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Microbial diversity of the soil, rhizosphere and wine from an emerging wine-producing region of Argentina

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ABSTRACT

The aim of this study was to characterize the microbial communities of Malbec vineyards recently established in a re-emerging wine region of Argentina. We studied the wine microbiota at different fermentation stages and the soil and rhizosphere microbial communities of two vineyards. A next-generation sequencing (NGS) approach was used to identify bacterial and fungal communities. The soil and rhizosphere samples showed a predominance of the phyla *Proteobacteria* and *Actinobacteria*. The order *Rhizobiales* stood out in the soil and rhizosphere of the two vineyards analyzed. Members of this order are recognized for their plant-growth promotion properties. Regarding fungal communities, *Ascomycota* and *Basidiomycota* were the most abundant phyla. The high abundance of the genus *Ilyonectria* in one of the vineyards may have impact on the health of vines. In wine samples, we detected low levels of lactic acid bacteria and the persistence of acetic acid bacteria (AAB) throughout the fermentation process, although there were no discernible effects on the acidity of wine. The results achieved could allow winemakers to improve the vineyard management practices and the fermentation process to favor the growth of microorganisms potentially beneficial for the health of the vines and the wine quality, while maintaining the regional microbial biodiversity.

1. Introduction

In the wine industry, the growing demand from consumers for distinctive regional flavors has led to the concept of "terroir", defined as the set of physical (climate and soil texture) and biological (variety of the grape and surrounding biodiversity) characteristics, as well as local viticulture and winemaking techniques, which, together determine the sensory attributes of the wine (Belda, Zarraonaindia, Perisin, Palacios, & Acedo, 2017; Liu, Zhang, Chen, & Howell, 2019). The bacterial and fungal communities of wines are shaped not only by the grape variety but also by geographical and climatic factors, through the growth of the vines (Bokulich, Ohta, Richardson, & Mills, 2013; Bokulich, Thorngate, Richardson, & Mills, 2014). In addition, the microbial communities from different geographical locations show significant differences at the genetic level (Knight, Klaere, Fedrizzi, & Goddard, 2015), as well as at the phenotypic level (Belda et al., 2016; Bokulich et al., 2016), which differentially affect the characteristics of the wine. The role of microbial interactions in the health of the vine, the quality of the fruit, and the quality of the wine has been studied (Barata, Malfeito-Ferreira & V. Loureiro, 2012) and extensively reviewed by Liu et al. (2019).

Argentina is currently the fifth producer after Italy, France, Spain and the USA, accounting for 5% (13.0 mhl) of the world's wine production (OIV, 2019). The main wine-producing regions in this country are those located along the Andes mountain range, both in the northwest and southwest of the country. In the center of the country, several other regions, including Buenos Aires province, are currently developing the activity, although these regions account for only 0.1% of the wine produced in Argentina (INV-Instituto Nacional de Vitivinicultura,). In Buenos Aires province, viticulture was a thriving agro-industry until the middle of the 20th century (Ospital, 2003), but was finally relegated for economic, political, and social reasons. Since the early 2000s, its recovery has begun through ventures that to this date show different degrees of development, with only 50 vineyards and 149 cultivated ha scattered in a total area of 307.571 km^2 (118,754 sq mi) (INV-Instituto Nacional de Vitivinicultura,).

Traditionally, the analyses of microbial diversity have been based on

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isolation methods and further characterization of the colonies obtained, neglecting the non-culturable fraction of the microbes, especially in complex microbial ecosystems. Nowadays, next-generation sequencing (NGS) techniques allow better characterization of the microbial diversity of complex ecosystems, including food samples (Ercolini, 2013; Galimberti et al., 2015; Morgan, Du Toit, & Setati, 2017). NGS technologies have been used to prove that the consortia of fungi and bacteria involved in wine fermentations correlate with the chemical composition of finished wines. In addition, these technologies allow predicting the microbiome-metabolome associations and implications for regionality (Bokulich et al., 2016; Liu et al., 2019; Wang, Hopfer, Cockburn, & Wee, 2021), monitoring seasonal changes in the microbiota present in the vineyard (Bokulich et al., 2013, 2014; Stefanini & Cavalieri, 2018), and monitoring how the different strategies for managing the microbial resources associated with alcoholic fermentation affect the spontaneous malolactic consortium (Berbegal et al., 2019). The microbiome of local soils is also important in the definition of "terroir", which, as mentioned above, is also influenced by the local climate and the characteristics of vineyards (Burns et al., 2015, 2016; Vega-Avila et al., 2015). Furthermore, Zarraonaindia et al. (2015) proved the potential of soil as a reservoir of bacteria that colonize leaves, flowers, grapes, and vine roots.

To our knowledge, only few studies have assessed the diversity of microbial communities associated with Argentine vineyards by using NGS (Oyuela Aguilar et al., 2020; Vega-Avila et al., 2015), all of them focusing on the main wine-producing regions of the country. The two vineyards involved in the present study are located in the southwest of Buenos Aires province, a non-traditional wine-producing region of Argentina. Both wineries have been operative since 2003. Although the agricultural practices and the climate are similar in both vineyards, other characteristics such as soil physicochemical properties are different. In Saldungaray, some vines show an early decline in productivity, according to the winery owner, who also reported a slowdown in malolactic fermentation throughout the years.

A better understanding about the consortia of the soil and rhizosphere microorganisms of the vines and of those involved in the fermentation processes during vinification will help to reveal their contribution to the regional characteristics of the wines. Furthermore, this knowledge could lead winemakers to improve vineyard management practices and the fermentation process to enhance the quality of the wine.

Based on the above, the aims of the present study were: (1) to characterize the bacterial and fungal communities of the soil and rhizosphere from two distinctive vineyards of the Malbec variety in a winegrowing region considered as re-emerging, focusing on microorganisms that are potentially beneficial or detrimental to the health of the grapevines, and (2) to assess the microbial diversity throughout the fermentation process of the Malbec musts and wines of one of the wineries. An NGS approach with massive sequencing of phylogenetic marker amplicons was used to identify partial sequences of the 16S rRNA gene of bacteria and the internal transcribed spacer ITS1 of fungi.

