iVES OENO One vine & wine

DOI: https://doi.org/10.20870/oeno-one.2024.58.4.8280

OPEN ACCESS

ORIGINAL RESEARCH ARTICLE

Soil-associated fungal and prokaryotic diversity influenced by stoniness, depth and vintage in a high-elevation vineyard

Daniela Mezzatesta[1,5,†], Mónica Oyuela Aguilar[2,†], Alex Gobbi[3,4,†], Mariano Pistorio[2], Lars Hestbjerg Hansen[4], Fernando Buscema[5], Patricia Piccoli[1], Federico Berli[1,*]

[1] Instituto de Biología Agrícola de Mendoza (IBAM), CONICET-Universidad Nacional de Cuyo, Facultad de Ciencias Agrarias. Almirante Brown 500 (5507) Chacras de Coria, Mendoza, Argentina.
[2] Instituto de Biotecnología y Biología Molecular (IBBM), CONICET-Universidad Nacional de La Plata, Facultad de Ciencias Exactas, Calles 49 y 115 (1900) La Plata, Argentina.
[3] CREA – Council for Agricultural Research and Economics, Research Centre for Agriculture and Environment, I-40128 Bologna, Italy.
[4] Department of Plant and Environmental Science (PLEN), University of Copenhagen, Frederiksberg 1821, Copenhagen, Denmark.
[5] Catena Institute of Wine, Cobos s/n (5509), Agrelo, Mendoza, Argentina.
[†] These authors contributed equally to this work.

*correspondence: fberli@fca.uncu.edu.ar

Associate editor: Gerardo Puopolo

Received: 27 August 2024

Accepted: 5 November 2024

Published: 20 November 2024

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ABSTRACT

Soil microbes are increasingly recognised as key contributors to wine terroir, playing crucial roles in soil health and nutrient cycling. Fungi and prokaryotes interact with soils, influencing physical and chemical properties and mediating nutrient availability for roots. These microorganisms also impact vine performance and wine quality. Viticulture is expanding to higher elevations due to their cooler temperatures; Mendoza's mountainous regions are of particular interest being, characterised by vineyards with heterogenous soil stoniness. However, the effect of this variability on soil-associated microbial communities remains unclear. This study explores microbial populations (alpha and beta diversity, taxonomical composition, and their relationship with soil physicochemical properties) in soils of contrasting stoniness, at different depths, vintages and sample types. A Malbec vineyard with heterogenous soil stoniness was selected, with two experimental sites 30 m apart containing stony soil (SS; 77 % stoniness) and non-stony soil (NS; 0 % stoniness), respectively, and which were managed identically. Samples were collected from two depths (0.3 m and 0.6 m) during two vintages (2017 and 2018), from bulk and rhizosphere. Amplicon sequencing targeted the V3-V4 region of the 16S rRNA gene (prokaryotes) and the ITS1 region (fungi). Results showed that soil type significantly influences fungal populations, with less effect on prokaryotes. Vintage, reflecting annual changes in weather and viticultural practices, was the most significant factor affecting microbial communities. Depth was particularly important for fungi, while the sampling type (bulk or rhizosphere) had no significant impact on the microbiome within the same soil profile. Certain soil components were found to influence microbial communities: pH affected prokaryotes, while calcareous content specifically influenced the *Proteobacteria* phylum. Additionally, five fungal orders were more abundant in the stony soils, though some remained unidentified. These findings provide a baseline for understanding microorganisms in contrasting soils types of a high-elevation vineyard, and they highlight the role of microbial diversity in supporting unique soil-plant-environment interactions.

KEYWORDS: metabarcoding, grapevine, soil microbiome, terroir, Argentina, Mendoza

INTRODUCTION

Over the last two decades, particularly since next generation sequencing (NGS) technologies have become widely available, research on grapevines (*Vitis vinifera* L.) has increasingly focused on plant-microbe interactions to understand their impact on crop health and the association between grape quality and cultivation sites (*i.e*., the *terroir* concept). The grapevine microbiome comprises a vast group of filamentous fungi, yeasts and bacteria, including plant growth-promoting rhizobacteria (PGPR), all microbes which, together with other biotic and abiotic factors, engage in complex metabolic exchanges with their hosts.

Soil is the primary reservoir of microorganisms (Zarraonaindia *et al*., 2015) and its microbial diversity pattern impacts grape juice and the final wines (Bokulich *et al*., 2016; Belda *et al*., 2017). Therefore, it has proved to be an essential element of terroir. Microbial diversity is influenced by several environmental factors, such as climate, soil composition and topography (Bokulich *et al*., 2014; Gobbi *et al*., 2022), particularly diverse in mountainous regions with high-elevation vineyards. Edaphic factors, such as phosphorus, pH, carbon and nitrogen availability are key factors for microbial compositions (Bassoi *et al*., 2003; Steenwerth *et al*., 2008; Eilers *et al*., 2012). In addition, endophytic microorganisms can exhibit high specificity to the genotype of their host plants (Marasco *et al*., 2018). In grapevines, soil-associated microbes are affected by root exudates which provide nutrients and promote microbial selection and competition among different types of bacteria (Philippot *et al*., 2013; Steenwerth *et al*., 2008). Annual microbial shifts within and between vineyard locations have been attributed to different vintages (Liu *et al*., 2019; Oyuela Aguilar *et al*., 2020), with variations in soil properties further influencing these changes, sometimes even more than the grapevine cultivar (Zarraonaindia *et al*., 2015). The effect of different vintages may reflect annual changes in weather conditions and viticultural practices, such as irrigation, fertilisation, pathogen control and soil management. Irrigation, especially in arid and semi-arid regions, shape this underground ecosystem by influencing how the root system deals with water stress (Bassoi *et al*., 2003).

