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Exploring microbial variation in Grenache vineyards of different ages: A case study from Ribera del Jiloca

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ABSTRACT

This study investigates how vineyard age influences microbial diversity in both soil and grape surfaces in the Ribera del Jiloca region of Spain. Three Grenache vineyards of different ages—Inclinada (72 years), Emparrado (31 years), and Banarro (4 years)—were compared using amplicon sequencing and Sanger sequencing of samples collected from soil and grape berries before and after harvest. The analysis identified several bacterial and fungal phyla: bacterial phyla included Proteobacteria, Actinobacteriota, Bacteroidota, and Planctomycetota, while fungal phyla comprised *Ascomycota*, *Basidiomycota*, and *Mortierellomycota*. The research reveals that older vineyards harbour more diverse and complex microbial communities, particularly fungi and yeasts with oenological relevance. These findings support the idea that vineyard age contributes to the unique microbial terroir, potentially enhancing wine quality and offering insights for sustainable viticulture and winemaking practices.

KEYWORDS: microbiome, Grenache, Aragon, yeast, bacteria, fungi, sequencing, amplicon sequencing

INTRODUCTION

The microbiome in the vineyard is directly related to the expression of "terroir" in wine, as soil and grape microbiota can differentiate wine regions (Gobbi *et al.*, 2022; Liu *et al.*, 2019; Liu *et al.*, 2020). Metabolites produced by fungal microbes found in soil and berries affect wine aromas, helping to differentiate wines from different regions. For example, the peppery notes found in Australian vineyard-grown Shiraz wines are produced by fungal microbiota that accumulate the terpene rotundone in the grapes (Gupta *et al.*, 2019). The microbiome includes not only fungal microbes but also bacterial communities that may make a minor contribution to wine aromas (Liu *et al.*, 2020). The prevalence of bacterial communities in soil may change due to soil management practices, while fungal microbiota may initially be associated with geographic changes (Coller *et al.*, 2019).

In addition to the microbial communities in the soil and berries, the microbiome of plant roots plays a crucial role in the overall health and growth of the vine. The microbiome of plant roots is more abundant than that of external organs and soil surfaces (Mezzasalma *et al.*, 2018) because the conditions and nutrients in the subsoil favour their presence. Microbes near roots are particularly important because microorganisms influence the chemical and nutritional properties of soils; the microbiome contributes to plant health and growth; microorganisms can play an important role in fermentation (Oyuela Aguilar *et al.*, 2020).

Furthermore, the underground environment serves as a significant bacterial reservoir, impacting the microbial dynamics above ground. The underground works as a bacterial reservoir as the colonies here are larger than those found in the aboveground (Zarraonaindia *et al.*, 2015). Bacterial populations in the rhizosphere can be beneficial, neutral, or harmful for the vine. The main benefits are preventing the invasion by pathogenic microorganisms and promoting plant growth (Bettenfeld *et al.*, 2022). Interactions between microbes and plants are responsible for the balance of vineyard ecosystems (Köberl *et al.*, 2020), and there is growing interest in understanding and enhancing healthy synergistic interactions between species in these ecosystems. The ecology of the vineyard in terms of yeast populations depends on the time before harvest that is analysed. Leaves and topsoil are often rich in basidiomycetous yeasts, while grapes are prone to low-fermentation ascomycetous communities at the beginning, and high-fermentation and osmo-resistant populations are often found before harvest (Barata *et al.*, 2012).

Among the various factors influencing microbial populations in vineyards, several key elements have been identified. Among all the factors conditioning the microbial populations in the vineyard, climatic conditions, grape variety, soil composition and viticultural practices are most commonly described. However, others, such as vineyard size and age, have also been recently considered (Coller *et al.*, 2019; Gobbi *et al.*, 2022; Gupta *et al.*, 2019; Liu *et al.*, 2019; Liu *et al.*, 2020; Mezzasalma *et al.*, 2018). Several studies have investigated how vine age affects vine development and wine quality, claiming that old vines can result in wines with berry scents and fruit flavours (Reynolds *et al.*, 2008). This dependency could have microbial populations as a link in the chain. Although most authors conclude that there is a dependence between microbiological populations and plant age, the direction of this dependence is still uncertain.

To gain a deeper understanding of these microbial dynamics, advanced molecular techniques are employed. While simple microbiological analyses, such as culture-based methods, help characterise the microbial properties of yeast, fungi, and bacteria, sequencing can provide rapid and detailed genomic information on the entire microbiome of grapes and vines (Morgan *et al.*, 2017). These molecular techniques include the use of polymerase chain reaction (PCR) and the use of markers such as ribosomal RNA genes, among others, to identify species at higher resolution. These techniques can also be used in winemaking to determine the populations involved in alcoholic fermentation at different fermentation stages (Sun & Liu, 2014). Not all techniques can be used to identify the overall ecology of vines and grapes, so more than one technique may be needed to identify dominant and non-dominant species (Belda *et al.*, 2017). In this way, accurate characterisation of the microbial community, among other factors, could explain variability between vineyards.

Amplicon sequencing has revolutionised the field by providing comprehensive insights into microbial communities. Amplicon sequencing technologies, such as Illumina and PacBio, enable the sequencing of entire genomes rapidly and cost-effectively, allowing for a more detailed understanding of microbial diversity and function (Hardwick *et al.*, 2017). High-Throughput Sequencing (HTS), particularly metagenomics, is a powerful approach that involves sequencing DNA extracted directly from environmental samples, bypassing the need for culturing. This method provides a broad overview of the microbial community, including both dominant and rare species, and can reveal functional capabilities by identifying genes involved in metabolic pathways (Baudhuin, 2013). Metagenomics is especially useful in winemaking for monitoring microbial populations throughout the fermentation process, ensuring quality and consistency in the final product (Ercolini, 2013).

In the context of this study, specific correlations have been observed in Grenache vineyards. Correlations have been observed between changes in the microbiome of the soil and the berries of Grenache vineyards in Ribera del Jiloca, located in the northern part of Spain, in the provinces of Aragon, at two different reproductive phases. These changes, influenced by geographic location, vineyard location, altitude, and slope, may also be linked to variations in climatic conditions and soil nutrients. This study aims to explore how vine age influences the fungal and bacterial communities present in the soil and on the grape surfaces of three specific Grenache vineyards located within the Ribera del Jiloca region of Spain. Microbial communities were characterised using amplicon sequencing for bacteria and fungi, complemented by Sanger sequencing for yeast, to enhance taxonomic resolution. Our objective is to provide exploratory insights into microbial variation across vineyards of different ages under similar geographical and climatic conditions.