2. Material and methods

2.1. Management of wineries and climatic conditions

The two wineries under study were "Saldungaray" (38° 12′54.5 ″S 61° 46′36.3″ W, 194 m.a.s.l.) and "Al-Este" (38° 48′51.4 ″S 62° 41′39.4″ W, at sea level), both located southwest of Buenos Aires province, Argentina 147 km from each other. Both vineyards grow several varieties, among which is Malbec. Plots are laid out with a spacing of 2 m between rows; withing rows vines planted 1m apart, and trained in a vertical shoot position. The pruning system employed by both vineyards is bilateral cordon de Royat. The soil is covered with native grasses, and artificial drip irrigation system is used in both wineries. During the harvest, both wineries use the manual selection of grape clusters. Both vineyards are routinely treated with several agrochemicals: glyphosate acid (Round-up®), and the fungicides Captan®, Folpet®, Mancozeb®, Ziram®, and Zineb®.

Both wineries are in a region prone to droughts, with a climate that could be characterized as a transition between humid temperate and temperate semi-arid. The average maximum and minimum temperatures in Saldungaray during the pre-harvest months (December 2016 to March 2017) were 28.5 °C and 13.2 °C, respectively. Prevailing winds blow from the north and northwest. In the case of the Al-Este winery, the maximum and minimum temperatures in the same period were 32.8 °C and 19.3 °C, respectively, and the wind usually blows from the west. The region is characterized a seasonal distribution of rainfall with a predominance of summer rains. This was the case for Saldungaray, where the annual accumulated precipitation was 721.5 mm, with 313 mm (43.4%) falling during the pre-harvest months, whereas Al-Este winery had an annual accumulated rainfall of only 303 mm from April 2016 to March 2017, with an extremely scarce rainfall in the pre-harvest months (<20 mm).

2.2. Sample collection and preparation

One grapevine plot of Malbec cultivar per vineyard was sampled, with a surface of less than 3 ha each. A total of 15 samples were obtained: 12 samples of soil and rhizosphere (two vineyards with biological triplicates from each), and three samples of Malbec wine (vintage 2017).

2.2.1. Soil and rhizosphere samples

Soil samples were collected from vineyards of the Malbec variety, three weeks prior to the 2017 harvest, from the two wineries: "Saldungaray" and "Al-Este". To obtain representative samples, a central area of each vineyard was selected, avoiding marked differences in relief or erosion, perimeter fences, roads and loading and unloading areas. The soil and rhizosphere (the soil surrounding the roots) samples were taken adjacent to each vine (within a 40-cm radius from the base) at 20–30 cm deep. Three samples were collected in the same row, skipping three plants between each sample. Other six samples were collected using the same procedure in two different rows, with one row in between. All the nine samples were randomly mixed in groups of three to give combined samples to obtain biological triplicates (supplementary material S1).

Approximately 150–250 g of soil containing roots was placed in sterile stomacher bags (Nasco WHIRL-PAK®, USA), which were labeled and divided for chemical and microbiological analysis and stored refrigerated and separated, to avoid cross contamination. Once in the laboratory, samples were stored at −20 °C until processing.

The biological triplicates of soil were processed under aseptic conditions to separate the roots from the soil. The roots were removed and reserved, and the soil was separated and reserved for subsequent DNA extraction (soil samples).

The rhizosphere samples were obtained by gently shaking the roots with sterile clamp to detach the soil adhered to them and removing the remnant. The rhizosphere samples were grouped in the same way as the soil samples, to constitute three biological replicates per vineyard.

2.2.2. Wine samples and chemical composition

The wine samples were collected from the Saldungaray winery, where a slowdown in malolactic fermentation throughout the years has been reported, according to the following schedule: grape must (24 h after destemming and crushing) pH 3.80, L-malic acid 1.57 g/L; fermentation stage 1 (FS1, day six of the fermentation process, i.e. alcoholic fermentation in development) pH 3.88, L-malic acid 1.03 g/L; fermentation stage two (FS2, day 13 of the fermentation process, i.e. alcoholic fermentation finished) pH 3.93, L-malic acid 0.70 g/L. The final wine had 13.4% ethanol, with total SO_2 50 mg/L. The winemaking process of young red wines in the Saldungaray winery began with a cold pre-fermentation for 48–72 h and involved the use of the commercial yeast Uvaferm BC® (Lallemand Inc., Montréal, QC, Canada) as a starter

in alcoholic fermentation, which took place in concrete tanks for about 10 days, followed by pressing, and then malolactic fermentation for 25–40 days. Since no malolactic bacteria starter had ever been used in this winery, whenever malolactic fermentation occurs, it is spontaneous.

An aliquot of 35 mL of each wine sample was centrifuged for 15 min at 8000 rpm, and the pellets were washed with Tris-EDTA buffer (TE) (20: 2 mM). A sample of bulk soil of each vineyard was physico-chemically analyzed (Table 1) at the Laboratory of the Instituto de Suelos of the Instituto Nacional de Tecnología Agropecuaria (INTA, Hurlingham, Buenos Aires, Argentina).

2.3. DNA extraction

DNA from soil and rhizosphere samples was extracted with the FastDNA Spin Kit for Soil (MP Biomedicals, LLC, Solon, OH, USA), following the supplier's instructions. Regarding the wine samples, since the DNA extraction process was more challenging, the protocol had to be modified to obtain quality genomic DNA and to accomplish the quality and integrity criteria established for NGS techniques. Briefly, the pellet was washed twice with TE buffer (20: 2 mM) and then resuspended in PBS. Then, 1 mL was added to an Eppendorf tube with glass beads, with the following lysis conditions: MT Lysis Buffer, two cycles of 1:30 min in a bead beater, and incubation of 2 min on ice between each cycle.

The DNA obtained was visualized on a 1% agarose gel, stained with ethidium bromide (0.5 mg/mL), to control its integrity. In addition, absorbances at 260, 280 and 230 nm (NanoDrop® ND-1000 Thermo Scientific) were measured as an additional quality parameter to determine the total DNA concentrations and 260/280 and 260/230 absorbance ratios of each sample. Those that exceeded a concentration of 20 ng/μL and had a 260/280 ratio in the range of 1.7–2.1 and a 260/230 ratio in the range of 1.5–2.1 were selected.

