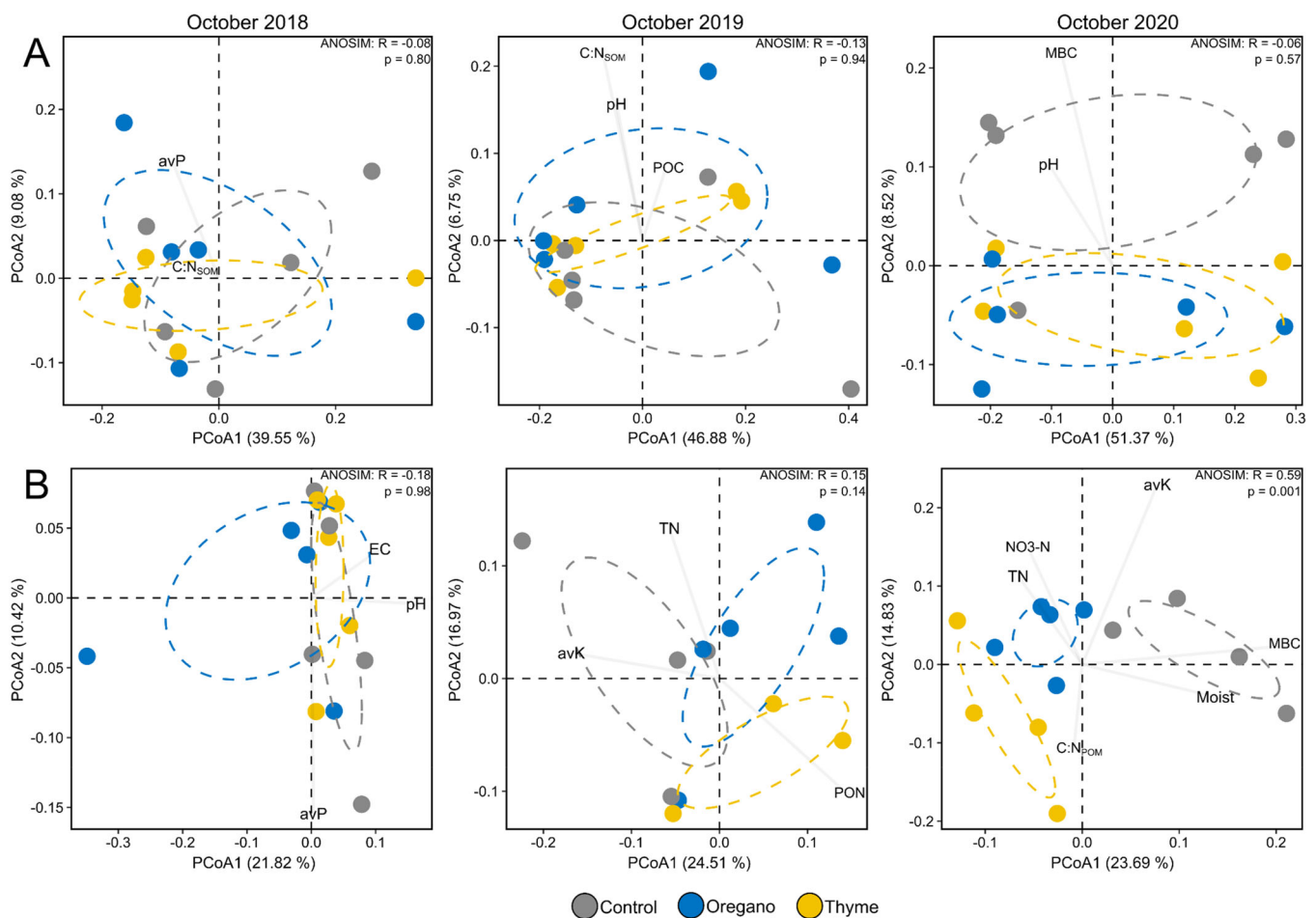


FIGURE 3 | Clustering of prokaryotic (A) and fungal (B) community structure derived from 16S and ITS amplicon sequences and based on principal coordinates analysis (PCoA) of the Bray-Curtis dissimilarity per sampling time and treatment. Control = grapevine monoculture; oregano = grapevine diversified with oregano and thyme = grapevine diversified with thyme. Included edaphic variables are significantly correlated to the community matrix and obtained from the “bioenv” analysis. The R- and p-values in the upper-right corner, obtained from the analysis of similarity (ANOSIM), indicate community similarity for low R values, while higher values reflect stronger dispersion and greater dissimilarity of the microbial community.

