International Journal of Food Microbiology 440 (2025) 111282

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Structure of culturable indigenous yeast population and genetic diversity of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts during spontaneous fermentation of Etna vineyards grapes

Nunzio Alberto Fazio [a], Warren Albertin [b], Isabelle Masneuf-Pomarede [b], Cinzia L. Randazzo [a], Cinzia Caggia [a,*]

[a] *Department of Agriculture, Food and Environment, University of Catania, Via S. Sofia, 100, 95123 Catania, Italy*
[b] *Univ. Bordeaux, Bordeaux Sciences Agro, Bordeaux INP, INRAE, OENO, UMR 1366, ISVV, F-33140 Villenave d'Ornon, France*

ARTICLE INFO

Keywords:
Indigenous yeasts
Spontaneous fermentation
Saccharomyces cerevisiae
Hanseniaspora uvarum, microsatellite genotyping
Sicilian grape cultivars

ABSTRACT

The microbial diversity of indigenous yeasts plays a fundamental role in the spontaneous fermentation of wines, contributing to the concept of *microbial terroir* and potentially influencing the sensory profile of the final product. This study explores the yeast ecology and genetic diversity of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts in four wineries located on two different sides of Mount Etna, a region of unique viticultural significance due to its volcanic soils and diverse microclimatic conditions. A total of 454 yeast isolates were obtained from spontaneous fermentations of different grape varieties, and identified as belonging to 18 distinct species. The spontaneous fermentation was characterized by an initial dominance of non-*Saccharomyces* yeasts, especially *Hanseniaspora uvarum* and *Metschnikowia pulcherrima,* followed by a gradual dominance of *S. cerevisiae* at later stages. Microsatellite genotyping revealed significant genetic diversity among *S. cerevisiae* strains, with some distinct genetic patterns associated with Italian winery environments. Additionally, *H. uvarum* exhibited significant genetic variation but lacked clear geographic clustering, suggesting complex ecological and enological interactions. Statistical analyses of microbial diversity indices indicated that vineyard-specific factors, including altitude, soil composition, and agronomic practices, may influence yeast community structure among the four wineries. These findings provide new insights into the microbial ecology of Etna wines and highlight the potential of indigenous yeast populations for maintaining and enhancing regional wine identity.

1. Introduction

Yeasts play a critical role in the fermentation process, converting sugars into ethanol and other metabolites, which directly influence the flavor, aroma, and overall quality of the final product (Berbegal et al., 2017). Traditionally, commercial yeasts have been used to properly carry out and ensure a safe fermentation performance. However, there is growing interest in the role of native yeasts naturally inhabiting vineyards and winery environments, as these microorganisms contribute to the concept of "microbial terroir," for which native microbial communities enhance the uniqueness and authenticity of wines (Lappa et al., 2020; Mas and Portillo, 2022). Native yeasts, including non-*Saccharomyces* genera such as *Hanseniaspora, Metschnikowia, Starmerella,* and *Pichia*, are predominantly found on grape surfaces at harvest (Liu et al., 2017). These species initiate the alcoholic fermentation (AF) process but are gradually outcompeted by *Saccharomyces cerevisiae* which, therefore is usually detected in low numbers in vineyards (Barata et al., 2012). The dominance of *S. cerevisiae* is due to the selective pressures of high ethanol levels, heat production, low pH, and nutrient depletion in the fermenting must (Albergaria and Arneborg, 2016). The initial activity of non-*Saccharomyces* yeasts can significantly contribute to the development of flavors and aromas, such as fruity and floral notes, enhancing the sensory traits of wine and producing metabolites that can modulate aroma complexity (e.g., organic acids, esters, terpenes, and higher alcohols) while reducing off-flavors (e.g., hydrogen sulfide, acetic acid) (Fazio et al., 2023; Wang et al., 2023). Factors such as soil composition, grape variety, vineyard altitude, and climatic conditions may influence the diversity and abundance of native yeast communities. This diversity impacts fermentation dynamics and helps to define the sensory profile of wines from specific regions (Cordero-Bueso et al., 2011). Although the

* Corresponding author.
E-mail address: cinzia.caggia@unict.it (C. Caggia).