The wine fingerprint has been studied in Argentina, primarily focusing on chemical and sensory features of grapevines across different scales and vintages, including the emblematic Malbec grape cultivar (Buscema & Boulton, 2015; Urvieta *et al*., 2018; Urvieta *et al*., 2021). Malbec, the most cultivated variety in Argentina, occupies 85 % of the irrigated viticulture surface in Mendoza (INV, 2023). However, research on its interaction with soil microbial communities is still being explored. Vega-Avila *et al*. (2015) and Oyuela Aguilar *et al*. (2020), using denaturing gradient gel electrophoresis (DGGE) and NGS technologies, respectively, evaluated different microbiomes from San Juan, Argentina, in vineyards with loamy soils at elevations of around 800 m above sea level (a.s.l.). Vega-Avila *et al*. (2015) found differences in bacterial communities associated with Syrah and agricultural practices (conventional *vs*. organic). Meanwhile, Oyuela Aguilar *et al*. (2020) found significant differences in fungal and bacterial composition between vintages and vineyard, with variances in pH, organic matter, carbon, nitrogen and phosphorus. In Mendoza, there are vineyards at higher elevations where soil heterogeneity at an intra-vineyard level is common due to alluvial origins, with contrasting stoniness playing a key role in terroir expression (Mezzatesta *et al.,* 2022). Salomon *et al*. (2014) and Salomon *et al*. (2017) isolated PGPR in a Malbec high-elevation vineyard (Gualtallary, Mendoza), and showed their role helping vines to cope with environmental stresses as high ultraviolet-B (UV-B) radiation, drought and *Botrytis cinerea*. Paolinelli *et al.* (2023) investigated rhizosphere microbial communities from two regions in Mendoza with contrasting elevations and identified *Ascomycota* and *Proteobacteria* as the dominant phyla for fungi and bacteria, respectively. The study further revealed that the primary factor influencing microbiome differences between these regions was the carbon-to-nitrogen (C/N) ratio.

In this study, we aimed to investigate at an intra-vineyard scale, how soil heterogeneity shapes microbial communities. We hypothesised that i) the structure of fungal and prokaryotic communities differs between sites of contrasting stoniness and varies depending on soil depth, vintage and sample type (bulk soil and rhizosphere), and ii) there is a relationship between microbial changes to the physicochemical characteristics of these contrasting soils.

MATERIALS AND METHODS

1. Site selection, experimental design and sample preparation

The experiment was conducted on own-rooted *Vitis vinifera* L. cv. Malbec in a commercial 23-year-old high-elevation vineyard located 1450 m a.s.l. in Gualtallary (69° 15' W and 33° 23' S), Mendoza, Argentina. The vines were trained in a vertical shoot positioning system (2 m x 1.2 m), pruned using the double Guyot method, protected by hail nets and drip-irrigated. Within the vineyard, two contrasting soil profiles were selected based on the overlap of four maps: electrical conductivity (EC) at 0.5 m and 1.5 m depth (Figures 1A-B), normalised difference vegetation index (NDVI; Figure 1C) and effective soil depth were determined by digging 70 holes per hectare (Figure 1D). As a result, two experimental plots separated by 30 m were chosen: a stony soil (SS) with < 0.45 m depth (77 % stones and a top slope position) and a non-stony soil (NS) with > 1 m depth (0 % stones and a foot slope position). Figure 1E and 1F show representative SS and NS soils, including the sampling distances from the vine and sampling soil depths. Homogeneity within the experimental plots were controlled measuring drip irrigation volumes and vegetative growth by trunk diameters (n = 30). A comprehensive description of the vineyard's geology and soil taxonomy is presented in Mezzatesta *et al*. (2022). Air temperature and rainfall data were registered from September

to March (2017 and 2018) by a meteorological station (PEGASUS EP 201, TECMES SRL, Argentina) located in the vineyard.

Sample collection and maintenance procedures are detailed in Oyuela Aguilar *et al*. (2020). Briefly, a total of 48 soil samples were collected one week before harvest from two vintages (2017 and 2018), comprising two sample types (bulk soil found at ~0.5 mm from the root system and rhizosphere soil directly adhered to the roots) taken from two soil types (SS and NS) at two depths (0.3 m and 0.6 m). For each condition type, three composite samples were prepared, each made from three subsamples (n = 3). Then, the samples were sieved (pore size 0.5 mm) to remove plant debris, and physicochemical analyses (organic matter, total nitrogen, available phosphorus, exchangeable potassium, calcium carbonate content, pH and EC) were performed based on Mezzatesta *et al*. (2022).

2. DNA Extraction

DNA was extracted from 400 mg of sieved soil samples using the FastDNA Spin Kit for Soil (MP Biomedicals, LLC, Solon, OH, USA), following the manufacturer's instructions. DNA concentration was quantified with a Qubit® 2.0 Fluorometer (Thermo Scientific™), and purity was assessed using a Nanodrop 2000 (Thermo Scientific™) spectrophotometer by measuring absorbance ratios of 260/280 nm and 260/230 nm.

3. Library preparation and sequencing

Library preparation for amplicon sequencing was performed using a two-step PCR approach recommended by Illumina. The prokaryotic community of each sample was analysed by targeting the V3-V4 region of the 16S rRNA gene, amplified with primers 341F and 806R (Gobbi *et al*., 2019). For the fungal community, the ITS1 region was targeted and amplified using the ITS1F and ITS2 primers from Del Frari *et al*. (2019). PCR conditions followed those described by Gobbi *et al*. (2019) for the prokaryotic community and Gobbi *et al*. (2020) for the fungal community. Sequencing was conducted on Illumina MiSeq platform using paired end 2x250 bp reads in a single run, with a V2 500-cycles reagent kit.

For fungi, all 48 samples were used, while for prokaryotes in 2017 only, 21 samples from SS and 23 samples from NS were analysed.

4. Bioinformatics

The reads generated were analysed using QIIME 2 v. 2019.7 (Bolyen *et al*., 2019) following a pipeline similar to that described by Gobbi *et al*. (2020). After demultiplexing, reads were denoised and dereplicated using DADA2 (Callahan *et al*., 2017). A multiple sequence alignment was performed with MAFFT (Katoh *et al*., 2013), and a phylogenetic tree was constructed using FastTree (Price *et al*., 2010). Alpha and beta diversity analyses were conducted using the q2-diversity plugin. Alpha diversity was measured using Phylogenetic Diversity (PD) from Faith (2015), while beta diversity was assessed with weighted UniFrac (Lozupone & Knight, 2005) and visualised through principal coordinates analysis (PCoA) plots generated by EMPeror (Vázquez-Baeza *et al*., 2013). After denoising the reads, taxonomy assignments were carried out with QIIME feature-classifier classify-sklearn (Bokulich *et al*., 2018), utilising a pre-trained Naïve-Bayes classifier with Greengenes v_13.8 (DeSantis *et al*., 2006) for 16S V3-V4 and UNITE (Nilsson *et al*., 2019) for ITS1. The raw reads are available in the sequence read archive (SRA) under Study Accession Number PRJNA838599.

