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Microbial inoculation shapes local and systemic grapevine microbiota and wine metabolites across ages and managements

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ABSTRACT

Given the established role of soil microbiomes in shaping plant traits, we hypothesized that alterations in rhizosphere microbial communities would impact grape berry microbiota and wine metabolite profiles along a controlled production chain. In this study, we investigated how a soil-applied bioinoculum influences root- and grape berry-associated prokaryotic and fungal communities and the chemical composition of wine. In a field study, a commercial bioinoculum was applied to grapevines in two vineyards located in the same site but differing in age and management practices. Over two growing seasons, we characterized bulk soil, rhizosphere, root, and grape berry microbiomes, analyzed the leaf ionome and the chemical composition of the resulting must and wines. Our results revealed that bioinoculum shaped the fungal community with a limited impact on the prokaryotic community and led to an increased abundance of plant growth-promoting microbes in the root endosphere. Integrated bioinformatic analyses revealed that bioinoculum treatment systemically altered berry-associated microbial communities, with downstream effects on must and wine metabolic composition. Notably, wines from treated plants exhibited higher acidity and polyphenol content. These results highlight that belowground microbiomes influence grape and wine metabolite profiles and underscore the potential of microbial inoculants to modulate wine quality.

1. Introduction

Grapevine (*Vitis vinifera* L.) is a perennial woody species and one of the most socio-economically important fruit crops cultivated worldwide, playing a central role in the economic stability of several countries (Legesse et al., 2025). Despite its economic importance, grapevine production is increasingly challenged by biotic and abiotic stressors, including nutrient and water limitations and pathogen diseases, which can reduce yield and compromise berry quality (Peng et al., 2023). Moreover, vineyards are highly climate-sensitive, and climate change, especially in Mediterranean regions, is predicted to exacerbate heat, water, and salinity stress, further affecting growth, yield, and wine quality (Sgubin et al., 2023; Bécart et al., 2022; Xyrafis et al., 2022; Yang et al., 2022).

To mitigate these stresses, viticulture relies on integrated management strategies, including grafting onto resistant rootstocks, targeted breeding, and timely application of chemicals (Williams et al., 2021; Chen et al., 2020; Bove et al., 2019; Schneider et al., 2019; Peressotti et al., 2010). Concurrently, the use of beneficial microorganisms, such as plant growth-promoting (PGP) bacteria and biological control agents, is

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emerging as a promising approach to enhance plant health and reduce chemical inputs, although their efficacy remains highly variable and context-dependent (Altieri et al., 2023).

Growing evidence indicates that the soil acts as a microbial reservoir for the plant-associated microbial communities which play critical roles in nutrient cycling, disease suppression, and overall plant fitness. In grapevine, the root-associated microbiome has gained particular attention for its potential influence, not only on plant performance, but also on wine quality (Mercanti et al., 2024; Zhou et al., 2021). Indeed, in grapevine, root associated microbial communities are included as emergent component of the wine *terroir*, which could influence grape berry composition and contribute to valuable traits valued for winemaking, including organoleptic complexity, influencing nutrient dynamics, and fermentation processes, linking them directly to wine quality and sensory attributes (Cobos et al., 2022; White, 2020; Liu et al., 2019; Vitulo et al., 2019; Zarraonaindia et al., 2015). This emergent paradigm of 'microbial terroir' has remarkable interest in microbiome engineering as a tool for modulating both plant performance and product identity (Gilbert et al., 2014).

An increasing body of evidence showed that microbial communities of grapevines are shaped by plant compartment (*e.g.,* rhizosphere, phyllosphere, carposphere), vineyard management practices, and environmental context (Coller et al., 2019). Notably, organic vineyard systems have been reported to harbour higher microbial diversity across root and aerial tissues compared to conventionally managed systems (Fournier et al., 2022). Despite extensive field-based studies, the composition and ecological functions of grapevine-associated microbiomes remain incompletely understood, particularly regarding their response to stress conditions. Moreover, the dynamics and cultivar-specific nature of microbial assemblages complicate efforts to define a core microbiome, limiting the capacity to design universally effective microbiome-based bioinoculants.

To address these knowledge gaps, Legesse et al. (2025) performed a comprehensive meta-analysis of grapevine-associated microbiomes spanning diverse cultivars and ecosystems. The analysis revealed a recurrent dominance of Actinobacteria and Proteobacteria, two bacterial phyla widely acknowledged for their ecological adaptability and diverse plant growth-promoting functions (Boukhatem et al., 2022; Bruto et al., 2014). These findings support the potential of these groups in the development of microbiome-informed strategies for sustainable viticulture.

The application of microbial biostimulants is gaining ground in agriculture as a means of promoting sustainability. Inoculation with PGP bacteria and arbuscular mycorrhizal (AM) fungi has been shown to improve plant nutrition and stress tolerance (Fiorilli et al., 2024; Singh et al., 2024). Viticulture is increasingly adopting these strategies to enhance grapevine health, improving nutritional and nutraceutical value of wine and reducing chemical inputs (Cataldo et al., 2022; Gabriele et al., 2016; Torres et al., 2016). These inoculants commonly include bacteria from the genera *Bacillus*, Pseudomonas, and Streptomyces, alongside beneficial fungi such as *Trichoderma* spp. and AM fungi (Langa-Lomba et al., 2023). Notably, AM fungi, key symbionts in terrestrial ecosystems that enhance plant mineral nutrient uptake in exchange for host-derived carbon (Bonfante and Genre, 2010), are commonly associated with grapevine roots (Trouvelot et al., 2015; Balestrini et al., 2010).

However, the persistence of bioinoculants, their integration into native soil-root microbial networks, and their downstream effects on grape and wine microbiota remain largely unexplored. In this study, we investigated the impact of a commercial microbial bioinoculant on grapevine-associated microbiota and the composition of wine metabolites from two vineyards (*cv*. Pigato), located at the same site but differing in plant age and management. In this work, we aimed to investigate whether the bioinoculum application impacts soil and root and grape berry microbiota, with a final impact on must and wine metabolome profiles.

2. Materials and methods

2.1. Experimental set-up and sample collection

The sampling sites were identified in two vineyards located in Albenga (Savona, Liguria, Italy). The two investigated fields were 50 m apart with same south-facing exposure and climate but of two different ages, 15-year-old (VO) and 2-year-old (VN) (VO - 44°04'33.7" N, 8°12'06.5" E; VN - 44°04'29.8" N, 8°12'13.2" E) (Fig. S1).

Agronomic management differed between the two vineyards: VO was cultivated using conventional farming methods, while in the newly planted vineyards (VN) plants were already inoculated with a commercial inoculum (MICOSAT F® VITE) at time of planting. Besides this initial treatment farming practices were the same in both sites. For each vineyard, a total of 200 grape plants (*Vitis vinifera* var. Pigato VCR 370, rootstock 110 R VCR 114 LU) were examined, where 100 plants were treated with commercial bioinoculum (MICOSAT F® VITE) (treated) and 100 plants non-treated (control).

The commercial bioinoculum used was MICOSAT F® VITE (CCS, Aosta, Italy), which contains (as stated on the producer's website https://www.micosat.it/prodotto/micosat-vite/, last accessed 30/07/2025), the following: *Trichoderma viride*, *T. harzianum*, *Pochonia chlamidosporia*, *Streptomyces spp. ST60*, *Streptomyces spp. SB14*, *Streptomyces spp. SA51*, *Bacillus subtilis BA41*, *Pseudomonas fluorescens PN53*, *Pseudomonas spp. PT65*, *Glomus spp. GB67*, *G. mosseae GP11*, *Glomus viscosum GC41* in the percentage of 40% crude inoculum (AM fungi), and 21.6% bacteria and saprotrophic fungi.

In the VO-treated and VN-treated vineyards, plants were inoculated twice annually, in March and July of both 2022 and 2023 (Fig. S2). At each application, 10 g of microbial inoculum were placed 30 cm below the soil surface, adjacent to the root zone. Root and soil samples were collected both years (2022 and 2023) directly in the field at the end of June (BBCH stage 7, flowering) and October (BBCH stage 9, ripening) (Fig. S2). For each selected plant, roots and surrounding bulk soil were excavated using a shovel, and subsamples were aseptically transferred into 50 ml Falcon tubes using a sterile scalpel. For each sample type (root and soil), five biological replicates per condition (vineyard, treatment, and phenological stage) were collected, with each replicate consisting of a pooled sample from three individual plants located at least 4 m from the vineyard edge (Fig. S2). Following collection, samples were temporarily stored in a refrigerated container, transported to the laboratory on the same day, and kept overnight at 4 °C.

Root-associated compartments, namely, the rhizosphere and root endosphere, were separated using a modified version of the protocol described by Bulgarelli et al., (2012). All washing steps were performed under sterile conditions using PBS-T buffer (130 mM NaCl, 7 mM $Na_2HPO_4{\cdot}12H_2O$, 3 mM NaH_2PO_4, 0.02% Tween 80). Processed root and rhizosphere samples were subsequently stored at –80 °C until DNA extraction. Vineyard soils (VO and VN) were also collected to perform physico-chemical analysis (Fig. S2).

2.2. DNA extraction from roots, soil, rhizosphere and grapes

After root compartment separation, roots were freeze-dried for 24 h and ground in liquid nitrogen using a Tissuelyzer instrument (QIAGEN). DNA was extracted from roots and soil/rhizosphere/bioinoculum slurry using the NucleoSpin Plant II Mini and the NucleoSpin Soil Mini kit (Macherey-Nagel, Düren, Germany), respectively, following manufacturer's instructions. DNA quantity and purity were assessed using a Nanodrop-1000 instrument (Thermo Scientific, Wilmington, Germany) and samples stored at –20 °C. DNA samples were adjusted to a final concentration of 10 ng/µl and sent to IGA Technology Services (Udine, Italy; http://igatechnology.com/) for marker gene amplification and sequencing. For Prokaryotic communities profiling the V4 16S region was targeted using primers pairs 515 F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806 R (5'-GACTACNVGGGTWTCTAAT-

3') (Parada et al., 2016; Apprill et al., 2015). For fungi, the ITS2 region was adopted as marker using primers pair fTIS7 (5'-GTGARTCATC-GAATCTTTG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Ihrmark et al., 2012).