that carbon and nitrogen inputs originate from plant shoot and root residues entering the soil ecosystem (Loveland 2003; Castellano et al. 2015; Chenu et al. 2019; Poelplau et al. 2021). Additionally, we observed that aromatic plants may also indirectly impact vineyard soil C and N budgets via acting as a physical barrier, which captures and retains a part of the dropped grapevine leaves in the end of the crop cycle. We estimated an extra input of 137–158 kg C ha⁻¹ and 3.5–4.1 kg N ha⁻¹ via this mechanism, which most likely contributed to the increasing trend in POC stocks in the diversification treatments. It can be expected that the trend towards increasing POC stocks and contents leads to an increase in substrate available for microbial growth (Cleveland et al. 2014; Witzgall et al. 2021).

However, aromatic plant growth went along with consistent reductions of EC, NH₄-N, avP and avK under both diversification treatments. Due to the relatively low mobility of NH₄-N, avP and avK (Marschner 1995), this impact was expected and points to the well-known fact that the observed growth of oregano and thyme plants is inevitably linked to nutrient uptake from soil, which is particularly strong in the rhizosphere

(Marschner et al. 2012; Kuzyakov and Blagodatskaya 2015). In addition, cation uptake is often associated with the release of protons (Custos et al. 2020), which coincided with the slightly lower soil pH values determined under both diversification plants. In contrast to the other nutrients, the concentration of NO₃-N was even partially elevated under diversification. The absence of NO₃-N depletion around roots has been previously reported, e.g. by Tinker and Nye (2000), and is explained by the high mobility of NO₃-N in soil so that concentration gradients do not form. Altogether, the observed impacts on soil nutrient levels (and to a lesser extent on pH value) clearly suggest a significant rhizosphere effect. It should be noted that by sampling the soil at a distance of < 10 cm from the plant row, the rooted soil was sampled. Being aware that rooted soil and rhizosphere soil are not identical (Kuzyakov and Razavi 2019), we assume that our sampling method has significantly included the rhizosphere, where a considerable interaction between aromatic plant roots and the soil microbiota is expected. Overall, the effects of the additional aromatic plants on the abiotic properties of the soil habitat are double-edged. On the one hand, the trend toward increased POC content and stocks suggests an enrichment of particulate organic matter, a key soil component