https://doi.org/10.1016/j.ijfoodmicro.2025.111282
Received 19 January 2025; Received in revised form 24 March 2025; Accepted 24 May 2025
Available online 26 May 2025
0168-1605/© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

use of Active Dry Yeast (ADY) can ensure fermentation reliability and, in some cases, enhance varietal aromas, preserving indigenous yeast populations could contribute to preserving microbial diversity, a relevant trait of regional wine identity and complexity (Drumonde-Neves et al., 2016; Viel et al., 2017; Feghali et al., 2019). Recently, several studies have provided a snapshot of yeast populations in wine-producing regions, through different techniques, such as Amplified Fragment Length Polymorphism (AFLP), Random Amplification of Polymorphic DNA (RAPD-PCR), mitochondrial DNA restriction analysis (mtDNA RFLP), and microsatellite analysis. Microsatellites, or Simple Sequence Repeats (SSRs), are short DNA sequences of 1–6 nucleotide motifs repeated in tandem, known for their high polymorphism due to variability in repeat numbers (Guillamón and Barrio, 2017), have been used as targets for genetic studies, including phylogenetic analysis of wine-related yeasts (Schuller et al., 2012; Albertin et al., 2016; Capece et al., 2016; Masneuf-Pomarede et al., 2016; Nisiotou et al., 2022; Vicente et al., 2023). Moreover, microsatellite analysis has been applied to trace the geographic and enological origins of *S. cerevisiae*, revealing unique genetic profiles associated with specific terroirs and environmental conditions. For example, sub-regional variations in yeast populations have been demonstrated to correlate with phenolic profiles in Pinot Noir grapes, underlining the impact of terroir on microbial diversity (Cheng et al., 2020). Microsatellite typing has also revealed differences in the distribution of *SSU1* promoter alleles between cellar and vineyard environments, highlighting genetic adaptations of *S. cerevisiae* to specific wine-related conditions (Marullo et al., 2020). Furthermore, indigenous yeasts, characterized through such a technique, have shown unique enological properties, supporting the production of wines that reflect regional origins (Zabukovec et al., 2020). Therefore, microsatellite markers have demonstrated a high capacity for discrimination, providing crucial insights into the geographic and oenological origins of microbial communities while elucidating the relationships among yeasts and their connections to regional terroir (Raymond Eder and Rosa, 2020).

Sicily, and particularly the area surrounding Etna volcano, represents a region of immense viticulture and oenological interest. The Sicilian vineyard area covers approximately 103,000 ha, with the Etna region occupying about 1200 ha, and it has been recognized as a historically significant viticultural zone (Bellia et al., 2022; https://thewinesofetna.com/, accessed on 17/12/2024). The unique geographical and climatic conditions of the highest European active volcano contribute to an exceptional diversity in grape varieties and wine types. The vineyards are spread across different altitudinal zones, ranging from low altitudes to over 1000 m above sea level, each with its own microclimate, soil composition, and different exposure to environmental elements. Such factors strongly influence the characteristics of grapes and actively shape their microbial ecosystems. Historically, in the Etna region have been produced wines with distinct sensory profiles, often attributed to the volcanic soils and the local varietals, such as Nerello Mascalese and Carricante (Ferlito et al., 2018).

However, the role of native yeasts in shaping wines produced in the Etna region has been poorly investigated, although some interesting investigations have been performed on Sicilian yeast biodiversity for wine fermentation (Di Maio et al., 2012a; Settanni et al., 2012). Given the significant oenological importance of the area, exploring its microbial diversity could provide valuable information to set up specific and unique starter cultures that would benefit regional winemaking practices.

This study aims to explore the genetic diversity among indigenous yeasts isolated from two distinct wine-producing sides on Mount Etna. These regions, despite being geographically close, differ in terms of altitude, soil composition, and microclimatic conditions. By isolating and identifying yeasts at strain levels and analyzing their genetic diversity through microsatellite markers, the present study attempts to provide a comprehensive snapshot of yeast ecology in such a unique environment, which has never been explored before.

2. Materials and methods

2.1. Sites and vineyards involved in the study

The study was conducted in the Etna region, focusing on four wineries located on two different slopes of Mount Etna: the southeastern and northern slopes (Fig. 1). These areas were selected for their contrasting geographical features, climatic conditions, and solar exposure, which are known to influence vineyard ecology and grape growth. The wineries chosen were Donnafugata (37°52′27"N, 14°59′00″E, 746 m a.s.l.) and Cusumano (37°53′10"N, 15°04′51″E, 514 m a.s.l.) on the northern slope, and Murgo (37°41′33"N, 15°07′36″E, 452 m.a.s.l.) and Barone di Villagrande (37°42′49"N, 15°07′09″E, 641 m a.s.l.) on the southeastern slope. Two of three grape samples of the same variety (1.5–2.0 kg each) were collected from different sites of vineyards, at the ripening stage from a healthy, undamaged bunch of grapes at the four wineries. The varieties included local cultivars commonly grown in the Etna region as: Nerello Mascalese, Nerello Cappuccio, Carricante, and Catarratto. To capture a representative diversity of yeast populations associated with different grape varieties and environmental conditions a total of 17 grape samples were collected (Table 1). Grapes were manually harvested and immediately placed in sterile bags and transported to the laboratory of Agricultural and Food Microbiology, at Di3A (University of Catania), under refrigerated conditions for further analyses.