MATERIALS AND METHODS

1. Vineyards location

Ribera del Jiloca is located to the northwest of the province of Teruel and to the southwest of the province of Zaragoza in Spain. The average altitude of the vineyards is between 700 and 900 meters above sea level. The average annual temperature is between 9.5 and 11 °C, with temperatures sometimes higher than 40 °C in summer, while rainfall is around 380 mm and 500 mm. The vineyards to be studied are three, all of which have the Grenache variety planted: Inclinada (41° 9' 38.88" N 1° 31' 17.77" W), Emparrado (41° 9' 51.37" N 1° 32' 19.11" W), and Banarro (41° 10' 41.91" N 1° 27' 34.28" W). The description of each vineyard is shown in Table 1.

2. Soil sampling

Sampling occurred in June 2019 and October 2019. Samples were taken from the three vineyards at three different points in each vineyard. Each sample was collected 5 cm from the chosen vine and at a depth of 10 cm from the soil. Samples (25 g) were homogenised, placed in sterile bags, and transported in a refrigerator to the laboratory. In total, 200 mg of soil was weighed and diluted in 800 µL of sterile water, in triplicate. Samples were shaken for 30 minutes at 150 rpm.

TABLE 1. Viticulture characteristics of the studied vineyards.

Vineyard	Inclinada	Emparrado	Banarro
			
Area	1 ha	0.8 ha	3.7 ha
Plantation year	1959	1990	2017
Grape variety	Grenache	Grenache	Carnacha
Rootstock	R110	R110	R110
Trellising system	Gobelet	Cordon Royat	Gobelet
Irrigation installed	No	No	No
Fertilisation	Earthworm humus	Earthworm humus	Earthworm humus
Grape production	2,000 kg/ha	3,000 kg/ha	Unknown
Soil	Degraded slate	Degraded slate and clays	Clay
Altitude[1]	860 m	820 m	860 m
Physico-chemical parameters			
pH	7.77	8.48	8.42
Sand (%)	56	48	36
Silt (%)	20	16	24
Clay (%)	24	36	40
Chemical balances			
C/N rate	7.07	6.56	6.27
Organic matter (ppm)	7,300	7,900	9,700
Nitrogen (ppm)	600	700	900
Potassium (ppm)	121.2	269.8	258.0
Magnesium (ppm)	111.9	149.6	210.4
Calcium (ppm)	2,090.2	4,220.4	4,489.0
Sodium (ppm)	16.10	11.50	20.70

[1] Average altitude of each vineyard in meters above sea level.

Using the soil wash water as inoculum, we prepared dilutions for colony isolation and further analysis with 16S/ITS sequencing and next-generation sequencing described hereafter.

3. Berries sampling

A total of 150 grape berries were randomly collected from each of three vineyards, selecting berries from different vines and various positions within the clusters. The samples were placed in sterile bags and transported to the laboratory under refrigerated conditions. Ten berries were randomly selected and transferred to a beaker containing 100 mL of sterile water. The mixture was agitated at 150 rpm for 30 minutes.

4. Microbial count and isolation

Yeast and fungi were first grown on glucose chloramphenicol agar (GCA) medium and a variation of the yeast extract peptone dextrose agar (YEPD) medium. To avoid the proliferation of bacteria on the plates, streptomycin was added to the YEPD medium while still liquid. To reduce the proliferation of fungi and isolate just yeast strains, diphenyl crystals were used in YEPD and GCA growing media. One milligram of diphenyl per 100 mL of media was added when both media were at 60 °C. One millilitre of serial decimal dilutions of samples (berry must or soil wash water) was spread-plated. Plates were kept at a constant 28 °C for three days.

Plate count agar (PCA) supplemented with nystatin (50 mg/L) after sterilisation was used to determine aerobic bacteria. Chromogenic and lysine agar were prepared from commercial preparations. The lysine medium (1 g/L of L-lysine) was prepared after the suspension of 6.6 g of lysine medium powder per 100 mL of distilled water; 0.84 mL of a potassium lactate solution (60 % *w/v*) were added to the 100 mL; the solution was boiled to dissolve the medium completely, cooled to 50 °C, and the pH adjusted to 5 with a 10 % *(w/v)* solution of L-lactic acid. Chromogenic agar was prepared with 45.9 g of powder in 1 L of distilled water; the mixture was continuously stirred while heating the solution and boiling for at least 1 minute until complete dissolution of the crystals. One millilitre of serial decimal dilutions of samples (berry must or soil wash water) was pour-plated in selective media and incubated for six days at 30 °C. All media were obtained from Pronadisa (Barcelona, Spain). Isolated grown colonies were kept at –80 °C with 20 % glycerol.

5. Sanger sequencing of isolated cultures

Total DNA was extracted from the collected samples. For that, an isolated culture sample was diluted to 100 µL with water, heated at 94 °C for 10 minutes, cooled, and centrifuged at 10,000 g for 2 minutes. Then, 2 µL of the supernatant (5–100 ng DNA template) was transferred to a PCR tube containing 50 µmol/L deoxynucleotides, 10X reaction buffer, 0.5 µmol/L of each primer and 35 U/mL of Taq™ DNA polymerase (TaKaRa, Tokyo, Japan) in a final volume of 50 µL. Yeast and fungi were identified using fungus-specific universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-GCATATCAATAAGCGGAGGA-3′) (Perpetuini *et al.*, 2022).

PCR amplification was carried out in a Biometra thermocycler (Biometra, Göttingen, Germany). The amplification conditions were as follows: pre-denaturation at 94 °C for 2 minutes; then, denaturation at 94 °C for 30 seconds; annealing at 55 °C for 30 seconds; extension at 72 °C for 1 minute, repeated for 35 cycles. PCR products were run on a 1.6 % agarose gel (Pronadisa, Laboratorios Conda S.A., Madrid, Spain) in 0.5 × TBE (45 mmol/L tris-borate, 1 mmol/L EDTA) buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 mg/L) and visualised under UV light. A 100-bp DNA ladder marker (Roche Molecular Biochemicals, Mannheim, Germany) served as a size standard.

The qualified DNA amplicons were diluted twice with water, and the primer (final concentration of 1 pmol/µL) was sequenced by Secugen (Madrid, Spain; www.secugen.es). Sequences were identified by comparing the query sequence with those in the National Center for Biotechnology Information (NCBI) database using the BLAST platform.