To profile the grape carposphere microbiota, 400 g of grape berries were ground and centrifuged at 9000 rpm for 10 min, rinsed twice with EDTA 50 mM and frozen at –20 °C until DNA extraction. A FastPrep-24 instrument (MP Biomedicals, Illkirch, France) was used for DNA extraction: 200 µl of glass beads (acid-washed, Ø 0.1 mm, Sigma, Lyon, France) and 1 ml of EDTA 0.05 M were added to the frozen pellet. The protocol described by Zott et al., (2010) was followed until complete extraction. DNA was stored at −20 °C. FR1 and FF390 primers were used to target eukaryotic 18S rDNA (FF390, 5'-CGATAACGAACGAGACCT-3'; FR1, 5'-AICCATTCAATCGGTAIT-3'), while 515 F and 806 R were used to target prokaryotes.

First, PCR amplifications were performed by appending to primers the Illumina overhang sequences with the following thermal protocol: 3 min at 95 °C, 35 cycles at 98 °C for 30 s, 52 °C for 30 s (annealing) and 72 °C for 60 s, and a final extension of 8 min at 72 °C. The 2X KAPA HiFi HotStart Ready Mix (Roche, Bâle, Suisse) kit was used, setting reactions in a final volume of 25 µL and using 2.5 µL of diluted DNA template (5 ng/µL). After amplification, libraries were constructed by adding Illumina sequencing adapters with the Nextera®XT Index Kit at the Genome Transcriptome Platform of Bordeaux (Bordeaux, France). Libraries were sequenced on a MiSeq Instrument (Illumina, CA, USA) with a 2 × 300 bp sequencing layout.

2.3. Bioinformatic analysis

Amplicon libraries were inspected for quality using FastQC v0.11.9 and multiQC v1.11 software and raw reads imported into QIIME 2 (Quantitative Insights Into Microbial Ecology) v2023.9 (Bolyen et al., 2019) for denoising, Amplicon Sequence Variants (ASVs) detection and taxonomy mapping. First, primers were fully removed from reads using the cutadapt 'trim-paired' plugin discarding untrimmed sequences. For ITS2 libraries the full-length ITS2 region was selected using ITSxpress plugin with the built-in fungal database to increase taxonomic resolution (Tedersoo et al., 2022). Clean reads were denoised and merged into ASVs using DADA2 plugin (Callahan et al., 2016) in 'pooled' chimera method detection and applying a reads truncation of 176 and 174 bp based on quality profiles for R1 and R2 sequences, respectively. No reads truncation was applied for ITS2 libraries (–p-trunc-len 0). Variants were then taxonomically annotated using a Naïve-Bayes classifier (Bokulich et al., 2018) using the 'feature-classifier classify-sklearn' plugin. The SILVA v138 database (99% clustering) (Quast et al., 2013) pre-formatted for QIIME and the UNITE + INSDC v10 database in dynamic mode (Nilsson et al., 2019) were used as reference databases for 16S and ITS2 libraries, respectively. Tables were further taxonomy-filtered to obtain the final feature table analyzed. For the 16S dataset, ASVs matching organellar (mitochondria and chloroplast) rRNA, or without any match (unassigned at the domain level) were removed while for ITS2 libraries non-fungal sequences were discarded. The obtained feature tables were imported into R v4.2.1 environment (R Core Team, 2024). α- and β-diversity analyses were performed using 'phyloseq' v1.40.0 (McMurdie and Holmes, 2013), 'vegan' v2.6–2 (Oksanen et al., 2025), and 'QsRutils' v0.1.5. The ASVs count table was first filtered by removing low-abundance ASVs using 'HTSFilter' v1.36.0 (Rau et al., 2013) and then normalized with a rarefaction-free approach using DEseq2 v1.36.0 (Love et al., 2014) for β-diversity and differential abundance analysis.

PERMANOVA and pairwise PERMANOVA analyses were performed using the adonis function of the R package 'vegan' and the package 'pairwiseAdonis' v0.4.161, respectively. Principal coordinate analysis (PCoA) was performed by multidimensional scaling (MDS) of Bray–Curtis distance matrices using the 'cmdscale' R function. Compartment enrichment and differential abundance analyses were performed using DESeq2 package applying a zero-tolerant geometric mean formula, as detailed in phyloseq package vignettes, and adopting an FDR threshold of 0.05 to define enriched/depleted taxa. Phylogenetic heatmaps were obtained using the ggtreeExtra R package v1.18.0 (Xu et al., 2021) keeping only highly abundant (relative abundance > 5%) and most enriched/depleted (FDR≤0.01) taxa annotated at least at family level. Briefly, ASV sequences of differentially abundant taxa were aligned using MAFFT (Katoh and Standley, 2013) (default parameters), and approximately-ML phylogenetic tree obtained with IQ-TREE (Nguyen et al., 2015) using the 'align-to-tree-mafft-iqtree workflow in QIIME2's 'q2-phylogeny' plugin.

2.4. Harvesting and vinification

During the harvest season of both years (2022 and 2023), a randomly selected subset of grapes from the 200 treated and the 200 non-treated plants in both vineyards was selected to produce must and wine for further chemical analysis (Fig. S2).

For each condition, four independent micro-vinifications were carried out in 1 L glass flasks at Azienda Lupi (Pieve di Teco, Imperia, Liguria, Italy). Grapes were hand-pressed, and the resulting must was passed through a sieve and subjected to static clarification overnight at 15 °C. The clarified must was then racked, inoculated with a commercial *Saccharomyces cerevisiae* strain (LAFFORT ZYMAFLORE® X5, 20–30 g/h), and sulphur dioxide (5 mg/L), then fermented for 10–12 days at 15–20 °C. Upon completion of fermentation, the wine was decanted and stored at 4 °C for subsequent analyses. Must was collected before alcoholic fermentation and immediately frozen at –20 °C. Obtained wine samples were then kept at 5 °C until chemical analysis. For each condition, 300 of the largest berries were selected, weighed, and used as a proxy for plant yield.

2.5. 2.5 Nuclear magnetic resonance (NMR) spectroscopy analysis and processing

The sample preparation was carried out by BTpH combined-pH titration unit by Bruker. The wines were diluted with 10% potassium-phosphate-buffer in D_2O. 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP) was used as an internal standard for referencing the chemical shift to 0 ppm. The final pH of the solutions was adjusted to 3.10 ± 0.02. For example, 900 µL of wine was mixed with 100 µL of buffer and the pH adjusted to the same pH of the wine reference, exactly 3.10 (± 0.02 pH units) with 1 M NaOH or HCl. This mixture was filtered by 0.22 µm filter and 600 µL was transferred into a 5 mm NMR tube and measured directly.

^{1}H NMR spectra were recorded on a Bruker Advance HD operating at 400.13 MHz for ^{1}H and Wine-Screener application. Acquisition of spectra was carried out with TOPSPIN software (version 3.2). The spectrometer transmitter was locked to D_2O frequency using a mixture H_2O:D_2O (9:1), and all the spectra were acquired at 300 ± 0.1 K. The ^{1}H NMR spectra were recorded with the standard pulse sequence for multiple suppression of water and ethanol (noesygpps1d.comp1 program pulse). The spectral window was 20.55 ppm, and data were collected into 64k data points after 32 scans plus 4 dummy scans. The relaxation delay (d1) was set to 4 s, and the receiver gain (RG) 16. The spectra were acquired using TOPSHIM tools and the NMR SampleTrack that allows the automatic analysis of several samples. The quantification analysis was performed by Wine-Profiling application of Bruker BioSpin GmbH & Co. KG (Version 4.0.13).

Metabolite analysis was performed by means of multivariate data analysis tools under MATLAB environment (2021a, Mathworks, Natick, MA, USA) as described in Cavallini et al., (2023), (2021). The full spectral data were explored using principal component analysis (PCA, Bro and Smilde, 2014) and were processed using multivariate curve resolution (MCR, de Juan et al., 2014) applied to manually-defined individual intervals to obtain a dataset of relative concentrations of

specific metabolites. All group-specific MCR models were fitted using the non-negativity constraint applied to both the profiles and concentrations, to ensure interpretability and adherence to the chemical phenomena underlying the signals. The resolved metabolites were tentatively identified based on personal knowledge and literature sources, as well as using the digital library of the Profiler GUI of the software Chenomx NMR Suite (version 9.02, Chenomx Inc., Edmonton, Alberta, Canada).

The raw data were aligned using *icoshift* (Savorani et al., 2010), operated both globally and on intervals, up to three consecutive times to better refine the alignment of specific signals. The raw data were initially explored to spot and correct possible quality issues and errors, such as samples whose spectral profile was clearly incoherent with the others, possibly due to acquisition errors or non-homogeneous experimental settings. Inspection of the original NMR spectra from the four vinifications revealed a high abundance of acetic acid (singlet at 2.08 ppm) and lactic acid (doublet at 1.40 ppm and quartet at 4.31 ppm), clearly indicating wine acidification processes in these samples. Consequently, these vinifications were excluded from further comparative analyses, since they were not representative of typical metabolite profiles observed in the remaining samples. The metabolomics data have been deposited to MetaboLights (Yurekten et al., 2024) repository with the study identifier MTBLS13342.

2.6. Soil analysis

Physicochemical soil parameters of six samples collected in both VN and VO vineyards, were measured according to the official Italian methods (Colombo and Miano, 2015). Briefly, the soils were dried at room temperature and the skeleton was removed by sieving soil samples to 2 mm, while the fraction < 2 mm was characterized for main physicochemical parameters. These included the soil texture, obtained by using the pipette method, carbon (C) (organic, inorganic, and total), total nitrogen (N), available (Olsen) phosphorus (P) as in Li et al. (2023). Additionally, CEC (Cation Exchange Capacity), and exchangeable potassium (K), magnesium (Mg) and calcium (Ca) were measured as detailed in Colombo and Miano (2015). The complete characterization is reported in Table S1.