2.2. Isolation of indigenous yeasts

Samples of ripe and undamaged grapes were manually destemmed, crushed, and naturally fermented with the skin, in the laboratory, under aseptic conditions. All the fermentations were conducted in sterile flasks (2.0 L) covered with cotton plugs under a controlled temperature of 20 °C. Sampling was performed during spontaneous fermentation on the same day (grape must after crushing), after 3 and 10 days. At each stage, aliquots of several dilutions (from 10^{-1} to 10^{-6}) were spread on Wallerstein Nutrient Broth (WLN) agar plates (Biolife Italiana Srl, Milano, Italy) supplemented with 100 mg/L of chloramphenicol to inhibit bacterial growth (Pallmann et al., 2001). Colony selection was based on both morphology and relative frequency on the plates, considering color, texture, margin shape, and growth dynamics. Colonies were proportionally selected from multiple dilution levels to ensure the inclusion of both dominant and less abundant species, thereby reducing the potential overrepresentation of fast-growing yeasts. For each sample, 15–25 non-*Saccharomyces* colonies were isolated using this approach, leading to a total of 454 isolates. All isolates were purified on yeast extract peptone dextrose (YPD) agar (containing 2 % glucose, 2 % peptone, and 1 % *w/v* yeast extract) for subsequent identification and stored in 20 % glycerol at −80 °C for further analyses.

2.3. Identification of isolates by MALDI-TOF MS Biotyper

Yeast identification was performed using MALDI-TOF MS Biotyper, as previously described by Windholtz et al. (2021). In detail, biological material obtained from a freshly grown single colony on YPD agar was transferred onto an MSP 96 polished steel target plate (Bruker, Karlsruhe, Germany) and allowed to air-dry at room temperature. After drying, 1 μL of 70 % formic acid was applied to each sample, followed by an additional drying step. Subsequently, a 1 μL aliquot of the α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Bruker, Germany) was added to promote crystallization. Mass spectra were acquired using the MicroflexTM LT/SH MALDI-MS System (Bruker Daltonics, Bremen, Germany) with the support of Flex Control (Version 3.1), MTB Compass (Version 3.1), and MALDI-BiotyperTM software. This technique relies on generating unique mass spectra from each sample and comparison was performed with profiles stored in the Biotyper database, extended by an oenological laboratory-specific database, for microorganism identification (Windholtz et al., 2021). Instrument calibration was ensured

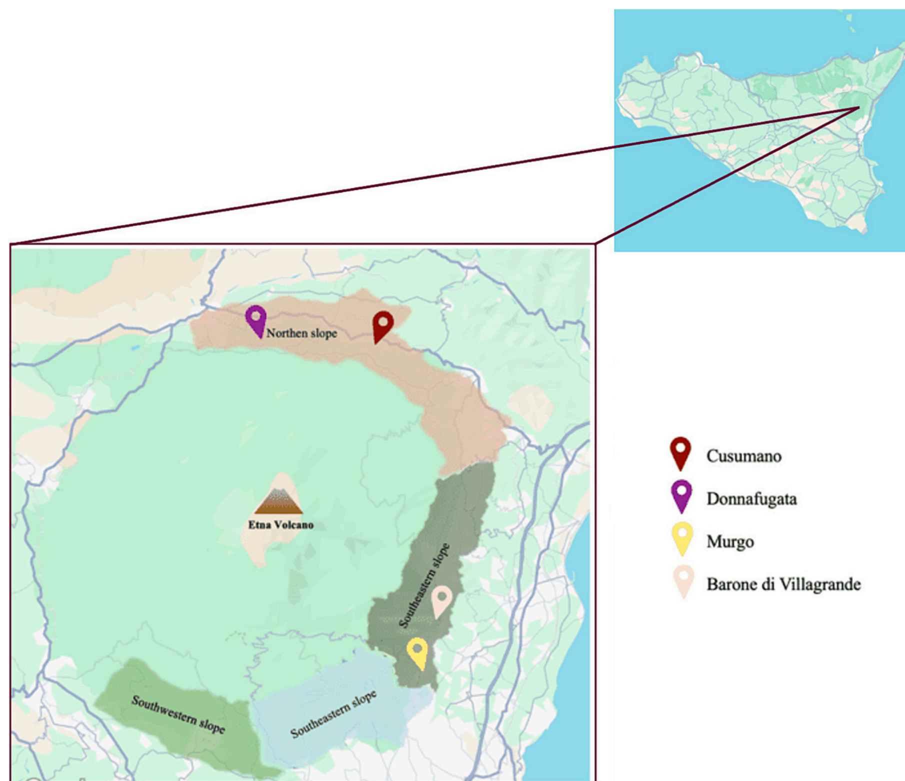

Fig. 1. Different sides of Etna regions and location of wineries participating in this study.