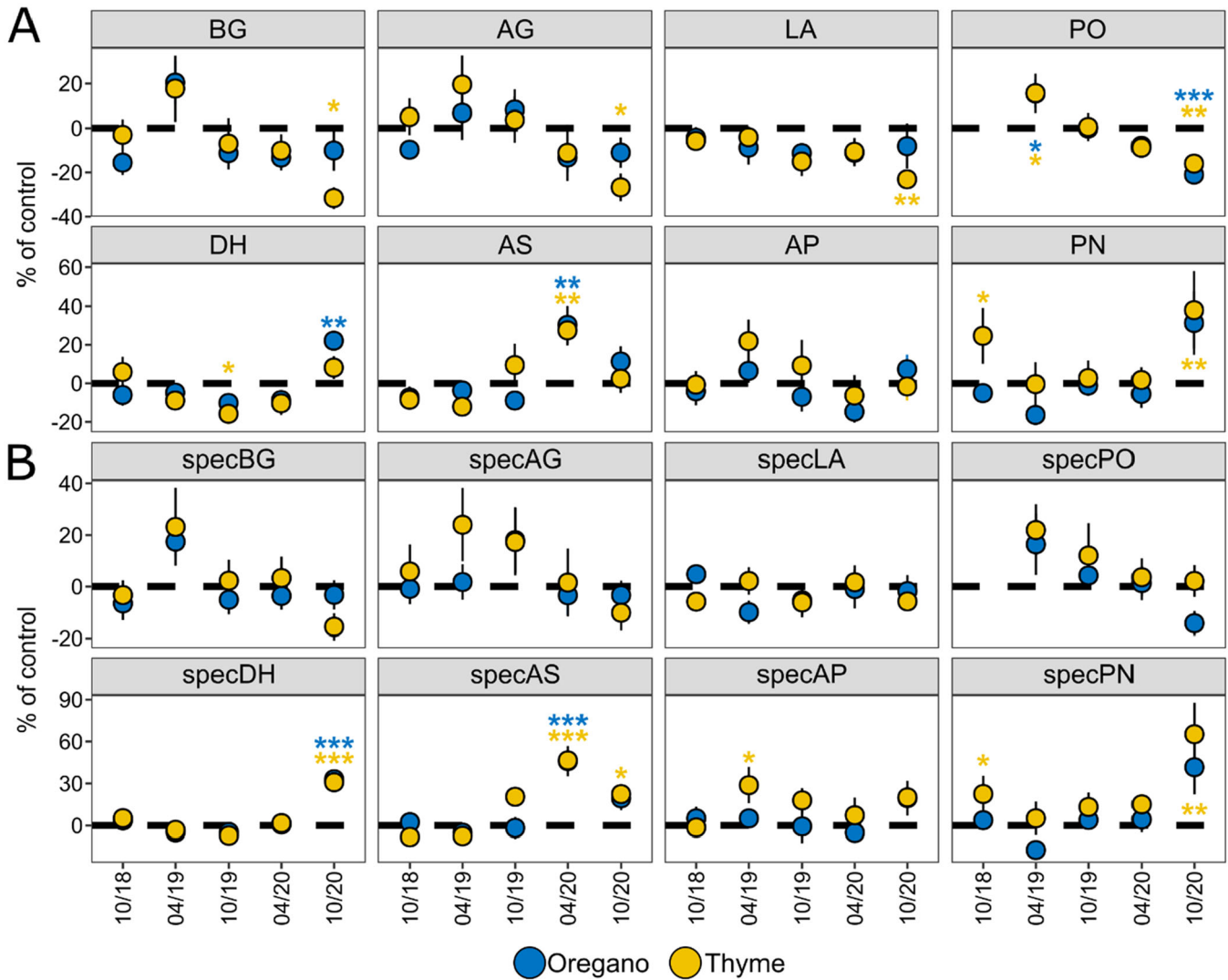


FIGURE 4 | Effect of vineyard diversification with oregano (blue) or thyme (yellow) on the relative change (% of control) of total (A) and biomass-specific (B) biological activities as compared to the control over time. BG = β -D-glucosidase, AG = N-acetyl-glucosaminidase, LA = leucine-aminopeptidase, PO = peroxidase, DH = dehydrogenase, AS = aryl-sulfatase, AP = acid phosphomonoesterase and PN = potential nitrification. Data points represent mean values and vertical lines the standard deviation. Asterisk represent significance levels at $p \leq 0.05$ = *, $p \leq 0.01$ = ** and $p \leq 0.001$ = ***. Activity of PO was not determined in 10/2018.

used by microbiota (Witzgall et al. 2021). On the other hand, the observed reductions in mineral nutrients and water may imply root-microbiota competition for these resources. In combination, these root-associated changes may also contribute to greater soil habitat heterogeneity, likely by promoting spatial variability in organic matter distribution, nutrient availability, and physical properties in the rhizosphere (Nunan 2017; Tecon and Or 2017). Such heterogeneity could, in turn, positively influence soil microbial diversity by creating distinct microhabitats and enhancing the conditions for different microbial communities to thrive (Chalmandrier et al. 2019; Signorini et al. 2021).

4.2 | Impact on the Microbial Biomass

MBC and respiration were significantly reduced under both diversifications (Figure 1 and Supporting Information S1: Table S1). Since the absolute values of both parameters in our

study, ranging on average from 365.7 to $521.5 \mu\text{g g}^{-1}$ for MBC and from 0.41 to $0.91 \mu\text{g CO}_2\text{-C g}^{-1} \times \text{h}^{-1}$ for respiration, clearly exceeded mean values of $325 \pm 159 \mu\text{g g}^{-1}$ and $0.29 \pm 0.11 \mu\text{g CO}_2\text{-C g}^{-1} \times \text{h}^{-1}$ reported for various agricultural soils derived from the same parent material in the same region (Ortner et al. 2022), we do not consider this reduction to be particularly negative. The observed MBC variations were best explained by the parameters TN, POC, PON, EC, avK, avP and gravimetric soil moisture, as indicated by the GLMM applied (Table S5), pointing to the aforementioned interplay of microorganism-promoting and inhibiting changes the soils with plant diversification. Hence, the consistent reduction of EC, avK, avP and gravimetric soil moisture under oregano and thyme had a particularly strong impact on the soil microbial community through root-microbe competition for those resources. Although the rhizosphere usually exhibits increased microbial abundance resulting from the input of labile C (Kuz'yakov and Blagodatskaya 2015), the concomitant root nutrient uptake can also reduce microbial growth (Bonkowski et al. 2000).

Therefore, we suggest that aromatic plants have outcompeted some of the microbial population.