2.7. Leaves nutrient content

Mineral nutrients were measured on five fully expanded leaves for each treatment. Leaves were collected in the field, frozen at –80 °C the same day, freeze dried overnight and ground to powder with mortar and pestle in liquid nitrogen (Fig. S2). Thirty mg of leaf powder were extracted in 6 ml of 67% nitric acid using a Multiwave GO Plus microwave digestion system equipped with polytetrafluoroethylene (PTFE) microwave digestion vessels (Anton Paar, Graz, Austria). Extracts were then diluted 1/100 in 18 MΩ ultrapure water and ionome profile analysed using an Agilent 7850 ICP-MS system equipped with a SP4 autosampler (Agilent Technologies Inc., Santa Clara, CA, USA). ICP-MS measurements were done in He mode (4.3 l/min) using High Matrix Introduction (HMI) mode and a 10 ppm Internal Standard mix (Agilent 5183–4681). Calibration standards were prepared for 27 elements using a multi-element calibration standard (Agilent, p/n IMS-102) plus the addition of single element standards (Agilent) for P (p/n ICP-415), S (p/n ICP-016), B (p/n ICP-005) and Mo (p/n ICP-042). All the elements were calibrated from 10 ppb to 10 ppm except for S for which 50 and 100 ppm calibration points were added. Data was acquired and exported using the 7850 Agilent ICP-MS MassHunter software.

2.8. Multivariate modeling and statistical analysis

The obtained datasets were integrated using Data Integration Analysis for Biomarker discovery using Latent Components (DIABLO, mixOmics; Singh et al., (2019), which implements a multi-block sparse partial least squares discriminant analysis (sPLS-DA). The model, which performs a supervised multivariate integrative classification, was used to identify multi-omics signatures associated with the bioinoculum treatment. The model was estimated considering microbial communities abundances (rhizosphere, root endosphere and carposphere), leaf ionome at reproductive phenological stage of the year 2023 as well as metabolome profiling of wine obtained from grapes collected at the same time point. Microbial community data from the selected time point were filtered for low-abundance taxa using HTS-filter, as previously detailed, aggregated at the genus level (removing unassigned and uncultured genera), and normalized with the centered log-ratio transformation (clr) calculated on relative abundances with the 'microbiome' R package v.1.30 (Lahti and Shetty, 2017) which accounts for compositional data by reducing interdependence between taxa reducing spurious correlations. Fungal and prokaryotic normalized microbial community data were pooled together by compartment in the same block. Leaf ionome data was $\log_{10}$-normalized and scaled while NMR metabolome data was log-10 scaled. The five blocks obtained were then used to fit a sPLS-DA model with DIABLO. A preliminary five-omics model was run using 'block.splsda()' function in mixOmics v6.32 (Rohart et al., 2017) setting the treatment factor as covariate, 'ncomp = 8, max.iter= 1000, tol= 1e-06, near.zero.var = T' and assuming 0.1 as expected similarity between blocks to maximize classification power as recommended by the mixOmics package vignettes. Number of components to be included in the final model was tuned using the perf() function setting 'validation = 'Mfold', folds = 7, nrepeat = 50' and feature selection tuning performed with tune.block.splsda() function setting 'validation = 'Mfold', folds = 5, nrepeat = 10, dist= "centroids.dist" ' testing variables in the interval between 2 and 20 at steps of 4 (for microbiota abundance data in all compartments) and in the interval between 2 and 10 at steps of 2 for leaf ionome and wine metabolome. The final model was drawn using 'block.splsda()' setting the number of selected variables and components as resulting from the previous tuning steps.

Graphical elaborations were performed using ggplot2 v3.3.6 package (Wickham, 2016). Heatmaps were plotted using ComplexHeatmap package v.2.12.1 (Gu, 2022). Plots from DIABLO analysis were obtained using mixOmics built-in graphical functions.

3. Results

To assess the impact of the application of a commercial bioinoculum composed by PGP microorganisms (prokaryotes and fungi) on grapevine soil- and root- associated microbiota, 16S and fungal ITS2 DNA metabarcoding analysis on bulk soil and grapevine rhizosphere and endosphere collected at two timepoints (flowering and ripening) in two consecutive years, 2022 and 2023, and in two different vineyards were performed. A total of 45,266,221 and 48,609,363 reads for 16S and fungal ITS2 markers were obtained, respectively. After primers removal, sequence denoising, ASV calling, and removal of non-target sequences (plant organellar DNA and non-fungal sequences), a total of 32,224,965 and 39,224,540 fragments were retained and used for subsequent analyses for 16S and ITS2 markers, respectively. A total of 26,696 16S ASV (bASVs) and 13,783 ITS2 ASVs (fASVs) were obtained, optimally covering diversity for both markers (Fig. S3, Dataset S1).

The two vineyards (VN and VO) analyzed in this study exhibited similar pH and soil texture, being located in proximity (50 m apart), with same south-facing exposure and climatic conditions (Table S1).

However, going to organic matter and nutrient presence, they exhibited some differences, likely due to their distinct agronomic managements and plant ages (Table S1). VO was managed under standard viticultural practices, whereas VN had previously been cultivated with basil, a plant known to reduce soil microbial diversity, the land was subsequently converted to vineyards and, upon planting, inoculated with a commercial bioinoculum.

More in detail, organic C and N presence were higher in the VO soil,

probably due to the stable management, while in VN soil, as basil cultivation implies tillage, ploughing and harrowing, this may have reduced the amount of organic matter in the soil over time. Basil cultivation on VN may also have implied fertilization, which could be the cause of the higher C/N values and of higher available P and K presence in VN soil.

3.1. *Bioinoculum application reshapes root-associated fungal communities with minor effects on prokaryotes*

None of the experimental factors significantly influenced fungal and prokaryotic α-diversity (Fig. S4), except for bioinoculum application, which led to increased species richness and diversity of VO rhizosphere prokaryotic community (Fig. S4b).

The major drivers of microbial communities assembly associated with grape roots were compartment, site, and phenological stage, which in this sequence, significantly shaped both fungal and prokaryotic communities (Table S2, Fig. 1a, b). Summarized results from multivariate community analysis are reported in Table 1. Interestingly, the fungal community assembly was significantly influenced by the microbial inoculum application while no effect was observed for Prokaryotes (Table S2). The root endosphere prokaryotic community was primarily composed of Alphaproteobacteria, Gammaproteobacteria, and Actinobacteria at both timepoints in both treated and control samples. In the rhizosphere, Alphaproteobacteria and Gammaproteobacteria were the most abundant classes in treated and control conditions. An increase in the relative abundance of Bacilli was observed during the flowering stage in the rhizosphere of VN control samples, while Bacteroidia increased during the ripening stage in both control and treated samples. In the rhizosphere of both treated and control conditions of VO, a higher abundance of Vicinamibacteria was detected compared to the same conditions of VN. In the soil, the bacterial relative abundance follows the same trend for both treated and control samples, with a decrease in Alphaproteobacteria compared to the other compartments. Vicinamibacteria and Nitrososphaeria were more abundant in VO, whereas other bacterial classes were more abundant in VN (Fig. 1c).

The root endospheric fungal community was dominated by Sordariomycetes (Ascomycota) with similar abundances in both vineyards and treated/control conditions at both phenological stages. Conversely, Dothideomycetes (Ascomycota) increased in treated samples in both VN and VO, Agaricomycetes (Basidiomycota) in VO control and VN treated samples, and Pezizomycetes (Ascomycota) in the control samples of the ripening stage in VN. An increase in arbuscular mycorrhizal fungi (Glomeromycotina) was also observed at the flowering stage in both treated and control samples of VN compared to other timepoints and the VO site. In the rhizosphere, Dothideomycetes and Sordariomycetes were predominant at both timepoints in both vineyards and in both treatments, with an additional increase in Tremellomycetes (Basidiomycota) in control and treated samples of VO. In the soil, the fungal community was dominated by Dothideomycetes in VN treated samples, and Mortierellomycetes (Mortierellomycotina) in control samples of both vineyards, with a noticeable increase in Tremellomycetes in control and