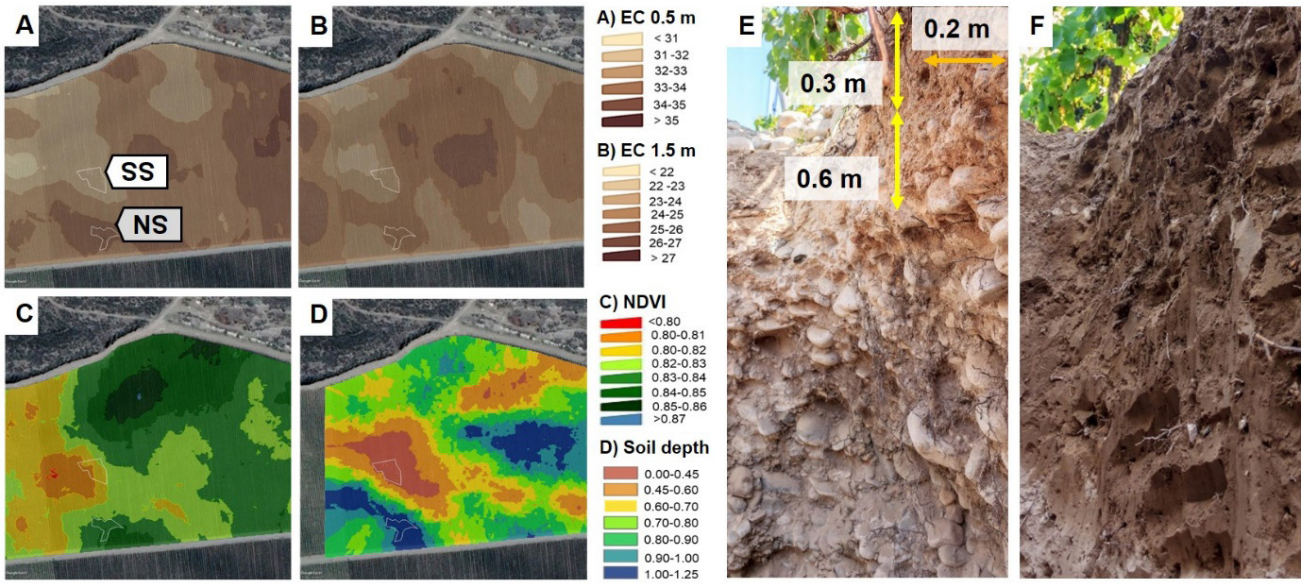


FIGURE 1. Maps used to select the experimental plots within the vineyard in stony soil (SS) and non-stony soil (NS): Electrical conductivity (EC, mS/m) at depths of 0.5 m (A) and 1.5 m (B), Normalised Difference Vegetation Index (NDVI; C) and effective soil depth (m) by digging (D). Representative profiles of SS, showing sampling distances from the vine and sampling soil depths (E) and NS (F).

TABLE 1. Description of sequence data for microbial DNA extracted from soil samples, including rhizosphere and bulk soil at depths of 0.3 m and 0.6 m and collected from stony soil (SS) and non-stony soil (NS) across two vintages (2017 and 2018).

		Fungi			Prokaryotes		
	Input	Filtered	Denoised	Non-chimeric	Filtered	Denoised	Non-chimeric
SUM	9,110,150	7,278,506	7,153,102	6,924,603	5,267,378	4,300,348	4,127,815
Average	159,827	127,693	125,493	121,484	94,06	76,792	73,711
Stdev	88,128	66,311	64,268	58,145	49,792	36,938	35,778

TABLE 2. Alpha diversity results for fungal and prokaryotic communities, categorised by depth, sample type and vintage for both stony (SS) and non-stony (NS) soils. The Table includes the sample sizes (n) and p-values obtained from Kruskal-Wallis pairwise comparisons.

		Fungi								
		SS *vs.* NS			SS			NS		
		N	H	q-value	n	H	q-value	n	H	q-value
Depth (m)	0.3	12	0.00	1.0000	12	2.08	0.2234	12	5.07	0.0730
	0.6	12	1.47	0.2704						
Sample type	B	12	0.00	1.0000	12	2.08	0.5453	12	0.00	1.0000
	R	12	1.47	0.5453						
Vintage	2017	12	0.65	0.4189	12	8.67	0.0117*	12	7.36	0.0133*
	2018	12	0.65	0.4189						
		Prokaryotes								
Depth (m)	0.3	11; 9	0.64	0.6375	9; 12	0.05	0.8311	11; 12	0.06	0.8311
	0.6	12	3.41	0.2642						
Sample type	B	12	3.41	0.3880	9; 12	0.61	0.5981	11; 12	0.46	0.5981
	R	11; 9	0.24	0.6214						
Vintage	2017	11; 9	1.21	0.3247	9; 12	3.96	0.0932	11; 12	4.13	0.0932
	2018	12	2.61	0.1590						

B w= bulk; R = rhizosphere; SS = stony soil; NS = non-stony soil.
*q value < 0.05.

5. Statistics

Statistical analysis of the microbial dataset was conducted using QIIME2 v2019.7 (Bolyen *et al*., 2019) and XLSTAT (Addinsoft, 2021). Differences in alpha diversity were assessed using Kruskal-Wallis test, with significance determined by a q-value threshold of 0.05 for multiple comparisons. Beta diversity was evaluated for statistical significance using PERMANOVA with 999 permutations. Analysis focused on the effects of depth and vintage as key factors, irrespective of samples type (rhizosphere *vs*. bulk), since it was not a differential variable when comparing sample type across NS and SS. Statistically significant taxa were identified using ANCOM (Mandal *et al.,* 2015). To explore the relationship between soil physicochemical characteristics and the relative abundance of identified species, canonical correspondence analysis (CCA; Ter Braak, 1986) were performed using 1000 permutations. Soil physicochemical data from different sites, depths and vintages were analysed using ANOVA, after verifying normality and homoscedasticity (Di Rienzo *et al*., 2018).