From the higher primary productivity of oregano, one could expect a stronger nutrient reduction and hence, root-microbe competition under oregano. On the other hand, a higher primary productivity may also allocate more easily available C into the rhizosphere (Kuzyakov and Domanski 2000). In fact, MBC reduction was less pronounced under oregano (−5% to −9%), whereas it steadily increased under thyme (reaching −19% in October 2020). This suggests that microbiota were less limited under oregano, either via a higher availability of easily available C or mineral nutrients, when compared to thyme.

At the plant level, only a few carbon- and nitrogen-related traits differed notably between oregano and thyme, while others showed minimal or inconsistent variation (Supporting Information S1: Table S3). Although rather unlikely, we cannot rule out that these differences also have contributed to the stronger MBC reduction under thyme, as for example, higher C:N ratios typically go along with a lower biodegradability of litter (Chomel et al. 2015, 2016). However, the continuously progressive reduction of MBC under thyme over time could also be due to an increasing accumulation of inhibitory compounds, such as terpenes released from the aromatic plants into the rhizosphere and soil (Adamczyk et al. 2015). A particularly strong inhibition of terpenes derived from *Thymus capitatus* on soil microbial biomass and respiration was observed by Jouini et al. (2020). Investigating dose-related inhibitory effects of thymol and carvacrol as typical representatives of thyme and oregano-derived terpenes in the vineyard soil, we could show that several of the selected soil microbial enzyme activities were negatively affected by the compounds. However, in a concentration range of around 1–14 $\mu\text{g g}^{-1}$ that is typical for soils with aromatic plants (Asensio et al. 2008), only the activities of two out of five enzymes (i.e., DH and AG) were significantly reduced by approximately 10% (Thiele-Bruhn et al. 2024).

4.3 | Impact on Microbial Community Diversity and Composition

In general, the prokaryote and fungal alpha diversity indices were comparable to those reported in other studies of vineyard soils worldwide (Coller et al. 2019; Gobbi et al. 2022). While significant differences among the treatments were rare, the significantly lower fungal Shannon index and Hill number under oregano (compared to control) in October 2020 (Table 2) align with previous studies that have found reduced microbial alpha diversity in the rhizosphere compared to bulk soil (Mendes et al. 2013; Philippot et al. 2013; Idbella et al. 2024). In contrast to the control treatment, in which the soil under the grapevines was managed by mechanical tillage, the treatments with thyme and oregano developed a dense root system in the soil. The findings on alpha diversity in the context of rooted soil indicate that the microbiota of the rhizosphere, in contrast to those in non-rooted soil, are confronted with altered biotic and abiotic properties, i.e. competition for water and nutrients by the roots as well as gradients in pH, nutrients and substrates (Ling et al. 2022).

Multivariate analysis also revealed that differences in community composition between treatments increased over the years, going along with the growth of thyme and oregano (Dittrich et al. 2021), although clearly different microbial communities were only observed in 2020 (Figure 4). Combined with the results on species richness discussed above, this demonstrates that diversification-induced heterogeneity does not necessarily lead to an increase in species richness or alpha diversity, but instead has a clear impact on microbial community composition. This finding aligns with another study showing that soil heterogeneity within a vineyard affects community composition without necessarily influencing microbial alpha diversity (Signorini et al. 2021). In the field experiment, the fungal community responded more distinctly towards the applied treatments, forming three clusters, while the prokaryotic community of both diversifications formed a uniform cluster distinct from the control treatment. This indicates that while the prokaryotic community is affected by the abiotic habitat properties of the rhizosphere, the fungal community even shows a more nuanced, plant-specific interaction and change in community structure. Such a differentiated response of fungi and prokaryotes has also been observed in other studies and may be explained by a closer biotic linkage of fungi to plant roots compared to prokaryotes (Peay et al. 2013; Mommer et al. 2018; Gan et al. 2022; Labouyrie et al. 2023).

In this context, the assignment of fungal OTUs by FUNGuild revealed a consistent enrichment of AMF abundance under diversification (Figure 2). This was also confirmed by means of neutral lipid fatty acid analysis (Supporting Information S1: Figure S1). An improved establishment of AMF in agricultural systems is typically realised through increased plant cover, a lower mechanical disruption of the hyphal network by tillage, and decreases in avP (Grant et al. 2005). All these factors apply to both diversification strategies, highlighting the potential of crop diversification to enhance the abundance of AMF, which could benefit grapevine growth and stress resilience (Trouvelot et al. 2015). While AMF are known to improve nutrient supply to crops, a direct link between grapevine nutrition and AMF abundance in our study has yet to be established.