2.4. Sequencing

The genomic DNA samples were sent to Macrogen Korea (Seoul, Rep. of Korea), where the amplicon libraries were prepared (Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2). The hypervariable region V3–V4 of the 16S rRNA gene from prokaryotes obtained using primers Bakt_341F: 5′-CCTACGGGNGGCWGCAG-3′ and Bakt_805R: 5′-GACTACHVGGGTATCTAATCC-3′ and the fungal internal transcribed spacer ITS1 domain from fungi obtained using primers ITS1: 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS2: 5′-GCTGCGTTCTTCATCGATGC-3' (Macrogen, default primers) were sequenced by Illumina (MiSeq). Sequences paired-end with 300 bp of length were obtained.

2.5. Sequence analysis

Raw sequences fastq files were demultiplexed, chimeric sequences were filtered and sequence ends were treated, to remove low-quality regions, using QIIME2 (Callahan et al., 2015). Also, mitochondrial and chloroplast DNA were removed (McDonald, Clemente, et al., 2012). The OTUs table was obtained using DADA2 (Callahan et al., 2015). The variation across samples was normalized by rarefying to two different reading depths: to include the wine, soil and rhizosphere sequences into the analysis, the reading depth was set up to 2895 for the 16S rRNA sequences and to 35908 for the ITS sequences, whereas to include only the soil and rhizosphere sequences into the analysis, the reading depth was set up to 6919 for the 16S rRNA sequences and to 92268 for the ITS sequences. The fidelity in the reading depth was evaluated by means of rarefaction curves (qiime diversity alpha-rarefaction of QIIME).

For the alpha and beta diversity analysis, OTUs were assigned and the Shannon index and distance matrix, according to unweighted unifrac distance matrix (UniFrac), were calculated. Statistical analysis was performed using PERMANOVA (test pseudo-F) for beta diversity and a non-parametric Kruskal-Wallis test (Kruskal & Wallis, 1952) for alpha diversity.

Further, the 16S rRNA OTUs were taxonomically analyzed with the Greengenes classifier (v 13_6) (DeSantis et al., 2006; McDonald, Price, et al., 2012), whereas the ITS OTUs were taxonomically analyzed with the UNITE classifier v 8.2 (Abarenkov et al., 2020), using "q2-feature-classifier" plugin (Bokulich et al., 2018; Pedregosa et al., 2011), with which the final taxonomic analysis was also performed, for both the 16S rRNA and ITS sequences.

2.6. Statistical analysis

The taxonomic results obtained for each biological replicate were grouped by means of different taxonomic levels (phylum, class, and order levels). Bar-plot graphics were performed only with the OTUs that showed a relative abundance greater than 0.5% in all three replicates (GraphPad 6.0), and one-way ANOVA and Tukey's test were used to compare the relative abundances between the soil and rhizosphere samples (Statistix 8).

Table 1
Chemical and texture characterization of the soils from the Saldungaray and Al-Este vineyards.

	Metodology	Identification	Saldungaray Soil	Al-Este Soil
Chemical analysis	Conductimetric (IRAM-SAGPyA 29579)	Electric conductivity (mS/cm)	[a]0.73 ± 0.16	[b]2.57 ± 0.40
	Calculation (IRAM-SAGPyA 29578 : 2009)	Water saturation (% v/w)	[a]40.23 ± 2.23	[a]35.00 ± 0.00
	Potentiometric (IRAM – SAGPyA 29574)	pH 1:2.5 water	[a]8.13 ± 0.15	[b]8.47 ± 0.15
	Potentiometric (IRAM – SAGPyA 29574)	pH 1:2.5 CaCl2 0.01 M	[a]7.45 ± 0.16	[a]7.5 ± 0.00
	Potentiometric (IRAM – SAGPyA 29574)	pH 1:2.5 KCl2 1 M	[a]6.90 ± 0.25	[a]7.07 ± 0.06
	Calculation (according to Read J W, Ridgell R H (1921))	Organic material (% w/w)	[a]1.38 ± 0.16	[a]0.75 ± 0.10
	Standard Environmental quality - Soil quality (IRAM-SAGPyA 29571–3: 2016)	Organic Carbon (% w/w)	[a]0.80 ± 0.09	[b]0.44 ± 0.06
	Modified Kjeldahl method (IRAM-SAGPyA 29572:2019)	Organic Nitrogen (% w/w)	[a]0.10 ± 0.02	[b]0.05 ± 0.00
	Calculation	C/N Relation (s/u)	[a]8.13 ± 0.67	[a]9.17 ± 0.96
	Bray Kurtz method (IRAM-SAGPyA 29570–1:2010)	Assimilable phosphorus (% w/w)	[a]11.87 ± 4.46	[b]29.27 ± 9.03
Texture characterization	Determination of soil texture (IRAM-SAGyP 29581)	Clay <2 μm (% w/w)	20.27 ± 2.31	5.50 ± 0.87
		Total slime 2–50 μm (% w/w)	31.43 ± 1.14	10.50 ± 1.80
		Very fine sand-I 50–100 μm (% w/w)	15.80 ± 2.23	14.19 ± 0.81
		Fine sand 100–250 μm (% w/w)	10.17 ± 1.22	65.58 ± 2.06
		Medium sand 250–500 μm (% w/w)	0.50 ± 0.10	3.30 ± 0.05
		Gross sand 500–1000 μm (% w/w)	0.37 ± 0.12	0.59 ± 0.10
		Very gross sand 1–2 mm (% w/w)	0.13 ± 0.06	0.34 ± 0.15
		Carbonates	n.r.	–

3. Results and discussion

As above-described, total genomic DNA with preserved integrity and without contaminant solvents was obtained from 12 soil and rhizosphere samples from Malbec cultivars (two vineyards, with biological triplicates from each), and three samples of Malbec wine from the Saldungaray winery (at different fermentation stages; vintage 2017). The vineyards, located in the southwest of Buenos Aires province, were selected due to their location in a non-traditional wine-producing region of Argentina. Both vineyards shared some characteristics: drip irrigation system, pruning system, grape variety (Malbec), age (less than 20 years). Both have well-drained soil, and the climatic conditions of the region (wide thermal amplitude, low rainfall, and regular winds) are overall favorable for viticulture, although drought has strongly impacted the viticulture and other agricultural activities for years (Andrade, Laporta, & Iezzi, 2009; Scarpati & Capriolo, 2013). The year 2017 was particularly challenging for the Al-Este winery, with practically no rainfall in the pre-harvest months, although an efficient drip irrigation system prevented major losses.