Table 1
Vineyard locations and grape varieties were sampled from the four wineries on the northern and southeastern slopes of Mount Etna. The table lists each winery with its geographical slope, coordinates, elevation (m.a.s.l.), grape varieties collected, and the number (#) of samples obtained from each.

Winery (Abbreviation)	Slope	Coordinates (Lat., Long.)	Elevation (m.a.s.l.)	Grape Variety (#)
Donnafugata (DF)	Northern	37°52′27"N, 14°59′00″E	746	Nerello Mascalese (3) Carricante (3)
Cusumano (CU)	Northern	37°53′10"N, 15°04′51″E	514	Nerello Cappuccio (3)
Murgo (MU)	Southeastern	37°41′33"N, 15°07′36″E	452	Nerello Mascalese (2) Carricante (2) Catarratto (2)
Barone di Villagrande (BA)	Southeastern	37°42′49"N, 15°07′09″E	641	Carricante (2)

through the inclusion of the Bruker Bacterial Test Standard (BTS) (Bruker, Germany) on each plate. Identification scores were interpreted based on the manufacturer's guidelines: scores above 2.3 indicated a highly reliable species-level match, scores between 1.7 and 2.0 corresponded to genus-level identification, and scores below 1.7 were deemed unreliable (Condina et al., 2019).

2.4. Genotyping characterization

For genotyping analysis, we focused on both *S. cerevisiae* (*Sc*) and *Hanseniaspora uvarum* (*Hu*) for their significant enological relevance and for being the most frequently identified species at the winery level, respectively. For *S. cerevisiae* genotyping, each colony, previously cultivated on YPD agar medium (2 days at 26 °C), was suspended in 100 μL of MilliQ and used as a DNA template. Subsequentially, two multiplex PCR reactions targeting 15 microsatellite loci (Supplementary Table S1, Legras et al., 2005) were performed. In detail, the two reactions contained, for each sample, 0.8 μL of multiplexed primers, 5 μL of QIAGEN Multiplex PCR Kit Master Mix, and 3.20 μL of MilliQ water. PCRs were run in a final volume of 10 μL, including 1 μL of cell suspension. The PCR protocol consisted of an initial denaturation at 95 °C for 15 min, followed by 35 cycles at 95 °C for 30 s, 57 °C for 2 min, and 72 °C for 1 min, with a final extension step at 60 °C for 10 min (Börlin et al., 2020). In addition, other isolates, from different wineries, located in different Sicily areas, were included for a total of 29 isolates; in detail 3 isolates from Caltanissetta province and 2 isolates from Agrigento province were added. Commercial strains RB2 and QA 23 were also tested since they are the most used starters for Etna winemaking.

Microsatellite amplification for *H. uvarum* was performed using two multiplex PCR reactions targeting 10 microsatellite loci (Supplementary Table S2, Albertin et al., 2016). Among the ***H. uvarum*** population, **40 isolates** were selected, taking into consideration the isolation matrices. Each yeast colony was diluted in 20 mM NaOH and heated at 94 °C for 10 min, yielding DNA templates for subsequent PCR reactions. The PCR reactions were performed in a 15 μL final volume, consisting of 1 μL of DNA template, 0.05 μM forward primer, 0.5 μM reverse primer, 0.5 μM labeled primer, and 1× Taq-&GO polymerase mix (MP Biomedicals, Illkirch, France). Universal M13 primers were fluorescently labeled with FAM-, HEX-, PET-, or NED-dyes (Eurofins MWG Operon, Les Ulis, France). A touch-down PCR protocol was applied using an iCycler thermal cycler (Bio-Rad, Hercules, CA). The cycling conditions included an initial denaturation step at 94 °C for 1 min, followed by 10 cycles of 94 °C for 30 s, annealing at Tm + 10 °C (decreasing by 1 °C per cycle until Tm was reached) for 30 s, and extension at 72 °C for 30 s. This was followed by 20 additional cycles at 94 °C for 30 s, Tm for 30 s, and 72 °C

for 30 s, with a final extension step at 72 °C for 2 min.

PCR products were analyzed by capillary electrophoresis using an ABI3730 sequencer (Applied Biosystems) and the 600LIZ size standard (GENESCAN). The resulting electropherograms were processed with GeneMarker software (v2.4.0) to determine the sizes of the microsatellite amplicons, which were subsequently used to investigate genetic relationships among strains.