RESULTS

1. Physicochemical description and vintage characterisation

The primary physicochemical difference between the contrasting soil profiles within the study vineyard, apart from soil stoniness, was the content in calcium carbonate ($CaCO_3$). At SS, it was approximately 3 %, compared to less than 0.3 % in NS at a depth of 0.6 m (Table S1). Below this depth, SS became increasingly stony, with 77 % stone content and $CaCO_3$ depositing and forming a hard compact layer (Figure 1E). Exchangeable potassium content increased with depth across all soil profiles. Other measured variables varied between the sites depending on the vintage, that is seasonal weather conditions and vineyard management practices. The 2017 and 2018 vintages exhibited similar temperatures from September to March, except for the minimum temperature in 2018, which was 0.54 °C lower (Table S2). Additionally, the 2018 vintage experienced 100 mm less rainfall, representing a 50 % reduction compared to the previous vintage.

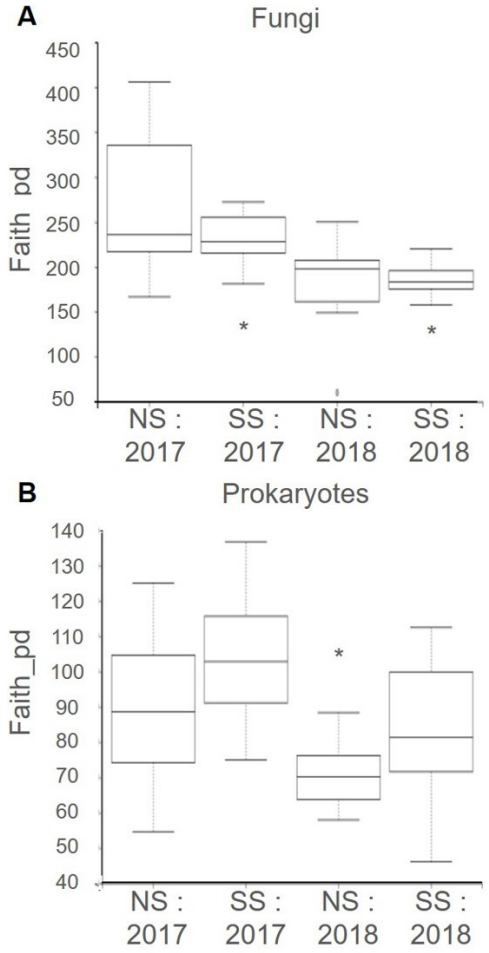


FIGURE 2. Phylogenetic diversity (Faith index) of fungal and prokaryotic communities, across different soil profiles (SS and NS) and vintages (2017 and 2018). Comparisons were made at soil-type and vintage level. There is no discrimination between type of sample (rhizosphere and bulk) and depth (0.3 m and 0.6 m). Asterisk represents outliers.

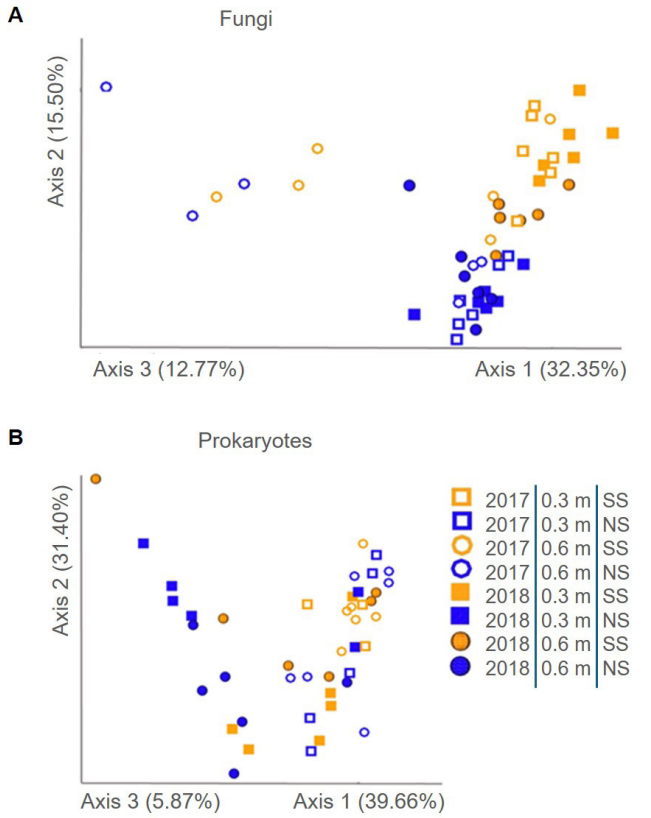


FIGURE 3. Principal Coordinates Analysis (PCoA) of Beta diversity based on unweighted UniFrac results for fungal (A) and prokaryotes (B) communities in SS and NS soils. Data is shown for two vintages (2017 and 2018) and two sampling depths (0.3 m and 0.6 m). There is no discrimination between type of sample (rhizosphere and bulk).

2. Microbial DNA sequencing data description

As indicated in Table 1, a total of 7,278,506 reads was obtained for ITS1, and 5,267,378 reads for the 16S rRNA gene. After denoising, 6,924,603 Amplicon Sequence Variants (ASVs) were identified for ITS1, and 4,127,815 non-chimeric ASVs for 16S rRNA, which were used for downstream analysis.

3. Diversity of the microbial communities

3.1. Intra-soil profile diversity (alpha diversity)

The Kruskal-Wallis pairwise analysis of phylogenetic distances revealed significant differences in fungal communities between the 2017 and 2018 vintages, with a Bonferroni corrected p-value of q = 0.0133 for NS and q = 0.0117 for SS. In contrast, no significant differences were observed in prokaryotic communities (q = 0.0932). Depth (0.3 m *vs*. 0.6 m) did not significantly affect either microbial community (Figure 2; Table 2).

3.2. Inter-soil profile diversity (beta diversity)

The PERMANOVA pairwise comparisons of unweighted UniFrac distance showed that fungal communities were significantly influenced by soil depth, vintage and sample type (q < 0.05). For fungi, depth emerged as the most influential factor across both soil profiles, with vintage being significant only in the SS profile. By contrast, no significant within-site differences were observed in terms of prokaryotic communities (Table 3), with vintage being the sole significant factor when comparing across depths and vintages within soil profiles (SS q = 0.0220, NS q = 0.0060).