The analysis of the soil texture showed differences, with higher clay and silt content in the Saldungaray vineyard (Loam soil) than in the Al-Este vineyard (loamy sand soil) (Table 1). We also found differences in assimilable phosphorus (P) content (Table 1), which is much higher in Al-Este vineyard and reveals a long history of P fertilization. Carbon and organic nitrogen content are lower in the Al-Este than in the Saldungaray vineyard.

3.1. Diversity

The prokaryotic diversity was studied through massive sequencing of the variable region V3–V4 of the 16S rRNA gene, obtaining 2,673,796 readings, 151,325 of which exceeded the quality filters (free of chimeric sequences, chloroplasts, and mitochondrial DNA) and with minimum values of readings per sample of 2895 and maximum of 15,931, and a total of 5449 OTUs. The diversity of the fungal microbiota was studied by massively sequencing the ITS intergenic marker, obtaining 2,703,697 readings, 1,694,740 of which exceeded the quality filters (free of chimeric sequences), and with values minimum of readings per sample of 35,908 and maximum of 170,948, and a total of 3397 OTUs.

The stabilization of the rarefaction curves indicates that the sampling was approaching its saturation (Rodriguez-r & Konstantinidis, 2014) and that the sequencing depths were adequate to characterize the prokaryotic and fungal populations, providing good coverage of the communities and ensuring adequate representation of the diversity and taxonomic classification in the results (supplementary material S2).

The within-sample species richness (alpha diversity) of each sample and for each genetic marker (16S and ITS) was evaluated through the estimation of the Shannon index and the abundance of unique OTUs, which showed that the richness of the soil and rhizosphere samples was statistically higher ($p < 0.05$) than that of the wine samples (using a reading depth of 2895 and 35908 respectively) for both bacterial and fungal communities (Fig. 1). These results were attributed mainly to the adverse conditions involved in the fermentation process, such as the high concentration of ethanol, presence of SO2, nutrient shortage, etc., and which selects a minority group of microorganisms able to proliferate or at least survive in these conditions. To emphasize the comparison between the soil and rhizosphere samples, the analysis was repeated excluding the wine samples by increasing the reading depth to 6919 for the 16S marker and to 92268 for the ITS marker (data not shown). Although the number of OTUs and Shannon's index increased, no significant differences were found between the soil and rhizosphere samples, which agrees with that described by other authors such as Zarraonaindia et al. (2015). Regarding the comparison between the alpha diversity of the two vineyards, we observed no significant differences (Fig. 1). The principal coordinate analysis plots of the unweighted UniFrac phylogenetic distances (Fig. 2) suggested differences in the bacterial and fungal community composition for the different sample types (soil and rhizosphere of the two wineries, and wine samples) since the samples from the different wineries clustered separately. However, significance was proven only for wine samples using PERMANOVA tests among samples ($p < 0.05$). Despite the differences previously mentioned in some climatic factors or the texture and chemical properties of the soils, both vineyards showed similarities in the structure and composition of the bacterial communities of the soil and rhizosphere. In vineyards located even closer to each other than the ones studied in the present work, other authors found differences in the alpha and beta diversity of the microbial communities present in the soil, driven mainly by management practices, soil properties and climate (Bokulich et al., 2016; Burns et al., 2015, 2016; Knight, Karon, & Goddard, 2020; Vega-Avila et al., 2015).

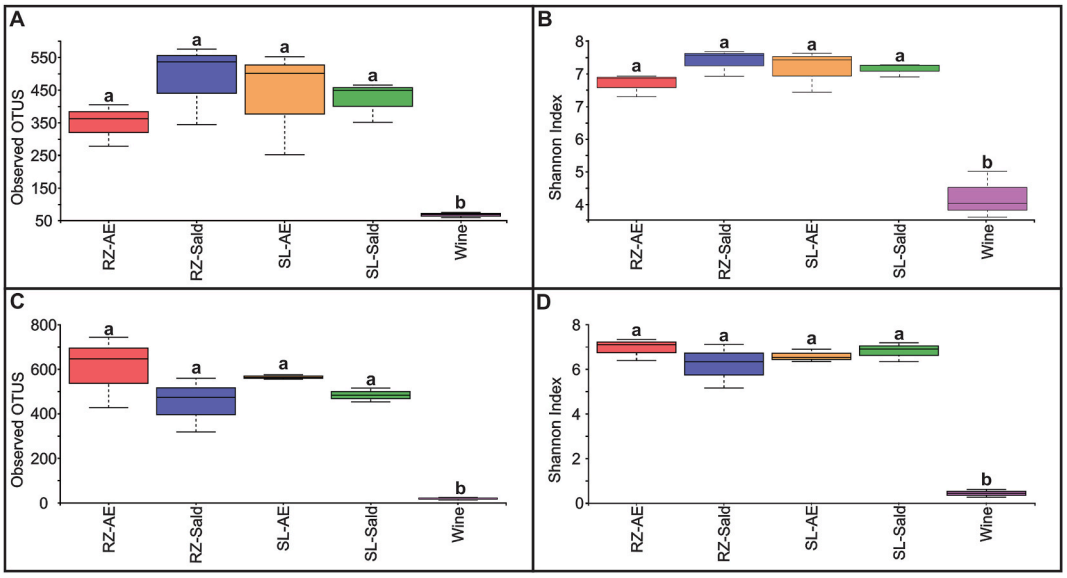

Fig. 1. Alpha diversity estimates based on observed OTUs and Shannon index for the soil (SL), rhizosphere (RZ) and wine samples from both the "Saldungaray" (Sald) and "Al-Este" (AE) vineyards. **A,B** Bacterial community (16S rRNA) at a reading depth of 2895. **C,D** Fungal community (ITS) at a reading depth of 35908. The results of the Kruskal-Wallis analysis are shown as letters in the corresponding data (different letters mean $p < 0.05$).