2.5. Statistical analyses

Diversity indices calculations were performed in R (R 4.3.3) using RStudio (2024.04.1 + 748) and the vegan package (Oksanen et al., 2013). Alpha diversity was assessed by calculating species richness and the Shannon diversity index for each winery. Richness quantified the number of species detected, while the Shannon index incorporated both richness and evenness and provided a comprehensive measure of microbial community complexity. These metrics were chosen to evaluate within-sample diversity and compare differences across groups (e.g., wineries and fermentation stages). Richness was computed as the count of non-zero species abundances, and the Shannon index was calculated using the diversity function from vegan. Statistical differences in alpha diversity indices were tested using a non-parametric **Kruskal-Wallis rank-sum test.** This test was chosen as it does not assume the normality of data and is robust against potential outliers and unequal group sizes. Statistical significance was defined at the level of $\boldsymbol{p < 0.05}$.

Beta diversity was analyzed to assess differences in microbial community composition across wineries and fermentation stages. Pairwise Bray-Curtis dissimilarity was calculated from the species abundance matrix, which quantifies differences in microbial composition based on relative abundances. To visualize differences in beta diversity, a Principal Coordinates Analysis (PCoA) was conducted on the Bray-Curtis dissimilarity matrix. PCoA was chosen as it reduces high-dimensional data into principal coordinates that best explain variation between samples. This allowed for a graphical assessment of clustering patterns based on the winery and fermentation stages. The statistical significance of observed differences in community composition was evaluated using Permutational Multivariate Analysis of Variance (PERMANOVA), implemented with the adonis2 function in the vegan package. PERMANOVA tests the effect of categorical variables (e.g., winery, fermentation stage) on the Bray-Curtis dissimilarity matrix, partitioning variance explained by each factor. The model included "Winery" and "Time" as fixed effects to evaluate the influence of these factors on microbial community structure. Statistical significance was determined based on 999 permutations, with the significance threshold set at $p < 0.05$. R-squared values were used to quantify the proportion of variance explained by each factor.

To investigate the genetic relationships between strains, the microsatellite datasets for *Sc* & *Hu* were analyzed independently using the Popprpackage in R (Kamvar et al., 2014). Dendrogram trees were drawn using Bruvo's distance and Neighbour Joining (NJ) clustering. Bruvo's distance considers the mutational process of microsatellite loci. For the *Hu* dataset, the data were combined with data from a previous study analyzing strains from France and South African vineyards (Albertin et al., 2016). For the *Sc* dataset, data were combined with a previous dataset encompassing 106 strains isolated from France, Greece, Spain, and Italy, as well as 35 commercial strains of oenological interest (from the ISVV database).

Diversity indexes were calculated using the vegan package, considering that the two strains were different when Bruvo's distance was strictly superior to 0. Multidimensional scaling was performed using the stats package. For the inference of population structure, the LEA package was used.

3. Results

3.1. Yeast screening

From the 17 analyzed samples, 454 yeast isolates were obtained and subsequentially stored at −20 °C in a 20 % glycerol medium solution and stored in YPD slope tubes respectively. The isolates were all identified through MALDI-TOF MS, at the species level, and results showed a great diversity of indigenous yeasts (Fig. 2). In detail, a total of 18 distinct species were detected: *Starmerella bacillaris*, *H. uvarum, Hanseniaspora guilliermondii, Hanseniaspora opuntiae, Hanseniaspora osmophila, Metschnikowia pulcherrima, Pichia terricola, Kluyveromyces marxianus, Meyerozyma guilliermondii, Pichia kluyveri, Pichia kudriavzevii, Trigonopsis californica, Candida membranifaciens, Naganishia albida, Aureobasidium pullulans, Torulaspora delbrueckii, Lachancea thermotolerans*, and *S. cerevisiae.*

The analysis of yeast communities across the four wineries (BA, CU, MU, DF) revealed variable temporal shifts over the three sampling points: T0 (grape juice), T3 (day 3), and T10 (day 10) (Fig. 2a). As shown in the bar chart, non-*Saccharomyces* species, as expected, dominated the initial fermentation phase (T0) in all wineries and some of them were able to survive until the 10th day of fermentation (T10). The *Hanseniaspora* genus was the species most prevalent detected in the unfermented grape juice samples (T0), with *H. uvarum* accounting for 29 % of the total yeast isolates identified at this sampling point (Table 2). Notably, *H. uvarum* exhibited dominance over other native yeasts in the samples from BA (40 %) and DF (31 %), whereas it was not isolated from the CU samples at T0, but detected at T3 and T10, suggesting that its initial presence may have been at very low levels, making isolation difficult. Instead, in the latter, *H. opuntiae* accounted for approximately 76.5 % of the isolates (13 out of 17 isolates). Additionally, *H. guilliermondii* was more abundant in the BA samples, representing 35.6 % of the total isolates.