The fungal sequences were aligned using Clustal Omega for pairwise sequence alignment.

6. Amplicon sequencing

For comprehensive DNA extraction and amplicon sequencing, 5 g of soil samples were supplied to Biome Makers (Valladolid, Spain; www.biomemakers.com). DNA extraction was performed with the DNeasy PowerLyzer PowerSoil kit from Qiagen (Becares & Fernández, 2017). To characterise both bacterial and fungal microbial communities associated with bulk soils and rhizosphere samples, we selected the 16S rRNA and internal transcribed spacer (ITS) marker regions. The libraries for both 16S and ITS were prepared using a two-step PCR, as described by Gobbi *et al.* (2019) and Liao *et al.* (2019). Negative controls were added to ensure that no cross-contamination occurred. Libraries were obtained by amplifying the 16S rRNA V4 region and the ITS1 region using custom primers, respectively (Becares & Fernández, 2017). All libraries were prepared following the two-step PCR Illumina protocol, where synthetic DNA sequences were used as a positive control to ensure reproducible results during sequencing. These were subsequently sequenced on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) using two 300-paired-end reads.

Primers were removed from paired-end reads using Cutadapt, and the trimmed reads were merged with a minimum overlap of 100 nucleotides. Sequences were then quality filtered with a maximum expected error of 1.0. Reads with single-nucleotide differences were clustered into amplicon sequence variants (ASVs) using Swarm, followed by the removal of *de novo* chimaeras and singletons. Taxonomy was assigned to ASVs via global alignment with 97 % identity against the SILVA 138.1 database for 16S sequences and the UNITE 8.3 database for ITS sequences (Bansal *et al.*, 2024). Phylogenetic trees were built with MEGA11: Molecular Evolutionary Genetics Analysis version (Tamura *et al.*, 2021).

7. Statistics

ANOVA and the least significant difference (LSD) test were used to analyse differences and determine the means and standard deviations. All calculations were performed with the program PC Statgraphics v.5 (Graphics Software Systems, Rockville, MD, USA). The cut-off for significance was $p < 0.05$. Heatmap analysis was carried out using the website https://www.bioinformatics.com.cn/en (accessed on 10 October 2024).

RESULTS

1. The viticulture aspects of the vineyards under study

The viticultural characteristics of the vineyards under study are summarised in Table 1. In addition to vineyard size and trellising system, edaphic factors such as soil pH and mineral composition may play a role in shaping the microbial communities associated with grape surfaces. This is supported by the variation observed in these parameters, particularly in the Inclinada vineyard, where both pH and mineral content are significantly lower. Moreover, the year of plantation is also worth noting. Given the known correlation between environmental conditions and the fungal and bacterial consortia inhabiting wine grapes, it is plausible that non-random microbial terroir contributes to regional diversity, alongside viticultural and geological factors (Bokulich *et al.*, 2014).

2. Amplicon sequencing

The amplicon sequencing technique was applied to soils from the three different vineyards before and after harvest in the same year. Amplicon sequencing identified yeast and bacteria, which were divided into phyla. Yeast and fungi were classified as *Ascomycota*, *Basidiomycota*, and *Mortierellomycota*, while bacteria were classified as Proteobacteria, Actinobacteriota, Bacteroidota, and Planctomycetota. Figure 1 summarises the phyla distribution of fungi and bacteria identified in June 2019 (before harvest) and in October 2019 (after harvest), before veraison and during harvest, in the three cultivars. As seen, the cultivar experiencing the greatest changes in microbiome phyla distribution during this period is Inclinada. The changes are especially observed in an increase in *Ascomycota* and Actinobacteriota, and a decrease in *Basidiomycota* and Proteobacteria. Statistical analysis confirmed that the increase in *Ascomycota* and Actinobacteriota in Inclinada after harvest was statistically significant ($p < 0.05$), while the decrease in *Basidiomycota* and Proteobacteria also showed significant differences ($p < 0.05$).

Yeast and fungi species corresponding to the phyla previously mentioned are listed in Table S1 (supplementary data). Biodiversity before and after harvest, corresponding to the three vineyards, is represented as Venn diagrams in Figure 2. The proximity of the different microorganisms obtained with amplicon sequencing is shown in a phylogenetic tree in Figure 3.

In all vineyards, the yeast diversity seems to decrease when the samples are taken after the harvest. In fact, some species known for their fermentation capacity (*Saccharomyces cerevisiae* being the most important) were only observed in Inclinada before harvest, while others, such as *Metschnikowia* sp., were observed in Inclinada both before and after harvest. There is a noticeable decrease in yeast diversity after harvest in all vineyards. However, Inclinada

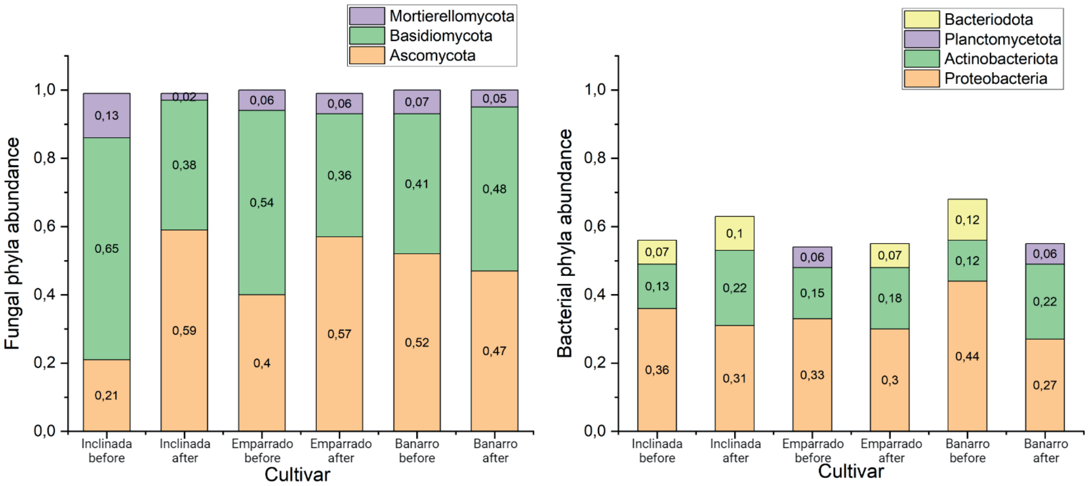

FIGURE 1. Changes in microbiome constitution before harvest and after harvest for the three cultivars under study. Distribution for fungi (left) and bacteria (right). Cultivars (x-axis) are shown with the same order for fungal and bacterial abundance as follows: Inclinada before, Inclinada after, Emparrado before, Emparrado after, Banarro before, and Banarro after.