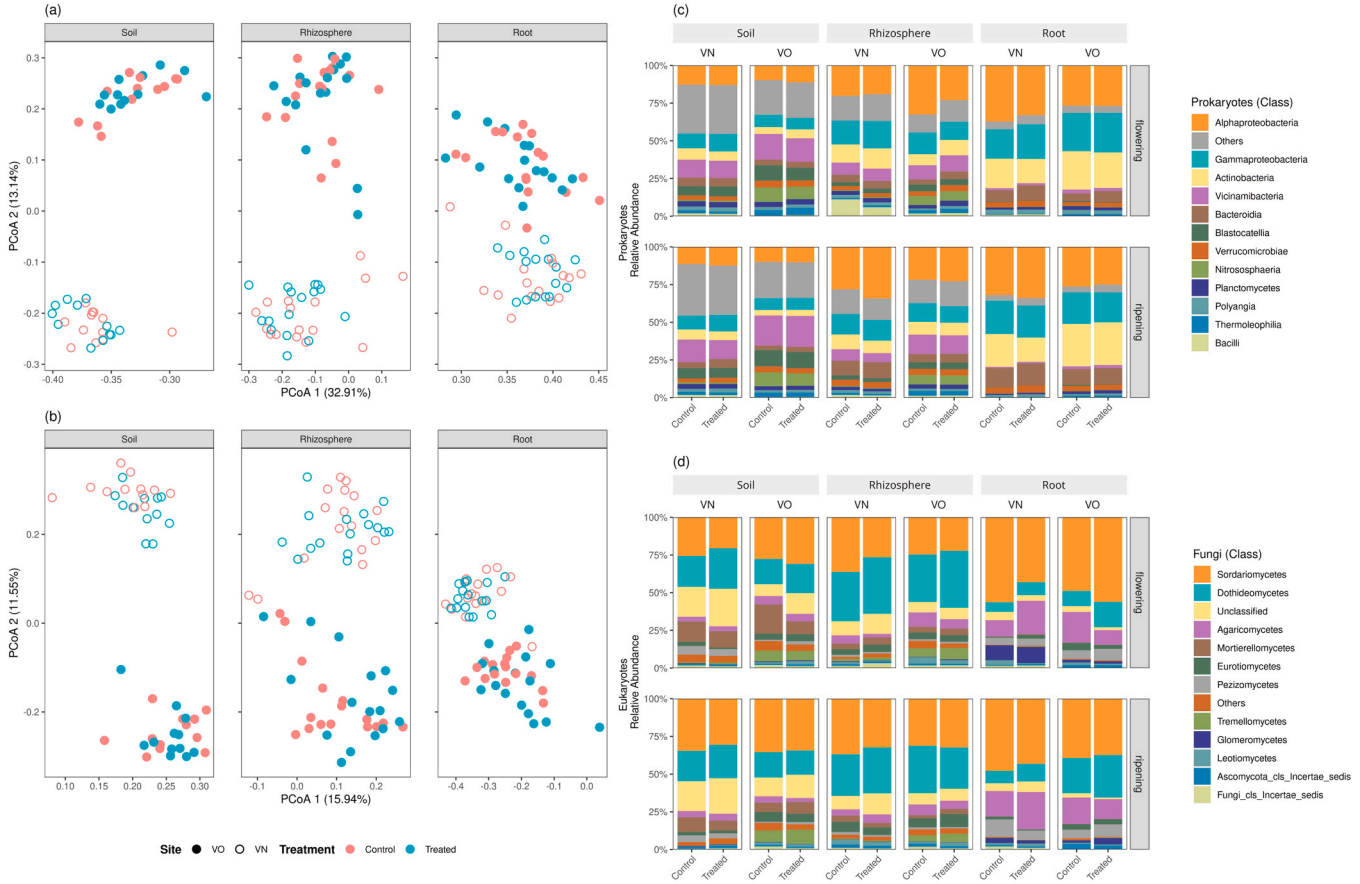

Fig. 1. Assembly and diversity of *Vitis vinifera* (Pigato) vineyard microbiota across compartments, stages, and treatment conditions. Prokaryotic and fungal communities were profiled in root endosphere, rhizosphere and bulk soil during the flowering and ripening phenological stage in two vineyards (sites), namely VO and VN, inoculated (treated) or not (control) with a commercial microbial inoculum. (a-b) Principal Coordinate Analysis (PCoA) plot of Bray–Curtis distances between samples by treatment of prokaryotic (a) and fungal communities (b) across the different compartments in both vineyards. Points represent single samples and are coloured by treatment. The fraction of the total variance explained by the projection is indicated in brackets along each axis. (c-d) Average relative abundances of prokaryotes (c) and fungi (d) at the class level.

Table 1
Summary of PERMANOVA analysis on root- and grape-associated microbial communities. Significant p-values ($p < 0.05$) are reported in bold.

Plant district	experimental factor	R^2		p-value	
		Prokaryotes	Fungi	Prokaryotes	Fungi
Root-associated	site	0.115	0.0994	**0.0001**	**0.0001**
	treatment	0.0038	0.0093	0.1684	**0.0031**
	year	0.0567	0.0406	**0.0001**	**0.0001**
grape-associated	site	0.3617	0.1583	**0.0033**	**0.043**
	treatment	0.131	0.0723	**0.0546**	0.275

treated samples of VO (Fig. 1d).

Overall, our results indicate that root-associated microbial communities were primarily structured by site, compartment, phenology, and year, while bioinoculum application selectively impacted fungal communities in both vineyards and increased rhizosphere bacterial α-diversity in VO.

3.2. Bioinoculum application increased the abundance of PGP microorganisms in the root endosphere

The analysis of differential abundance at the ASVs level (Fig. 2) revealed that the bioinoculum application had the most substantial impact on root endosphere communities in VO, as indicated by a higher number of differentially abundant taxa for both prokaryotic and fungal communities.

Venn diagrams indicate that ASVs enriched under treated conditions in both vineyards were also detected in the bioinoculum for both bacteria (Fig. S5a-c) and fungi (Fig. S5d-f) with proportion variable across vineyard and compartment.

In soil samples, taxa that belong to *Chthoniobacter* (Fig. S5a) and the order Pleosporales (Ascomycota) (Fig. S4d) were identified in the bioinoculum and were also consistently detected in treated plots of both VO and VN vineyards. In the endosphere, shared enriched taxa between the two vineyards and the bioinoculum included Vicinamibacteraceae, *Skermanella*, *Luteitalea*, Comamonadaceae, and Actinomycetospora (Fig. S5c), along with *Solicoccozyma terricola* (Basidiomycota), Didymellaceae (Ascomycota), and *Lectera longa* (Ascomycota) (Fig. S5f). In the rhizosphere, no ASVs were shared between vineyards and the bioinoculum (Fig. S5b,e), suggesting that the inoculum components recruited in the two vineyards are different, and that the bioinoculum mainly influenced the root endosphere and soil compartments, leading to a differential recruitment across plant compartments.

Bioinoculum application led to a significant increase of *Pseudomonas* spp. and *Skermanella* spp. in the root endosphere of both VN and VO treated vineyards (Fig. 2a), which also occurred in the bioinoculum formulation. In contrast, members of Vicinamibacteraceae (Acidobacteriota) and WD2010 soil group (Planctomycetes) are consistently reduced in the endosphere of both vineyards (Fig. 2a).

Concerning fungi, ASVs affiliated with Mortierellomycota and Glomeromycotina (Glomeromycota *sensu* UNITE v10), recognized for their roles in promoting soil health and establishing plant symbiosis, were enriched in treated conditions of VO (Fig. 2b). Fungi belonging to the order Pleosporales (Ascomycota) and of the species *Cystofilobasidium macerans* (Basidiomycota) increase significantly in the endosphere of both vineyards with the treatment (Fig. 2b). These fungi can be found in the soil and have saprotrophic abilities. While *Zopfiella* (Ascomycota) are reduced in both vineyards (Fig. 2b).

Together, these results highlight the local impact of the bioinoculum on the root endosphere, with a significant increase of taxa belonging to PGP microorganisms in treated conditions and also suggest a selective recruitment of bioinoculum-derived microorganisms across compartments and sites.

3.3. The application of the bioinoculum had a systemic effect altering grape berries-associated microbiota

To assess the systemic impact of soil bioinoculum application, the grape berry-associated microbiota was characterized using 16S and 18S rDNA metabarcoding from samples harvested in 2023. Sequencing yielded a total of 1539,196 and 1469,741 raw reads for the prokaryotic (16S) and fungal (18S) markers, respectively. Following primer removal, sequence denoising, ASV inference, and exclusion of non-target sequences (including plant organellar and non-fungal reads), 257,183 (16S) and 1124,087 (18S) high-quality fragments were retained for downstream analyses. This resulted in the identification of 2691 prokaryotic ASVs (bASVs) and 108 fungal ASVs (fASVs), with rarefaction curves indicating adequate sequencing depth for both datasets (Fig. S6, Dataset S2).

Grape samples were collected in September 2023 from both vineyards, including berries from bioinoculum-treated (treated) and untreated (control) plants. In both locations, treated plants exhibited a significant increase in grape berry weight (Fig. 3a), suggesting a systemic physiological response to the soil bioinoculum, which was more significant for VO rather than VN samples.

Consistent with observations in root-associated compartments, the vineyard site was the principal driver of fruit-associated microbial β-diversity for both bacterial and fungal communities (Table 1 and Table S3). While the bioinoculum application showed a clear separation of the prokaryotic community assembly between treated and control was observed in the VN vineyard (Fig. 3b), it did not significantly alter the berry fungal community composition in either vineyard (Fig. 3c).

The effect of the bioinoculum on α-diversity was opposite in the two vineyards. Prokaryotic communities in the VO exhibited increased Shannon diversity index following treatment (Fig. 3d). In contrast, no significant differences in α-diversity were observed in VN for Prokaryotes and for the fungal community (Fig. 3e) in both sites.

Bacilli, Gammaproteobacteria and Alphaproteobacteria were the dominant bacterial classes in all conditions (Fig. 3f).

In VN grapes prokaryotic communities under control conditions showed an increase in Gammaproteobacteria. Interestingly, we also found treatment-induced variations of low-abundance members (<5% in relative abundance) of the community (Fig. 3g). Here, Bacteroidia, Negativicutes, Phycisphaera, Planctomycetes and Blastocatellia decreased with the treatment whereas Actinobacteria and Gemmatimonadetes increased compared to the control (Fig. 3g).

In VO, grape microbiota from treated plants showed a decrease of Bacilli and, among the low-abundance taxa, an increase of Alphaproteobacteria Actinobacteria, Bacteroidia, Negativicutes, Chloroflexia, Planctomycetes, KD4.96 (phylum Chloroflexi) and Vicinamibacteria, compared to the controls. Notably, most of these taxa were not found in control samples (Fig. 3g). The presence of Alphaproteobacteria, Actinobacteria, and Bacteroidia was also notable within the root-associated microbiota, with an abundance of Bacilli in the rhizosphere of VN both treated and control samples during flowering (Fig. 1c). *Lactobacillus agilis* was present in both VN and VO treated samples; in contrast, no fungal taxa were found to be enriched in both VN and VO treated samples compared to the control (Table S4).