M. pulcherrima was the second most abundant species found in grape juice, initially in must from DF grapes, where it accounts for approximately 45.2 % of the isolates. Moreover, 4 colonies were also obtained from MU, while it was completely absent in BA and CU samples. Furthermore, the yeast-like fungus of the genus *Aureobasidium,* previously known as a potential colonizer of freshly pressed grape juice (Onetto et al., 2020), was identified in MU (5) and DF (6) samples,

After 3 days of fermentation (T3), the genus *Hanseniaspora* continued to play a predominant role, though its overall prevalence declined compared to the fresh grape juice. *H. uvarum* remained dominant in DF samples, where it accounted for approximately 49.1 % of the total yeast isolates (28 out of 57 isolates), whereas in the MU sample, it was detected at the lowest levels, lower than the initial value, representing only the 3.3 % of isolates (1 out of 30 isolates). Furthermore, *H. opuntiae* demonstrated a marked dominance in CU samples, where it represented 69 % of the isolates (29 out of 42). *H. guilliermondii* showed modest levels of persistence, particularly in MU samples, where it accounted for 33.3 % of the total isolates (10 out of 30), and in BA, where it accounted for 65 % of the total isolates (13 out of 20), while in DF sample it significantly decreased, suggesting competitive exclusion by other yeast species during this fermentation stage.

M. pulcherrima remained vital at T3, particularly in DF musts, where it accounted for 28 % of the total isolates (16 out of 57). In MU samples, its abundance increased to 33.3 % of the isolates (10 out of 30), suggesting its capacity to thrive in the first stage of alcoholic fermentation, while it was absent in BA and CU samples. The genus *Aureobasidium*, previously detected at T0, was no longer present in any condition starting from T3, suggesting that it was likely an early colonizer suppressed by the following fermentative stages.

By the tenth day of fermentation (T10), some indigenous species were still detected. *H. uvarum* was dominant in DF samples, where it accounted for 55.3 % of the total isolates (21 out of 38), in BA, with 33.3 % (6 out of 18) and in CU with 20 % (4 out of 20). On the other hand, a