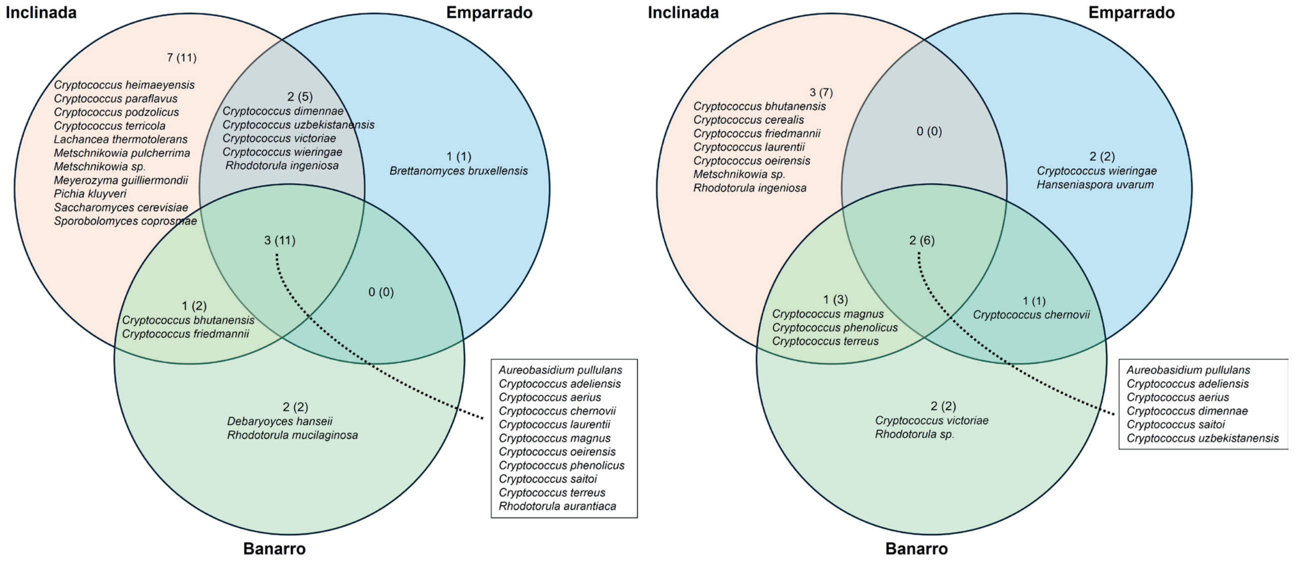

FIGURE 2. Venn diagrams of yeasts and fungi identified by amplicon sequencing in soils of the three vineyards (Inclinada, Emparrado, and Banarro) before (left) and after (right) harvest. Numbers represent shared and unique genera, and numbers in brackets represent shared and unique species.

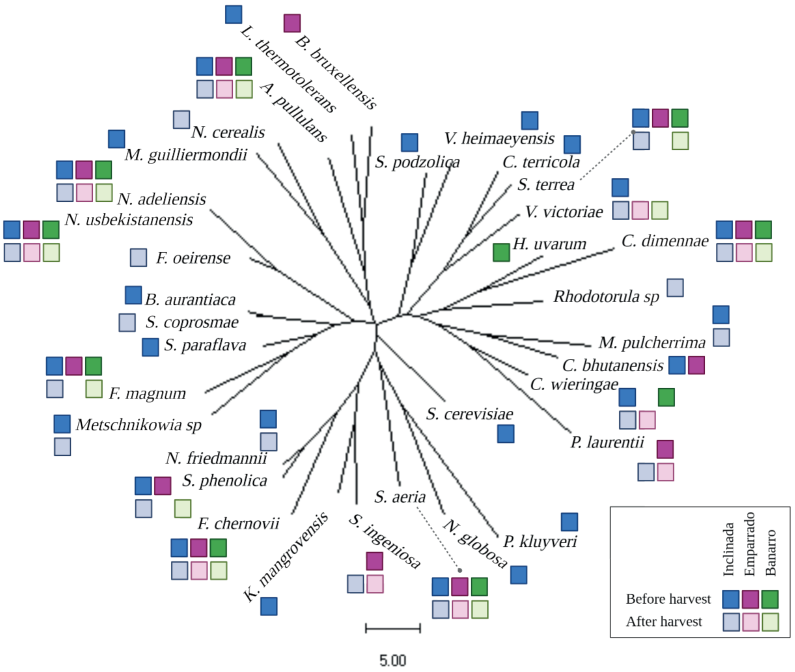

FIGURE 3. Phylogenetic tree for yeast and fungi built up after amplicon sequencing with MEGA11.

maintains a higher diversity compared to Emparrado and Banarro, even after harvest. Moreover, according to the amplicon sequencing results, the fungi listed in Table S1 and plotted in the phylogenetic tree differ based on the age of the vineyard; yeast genera and species diversity are much higher in the older "Inclinada" vineyard (Figure 2). The diagrams highlight both shared and unique genera andspecies among the vineyards. Inclinada, being the oldest vineyard, has the highest number of unique species, indicating a richer and more complex microbial ecosystem.

In other words, the younger the vineyard under research, the less decrease in yeast colonies after harvest there is, according to the observed percentages. In this regard, the population in Banarro, the youngest cultivar, seemed constant throughout the year, while the other two increased *Ascomycota* microorganisms and decreased *Basidiomycota*.

From the Sanger sequencing of isolated cultures, the growing media allowed us to isolate yeast colonies from soil and berries. We preliminarily identified the microorganisms according to the medium in which they were able to grow, and the colour observed in the differential chromogenic agar medium (Table 2).

The ITS sequencing performed afterwards identified eight yeast species from at least 20 isolates, as shown in Figure 4. Most of these isolates were obtained from berries.

TABLE 2. Growing media and isolated colonies. The symbols (+/-) indicate growth or absence of colonies in growing media. The chromogenic medium compares the colour displayed by each species by enzymatic activity.