Grape fungal communities were taxonomically less complex. In VO it

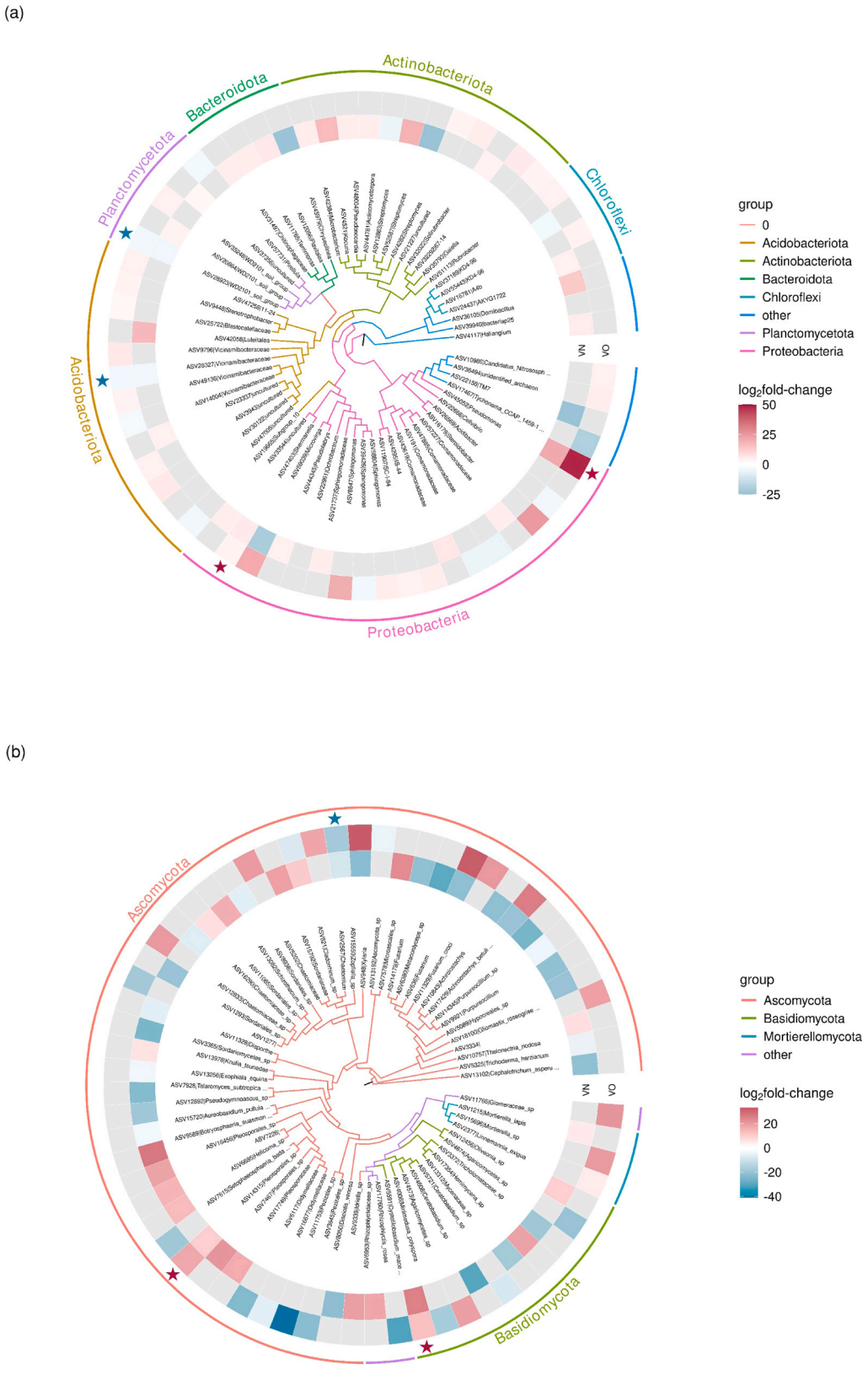

Fig. 2. Differential abundance of prokaryotes and fungi in treated *versus* control conditions in both vineyards. Circular phylogenetic tree and heatmap of the bacterial (a) and fungal (b) ASVs with higher or lower microbial loads in the endosphere of both vineyards treated plants compared with controls. Only ASVs with a relative abundance higher than 5% (FDR<=0.01) and classified at least at the family rank are displayed. Maximum likelihood (ML) phylogenetic trees were constructed using differentially abundant ASV sequences and tree branches were coloured according to taxonomy (*sensu* UNITE v10 for Fungi, and SILVA v138 for prokaryotes) at the phylum level. The heatmaps show log_2fold-change values indicating ASVs enriched (red) and depleted (blue) in treatments compared with the control. Stars represent ASVs consistently enriched or depleted compared to the control in both vineyards. VO, old vineyard; VN, new vineyard.

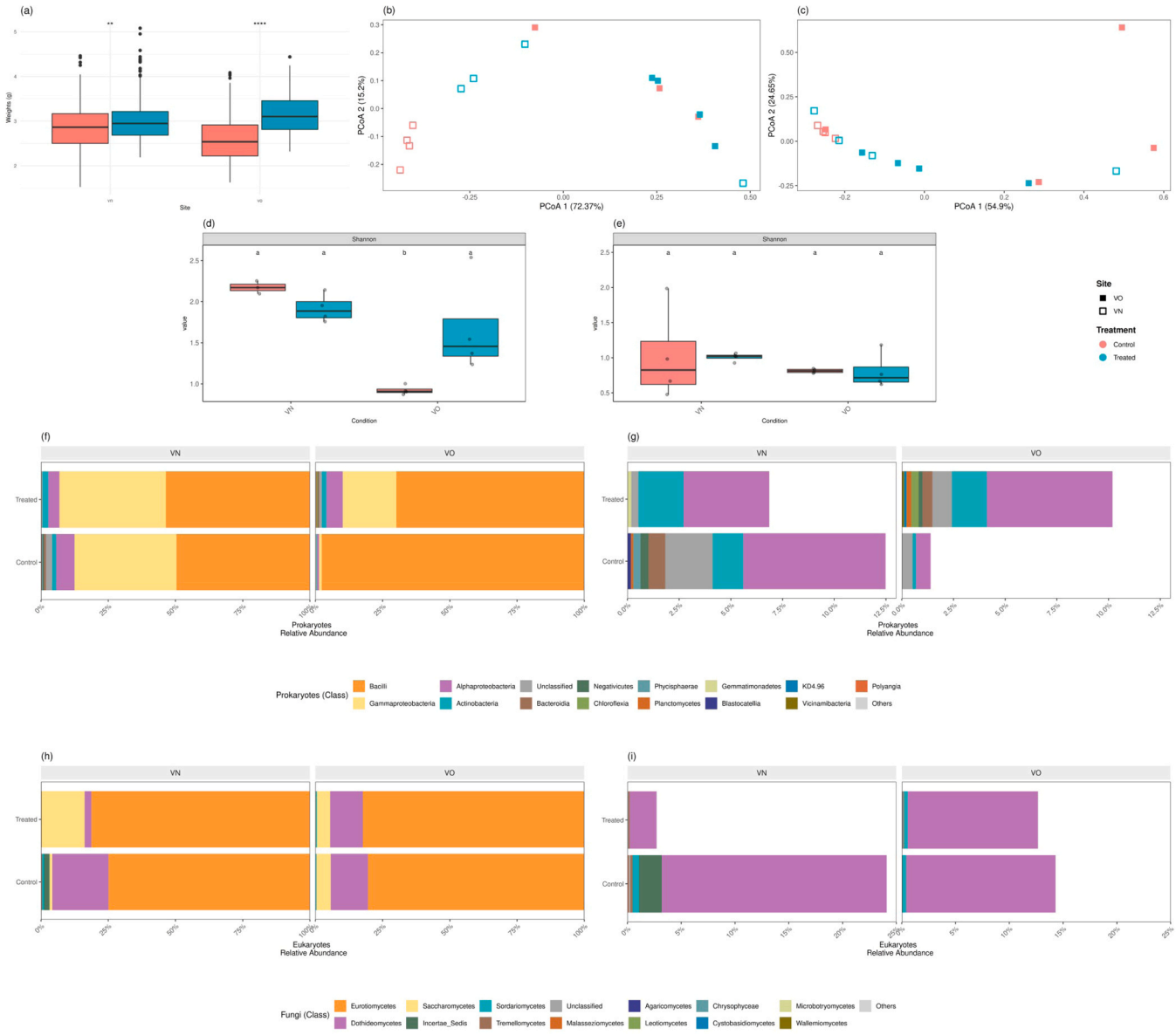

Fig. 3. Assembly and diversity of fruit-associated microbiota in *Vitis vinifera* var. Pigato under bioinoculum treatment conditions. (a) Fresh biomass of grapes collected from both vineyards (VO, old vineyard, VN, young vineyard), Boxplots display the median (horizontal line), the quartiles (boxes) and 1.5 × interquartile range (whiskers). Asterisks indicate significant differences between sites and treatment according to the post hoc Tukey's test ($p < 0.05$) after ANOVA. (b-c) Principal Coordinate Analysis (PCoA) plot of Bray–Curtis distances between samples by treatment of prokaryotic (b) and fungal communities (c) in both vineyards. Points represent single samples and are coloured by treatment. The fraction of the total variance explained by the projection is indicated in brackets along each axis. (d-e) Shannon diversity index in prokaryotic (d) and fungal (e) libraries by treatments and sites in grapes. Points represent single samples and are coloured by treatment. Boxplots display the median (horizontal line), the quartiles (boxes) and 1.5 × interquartile range (whiskers). Asterisks indicate significant differences between sites and treatment according to the post hoc Tukey's test ($p < 0.05$) after ANOVA. (f, h) Average relative abundances of prokaryotes (f) and fungi (h) at the class level. (g, i) Occurrences of lowly abundant taxa (<5%) of prokaryotic (g) and fungal (i) community at the class level.

is mainly composed of Eurotiomycetes, Saccharomycetes and Dothideomycetes (Fig. 3h) in both control and treated conditions. Only Dothideomycetes were more abundant in control samples, whereas Tremellomycetes increased in treated samples (Fig. 3h, i). Also, in VN a higher number of fungal classes were observed. Grape-associated communities of treated plants showed an increase in Eurotiomycetes and Saccharomycetes compared to controls, and a decrease in Dothideomycetes abundance as well as of Sordariomycetes, Tremellomycetes and Malasseziomycetes (Fig. 3h,i). Differences in relative abundance between treated and control grape communities in VN, suggested a specific response to the bioinoculum at the fruit level. Interestingly, most of these taxa, such as Dothideomycetes and Sordariomycetes, were abundant across all root-associated compartments, whereas Eurotiomycetes were also more abundant in the rhizosphere of VN and VO, and in VO soil and roots microbiota and Tremellomycetes were also highly abundant in the soil and rhizosphere microbiota of both VO and VN (Fig. 1d).

Overall, as expected, the grape-associated diversity was lower than the root-associated microbial community and largely shaped by vineyard site. Nonetheless, treatment induced some measurable effects, including increased berry weight and alterations in both α- and β-diversity.

3.4. Bioinoculum induced changes in leaf nutrients content and wine metabolites

To investigate the impact of soil-applied bioinoculum on plant physiology at systemic level, we analyzed leaf nutrient profiles as a proxy of plant health. Concurrently, comprehensive metabolite profiling of the wines produced from grapes harvested from treated and control plants at both sites was performed to assess the influence of the inoculated microbial consortium on grapevine secondary metabolism and wine quality.

The analysis of leaf nutrients content revealed treatment-dependent differences in both vineyards. In VN, application of the bioinoculum resulted in a significant increase in aluminium (Al), barium (Ba), potassium (K), and strontium (Sr) levels, while concentrations of arsenic (As) and boron (B) were reduced (Fig. S7). Conversely, in VO, the bioinoculum treatment led to elevated levels of As and B, along with a reduction in magnesium (Mg) content (Fig. S7). Although not statistically significant, the bioinoculum treatment was associated with a trend toward increased copper (Cu) and sulphur (S) content in both vineyards (Fig. S7). By contrast, phosphorus (P) levels did not differ among treatments or between vineyards (Fig. S7).