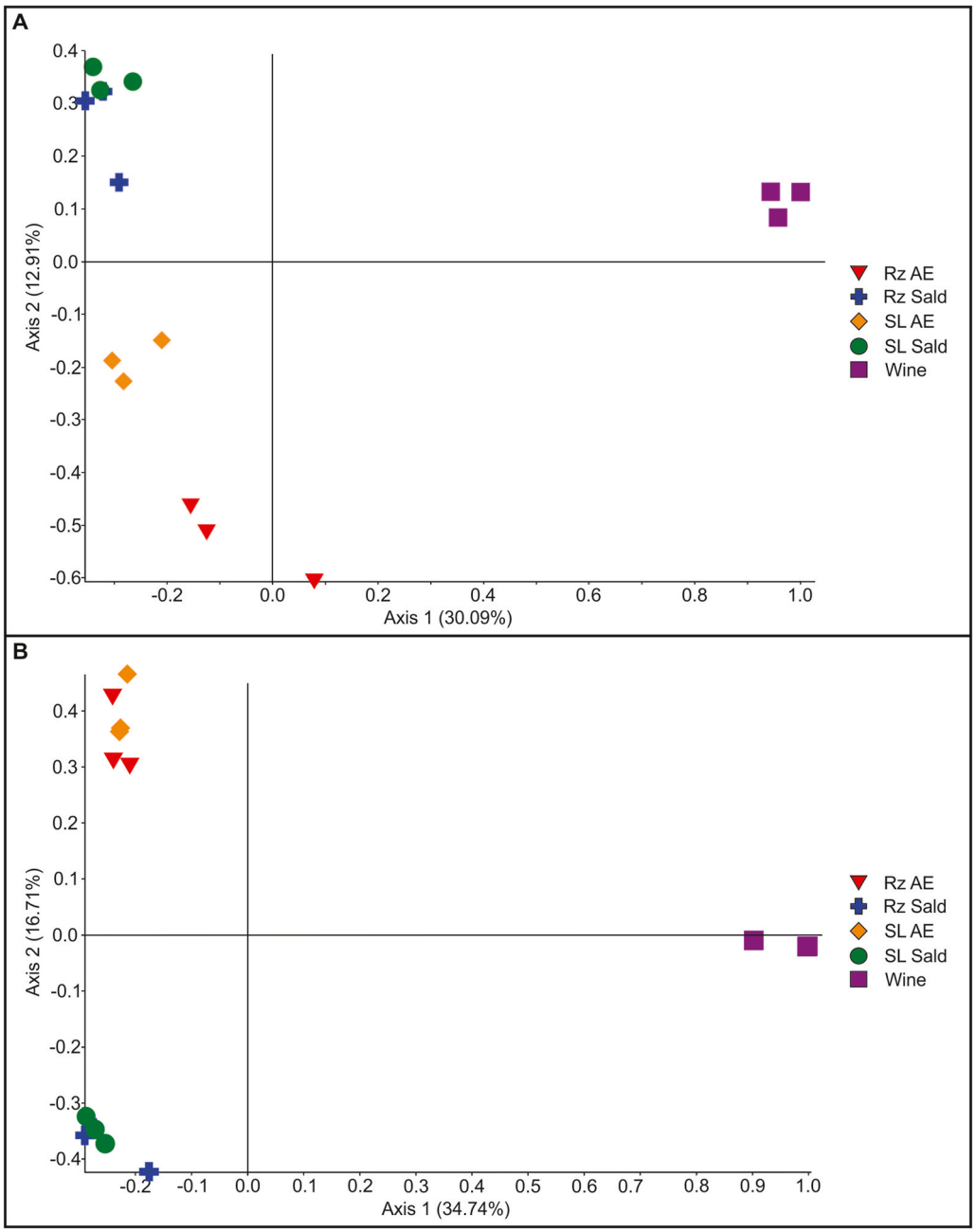

Fig. 2. Principal coordinate analysis (PCoA) for both wineries, "Saldungaray" (Sald) and "Al-Este" (AE), among soil (SL), rhizosphere (RZ), and wine samples based on unweighted UniFrac distances for bacteria (16S rRNA) at a reading depth of 2895 **(A)**, and fungi (ITS) at a reading depth of 35908 **(B)**.

3.2. Bacterial communities

The five most abundant phyla accounted for almost 90% of the total bacterial community. *Proteobacteria* and *Actinobacteria* were the most abundant prokaryotic phyla associated with all the samples studied in the two vineyards (28.4%–55.7% of OTUs and 27%–40.1% respectively). The phyla *Chloroflexi* and *Acidobacteria*, which were almost absent in the wine samples, were also abundant in the soil and rhizosphere samples (6.9%–16.8% and 2.9%–8.3% respectively). *Planctomycetes* were relatively more abundant in the soil and rhizosphere samples (2.6%–6.1%) than in the wine samples (1,3%–2.9%); however, they were almost absent in the rhizosphere samples of Al-Este vineyard. Although *Firmicutes* and *Bacteroidetes* were important phyla in wine, specially at fermentation stage two (FS2), their members were scarcely recorded in the soil or rhizosphere samples. The least abundant phyla present in almost all the soil and rhizosphere samples, and not recorded in wine, were mainly *Gemmatimonadetes*, *Armatimonadetes*, *Verrucomicrobia*, *Candidatus Saccharibacteria* (TM7) and *Parcubacteria* (OD1). Of the total bacterial OTUS obtained, only a minority could not be classified at the phylum level. Other authors reported somewhat similar results, with soil bacterial communities and root-associated communities largely dominated by *Proteobacteria* spp. and varied abundance of *Actinobacteria* spp., *Acidobacteria* spp., *Bacteroidetes* spp., *and Planctomycetes* spp. (Gupta, Bramley, Greenfield, Yu, & Herderich, 2019; Oyuela Aguilar et al., 2020; Vega-Avila et al., 2015; Zarraonaindia et al., 2015).