Cultivar	YEPD 28 °C	YEPD 37 °C	Lysine	AGC	Chromogenic	Yeast species
Inclinada	+	-	+	+	Red	*Aureobasidium pullulans*
	+	-	+	+	White	*Metschnikowia pulcherrima*
	+	-	+	+	Cream	*Meyerozyma guilliermondii*
	+	-	+	+	Cream	*Rhodotorula* spp.
	+	-	+	+	Red	*Hanseniaspora uvarum*
Emparrado	+	+	+	-	-	*Solicoccozyma terrea*
	+	-	+	+	White	*Metschnikowia pulcherrima*
	+	-	+	+	Red	*Hanseniaspora uvarum*
	+	-	+	+	Cream	*Meyerozyma guilliermondii*
	+	-	+	-	Red	*Aureobasidium pullulans*
Banarro	+	-	+	+	Red	*Aureobasidium pullulans*
	+	-	+	+	Red	*Hanseniaspora uvarum*
	+	-	+	+	White	*Metschnikowia pulcherrima*

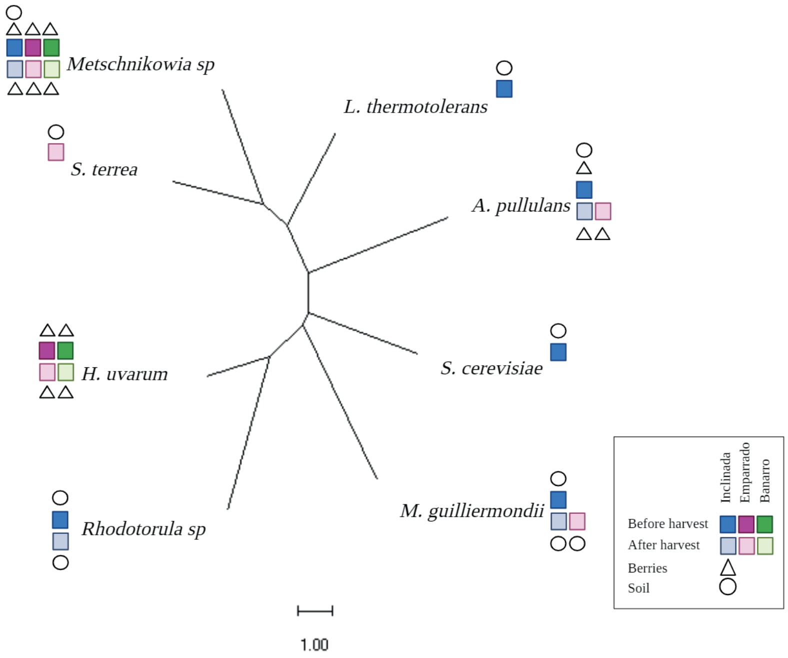

FIGURE 4. Phylogenetic tree for yeast and fungi built after Sanger sequencing with MEGA11.

The frequency with which yeast species were identified by Sanger sequencing in the three vineyards is described in Table S2 (supplementary data) and Figure 5. The Table shows the number of isolates obtained for each of the cultivars, which is also represented as a heatmap in Figure 5. The cultivar Inclinada shows the highest diversity, with a significant number of isolates both before and after harvest. Species such as *Aureobasidium pullulans*, *Metschnikowia pulcherrima*, and *Hanseniaspora uvarum* are prevalent. Next in number of isolates and diversity of microorganisms is the cultivar Emparrado, with five different species identified. This vineyard also exhibits a diverse microbial population, though slightly less than Inclinada. It has a notable presence of *Metschnikowia pulcherrima* and *Hanseniaspora uvarum*. Lastly, the youngest vineyard, Banarro, shows the least diversity. It primarily features *Aureobasidium pullulans* and *Hanseniaspora uvarum*. Nonetheless, it is important to mention that in all three cultivars, the number of isolates identified with this technique is higher or equal after harvest than before. These results show inconsistency with respect to the massive sequencing, where more isolates were identified before harvest, and therefore, the diversity was lower than during the pre-harvest analyses. The molecular sequencing may not reflect the ecology of the vineyards, as the results depend on the growing conditions, the dilutions, and the inhibition against undesired microorganisms in each growing medium.

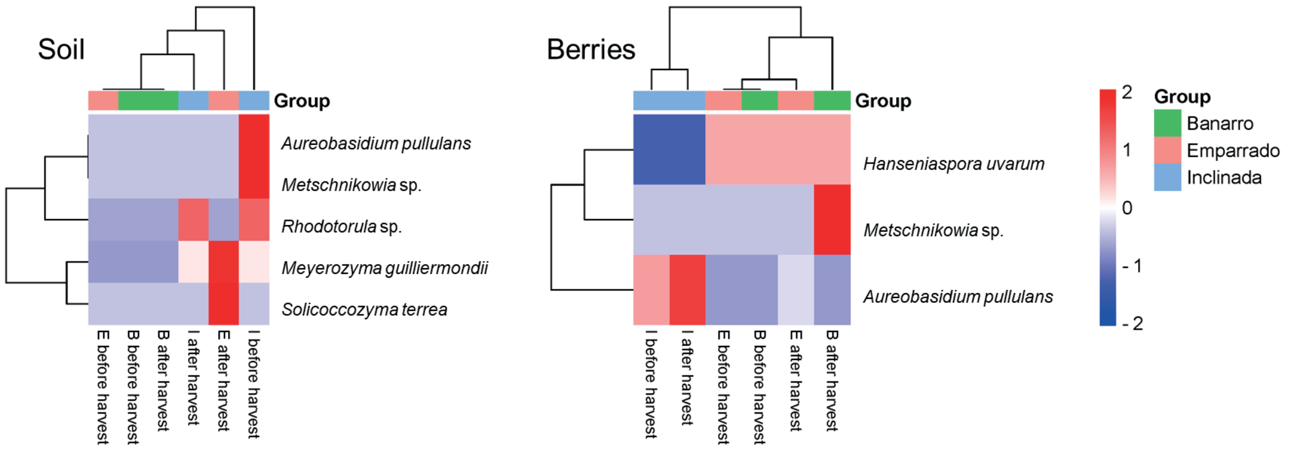

FIGURE 5. Heatmaps for the microorganisms isolated in soils (left) and berries (right) of the three vineyards (Inclinada, Emparrado, and Banarro) before and after harvest, and identified using Sanger sequencing of isolated cultures.

To quantitatively evaluate biodiversity, the Shannon index is presented in Table 3. A higher index value indicates greater biodiversity within the cultivar. This index accounts for both species' richness and their relative abundances, providing a comprehensive measure of which cultivars exhibit a higher number of amplicon sequence variants (ASVs) alongside increased species diversity. According to ANOVA results, significant differences were observed in fungal diversity (Shannon index) between vineyards and sampling times ($p < 0.05$). Banarro showed the highest fungal diversity before harvest (3.57), while Inclinada exhibited the highest value after harvest (3.68). LSD tests confirmed that the decrease in fungal diversity in Banarro after harvest was statistically significant ($p < 0.05$), whereas Inclinada maintained or slightly increased its diversity. These results suggest that older vineyards like Inclinada may sustain a more stable fungal community throughout the harvest period, while younger vineyards such as Banarro experience greater fluctuations.