Untargeted NMR-based metabolomic profiling was performed on samples of all wines obtained from the 2023 vintage. The PCA ordination plot obtained from the NMR dataset highlighted the presence of groupings that could be related to the individual vinifications. This situation was partially expected, as each vinification corresponds to a real individual batch, which can be characterized by its own specific features (Fig. 4a) which can be possibly also magnified by the reduced fermentation volume (see Materials and methods, 2.4). Nevertheless, the distribution of samples in Fig. 4a closely reflects the four experimental conditions defined by site (VO/VN) and treatment (treated/control) combinations. Within each of these groups, samples are further differentiated according to individual vinifications, indicating that PC1 and PC2 capture the main sources of variation in the dataset (64.22% of the total variance explained by these two inspected components), encompassing both broad group separations and finer distinctions among vinification replicates. The directions of separation are consistent and must be interpreted by inspecting the metabolites shown in the loadings plot of the same PCs (Fig. 4b), suggesting a distinct compositional profile among treatments. The distribution of VN wine samples differs from that of VO samples and between treatments. The representation of wine metabolites quantification is reported in Fig. 4c. Differences in sample distribution could be largely attributed to variation in the quantity of key chemical classes, including acids, alcohols, carbohydrates, and amino acids (Fig. 4a,b).

Among the identified chemical classes, acids are the most abundant. Most of the measured acids show positive loadings along PC2, indicating generally higher concentrations in VN-treated samples compared to VN-control samples. VN-treated wines are notably enriched in citric acid, malic acid, and fumaric acid (Fig. 4c). Similarly, in VO treated samples exhibit higher acid content than controls, with increased levels of acetic acid, succinic acid and tartaric acid. Both treated wine samples in VO and VN show higher levels of lactic acid, ethyl acetate, succinate acid and gallic acid and lower pyruvic acid content (Fig. 4c).

Only a few alcohols could be reliably identified and quantified. Among these, ethanol levels are higher in samples with negative PC2 scores and decrease along the PC2 axis, indicating in VN a lower ethanol content in treated samples compared to control ones. These differences correlate with sugar content in the grape must (Fig. S8), suggesting a fermentative origin for the elevated ethanol levels. A similar, although less pronounced, decrease is also observed in treated *vs* control samples in VO (Fig. 4c). In VN, control samples show elevated levels of phenyl alcohol and, to a lesser extent, isopentanol compared to treated samples, whereas the opposite trend is observed in VO. Additionally, samples

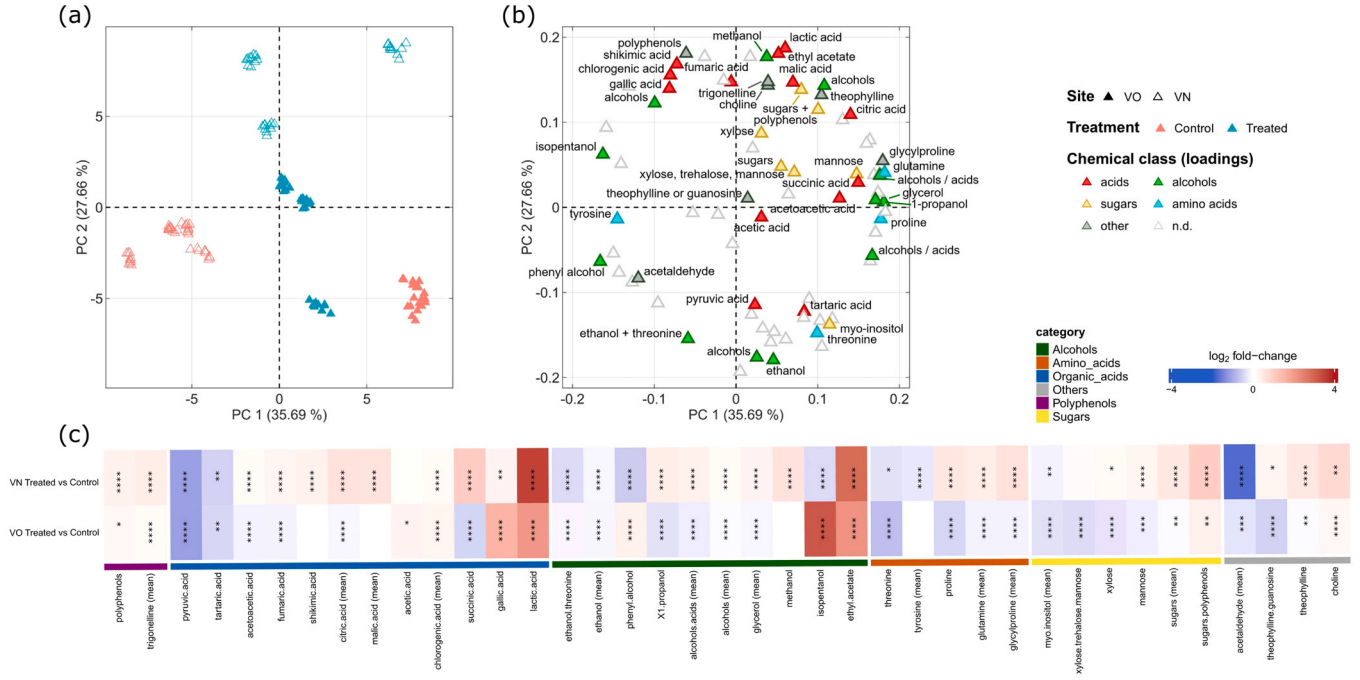

Fig. 4. Metabolite profile of two *Vitis vinifera* var. Pigato vineyard's wine samples under microbial inoculation conditions. (a) Principal Coordinate Analysis (PCoA) between samples, grouped by treatment and vineyard. (**b**) Corresponding loadings for both vineyards (VN, new vineyard and VO, old vineyard) under treated and control conditions. Points represent individual wine samples and are colored by treatment; filled and empty triangles indicate different vineyard sites. The percentage of total variance explained by each axis is shown in brackets. (**c**) Heatmap plot showing log_2fold-change values (treated *vs.* control) of wine metabolites detected in the two vineyards (VN, VO). Blue and red colors depict a decrease and increase in metabolite levels, respectively. Asterisks indicate significantly-supported differences between each metabolite levels in treated versus control condition according to *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Metabolite profiles of wines produced from treated and untreated vines and micro-vinified in 2023. Similar patterns were observed in the corresponding analyses performed in 2022.

with positive PC1 scores are associated with higher concentrations of 1-propanol and glycerol.

Treated samples from VN show generally elevated sugar levels, with most identified carbohydrates (xylose, trehalose, and mannose), corresponding to positive PC1 and PC2 loadings (Fig. 4b). The only exceptions are two signals corresponding to myo-inositol, which are associated with negative PC2 loadings which correlate with both treated and control samples in VO. In contrast, VN control samples exhibit lower sugar content. Interestingly, both treated samples from VN and VO exhibit higher levels of sugars polyphenols content compared to the related control samples (Fig. 4c).

Glutamine, and proline seem very consistently located at positive PC1 loadings, thus characterizing most of the VO control samples and VN treated. The wine samples from treated plants in VO are characterized by higher contents of threonine. Tyrosine is identified by two distinct signals, each associated with control and treated samples in VO, respectively.

The wine samples from treated plants were also associated with higher levels of glycylproline, trigonelline and choline (Fig. 4b).

Collectively, the data indicate that wines from control plants in VN are enriched in phenyl alcohol and tyrosine, while wines from controls in both vineyards exhibit higher levels of acetaldehyde, pyruvic acid, and ethanol (Fig. 4c). In contrast, treated samples from VN show increased concentrations of organic acids and sugars compared to their corresponding controls (Fig. 4c). Considering treated groups on both vineyard sites, the obtained wines display increased levels of ethyl acetate, gallic acid, lactic acid, chlorogenic acid, polyphenols, theophylline, and trigonelline (Fig. 4c). The higher concentration of lactic acid