As shown in Table 3, sample type was only significant for fungal communities between soil profiles and not within soil profiles (SS and NS), and it was not relevant for prokaryotes.

The three axes of the PCoA plot accounted for a total of 60.62 % and 76.93 % of the variability in fungal and prokaryotic communities, respectively, as shown in Figure 3. Fungal communities exhibited a clear separation between soil profiles at different depths and in different vintages. Prokaryotic communities did not show the same trend.

TABLE 3. β-diversity PERMANOVA pairwise comparisons of fungal and prokaryotes communities based on unweighted UniFrac distance and considering depth, sample type and vintage. Comparisons were made both between sites (SS and NS) and within-each site. Bonferroni corrected p-values (q-values) are presented.

		Fungi								
		SS *vs.* NS			SS			NS		
		n	pseudo-F	q-value	n	pseudo-F	q-value	n	pseudo-F	q-value
Depth (m)	0.3	24	11.67	0.0015*	24	5.32	0.0015*	24	3.84	0.0024*
	0.6	24	4.25	0.0050*						
Sample type	B	24	8.71	0.0020*	24	1.38	0.2120	24	2.19	0.0612
	R	24	2.99	0.0405*						
Vintage	2017	24	2.91	0.0018*	24	2.90	0.0180*	24	1.91	0.0950
	2018	24	7.71	0.0020*						
		Prokaryotes								
Depth (m)	0.3	20	1.86	0.2310	21	2.84	0.0660	23	1.54	0.2340
	0.6	24	2.94	0.0660						
Sample type	B	24	1.52	0.7420	21	0.54	0.7420	23	0.51	0.7420
	R	20	0.80	0.7420						
Vintage	2017	20	2.22	0.0924	21	2.98	0.0220*	23	6.00	0.0060*
	2018	24	1.82	0.1410						

B = bulk; R = rhizosphere; SS = stony soil; NS = non-stony soil.
*q value < 0.05.

TABLE 4. Relative abundance (%) of fungal taxa in rhizosphere and bulk soil samples from SS and NS at 0.3 m and 0.6 m depth over two vintages (2017 and 2018).

	SS		NS		SS		NS	
	2017	2018	2017	2018	0.3	0.6	0.3	0.6
Ascomycota	52.64	58.93	55.14	55.06	49.35	62.22	62.04	48.16
Unidentified fungi	29.86	15.71	21.88	13.44	27.43	18.14	12.81	22.52
Mortierellomycota	7.75	11.50	12.54	16.72	10.3	8.95	13.28	15.99
Basidiomycota	7.06	10.98	8.46	12.90	9.53	8.51	10.57	10.79
Olpidiomycota	0.13	0.14	0.66	0.22	0.18	0.09	0.30	0.58
Glomeromycota	1.12	1.16	0.40	0.47	1.15	1.13	0.17	0.69
Rozellomycota	0.98	1.07	0.36	0.64	1.53	0.51	0.58	0.42
Chytridiomycota	0.11	0.22	0.28	0.39	0.20	0.13	0.13	0.54
Kickxellomycota	0.28	0.25	0.14	0.03	0.30	0.23	0.09	0.09
Calcarisporiellomycota	0.04	0.03	0.11	0.04	0.02	0.05	0.01	0.15
Mucoromycota	0.02	0.01	0.02	0.04	0.01	0.02	0.01	0.05
Zoopagomycota	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.01
Entomophthoromycota	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Blastocladiomycota	0.01	0.00	0.00	0.03	0.00	0.02	0.00	0.02
Basidiobolomycota	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

3.3. Taxonomical composition of the microbial communities

In fungal communities, the most abundant phyla were *Ascomycota*, Morteriellomycota and *Basidiomycota*, with *Ascomycota* being the predominant group, constituting ≥ 50% of the total abundance. The remaining 11 identified taxa were present at less than 1 % abundance, except for *Rozellomycota*, which reached 1.07 % in SS at a depth of 0.3 m, and *Glomeromycota*, which was found at 1.14 % in SS soil (Figure 4A). *Chytridiomycota* showed an increase in relative abundance at 0.6 m depth in NS, although it remained below 1 % (Table 4).

The structure of prokaryotic communities is presented in Figure 4B. The top eight most abundant phyla

include *Proteobacteria*, *Crenarchaeota*, *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Bacteroidetes* and *Verrucomicrobia*. *Nitrospirae*, OD1, GN02, *Chlorobi* and *Parvarchaeota* were present at less than 1 % abundance. TM7 showed an abundance of 1.6 % in SS in 2017 and 3.3 % in 2018, while in 2018 it was only detected in NS at 4.2 %. In the same year, TM6 was found at a 2.2 %. *Acidobacteria* and *Actinobacteria* were more prevalent in SS across both vintages, while *Firmicutes* were more abundant in NS, specifically at 0.6 m depth in 2017. Among the least abundant taxa (TM6, *Nitrospirae*, OD1, GN02, *Chlorobi*, and *Parvarchaeota*), higher relative abundance was observed in NS compared to SS in both vintages (Table 5).

3.4. Effect of soil type physicochemical parameters on microbial communities

Canonical Correspondence Analysis (CCA) for fungi and prokaryotes explained 78.76 % and 83.4 % of the data variability, respectively (Figure 5), with a constrained inertia of 41.9 % and 41.5 % (Table S3). The vintage was the most significant factor affecting the distribution of both fungal and prokaryotic communities. Sampling depth influenced only the fungal communities, with specific effects observed in *Ascomycota* at 0.3 m in SS and *Olpidiomycota* at 0.6 m depth. *Basidiobolomycota* and *Mortierellomycota* were associated with sand content and electrical conductivity in NS. *Glomeromycota* and Zoopagomycota were found to be more prevalent in SS, correlating with higher nitrogen, organic matter and C/N ratios. Phosphorus and pH were not significant factors when differentiating the soil SS and NS profiles.

For prokaryotes, (in addition to vintage) organic matter, nitrogen, and EC were influential in differentiating phyla, particularly *Bacteroidetes*, TM7, *Parvarchaeota*, *Chlorobi* and TM6. Clay content and pH also played a discriminant role, with *Nitrospirae*, *Actinobacteria*, *Chloroflexi* and *Acidobacteria* showing significant associations. The second axis of the CCA indicated a relationship between sand content and *Firmicutes*. $CaCO_3$ had an effect on the relative abundance of *Proteobacteria* in the prokaryotic community, but not on fungi.