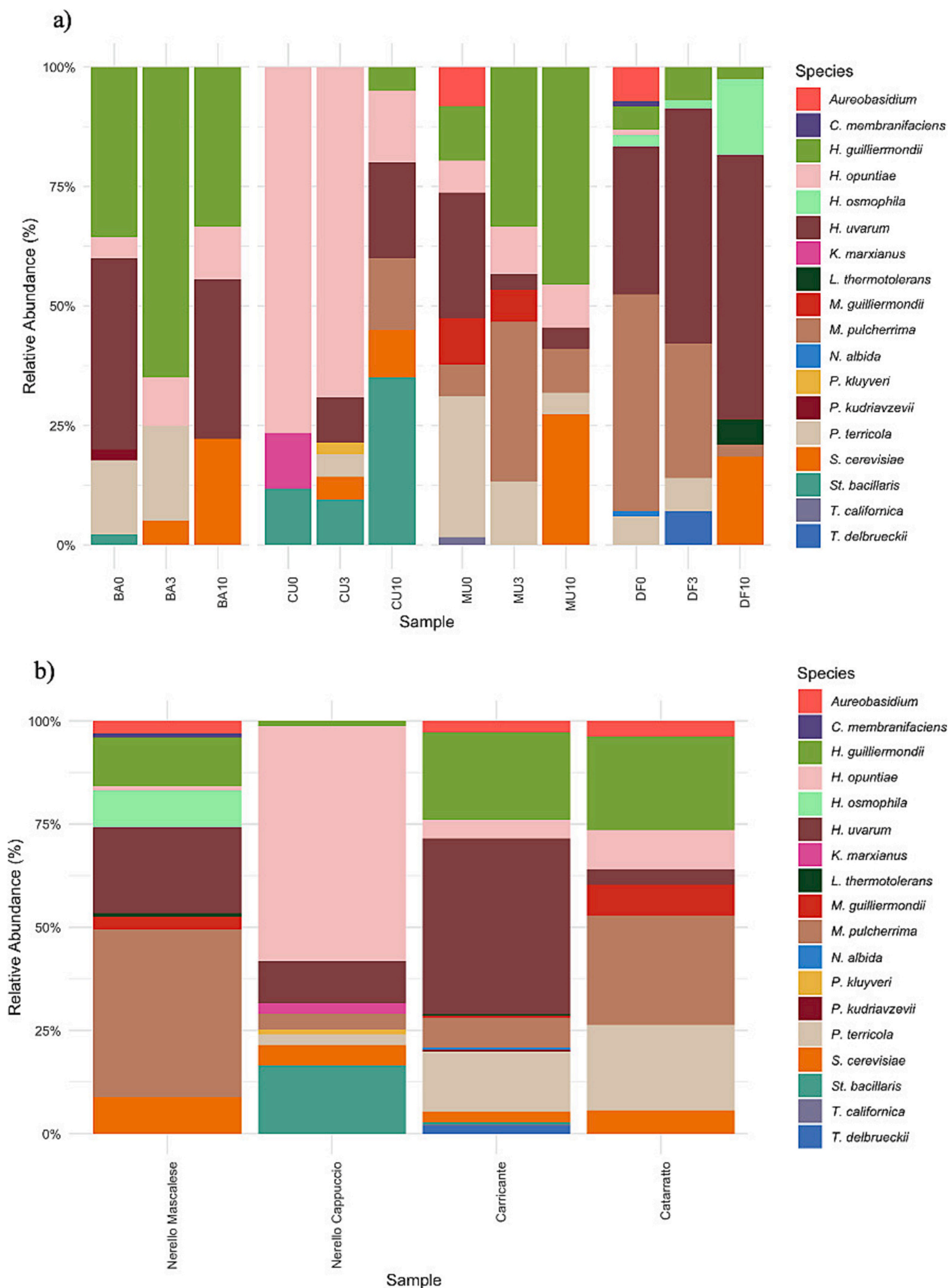

Fig. 2. Relative abundance of yeast species showing: a) the relative composition in must samples collected from different wineries (BA, CU, MU, DF) at three time sampling points (0, 3, and 10 days); b) the relative composition of species across the analyzed grape varieties: Nerello Mascalese, Nerello Cappuccio, Carricante, and Catarratto.

drastic decrease was observed for MU, where only 1 isolate was detected.

H. guilliermondii showed increased persistence in MU, where it represented 45.5 % of the total isolates (10 out of 22), while it decreased in BA (6 out of 8) and in DF (1 out of 38) samples. *H. opuntiae*, which was highly dominant in CU at T3, exhibited a severe decline by T10 (3 out of 20).

M. pulcherrima which seems to play a significant role at T0 and T3 in DF, declined drastically by T10, representing only 2.6 % of the isolates (1 out of 38), confirming its poor ethanol resistance. Other yeast species played minimal roles by T10. *H. osmophila* showed a late-stage increase in DF, where it represented 15.8 % of the isolates (6 out of 38). *L. thermotolerans* also emerged exclusively in DF at this stage, albeit at a very low level (5.3 %, 2 out of 38).

Interestingly, *St. bacillaris* showed a primary persistence exclusively in CU samples across all fermentation stages, being the dominant species, with 7 isolates out of 20, over *S. cerevisiae* at T10.

As expected, the emergence of *S. cerevisiae* at the late stage of fermentation marked a critical transition in the microbial community. This species, absent at earlier time points and detected at low concentration at the initial stages of fermentation, became much more consistent at T10 but still at low density. In detail, 7 isolates were detected in DF samples, 6 in MU, 4 in BA, and 2 in CU.

Zooming on microbial composition in different grape cultivars,

Table 2
Species distribution among isolates identified in grape juice samples from different wineries (Barone di Villagrande – BA; Cusumano – CU; Murgo – MU; Donnafugata – DF) and fermentation stages (T0, T3, and T10).

	T0				T3				T10			
Species	BA	CU	MU	DF	BA	CU	MU	DF	BA	CU	MU	DF
St. bacillaris	1	2	0	0	0	4	0	0	0	7	0	0
H. uvarum	18	0	16	26	0	4	1	28	6	4	1	21
H. guilliermondii	16	0	7	4	13	0	10	4	6	1	10	1
H. opuntiae	2	13	4	1	2	29	3	0	2	3	2	0
M. pulcherrima	0	0	4	38	0	0	10	16	0	3	2	1
P. terricola	7	0	18	5	4	2	4	4	0	0	1	0
K. marxianus	0	2	0	0	0	0	0	0	0	0	0	0
M. guilliermondii	0	0	6	0	0	0	2	0	0	0	0	0
H. osmophila	0	0	0	2	0	0	0	1	0	0	0	6
P. kluyveri	0	0	0	0	0	1	0	0	0	0	0	0
P. kudriavzevii	1	0	0	0	0	0	0	0	0	0	0	0
T. californica	0	0	1	0	0	0	0	0	0	0	0	0
C. membranifacies	0	0	0	1	0	0	0	0	0	0	0	0
N. albida	0	0	0	1	0	0	0	0	0	0	0	0
Aureobasidium	0	0	5	6	0	0	0	0	0	0	0	0
T. delbrueckii	0	0	0	0	0	0	0	4	0	0	0	0
L. thermotolerans	0	0	0	0	0	0	0	0	0	0	0	2
S. cerevisiae	0	0	0	0	1	2	0	0	4	2	6	7
Total	45	17	61	84	20	42	30	57	18	20	22	38