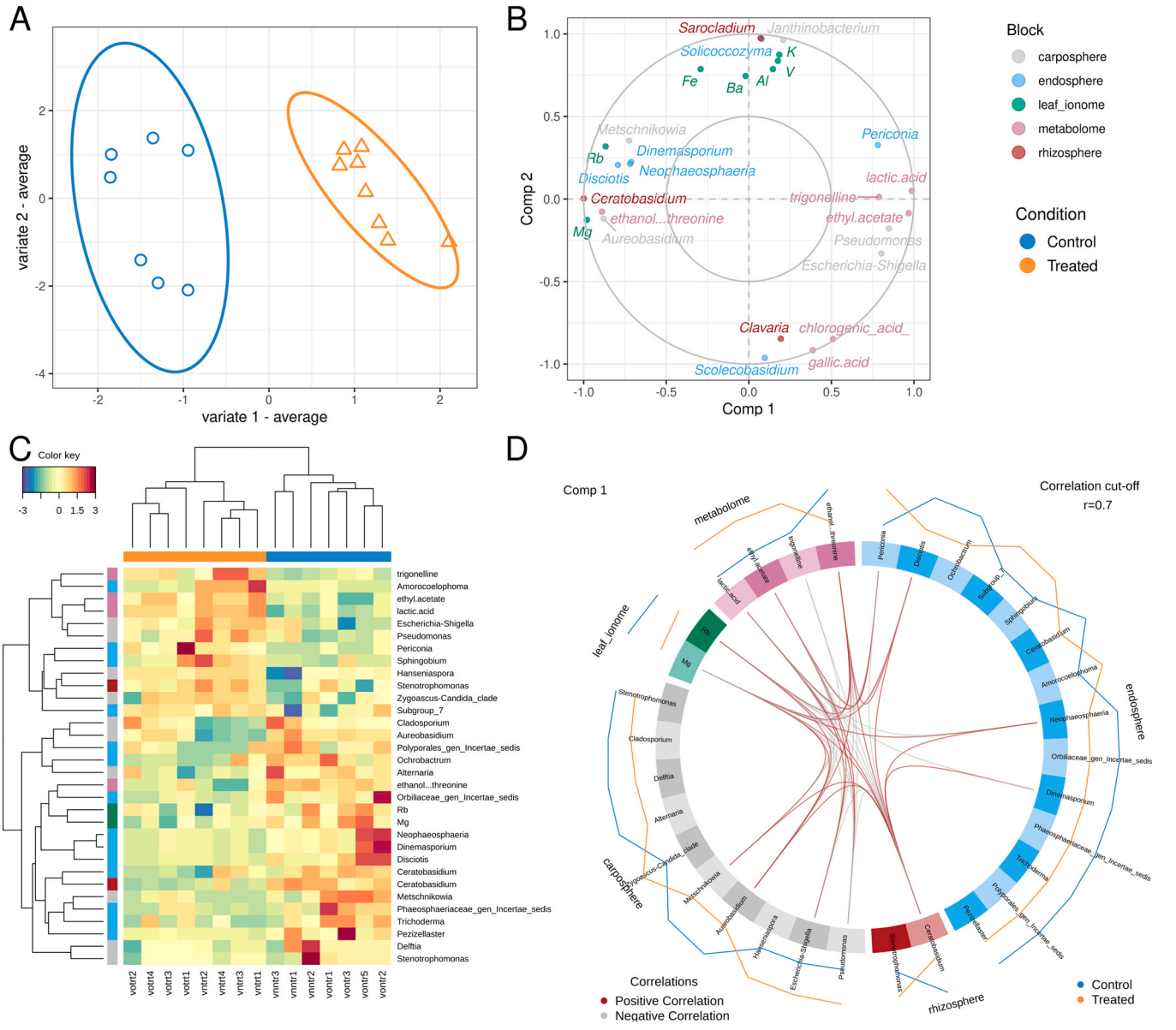

Fig. 5. Relationships between variables across datasets obtained as resolved by DIABLO (multiblock sPLS-DA). (**A**) Samples plot based on consensus components (average variates) across blocks. The plot illustrates differences across treatments resulting from multi-omics data integration. (**B**) Correlation circle plots showing the projection of selected discriminant variables across blocks with the first two components. The graph shows the pairwise correlation of each variable within each of the integrated dataset (block) with model components 1 and 2. Variables from different blocks are highlighted with different colors according to the legend. (**C**) Clustered image map (CIM) showing sample similarities (columns) and co-abundance clusters of variables across each block (rows) above 0.7 correlation threshold. (**D**) Circos plot illustrating direct correlations between variables across different blocks; outer tracks indicate average abundance of each variable within each block in treated and control samples: only inter-block correlations higher than 0.7 are shown.

correlates with increased malic acid levels in grape must from treated samples in both vineyards (Fig. S8).

Thirty-six integrated signals are not assigned, and their distribution across the principal components space seem rather homogeneous (Fig. 4b). Notably, a denser cluster of these unassigned signals is located in the region defined by positive PC1 and negative PC2 loadings, which corresponds to the positioning of wine samples from VO.

In summary, based on these results, vineyard treatment with the bioinoculum appears to have more influence on wine obtained from VN rather than VO. However, treatment induced in both vineyards an increase in the content of specific wine-relevant compounds, such as polyphenols, gallic acid, and lactic acid in comparison with their controls.

3.5. System-level data integration reveals bioinoculum multi-omic signature

Data obtained from all the previous analyses were integrated using sPLS-DA (DIABLO) multivariate supervised modelling (see Materials and methods, 2.8), looking for signatures related to the bioinoculum applied, at local (rhizosphere, root endosphere) and systemic level (leaf ionome, grape carposphere), and on the obtained wine. The model well-discriminated treatment conditions with the first model component of each of the provided dataset (blocks, Fig. S9) suggesting a coordinated and consistent response to the treatment across all blocks considered. In particular, the integration of the five omics dataset provided a list of 14 root endosphere, 2 rhizosphere, 10 carposphere taxa, 2 leaf elements and 4 wine metabolites highly discriminative for treatment and highly correlated to each other (Fig. 5). Among those interactions, the model highlighted that variation in ethyl-acetate, lactic acid, trigonelline and ethanol previously mentioned are significantly correlated with specific taxa occurring in root-associated compartment and carposphere. The higher amount of lactic acid and ethyl-acetate measured in wines obtained from treated plants, seems to be correlated with the abundance of *Periconia* genus in the root endosphere and of that of Enterobacteraceae (*Escherichia-Shigella* complex), and *Pseudomonas* in the carposphere. By contrast, the decrease in ethanol and threonine abundance in wine from treated plants was positively correlated with lower abundances of the genera *Disciotis* (root endosphere), *Ceratobasidium* (rhizosphere) and *Aureobasidium* (carposphere) in root endosphere and rhizosphere, respectively, and with the decreased amount of Mg in leaves. Notably, among other taxa with a lower degree of co-abundance and correlation we observed other interesting genera which increased in abundance under inoculum treatment including *Sphingobium* in roots and *Stenotrophomonas* in rhizosphere.

The fitted DIABLO model also highlighted interactions of variables correlated to the other components, highlighting that complex interactions occur across the five blocks considered not only according to the treatments but also in relation to other unmeasured environmental or plant variables. These interactions include correlations across a multitude of root-associated microbes such as Rhizobia and *Steroidobacter* to leaf elements and wine metabolites, including phenolic compounds such as chlorogenic acid and gallic acid (Fig. S10).

4. Discussion

Vineyard ecosystems harbour diverse and habitat-specific bacterial and fungal communities, with distinct assemblages reported across soils, roots, and epigeous organs (Aguilar et al., 2020; Del Frari et al., 2019a, 2019b), including leaves (Gobbi et al., 2022) and reproductive structures such as grapes, flowers, and musts (Martins et al., 2024; Zarraonaindia et al., 2015; Gilbert et al., 2014). Several studies have identified soil as a primary source of microorganisms colonizing the above-ground organs, including grape berries (Wagner et al., 2016; Zarraonaindia et al., 2015; Martins et al., 2013). These microbial communities play key roles not only in berry development but also in host transcriptome response, in the production of secondary metabolites and fermentation processes, thereby influencing the sensory and organoleptic qualities of wine (Liu et al., 2025; Mercanti et al., 2024; Balestrini et al., 2017).

In this study, we tested whether the application of a commercial bioinoculum to vineyard soils could influence grapevine-associated microbiota in soils, roots, and berries, and, in turn, impact must and wine metabolite composition (Fig. 6).

Consistent with previous studies, the root-associated prokaryotic core community was dominated by Proteobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Acidobacteria, and Firmicutes (Darriaut et al., 2022; Marasco et al., 2018; Teixeira et al., 2024). While the eukaryotic microbiome was characterized primarily by Ascomycota and Basidiomycota in both above- and below-ground tissues, Glomeromycota were detected, as expected, in grapevine roots (Teixeira et al., 2024; Bettenfeld et al., 2022; Darriaut et al., 2022; Berruti et al., 2017).

Experiments were carried out in two adjacent vineyards sites with different soil physicochemical features, plant age, and management practices at planting, factors that are acknowledged as major determinants of the plant-associated microbial community structure (Legesse et al., 2025; Berlanas et al., 2019; Dissanayake et al., 2018; Berruti et al., 2017). These vineyard-specific differences were reflected in root-associated microbiota, leaf nutrient composition, and wine metabolite profiles. Despite this heterogeneity, bioinoculum application induced comparable shifts in root-associated prokaryotic and fungal assemblages at both sites. Specifically, treatment promoted the enrichment of potential PGP bacteria, including *Pseudomonas spp.*, biocontrol-associated taxa such as *Skermanella* spp., and saprotrophic fungi belonging to Pleosporales (Ascomycota), together with the basidiomycete *Cystofilobasidium macerans*, within the root endosphere. The greatest response was observed in the VO site, where ASVs belonging to Mortierellomycota and Glomeromycota (Glomeromycotina), well-established contributors to soil health and plant symbiosis, were enriched. By contrast, as expected, the VN vineyard exhibited a more limited response, likely due to its prior inoculation with the same bioinoculum at planting. Interestingly, root-associated fungal community assembly was significantly affected by microbial inoculum application, whereas a much weaker response was observed for prokaryotes. This trend aligns with previous studies indicating that beneficial fungal inoculants, such as *Trichoderma*, can enhance fungal abundance and alter fungal community composition, while their effects on bacterial communities are generally less pronounced and more context-dependent (Asghar et al., 2024; Asghar and Kataoka, 2021; Ros et al., 2017).

Application of the bioinoculum also exerted systemic effects on treated plants in both vineyards, influencing leaf nutrient status, berry-associated microbiota, and in turn wine metabolite composition. Notably, while changes in leaf nutritional profiles were site dependent, plants at both locations showed a significant increase in berry weight following treatment. This suggests that the observed effect may be linked to bioinoculum application but seems to operate independently of nutritional status, potentially reflecting alternative mechanisms such as hormone-mediated responses or enhanced water uptake triggered by shifts in root-associated microbial communities. Indeed, higher abundance of auxin-producing bacteria such as *Pseudomonas* spp., and *Bacillus* strains (Lata et al., 2024; Batista et al., 2021; Sah et al., 2021) and AM fungi which regulate auxin metabolism (Ma et al., 2024) and improve host water uptake (Kakouridis et al., 2022) were detected in root treated plants in both vineyards.