3. Microbiota with enological interest

Among the yeast and bacteria isolated from soil and berries, some species have oenological interest as they can provide particular features during winemaking. Species from the genera *Metschnikowia* and *Hanseniaspora*, as well as the species *Aureobasidium pullulans*, *Lachancea thermotolerans*, and *Saccharomyces cerevisiae*, together with the bacteria *Lactococcus lactis* and *Oenococcus oeni*, are listed in Table 4 as species with oenological interest. The main oenological features include the production of ethyl and acetate esters, lactic acid, or the modification of the body in wines by increasing the release of polysaccharides.

The yeast species with oenological interest identified by molecular sequencing are not equally distributed by vineyard, as can be seen in Figure 6 and Table 4 previously described.

Banarro has fewer strains with oenological potential, whilst Emparrado has the largest number of species with interest in the winemaking industry. These outcomes do not ensure that these strains would perform correctly and thrive in alcoholic fermentations.

DISCUSSION

1. Phyla/genera diversity

The results for fungi phyla identified in the cultivars are in line with Oyuela Aguilar *et al.* (2020), with ascomycetes, basidiomycetes, and mortierellomycetes being the most abundant. Ascomycetes are mentioned to be predominant in cultivar soils, and basidiomycetes are more predominant in pastures, which serves as a differentiating factor. Identified bacterial phyla include Proteobacteria, Actinobacteriota, Bacteroidota, and Planctomycetota. Inclinada shows a decrease in *Basidiomycota* and Proteobacteria before and after harvest.

Regarding the environments in which the yeast and bacteria proliferate, the classification given by Barata *et al.* (2012) could classify most ascomycetes as oxidative or copiotrophic fungi found in environments rich in nutrients, mainly sources of carbon, while basidiomycetes belong to oligotrophic microorganisms found in nutrient-poor and oxygen-rich environments. The degree to which those three groups develop is determined by the availability of nutrients, which varies during the plant cycle. After veraison, the distribution of nutrients on the berry surface plays a crucial role in maintaining the equilibrium between these groups.

As for phyla abundance, there are significant differences between vineyards observed around the globe. Climatic conditions may play an important role in this regard. In a study considering 13 different countries, the Proteobacteria showed to have the highest abundance in all countries under study, with ranges going from 18.8 % to 32.5 % in Portugal and Argentina, respectively (Gobbi *et al.*, 2022). The results observed in this analysis are within the values reported, with a slightly higher percentage observed in Banarro before harvest. Similarly, the second most abundant phylum was the Actinobacteriota, which is also in line with the observations given by Gobbi *et al.*, while planctomycetes and bacteroidetes showed abundances below 5 %. In two of the cultivars, Emparrado and Banarro, the population of planctomycetes before harvest

TABLE 3. Shannon's index for bacteria and fungi before and after harvest for each of the three cultivars.

Vineyard	Sampling time	Shannon's index bacteria	Shannon's index fungi
Inclinada	Before harvest	5.74	3.46
Inclinada	After harvest	6.40	3.68
Emparrado	Before harvest	5.50	3.10
Emparrado	After harvest	5.68	3.66
Banarro	Before harvest	6.26	3.57
Banarro	After harvest	5.37	3.21

TABLE 4. Yeast and bacterial species identified in Ribera del Jiloca vineyards with potential oenological relevance. The cultivar column indicates the specific vineyard from which each strain was isolated.

Isolates	Cultivar	Oenological interest	References
Aureobasidium pullulans	Inclinada (*b*, *s*)	Pectinases, glucanases, xylanases and proteases for clarification	(Bozoudi & Tsaltas, 2018)
	Emparrado *(b)*		
Metschnikowia sp.[1,2]	Inclinada *(s)*	High glycerol production, high β-glucosidase activity	(Arroyo *et al.*, 2010; García *et al.*, 2017a; García *et al.*, 2017b)
	Emparrado *(s)*		
	Banarro *(b)*		
Hanseniaspora sp.[1,2]	Emparrado *(b)*	Floral aroma and palatability	(Morata *et al.*, 2021)
	Banarro *(b)*	Hydrolyse terpenes	(Fernández-González *et al.*, 2003)
Lachancea thermotolerans[1,2]	Inclinada *(b)*	9 % *v/v* lactic acid	(Vaquero *et al.*, 2020)
		Acidity, high aroma intensity, ripe fruit (pear), and floral aroma notes	(García *et al.*, 2017a; García *et al.*, 2016; García *et al.*, 2017b)
Saccharomyces cerevisiae[1,2]	Inclinada *(b)*	High fermentation performance, low production of H_2S and SO_2	(Cordero-Bueso *et al.*, 2016)
Lactococcus lactis[1]	Banarro *(s)*	Bacteriocin nisin for controlling the growth of bacteria in wine	(Fernández-Pérez *et al.*, 2018)
Oenoccocus oeni[1]	Inclinada *(s)*	Malolactic fermentation	(Lerm *et al.*, 2011)
	Emparrado *(s)*		
	Banarro *(s)*		

Numbers indicate the sequencing method used for the identification: [1] amplicon sequencing and [2] molecular sequencing. Letters indicate whether the strains were isolated from berries *(b)* or from the soil *(s)*.