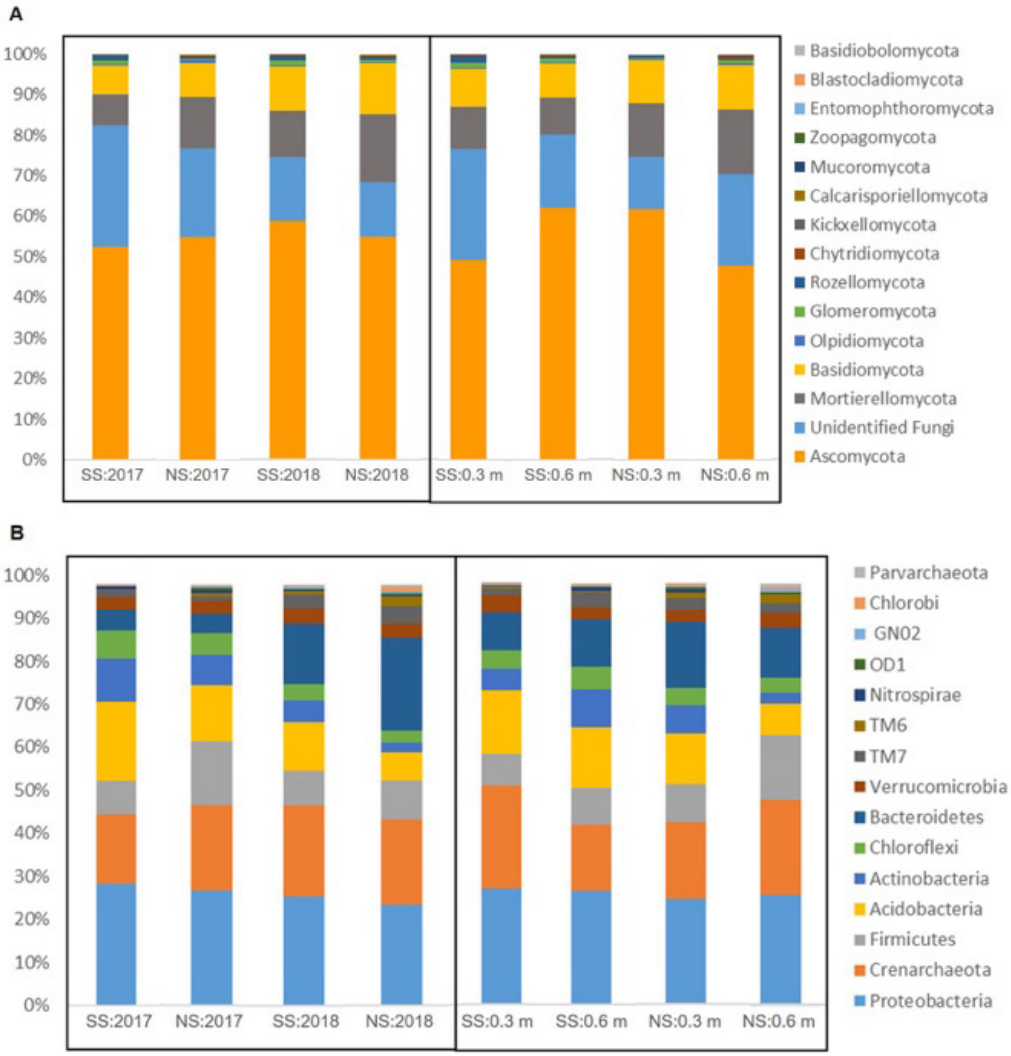


FIGURE 4. Taxonomical composition of fungal (A) and prokaryotic (B) communities and in SS and NS soils over two vintages (2017 and 2018) and at two depths (0.3 m and 0.6 m). There is no discrimination between type of sample (rhizosphere and bulk).

TABLE 5. Relative abundance (%) of prokaryotic taxa in rhizosphere and bulk soil samples from NS and SS at 0.3 m and 0.6 m depth over two vintages (2017 and 2018).

	SS		NS		SS		NS	
	2017	2018	2017	2018	0.3	0.6	0.3	0.6
Proteobacteria	28.89	25.81	27.11	23.95	27.36	26.96	25.02	25.94
Crenarchaeota	16.57	21.69	20.35	20.13	24.64	15.63	18.08	22.57
Firmicutes	7.84	8.33	15.10	9.41	7.43	8.64	8.99	15.55
Acidobacteria	18.94	11.47	13.51	6.57	14.94	14.47	12.21	7.35
Actinobacteria	10.22	5.24	7.18	2.38	5.16	9.03	6.60	2.57
Chloroflexi	6.79	3.67	5.12	2.74	4.51	5.38	4.13	3.60
Bacteroidetes	4.82	14.47	4.71	22.23	8.99	11.34	15.73	11.79
Verrucomicrobia	3.14	3.66	2.96	3.23	4.17	2.88	2.72	3.50
TM7	1.57	3.35	0.95	4.24	1.48	3.41	2.83	2.49
TM6	0.16	0.61	0.92	2.20	0.36	0.46	1.34	1.86
Nitrospirae	0.64	0.40	0.85	0.24	0.27	0.68	0.83	0.21
OD1	0.13	0.32	0.34	0.61	0.15	0.30	0.41	0.55
GN02	0.08	0.36	0.34	0.57	0.14	0.32	0.34	0.59
Chlorobi	0.14	0.26	0.32	0.81	0.28	0.16	0.51	0.65
Parvarchaeota	0.07	0.37	0.27	0.71	0.10	0.35	0.24	0.77

ANCOM analysis revealed distinct fungal communities that were more prevalent in SS compared to NS (Table S4). The identified taxa belong to the orders *Diversisporales, Sebacinales, Helotiales, GS10* and *Chaetothyriales. Diversisporales* and *Sebacinales* were identified as far as the family level (*Diversisporacea* and *Serendipitaceae*), while the other orders remain unidentified.