OIV results also showed similarities in the whole structure and composition of the bacterial communities in the soil and rhizosphere samples within each vineyard (Figs. 1 and 2). However, some differences became more evident when the taxa were compared one by one. Fig. 3-D shows that rhizosphere samples from Al-Este vineyard were significantly

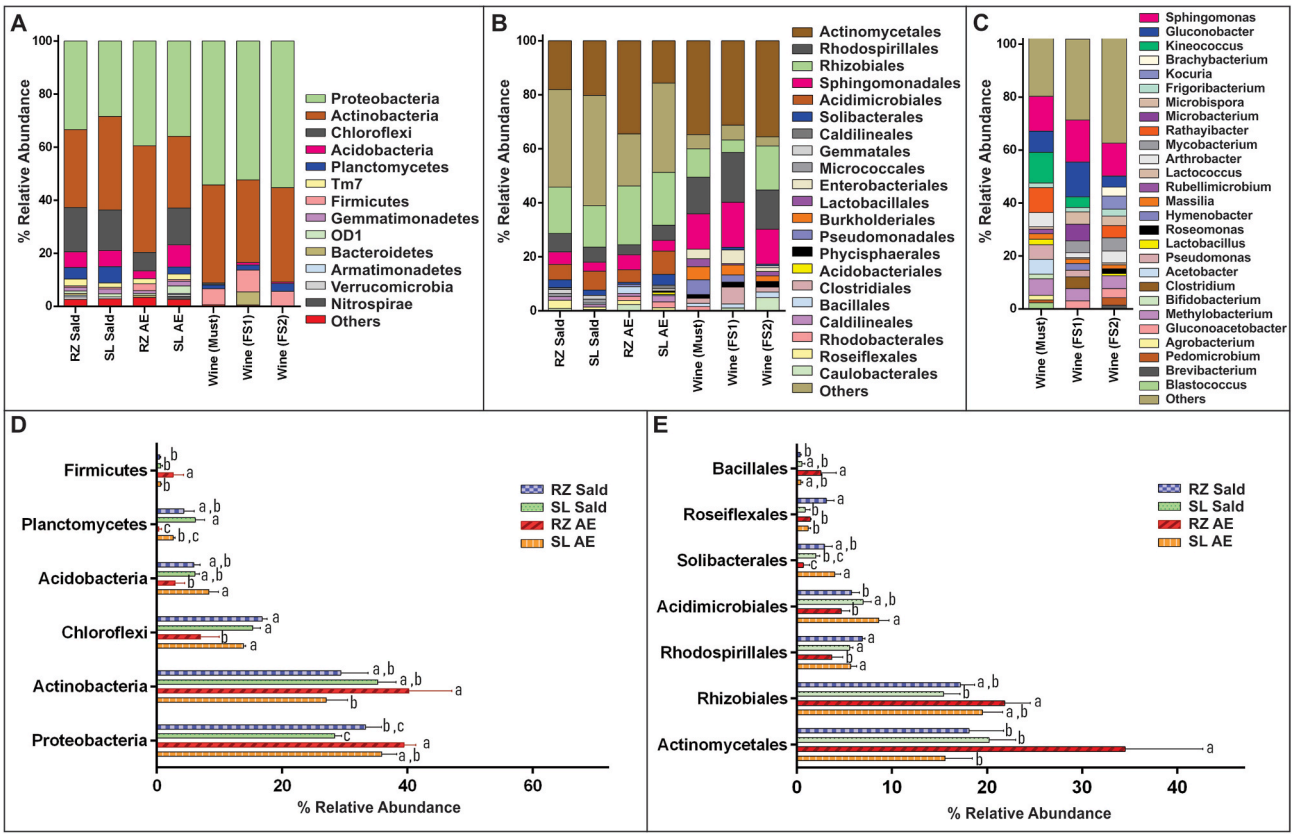

Fig. 3. Microbial structure of the bacterial community for the soil (SL), rhizosphere (RZ), and wine samples from both the "Saldungaray" (Sald) and "Al-Este" (AE) vineyards, at the phylum level **(A)**. The most abundant groups are shown at the order level **(B)** and at genus level for wine samples only **(C)**. Only OTUs showing a relative abundance ≥0.5% are shown. In addition, the "others" category includes minority (<0.5%) and unclassified OTUs. ANOVA results are shown for the relative abundances for each taxonomic group, with Tukey's test for multiple comparisons, at the phylum level **(D)** and at the order level **(E)**.

richer in *Actinobacteria* and *Firmicutes* and poorer in *Chloroflexi* and *Acidobacteria* than soil samples of the same vineyard. These results partially agree with those of Zarraonaindia et al. (2015), who also found differences in specific taxa between root or root-zone samples and soil samples. In contrast, the soil and rhizosphere samples from Saldungaray were more similar to each other (Fig. 3-D).

Regarding the composition of the bacterial communities between the two vineyards at the phylum level, we found a higher abundance of *Proteobacteria* and *Firmicutes* in the rhizosphere of Al-Este than in the rhizosphere of Saldungaray, whereas *Chloroflexi* and *Planctomycetes* were more abundant in the rhizosphere of Saldungaray than in the rhizosphere of Al-Este (Fig. 3-D). At the order level, various *Rhizobiales* belonging to *Alphaproteobacteria*, as well as *Actinomycetales,* were the most abundant in the soil and rhizosphere samples from the two vineyards, ranging from 15.4 to 21.8% and 15.6–34.5% respectively, the latter being significantly higher in the rhizosphere of Al-Este (Fig. 3-E). Members of these orders are recognized for their plant growth promotion properties, due to their ability to establish functional symbioses with legumes and non-legumes, and nitrogen fixation. This result suggests that these taxa are selected by the vine or that they outcompete other bacteria for colonization of the roots.

Significant enrichment in *Actinobacteria* in rhizospheres and root-endophytes during the drought has been shown for different plants (Naylor, DeGraaf, Purdom, & Coleman-Derr, 2017; Taketani, Kavamura, Mendes, & Melo, 2015). Whether the significant enrichment in *Actinobacteria* of Al-Este rhizosphere is related or not to the event of water shortage in 2017 is hypothetical, and further studies should explore this. This may also be related to the high P content in Al-Este vineyard, since the long-term use and type of P fertilizer influence the composition of soil microbial communities. Moreover, root exudation represents an important source of soil carbon for microorganisms and is influenced by the plant P status (Beauregard, Hamel, & St-Arnaud, 2010; Gumiere et al., 2019).

Other *Alphaproteobacteria*, like *Rhodospirillales* and *Sphingomonadales,* were also abundant in the soil and rhizosphere samples, ranging from 3.6% to 6.8% and from 3.3% to 5.5%, respectively (Fig. 3-B). The presence of Rhodospirillales, Solibacterales, and Roseiflexales was significantly higher in the rhizosphere of Saldungaray (Fig. 3-E).