was replaced by bacteroidetes after harvest and *vice versa*, respectively. In this study, no Acidobacteriota were identified in any of the three cultivars, as the 13-country study suggested as a third group of bacterial phyla in abundance. In terms of fungal population, the presence of *Cryptococcus* spp. in all Inclinada and Emparrado cultivars corresponds to having the highest abundance, as was also observed by Mašínová *et al.* (2017). This species is classified as an oxidative basidiomycete, or oligotrophic yeast, found in nutrient-poor environments in grapes and soil (Barata *et al.*, 2012), especially before harvest or earlier in the plant cycle. It is expected to have a higher abundance of these species as the nutrients only increase in the different vine organs towards/after harvest. The only difference in tendency was observed in Banarro, where the abundance of these basidiomycete yeasts was not only lower than the ascomycete genera but also higher after harvest. The difference in abundance may be related to other factors, such as low sensitivity to fungicides and the location in which this species may be found in the vine. Bruez *et al.* found a larger community of basidiomycetes in woody parts than in soil and reproductive organs like berries; therefore, the age of the cultivar may also play an important role in the growth of such microbial communities (Bruez *et al.*, 2016). The second most abundant phylum in all three cultivars, except for Banarro before harvest, is ascomycetes, with yeast species able to ferment sugars and therefore found in grapes and grape juices (Kachalkin *et al.*, 2015). The population of yeasts belonging to this phylum is more abundant in berries, as different studies carried out in different countries, such as the USA, Spain, and China, prove their abundance (Bokulich *et al.*, 2014; Dutra-Silva *et al.*, 2021; Wang *et al.*, 2015; Wei *et al.*, 2018). This phylum happens to be more abundant after harvest in Inclinada than in Emparrado and Banarro. The transition of yeast populations before and after harvest observed in Inclinada and Emparrado is as expected regarding the studies previously mentioned. Nonetheless, the results obtained in Banarro might be explained after considering other interactions that could not be retrieved with the use of either molecular or massive sequencing. What is clear is that the diversity of bacteria and yeast is firstly related to the

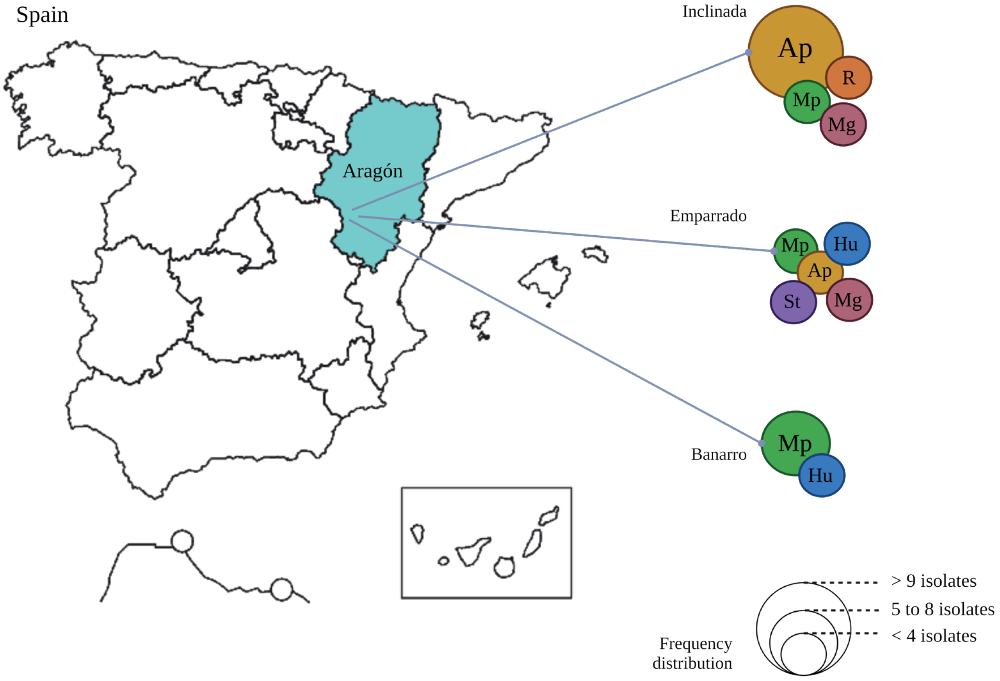

FIGURE 6. Frequency distribution of yeast species isolated by molecular sequencing from soil and berries in the three vineyards. Ap—*Aureobasidium pullulans*, Hu—*Hanseniaspora uvarum*, Mp—*Metschnikowia pulcherrima*, Mg—*Meyerozyma guilliermondii*, R—*Rhodotorula* spp., St—*Solicoccozyma terrea*.

grapevine compartments, with the soil, roots and rhizosphere being the most biodiverse, and decreasing biodiversity in woody parts, reproductive organs, and phyllosphere (leaves) as the least biodiverse compartment of all (Bettenfeld *et al.*, 2022). In terms of biodiversity found per cultivar in this study, and following Shannon's index, Inclinada had the highest value of all three cultivars, with a value of 6.93, considering 582 different microorganisms sequenced in soil. The overall index observed in Spain for prokaryotes is 9.2, while just for fungi is 5.2 (Gobbi *et al.*, 2022).

2. Diversity in Inclinada

As previously described, all three cultivars have similar ecology in terms of biodiversity and proportions of fungi and bacterial populations before and after harvest. Nonetheless, the Inclinada cultivar stands out from the rest of the cultivars for having the highest proportion of ascomycetes after harvest and for having species that have not been sequenced in the other two cultivars. *Lachancea*, *Metschnikowia*, *Pichia*, and *Meyerozyma* are some of the species found exclusively in the soil of the Inclinada vineyard. *Pichia* can form ascospores and produce killer toxins, inhibiting the growth of other fungi. *Metschnikowia* produces pulcherrimin, which acts as a chelator of Fe, so its presence can decrease the growth of some other species. Therefore, in terms of oenology applications, it is of great interest to have this biodiversity in the vineyard. This biodiversity is believed to positively improve the quality of wine as the microbial population, together with factors such as climate, water management, soil, altitude, etc., is considered to be part of the so-called "terroir" (Carrau *et al.*, 2020). The contribution to flavour that these species have may differentiate wines from different regions elaborated with the same variety.

3. Factors conditioning the biodiversity in vineyards

Besides biotic and environmental conditions, there are other factors that impact the biodiversity of fungal and bacterial communities in vineyards. Nonetheless, despite the effect that chemical and biological treatments may have on microbial populations, the microorganisms found in the cultivars, whether in soil, foliar tissue, or sexual organs, are more affected by environmental conditions than by vineyard management systems (Perazzolli *et al.*, 2014). The symbiotic associations between microbial communities and the vine not only provide optimal conditions for microorganisms to thrive but also allow the vine to strengthen and, in cases like with the Pinot noir variety, help the vine grow (Bao *et al.*, 2022). In this last case, bacteria from the genera *Pseudomonas* and *Rhizobium* are examples of microorganisms that help the vine grow.

Regarding biotic and environmental conditions, Bokulich *et al.* (2014) mentioned that changes, not only in weather or climate at a large scale but also variations observed at the microclimate level, impact grape microbial populations. Setati *et al.* (2012) reported that variations in microbial communities within the same vineyard are greater than the variations observed between two different vineyards due to changes in microclimate. The results for biodiversity observed in these three cultivars in Ribera del Jiloca have been assessed during sampling, and the comparison is based on inter-vineyard variations considering the time of year in which the samples were collected.