Also, the prokaryotic phyla *Acidobacteriota*, *Verrucomicrobiota* and *Crenarchaeota* were consistently enriched under both diversifications. Increases in *Acidobacteriota* and *Verrucomicrobiota* abundance have also been observed in a low-nutrient environment under conservation agriculture and may be related to the oligotrophic lifestyle of these phyla (Fierer et al. 2007; Ramirez-Villanueva et al. 2015; Schmidt et al. 2018; Liu et al. 2022). Considering this oligotrophic lifestyle, the increased relative abundance of these prokaryotic phyla implies a particular response towards the reduced mineral nutrient availability (avP, avK) determined, rather than to the increased POC level, under aromatic plants.

Regarding the archaeal phylum *Crenarchaeota*, a significantly positive response to agricultural management has been demonstrated (Bates et al. 2011; Zhalnina et al. 2013), and *Crenarchaeota* are considered as key players involved in soil nitrification (Leininger et al. 2006; Nicol and Schleper 2006). Assessing the sequencing data with the FAPROTAX functionality confirmed an increased abundance of taxa involved in

nitrification, such as the *Nitrososphaeria* class from the *Crenarchaeota* phylum. These findings and reports on *Crenarchaeota* at least coincide with the elevated $\text{NO}_3\text{-N}$ levels that were observed for numerous soil samples that were rooted by oregano or thyme. Since archaea generally grow slower compared to bacteria, they may only outcompete bacteria in environments where stress hampers bacterial growth (Valentine 2007). Therefore, the proliferation of *Crenarchaeota* under both aromatic plants may probably result from a decreased competition with other bacteria and could point to a partial inhibition of the bacteria, maybe by the antibacterial terpenes (Adamczyk et al. 2015; Thiele-Bruhn et al. 2024) released by the aromatic plants into the soil (Asensio et al. 2008). The takeover of microbial functions by archaea from inhibited bacteria has been previously shown (Kleineidam et al. 2010).

4.4 | Impact on Microbiological Activities

The changes in microbial biomass, diversity and community composition went along with differing rates of microbial respiration and enzyme activities in the different treatments (Figures 1 and 4). The significant reductions of the metabolic quotient ($q\text{CO}_2$), i.e. a lower release of CO_2 per unit MBC indicate a lower C mineralisation compared to the control. The metabolic quotient is generally negatively related to the C use efficiency of microbes (Ye et al. 2020) and considered critical for soil C storage (Anderson and Domsch 1993; Xu et al. 2017; Risch et al. 2023). Therefore, we assume an improved C use efficiency by microbes under aromatic plants, which will have contributed to the increased C sequestration. However, within a duration of the field study of only 3 years, this contribution will not have reached a measurable or even significant level.

A lower C mineralisation following diversification was also indicated by a lower activity of extracellular enzymes involved in organic C cycling (BG, AG and PO). These are typically positively correlated with total C contents, and released to depolymerise more recalcitrant biopolymers, such as cellulose (BG), chitin (AG) and lignin (PO) (Gooday 1990; Pérez et al. 2002; Sinsabaugh 2010; Burns et al. 2013). The activity of these enzymes were found to be in close relation to MBC in our study, so that the lower MBC in soils under diversification apparently explains our finding. However, a similar decrease in BG, AG and PO activity under diversification from early to later stages of the experiment was still evident when the data were related to MBC and expressed as biomass-specific activities. This suggests an altered substrate preference of the microbiota. This may be due to the fact, that aromatic plant roots exude low molecular weight substances, such as sugars and organic acids (Zhang et al. 2021a), which are considered to be easily available substrates and energy sources for microorganisms (Marschner et al. 2012). Therefore, it seems likely that microbial populations under oregano and thyme were less dependent on the less efficient acquisition of C and energy from biopolymers and the production of the required enzymes. The fact that AMF (which show an increased abundance under both aromatic plants) possess a decreased capability of genes encoding for plant cell wall-degrading enzymes and lack of genes encoding for BG (Frey 2019) may help explain the observed patterns in C-cycling enzymes, potentially in combination with competitive

interactions between AMF and free-living bacteria or fungi producing extracellular enzymes (Boer et al. 2005; Leifheit et al. 2015).