As expected, the taxonomic structure of the prokaryotic communities associated with the elaboration process of the wine analyzed showed remarkable differences with respect to the communities associated with the soil and rhizosphere. In all the samples of must and wine, the phylum *Proteobacteria*, especially the *Alphaproteobacteria*, was dominant, and the most abundant OTUs identified at the order level revealed an important presence of *Sphingomonadales* and *Rhodospirillales* (Fig. 3-A and 3-B). The latter includes potentially detrimental genera of AAB, such as *Acetobacter*, *Gluconoacetobacter* and *Gluconobacter*. Our results showed that *Acetobacter*, a major cause of spoilage in wine, was present only in the must sample. *Gluconobacter* and *Gluconoacetobacter* reached a peak during the FS1 (13.1% and 3.1% respectively) and remained high until the end of the fermentation process (FS2) in samples of the 2017 vintage (Fig. 3-C). None of the wine samples was spoiled at the end of fermentation or in the bottle.

Other authors, using culture-independent methods, have also reported that the AAB population remained high throughout the fermentation stages of several grape varieties, although not necessarily related to spoilage events. Andorrà, Landi, Mas, Guillamón, and Esteve-Zarzoso (2008), for example, showed that, in Cariñena grape vinifications, the AAB populations, unlike those of lactic acid bacteria (LAB), were relatively unaffected by yeast inoculation or sulphite addition. On the other

hand, in botrytized wine and Chardonnay, Bokulich, Joseph, Allen, Benson, and Mills (2012 and 2014 respectively) reported high abundance of *Gluconobacter* during fermentation with concentrations below 25 mg/L of SO2. Piao et al. (2015) studied wines organically and conventionally (*pied-de-cuve*) produced from Riesling grapes and found that, although the *Gluconobacter* population increased during the fermentation process in both musts, it became the predominant genus in organically produced wine during the late stage of fermentation, concluding that this could explain its greater susceptibility to spoilage. Pinto et al. (2015) also found AAB during the fermentation of different Portuguese wine appellations. In spontaneous fermentation of the Grenache variety, Portillo and Mas (2016) found that AAB were dominant until the end of fermentation, when they were replaced by LAB. In grape must from the Apulian autochthonous grape varieties *'Uva di Troia'*, Berbegal et al. (2019) found that the genera *Acetobacter* and *Gluconobacter* were associated with uncontrolled alcoholic fermentations (un-inoculated must and *pied-de-cuve*). Our results in Malbec wine support the evidence that AAB are abundant and dynamic, independently of the grape variety fermented (Portillo & Mas, 2016). The wines analyzed in the present study were not produced organically and underwent controlled alcoholic fermentation by inoculation of commercial yeasts. However, the persistence of AAB throughout the fermentation stages suggests the need to carefully control sulfite additions and oxygen exposure to prevent excess of volatile acidity production due to AAB in these particular Malbec vinifications. We found high relative abundance of *Sphingomonas* and *Methylobacterium* during all the stages of fermentation. These genera are grape epiphytes often recorded previously in wine, although their role in the quality of wine remains unknown (Bokulich et al., 2012, 2016; Piao et al., 2015; Zarraonaindia et al., 2015).

In the present study, the proportion of *Firmicutes* increased throughout the fermentation process of the 2017 vintage: 6.8% in must, 8.5% in FS1 and 9.7% in FS2. Among them, the most abundant OTUs corresponded to *Bacillales*, *Clostridiales* and *Lactobacillales*. Among *Lactobacillales*, only the genera *Lactobacillus* (must: 2.3%, FS1: 0%, FS2: 1%) and *Lactococcus* (must: 1.6%, FS1: 0.7%, FS2: 0.7%) were identified (Fig. 3-A, B and C), and no other LAB or other bacteria with known technological relevance were recorded. The low detection of LAB and the persistent presence of AAB and other several heterogeneous taxonomic groups throughout fermentation seem to be more typical of uncontrolled fermentation processes, according to descriptions by other authors (Berbegal et al., 2019; Bokulich et al., 2012; Piao et al., 2015) and could be related to the mentioned slowdown in malolactic fermentation throughout the years.

Our results highlight the great biological complexity of fermentation processes, particularly those that involve indigenous bacteria. The isolation and selection of autochthonous LAB from the wines themselves, with appropriate oenological traits, to formulate a malolactic fermentation starter could be a viable solution that has been proposed to this winery owners to control the fermentation process, providing greater stability by reducing potentially detrimental bacteria such as AAB, while also improving the organoleptic characteristics of the final wine.

3.3. Fungal communities

Regarding the fungal communities in the soil and rhizosphere samples, the taxonomic analysis allowed us to identify 7 phyla with relative