DISCUSSION

This study evaluated the effects of contrasting intra-vineyard soil stoniness, in combination with varying soil depth, vintage and sample type, on the fungal and prokaryotic communities associated with an own-rooted Malbec grape in a high-elevation commercial vineyard. Amplicon sequencing was performed and the microbiome analysis included comparisons of alpha and beta diversity, taxonomical composition and their relationship with soil physicochemical characteristics.

The experimental vineyard had been previously used to evaluate the impact of its contrasting soil profiles on Malbec root morphology and distribution and vegetative and reproductive expressions (Mezzatesta *et al*., 2022). It had been shown that in the stony soil (SS) of this vineyard, root depth was not limited by soil physical constraints, and that the first 0.45 m held most of the vine root system, likely associated with better availability of water and nutrients. In addition, in SS, fine roots (< 1 mm) had been found to be greater in quantity and patchily distributed in sites with higher contents of silt, clay and organic matter. By contrast, the non-stony soils (NS) of the vineyard, had a more uniform distribution in terms of total root size category throughout the soil profile and lower total root quantity. The root system plays a crucial role in nutrient availability through root exudates, which influences microbial dynamics by promoting microbial selection and competition (Philippot *et al*., 2013). Although not directly measured in the present study, there seems to be a correlation between the variable root systems and microbial composition.

In the present study, our observations indicate that physical and chemical differences within the soils are primary selective factors influencing fungal and prokaryotic shifts. Steenwerth *et al*. (2008) observed root turnover and exudation at greater depths to be influenced by stress-tolerant bacteria in response to increased rhizodeposition and labile carbon. They also found that microorganisms were more enriched in the surface soil layer between 1.6 m to 2.5 m depths, with variations between bulk and rhizosphere samples depending more on soil physicochemical properties than on depth alone. In our study, depth was a significant factor for fungi but not for prokaryotic communities. This is consistent with Liang *et al*. (2019), who found depth to be irrelevant for bacterial community variations in an intra-vineyard study.

Soil depth and vintage were more effective in differentiating fungal communities than sample type within soil profiles (SS and NS). This result agrees with Del Pilar Martínez-Diaz *et al*. (2019), who did not observe fungal community changes between bulk and rhizosphere soil samples in Tempranillo grapevines from five vineyards in La Rioja, Spain. By contrast, Zarraonaindia *et al*. (2015) identified sample type as being an important factor in microbial differences. When evaluating different soil characteristics across depths, we found that both fungal and bacterial communities were influenced by factors such as clay content, organic matter, nitrogen and soil EC. Similarly, Oyuela Aguilar *et al*. (2021) confirmed the impact of pH and other nutrients, such as

organic carbon, organic nitrogen, C/N ratio and phosphorus, on microbial communities in San Juan, Argentina.

Bacteria were particularly affected by pH, and *Proteobacteria* by calcareous content. The latter finding is important, since $CaCO_3$ was the most differential soil component in SS and NS; this may indicate a specific soil-bacterial interaction and supports the idea that microbes can serve as biogeographic markers of terroir (Bokulich *et al*., 2014; Bokulich *et al*., 2016; Belda *et al*., 2017; Gobbi *et al*., 2022).

The vintage was significant for both fungi and prokaryotes, though significant differences in prokaryotes were observed only within soil profiles, while fungi showed vintage effects only in the SS site. These differences may be due to annual variations in viticultural practices (which were identical on both soil sites), as well as meteorological conditions, rainfall and temperatures in 2017 and 2018. Differences in the structure of prokaryotic communities depending on elevation gradients and related to macro and micronutrient availability were found by Corneo *et al*. (2013). Burns *et al*. (2015) and Burns *et al*. (2016) demonstrated that changes in agricultural practices can variably affect microbial soil composition, even within the same vineyard. Soil fertilisation increased organic matter and total nitrogen in both soil types, which led to decreased pH values as a result of the salt content of the soil solutions (Sparks *et al*., 2020).

Alpha diversity analysis revealed that the 2018 vintage, characterised by higher soil fertility, exhibit significant differences in fungal communities but not in prokaryotes. This suggest that fungi are more sensitive to soil fertility changes than prokaryotes. Mezzatesta *et al*. (2022) demonstrated that the stony soils of this vineyard have lower water holding capacity and a shallower initial soil layer, in which grapevine roots were more concentrated. When comparing both soil profiles, the beta diversity analysis showed a significant effect of the vintage on fungal communities, with depth and sample type also contributing to these differences. However, depth and sample type did not influence prokaryotic communities. The vintage significantly affected prokaryotic communities at an intra-site-scale only. The impact of vintage on fungal communities was observed only in the SS and not in the NS.

Our study identified *Proteobacteria* and Ascomycetes as the dominant bacterial and fungal phyla, respectively, consistent with previous findings in the San Juan region by Vega-Avila *et al*. (2015) and Oyuela Aguilar *et al*. (2021). These phyla have been well-documented as being dominant in agricultural crops (Deacon *et al*., 2006; Beckers *et al*., 2017), with *Proteobacteria* often associated with alluvial soils (Schreiter *et al*., 2014)–a soil type also identified in our vineyard by Mezzatesta *et al*. (2022). *Ascomycota* and *Basidiomycota* phyla were present in abundances ranging from 59 % to 73 %, which aligns with reports of these