Consistent with observations in root-associated compartments, the vineyard site was the primary determinant of berry-associated bacterial and fungal communities. These microbial communities were dominated by prokaryotic taxa, including Bacilli, Gammaproteobacteria and Alphaproteobacteria, and by fungal taxa such as *Saccharomyces* and Eurotiomycetes, consistent with previous reports (Lorenzini and Zapparoli, 2020; Zhang et al., 2019; Mezzasalma et al., 2017; Wang et al., 2015; Barata et al., 2012). Nonetheless, bioinoculum application elicited

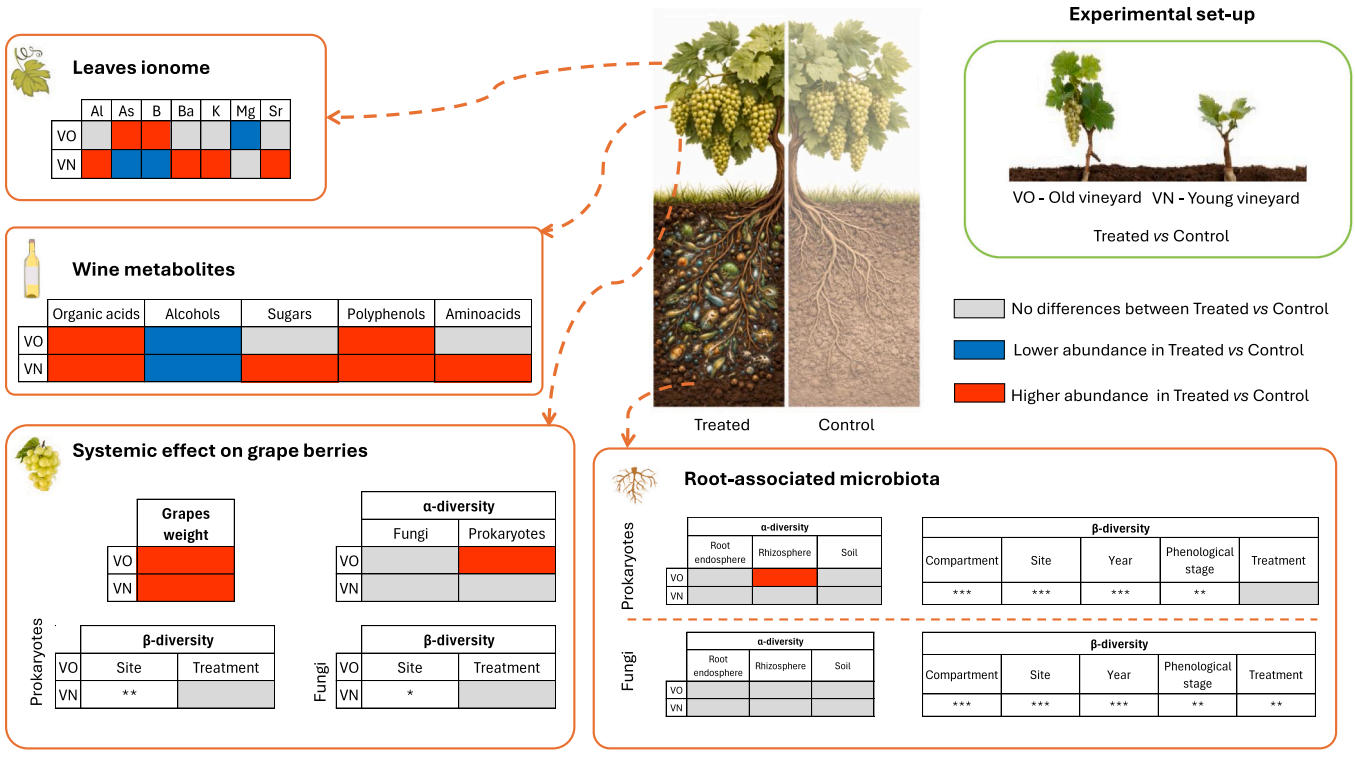

Fig. 6. Experimental design and main results of the impact of the treatment on grapevine-associated microbiota, leaf ionome, and wine metabolite profiles. Schematic overview of the experimental set-up conducted in two vineyards, a young vineyard (VN) and an old vineyard (VO), treated with a commercial bio-inoculum. The effect of the treatment was evaluated in both vineyards by investigating root-associated microbiota (soil, rhizosphere, and root endosphere), grape-associated microbiota, yield, leaf ionome, and wine metabolite profiles. Each panel reports treated conditions relative to their respective controls in both vineyards (VO and VN). For root- associated microbiota and its systemic effects in grape berries, treatment effects on α-diversity and β-diversity and grapes weight are shown. Statistical significance is reported at $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Heatmap colors indicate the direction of change (red/higher or blue/lower) in treated samples *vs* control, while grey denotes no differences. For wine, differences between treated and control samples in the two vineyards are reported for major metabolite classes (amino acids, polyphenols, sugars, alcohols, and organic acids). Leaf ionome analysis highlights variations in mineral elements between treated and control plants. Abbreviations: Al, aluminium; As, arsenic; B, boron; Ba, barium; K, potassium; Mg, magnesium; Sr, strontium. Vineyard images were generated using AI for illustrative purposes only.

site-dependent and common responses in microbial communities associated with grape berries. In particular, in VN vineyard, *Saccharomyces* were enriched in treated berries, while in VO vineyard, treated berries were enriched in Tremellomycetes, whereas at both sites, treated berries showed increased abundances of Dothideomycetes. Notably, both Tremellomycetes and Dothideomycetes were also enriched in treated soils and root-associated compartments, suggesting a systemic effect of bioinoculum application. These fungal classes, together with Saccharomycetes and Eurotiomycetes, have previously been reported as dominant ASVs during berry crushing and fermentation (Martiniuk et al., 2023). Regarding bacterial communities, grapes from both VN- and VO-treated plants were enriched in *Lactobacillus agilis*, a lactic acid bacterium previously reported to enhance wheat growth and triggering antioxidant activities, also under abiotic stress (Nawaz Khan et al., 2024). The enrichment of *L. agilis*, coupled with the elevated malic acid levels detected in the must of treated plants, likely underlies the increased lactic acid content observed in wines from both vineyards. Moreover, although further validation is needed, system-level data integration revealed previously unrecognized correlations between the abundance of the fungal genus *Periconia* in the root endosphere and *Pseudomonas* and *Enterobacter* in the carposphere with lactic acid and ethyl acetate levels in wine. Notably, *Pseudomonas* populations in grapes are known to vary according to vineyard management practices (Kecskeméti et al., 2016). By contrast, the ability of *Enterobacter* species to produce lactic acid has been documented (Thapa et al., 2017), providing additional support for potential microbial contributions to these metabolite shifts. Together, these findings suggest that bio-inoculum application may favor a more pronounced acidity profile, potentially enhancing wine freshness and structural balance.

With respect to the reduced ethanol levels in wines from treated plants, a positive correlation was observed with the decreased abundance in the carposphere of treated samples of *Aureobasidium*, a yeast-like fungus that represents a core component of the grape-associated fungal microbiota (Stefanini and Cavalieri, 2018). *Aureobasidium* species have been involved in increased accessibility to fermentable sugars influencing sugar metabolism and interacting with *Saccharomyces* and other yeasts involved in the fermentation phase (Watanabe and Hashimoto, 2023). We thereby speculate that its reduced abundance, associated with shifts in the microbial community, may contribute to altered juice composition that could impact the metabolism of yeasts with fermentative capacity, thereby impairing the fermentation process. This hypothesis would provide a plausible explanation for the lower ethanol concentrations and the higher level of sugars observed in wines from treated plants. Notably, wines from both VO- and VN-treated plants exhibited elevated levels of polyphenolic compounds, which are central to grape and wine quality through their impact on color, flavour, and mouthfeel. Beyond their contribution to organoleptic traits, polyphenols are also recognized for their antioxidant and cardioprotective activities, as well as their capacity to modulate the gut microbiota by promoting beneficial bacteria while suppressing pathogenic taxa (Velásquez et al., 2025; Gutiérrez-Escobar et al., 2021; Nash et al., 2018). Since this trend was consistently observed across two independent vineyards, it appears to be associated with the bioinoculum application. Plant-associated microbes are known modulators of secondary metabolism, particularly through activation of the phenylpropanoid pathway, which drives the accumulation of polyphenolic compounds (Kisiel et al., 2024). In

grapevine, colonization by AM fungi has been shown to upregulate key phenylpropanoid genes, including phenylalanine ammonia-lyase (PAL) and stilbene synthases, thereby enhancing the synthesis of protective stilbenoids such as resveratrol and pterostilbene (Bruisson et al., 2016). In this regard, Torres and colleagues (Torres et al., 2018, 2016) proposed that AM fungal inoculation may play an important role under future climate-change scenarios, by sustaining or even improving berry quality through enhanced phenolic content (e.g., increased anthocyanin content) and greater antioxidant activity. In addition, other PGPB such as *Pseudomonas*, *Bacillus*, and *Azospirillum* have been documented to enhance phenolic and flavonoid content in various crops (Jakubowska et al., 2025).

Overall, although the multi-omics integration revealed strong associations among microbial taxa, leaf elemental profiles, and wine metabolites, these relationships remain correlative, and further targeted analyses will be required to support causal interpretations.

5. Conclusion

Taken together, our data suggest a link between shifts in root-associated microbiomes and wine metabolite composition. We showed that soil application of a bioinoculum can locally modulate the root-associated microbiota and, in turn, systemically influence grape berry fungal communities and significantly affect must and wine chemical composition confirming the postulated hypothesis (Fig. 6). The observed correlations between rhizosphere and berry-associated microbial communities with key metabolites, indicate a potential connection between belowground microbial diversity and aboveground berry and wine traits. An alternative explanation may be that the effects of the bioinoculum on wine may arise from plant transcriptional and metabolic responses that alter berry juice composition and, consequently, wine metabolites.

In conclusion, our work suggested for the first time, the impact of root bioinoculum application on wine chemical composition across the entire wine production chain. As also emerged in recent studies, these findings confirm that *microbial terroir* plays a pivotal role in shaping wine quality and fingerprint and demonstrate that targeted microbial applications hold promise as sustainable tools to enhance grapevine resilience under increasing environmental stress. Unraveling the mechanisms underlying these belowground–aboveground interactions will be essential to fully harness their potential for viticulture and ecosystem sustainability.

Author contributions

BB and MC performed the experiments related to microbiota analysis, bioinformatics, multivariate modeling and statistical analysis. TM and CV carried out the sampling. NC and FS conducted multivariate analysis of wine metabolites. EP performed the soil analysis. IM-P and AP conducted the analysis of grape microbiota. AB was responsible for vinification. EL-R carried out the analysis of wine metabolites. SC, LL, MC, and VF designed the investigation. VF, BB, and MC wrote the manuscript. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Valentina Fiorilli: Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Nicola Cavallini:** Writing – review & editing, Methodology, Formal analysis. **Isabelle Masneuf-Pomarede:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Matteo Chialva:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Teresa Mazzarella:** Writing – review & editing, Methodology, Formal analysis. **Sergio Capaldo:** Writing – review & editing, Supervision, Conceptualization. **Francesco Savorani:** Writing – review & editing, Methodology, Data curation. **Eva López-Rituerto:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Beatrice Buffoni:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Luisa Lanfranco:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Cristina Votta:** Writing – review & editing, Methodology, Formal analysis. **Elio Padoan:** Writing – review & editing, Formal analysis. **Anaïs Poirier:** Writing – review & editing, Methodology, Investigation. **Alex Berriolo:** Writing – review & editing, Formal analysis.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2026.128505.

Data availability

Research link provided
NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1311317. Link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1311317?reviewer=q8d4f11a2nf2i2vso1r0a5g6gt. (MetaboLights)

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