Correspondingly, the growth of oregano and thyme increased the activity of other enzymes, i.e. DH, AS, AP and PN. The activity of the intracellular enzyme DH is increased when low-weight molecular substances are taken up into the microbial cell and metabolised (Shukla and Varma 2011). This at the same time requires the activation of other enzymes such as AS, AP and PN to obtain other elements to keep homeostasis (Bai et al. 2021). Additionally, abiotic properties of the rooted soil habitat may have affected the enzyme activities. Multivariate statistics revealed, for example, that the PN activity was closely related to the soil pH and OM content and OM elemental composition (TOC, TN, C:N_{SOM}), suggesting that the PN activity was increased due to the accumulation of POM in soil under aromatic plants. The enzymes DH, AS, AP and PN are known to exhibit increased activity in the rhizosphere, where variations in pH, OM and mineral nutrients may alter their activity (Kuzakov and Blagodatskaya 2015). Furthermore, because AS, AP and PN are directly involved in the cycling of the nutrients SO_4^{2-} , PO_4^{3-} and $\text{NO}_3\text{-N}$, they are also highly relevant for grapevine nutrition (Keller 2015). Hence, the diversification-induced activation of these enzymes may ultimately benefit grapevine nutrition and crop growth. Correspondingly, positive effects on grapevine nutrition or at least the compensation of negative effects due to the nutrient competition between grapevines and the aromatic plants were reported in our previous study (Dittrich et al. 2021).

5 | Conclusions

In this study, we investigated the impact of vineyard diversification through the introduction of two aromatic plant species, oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*), on soil microbial and abiotic properties. Our findings demonstrate that integrating these aromatic plants into a perennial vineyard ecosystem influences the soil environment and microbial communities in multiple ways. The introduction of aromatic plants led to a discontinuous reduction in nutrients and pH compared to the control, suggesting that the soil system was able to buffer these plant-induced chemical changes, especially near the root zones. We propose that the observed inputs of organic matter due to aromatic plant diversification may further enhance the soil's buffering and storage capacity, which could have additional benefits, e.g. for pollutant retention. Our results indicate that particulate OM is particularly sensitive to crop diversification, showing a noticeable increase under grapevines. This elevation of particulate OM likely promotes soil aggregation and erosion control. Furthermore, the aromatic plants effectively retained grapevine litter via their shoots, suggesting an overlooked but significant pathway through which diversification can enhance soil carbon content. We observed that the impact on soil microbial biomass varied by plant species, with thyme causing a more pronounced reduction. This suggests that competition for soil resources between aromatic plants and microorganisms may be intensified by thyme but mitigated by oregano, potentially due to plant-specific alterations to the soil environment or the release of toxic terpenes by the roots. In

general, the decrease in microbial biomass caused by the aromatic plants may represent an allelopathic strategy to improve growth conditions for both themselves and the grapevines in resource-limited soils. Future research on the allelopathic activity of aromatic plants could provide insights into their potential to suppress undesirable weed growth. The strong influence of microbial biomass on broad-spectrum SOM transformation activities (e.g., microbial respiration, BG, AG, PO) suggests that these processes are widespread among soil microorganisms. However, enzyme activities related to the conversion of the nutrients S, P and N were less dependent on microbial biomass, suggesting that they are more dependent on the specific community composition. The results also suggest that while diversification-induced soil heterogeneity does not affect alpha diversity, it clearly impacts microbial community composition. Further long-term studies are needed to elucidate how vineyard diversification influences plant-soil-microbe interactions over time, particularly the observed promotion of symbiotic and oligotrophic species. Understanding these dynamics will be crucial for assessing the effects on soil and grapevine health.

Author Contributions

Conceptualisation: Felix Dittrich. Methodology: Felix Dittrich. Software: Felix Dittrich, Luigi Orrù and Bei Liu. Formal analysis: Felix Dittrich, Luigi Orrù and Bei Liu. Investigation: Felix Dittrich. Data curation: Felix Dittrich, Luigi Orrù and Bei Liu. Writing – original draft: Felix Dittrich. Writing – review and editing: Felix Dittrich, Loredana Confara, Orrù, Bei Liu, Christoph C. Tebbe and Sören Thiele-Bruhn. Visualisation: Felix Dittrich. Supervision: Felix Dittrich and Sören Thiele-Bruhn. Project administration: Felix Dittrich and Sören Thiele-Bruhn. Funding acquisition: Sören Thiele-Bruhn.

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Ethics Statement

The authors confirm that they have adhered to the ethical policies of the journal.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data will be made available under [10.5281/zenodo.6906538](https://doi.org/10.5281/zenodo.6906538) on October 31, 2025.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.