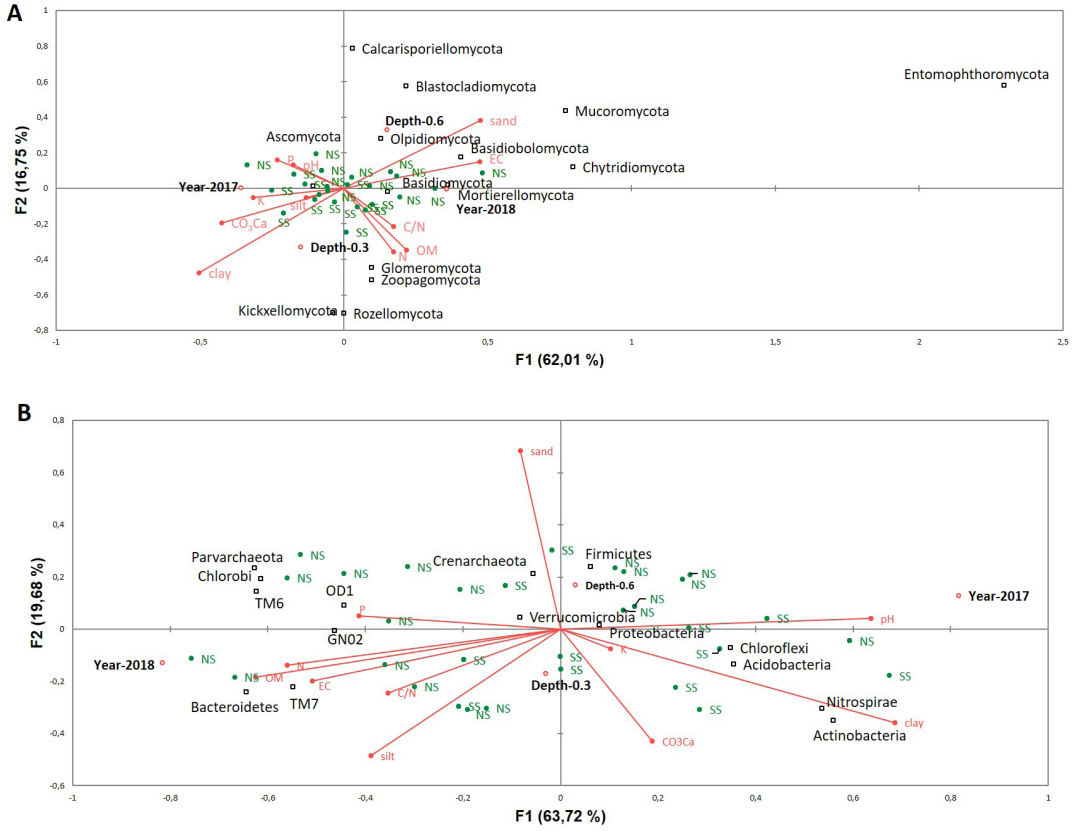


FIGURE 5. Canonical Correspondence Analysis (CCA) for fungal (A) and prokaryotes (B) communities and soil physicochemical data for bulk and rhizosphere samples at two depths (0.3 m and 0.6 m) across two vintages (2017 and 2018). There is no discrimination between type of sample (rhizosphere and bulk).

phyla constituting approximately 75 % of vineyard soils. This suggests that microbial selection is influenced by vine roots, which modulate microbial dynamics through root exudates (Del Pilar Martínez-Diaz *et al*., 2019). However, *Mortierellomycota* emerged as a significant second phylum in our study, increasing the observed fungal diversity to 67 % and 86 %. In 2018, *Bacteroidetes* proportionally increased, while phyla *Proteobacteria* slightly decreased, possibly reflecting higher fertilisation levels. This observation aligns with Eilers *et al*. (2012), who linked increased *Bacteroidetes* abundance to higher carbon availability. Conversely, *Verrucomicrobia* percentages remained stable across vintages, although there was a 45 % increase in SS at 0.3 m depth. This increase cannot be fully explained by organic matter content, as interactions between soil depth and vintage were not significant. *Verrucomicrobia* are known to thrive under lower carbon availability, though their ecological niches remain poorly understood (Eilers *et al*., 2012).

Actinobacteria and *Acidobacteria* abundances were higher in both soils in 2018, but their responses to depth varied between soil profiles. In NS, both phyla decreased to around 50 %, while in SS, *Acidobacteria* remained stable and *Actinobacteria* increased to 73 %. These findings suggest that depth-related changes in microbial communities can be site specific, as noted by Eilers *et al*. (2012).

Our analysis of microbial composition relative to soil components revealed that *Basidiobolomycota* and *Mortierellomycota* were more closely related to sand and electrical conductivity in NS, while the *Glomeromycota* were more influenced by nitrogen, organic matter and the C/N ratio in SS. The high stone content (77 %) of SS likely influenced water retention capacity and nutrient availability, affecting microbial communities differently to NS, where roots and nutrients were more evenly distributed (Swanepoel & Southey, 1989; Smart *et al*., 2006; Mezzatesta *et al*., 2022). Previous observations have shown the impact of arbuscular mycorrhizal communities to be more relevant than vineyard management or grapevine phenological stages, with some taxa found at depths of between 0.5 and 0.75 m (Schreiner, 2020). Our ANCOM analysis showed that the *Diversisporacea* family was more abundant in SS than in NS.bacterie

These findings highlight that grapevine microbial composition across soil depths may relate to nutrient availability, root distribution and overall vine performance. Extensive research on regional grape expressions and wine typicity (Reynolds *et al*., 2013; van Leeuwen e*t al*., 2018; Urvieta e*t al*., 2021) has established a connection with Malbec wine chemistry (Buscema & Boulton, 2015; Urvieta *et al*., 2018) and biogeographic microbial markers (Bokulich *et al*., 2014; Bokulich *et al*., 2016; Gobbi *et al*., 2022). Our study supports the hypothesis that microbial composition in grapevine root zones influences vine adaptation, performance and wine attributes. Additionally, it underscores that factor like soil type (SS *vs*. NS) can significantly impact microbial community structure in the study area.

CONCLUSION

Our study demonstrates that soil type exerts a significant influence on the microbial structure of fungal populations across vintages, depths and sample types, whereas this effect was less pronounced in prokaryotes. We identified six fungal taxa at the order level that exhibited significant differences depending on soil stoniness, being more abundant in stony soil. The vintage (*i.e*., the annual variation in soil organic matter, pH and nitrogen levels due to viticultural management practices), was a substantial factor affecting both fungal and prokaryotic communities. These results provide a descriptive baseline of the microorganisms present in soils of contrasting stoniness in high-elevation vineyards, and supports the unique soil-plant-microorganism combination, even over short distances in vineyards with heterogeneous soil profiles. Further research is needed to elucidate the terroir effect and to thus connect microbial community structures to wine characteristics.

FUNDING

This research was supported by the Catena Institute of Wine (CIW), the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and the Horizon 2020 Programme of the European Commission through the Marie Skłodowska-Curie Innovative Training Network "MicroWine". This paper is part of D. Mezzatesta's doctorate fellowship funded by CONICET-CIW.

ACKNOWLEDGEMENTS

We extend our gratitude to L. Vila, F. Vicario, L. Alcanoni, M. Paredes and B. Iacono for their assistance with sampling and logistical support.

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