4. Age of vineyards

Venn diagrams and heatmaps show that the age of the vineyard appears to correlate with microbial diversity. Older vineyards like Inclinada have a more diverse microbial community, which could contribute to the unique terroir and potentially higher wine quality, and the changes in microbial populations before and after harvest suggest dynamic interactions influenced by environmental factors and vineyard management practices.

Ji *et al.* (2019) say that the richness in species of bacteria in the rhizosphere and Shannon's diversity index increase significantly with the plant's age (3 to 11 years old). According to Ji *et al.*, the diversity index increases from 2 to 3.5 as the age of the vineyard increases. The effect of other factors, such as microbial terroir conditioners, has been raised in addition to those associated with climate conditions, soil composition, or wine-growing practices. These include the size of the vineyards or the age of the strains (Gilbert *et al.*, 2014).

The relationship between the age of the vineyard and its vegetative and reproductive development is a subject widely studied. Some authors point out that, contrary to popular belief, old vineyards produce higher yields (Bou Nader *et al.*, 2019; Grigg *et al.*, 2018). They attribute this improvement to the fact that the plant's age favours resistance to water deficit and improves the balance between vegetative and reproductive growth. However, other authors point out that some diseases, such as leaf spots, are more common in older vineyards (Csótó *et al.*, 2020). The age of the plant also determines the presence of metabolites in the berries and the sensory attributes of the wine (Reynolds *et al.*, 2007). However, it is very difficult to assess the impact due to the contribution of multiple factors. Reynolds *et al.* (2008) observed that must from 4-year-old vineyards had higher concentrations of phenols and anthocyanins than that from 14- or 15-year-old vines, and concluded that the age of the vineyard has more repercussions on the composition of must and wine than on the grape composition.

In recent years, after including the microbiome in the concept of terroir, several studies have highlighted the role of grapevine genotype and rootstock in shaping microbial communities, particularly in the rhizosphere. For instance, Noceto *et al.* (2021) demonstrated that arbuscular mycorrhizal fungi tend to form highly specific symbiotic associations with grapevines, suggesting that the composition of these fungal communities may be strongly influenced by plant genotype. These findings support the idea that grape variety–rootstock combinations can significantly affect the structure and function of microbial reservoirs in vineyard soils. Although our study did not assess rootstock-specific effects, the observed microbial patterns may reflect underlying plant–microbe interactions shaped by vineyard age and plant material.

Moreover, several studies have been carried out correlating the microbiome, among other factors, with the age of the plant (Bettenfeld *et al.*, 2022; Pretorius, 2000). Andreolli *et al.* (2016) found that there is a greater richness of bacterial genera in 3-year-old vineyards than in 15-year-old ones, suggesting a higher potential for pathogen control in young plants. However, more authors found greater richness in older vineyards. Thus, Bruez *et al.* (2016) compared 42- and 58-year-old vines and claimed that the genus richness grows with age. In this line, Dissanayake *et al.* (2018) also found more diversity of fungi in 13-year-old vines than in 8- and 3-year-old vines. The same trend was found in species of bacteria in the rhizosphere (Ji *et al.*, 2019; Zhou *et al.*, 2021). Ji *et al.* (2019) observed that both metabolic activities and functional diversity of soil microbial communities increased significantly with the age of the vineyard (from 3 to 11 years of age). Other authors point out that this relationship may not be proportional across the entire plant age spectrum, as it appears that the highest incidence of diseases in plants occurs at the age of 15–20 years (Bruez *et al.*, 2016), and that the microbial richness seems to be reduced on plants over 20 years old (Schreiner & Mihara, 2009).

Ji *et al.* (2019), in their studies with vineyards of different ages, incorporated variables to relate not only the microbiota but also some components of the grapes. Thus, they found that grapes from 12-year-old vines presented fewer phenolic compounds, more tannins, and more acidic compounds than 3- and 6-year-old vineyards. The authors conclude that the age of the plant affects the microbial community of the rhizosphere and, therefore, the quality of the grape. To provide more insight into the above-mentioned studies, Manici *et al.* (2017) studied the microbiome in the rhizosphere of young plants that were planted to replace sick vineyards in old vineyards. They found that microbial diversity was the same in young plants as in the old ones in the same vineyard, which leads to the conclusion that the plant age itself is not the determining factor. However, this result is consistent with the hypothesis that the soil is the storage place of many microbial species, and that their diversity may be conditioned by the age of the plants that grew in it.

This study provides exploratory insights into the microbial diversity associated with Grenache vineyards of different ages within a specific area of Ribera del Jiloca. While the findings suggest that vineyard age may influence microbial complexity, particularly in fungal and yeast communities, we acknowledge the limitations in sample size and spatial coverage. The results are based on three vineyards and a limited number of soil and berry samples and, therefore, should be interpreted as preliminary observations rather than general conclusions about the entire wine-growing region. Future studies with broader sampling and replication are needed to validate and expand upon these findings.

CONCLUSIONS

The biodiversity observed in the three Grenache vineyards studied within Ribera del Jiloca aligns with patterns found in other subcontinental regions of the Iberian Peninsula, showing similar dominant fungal and bacterial phyla. Although the sample size and spatial coverage are limited, the study provides valuable exploratory insights into microbial richness and diversity in relation to vineyard age. The data suggest that older vineyards, such as Inclinada, may harbour more diverse and complex microbial communities, including fungi and yeasts with oenological relevance. This increased biodiversity highlights the potential for discovering strains that could enhance wine quality. Understanding these patterns, even at a localised scale, may inform future vineyard management strategies aimed at promoting microbial diversity and preserving terroir. These findings contribute to the growing body of knowledge on vineyard ecology in Spanish viticulture, offering preliminary data on the microbial dynamics of Ribera del Jiloca.

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Data availability

Raw files for fungal amplicons for each sample are available in NCBI under BioProject accession code PRJNA1179731.

Author contributions

Conceptualisation, J. A. S. L. and C. L.; methodology, C. L.; software, P. H. and C. E.; validation, C. L., C. E., and J. A. S. L.; formal analysis, P. H.; investigation, P. H.; resources, C. L.; data curation, P. H. and C. E.; writing—original draft preparation, P. H. and C. E.; writing—review and editing, C. L.; visualisation, P. H., C. E., and C. L.; supervision, C. L. and J. A. S. L.; project administration, C. L.; funding acquisition, J. A. S. L. and C. L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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