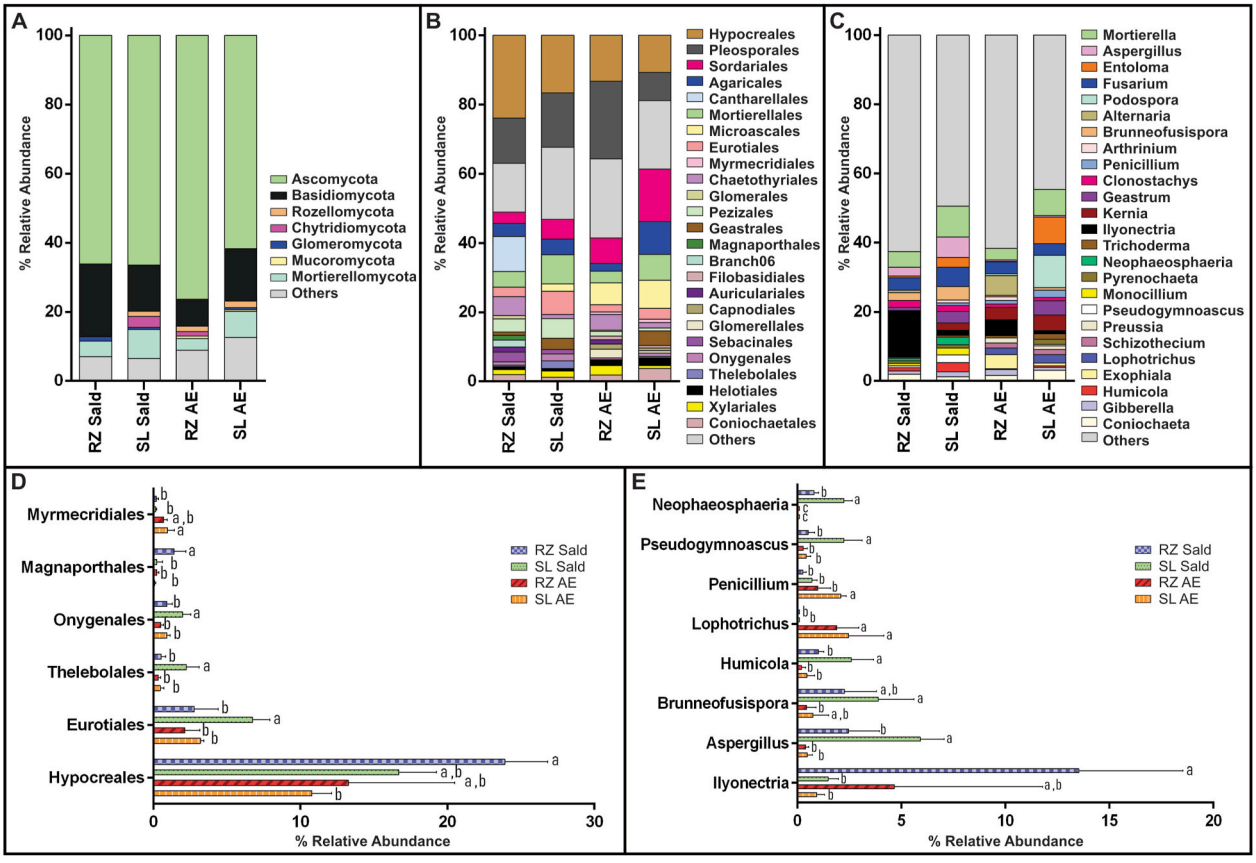

Fig. 4. Microbial structure of the fungal community for the soil (SL), rhizosphere (RZ), and wine samples from both the "Saldungaray" (Sald) and "Al-Este" (AE) vineyards, at the phylum level **(A)**. The most abundant groups are shown at the order level **(B)** and at genus level **(C)**. Only OTUs showing a relative abundance ≥0.5% are shown. In addition, the "others" category includes minority (<0.5%) and unclassified OTUs. ANOVA results are shown for the relative abundances for each taxonomic group, with Tukey's test for multiple comparisons at the order level **(D)** and at genus level **(E)**.

abundance ≥0.5%. *Ascomycota* was the most abundant group in all samples, representing from 61.6% to 76.3% of the OTUs. The phylum *Basidiomycota* was detected in a smaller proportion, from 7.8% to 21% of the total fungal OTUs. *Rozellomycota*, *Glomeromycota* and *Chytridiomycota*, as well as other less abundant phyla, were also detected. From 6% to 12% of the total fungal OTUs, depending on the sample, corresponded to minority (<0.5%) or unclassified OTUs (Fig. 4-A).

At the order level, the fungal communities of the soil and rhizosphere samples were dominated by *Pleosporales*, *Hypocreales* and *Sordariales*, among *Ascomycota.* Although we observed a higher relative abundance of *Hypocreales* in the rhizosphere of Saldungaray than in the soil and rhizosphere of Al-Este, these differences were not significant (Fig. 4-B and Fig. 4-D). A more detailed analysis of the OTUs showed that the predominance of *Hypocreales* in these samples is due to the high abundance of the genus *Ilyonectria* (Fig. 4-C) Several species of this genus or related ones cause black foot disease, a grapevine trunk disease, affecting young grapevines and other fruit crops worldwide (Akgül & Ahioğlu, 2019; Manici et al., 2018; Pintos, Redondo, Costas, Aguin, & Mansilla, 2018). This may be related to the report in Saldungaray vineyard of grapevines showing decline symptoms, since all the biological triplicates of the rhizosphere samples from there showed a high abundance of *Ilyonectria*, although further studies should be conducted in order to prove this. Another concern is the origin of the inoculum, which could be either the vineyard soils, where these species can survive as spores, or the nurseries that provide the plant material (Akgül & Ahioğlu, 2019; Pintos et al., 2018). Thus, prophylactic cultural practices should be considered, and disease management practices should be carried out.

Agaricales, *Geastrales* and *Cantharellales* were the most abundant orders among the phylum *Basidiomycota* (Fig. 4-B). However, none of them showed significant differences in relative abundance between the two vineyards. A few less abundant orders showed differences between the two vineyards (Fig. 4-D). Despite the already mentioned differences, our results showed that the general structure of the fungal communities of both Saldungaray and Al-Este vineyards appears to be similar.

The fungal communities of the wine were dominated by *Pleosporales*, almost exclusively *Saccharomyces*, from the beginning of fermentation (data not shown). Similarly, Andorrà et al. (2008) found in grape must that the *Saccharomyces* population was ten times higher than that of *Hanseniaspora,* and concluded that this result was due to the high degree of contamination with *Saccharomyces* in the winery environment. Our results seemingly agree with those findings, since the Malbec variety is the last to be harvested during vintage and its processing takes place with equipment, tanks and facilities previously used in the vinification of other varieties.

4. Conclusion

The current work is a preliminary analysis that contributes to the knowledge about both the bacterial and fungal communities associated with the soil and rhizosphere of grapevines in a region considered re-emerging for the Argentine wine production. Although the two wineries are located at a considerable distance from each other, no major differences were found in the microbial structures of the analyzed samples, which could be due to decades of intensive agricultural practices involving the use of agrochemicals such as pesticides and fertilizers, leading to the loss of biodiversity of their soils and rhizospheres. At this point, the implementation of conservation actions aimed to restore biodiversity might need to be considered. We only found significant differences in specific taxa, a fact that may have an impact on the plant growth promotion properties of the microbial consortia or even in the health of vines. These differences could be attributed to different physicochemical properties of the soil. In addition, the findings related to the persistence of AAB throughout fermentation stages reinforce the evidence accumulated about its abundance and dynamics, independently of the grape variety fermented, and suggest the need to prevent excess volatile acidity in these vinifications as well as to use autochthonous LAB in malolactic fermentation starters, in order to control the fermentation process and even improve the organoleptic characteristics of the final wine, while preserving its regional typicity. Further studies are needed to elucidate the role of the heterogeneous taxonomic groups of microorganisms in the quality and regional characteristics of the wines produced. Our results open possibilities for winemakers to improve both the vineyard management practices and the fermentation process by favoring microorganisms potentially beneficial for the health of the grapevines and for the quality of the wine, maintaining the regional microbial biodiversity.

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CRediT authorship contribution statement

Gabriel A. Rivas: Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Liliana Semorile:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Lucrecia Delfederico:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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