distinct differences were observed across the four varieties (Nerello Mascalese, Nerello Cappuccio, Carricante, and Catarratto), as shown in Fig. 2b. In detail, for Nerello Mascalese, a total of 101 yeast isolates were identified (Table 3), with the dominant species being *M. pulcherrima*, contributing 41 isolates. Other notable species included *H. uvarum* (21 isolates), *H. guilliermondii* (12 isolates), *H. osmophila* (9 isolates), *M. guilliermondii* (3 isolates), *Aureobasidium* (3 isolates), *C. membranifaciens* (1 isolates), and *H. opuntiae* (1 isolate). In Nerello Cappuccio, 79 isolates were identified, with a striking dominance of *H. opuntiae*, which accounted for 45 isolates. Other species included *St. bacillaris* (13 isolates) and *H. uvarum* (8 isolates), with a few minor contributions from species such as *M. pulcherrima* (3 isolates), *P. terricola* (2 isolates), *K. marxianus* (2 isolates), *H. guilliemondii* (1 isolate), and *P. kluyveri* (1 isolate). For Carricante, the analysis revealed a significantly higher abundance of yeast species, with 221 isolates identified. The dominant species was *H. uvarum*, with 94 isolates, followed by *H. guilliermondii* (47 isolates) and *P. terricola* (32 isolates). *M. pulcherrima* (16 isolates) and *H. opuntiae* (10 isolates) also contributed substantially, alongside minor representations from species such as *Aureobasidium* (6 isolates), and *T. delbrueckii* (4 isolates). *St. bacillaris, M. guilliermondii, P. kudriavzevii, T. californica, N. albida, L. thermotolerans* accounted with 1 isolate for each species. Finally, in Catarratto, 53 yeast isolates were identified, with *M. pulcherrima* being the most abundant species, accounting for 14 isolates. Other species included *H. guilliermondii* (12 isolates), *P. terricola* (11 isolates), *H. opuntiae* (5 isolates), *H. guilliermondii* (4 isolates), *H. uvarum* (2 isolates), and *Aureobasidium* (2 isolates). As shown above, *S. cerevisiae* was identified in low abundances across all spontaneous fermentation samples from different grape varieties, with 9 isolates in Nerello Mascalese, 4 in Nerello Cappuccio, 6 in Carricante, and 3 in Catarratto.

Table 3
Species distribution of isolates identified in grape juice samples among the grape varieties: Nerello Mascalese, Nerello Cappuccio, Carricante, and Catarratto.

Species	Nerello Mascalese	Nerello Cappuccio	Carricante	Catarratto
St. bacillaris	0	13	1	0
H. uvarum	21	8	94	2
H. guilliermondii	12	1	47	12
H. opuntiae	1	45	10	5
M. pulcherrima	41	3	16	14
P. terricola	0	2	32	11
K. marxianus	0	2	0	0
M. guilliermondii	3	0	1	4
H. osmophila	9	0	0	0
P. kluyveri	0	1	0	0
P. kudriavzevii	0	0	1	0
T. californica	0	0	1	0
C. membranifacies	1	0	0	0
N. albida	0	0	1	0
Aureobasidium	3	0	6	2
T. delbrueckii	0	0	4	0
L. thermotolerans	1	0	1	0
S. cerevisiae	9	4	6	3
Total	101	79	221	53

3.2. Diversity indices

The analysis of yeast diversity within and among the sampled vineyards revealed some variations in both alpha and beta diversity metrics across the different wineries and fermentation stages.

The species richness, abundance and Shannon diversity index were calculated for each winery, providing insights into the microbial community structure. Despite observed numerical differences in diversity indices (Fig. 3), statistical analysis using the Kruskal-Wallis test indicated these variations were not statistically significant ($p = 0.218$ for Shannon index; $p = 0.189$ for species richness). Overall, Murgo stands out as the winery with the highest diversity and richness values (Fig. 3), apparently reflecting a varied and well-distributed microbial community. Donnafugata follows with similar high values, suggesting a comparable but slightly less diverse ecological dynamic. In contrast, Cusumano and Barone di Villagrande showed lower diversity and richness, with the latter recording the lowest values.

In detail, at the beginning of spontaneous fermentation (T0), MU and DF samples exhibit the highest richness and abundance (Table 4), with Shannon index values indicating well-balanced and highly diverse microbial communities. BA samples show moderate richness and abundance at the initial stage, with a balanced diversity, while CU samples start with the lowest richness, abundance, and diversity, reflecting a sparse and uneven microbial population.

Over time, a general decline in abundance is observed across all wineries, accompanied by stabilization in richness. For example, DF samples, which initially exhibited the richest and most abundant community, stabilizes at six species by T3 and T10, but with a gradual reduction in evenness as indicated by the declining Shannon index. Similarly, MU samples maintain relatively high diversity, although its abundance decreases steadily, reflecting a shift in microbial population dynamics. BA samples showed a notable reduction in richness and

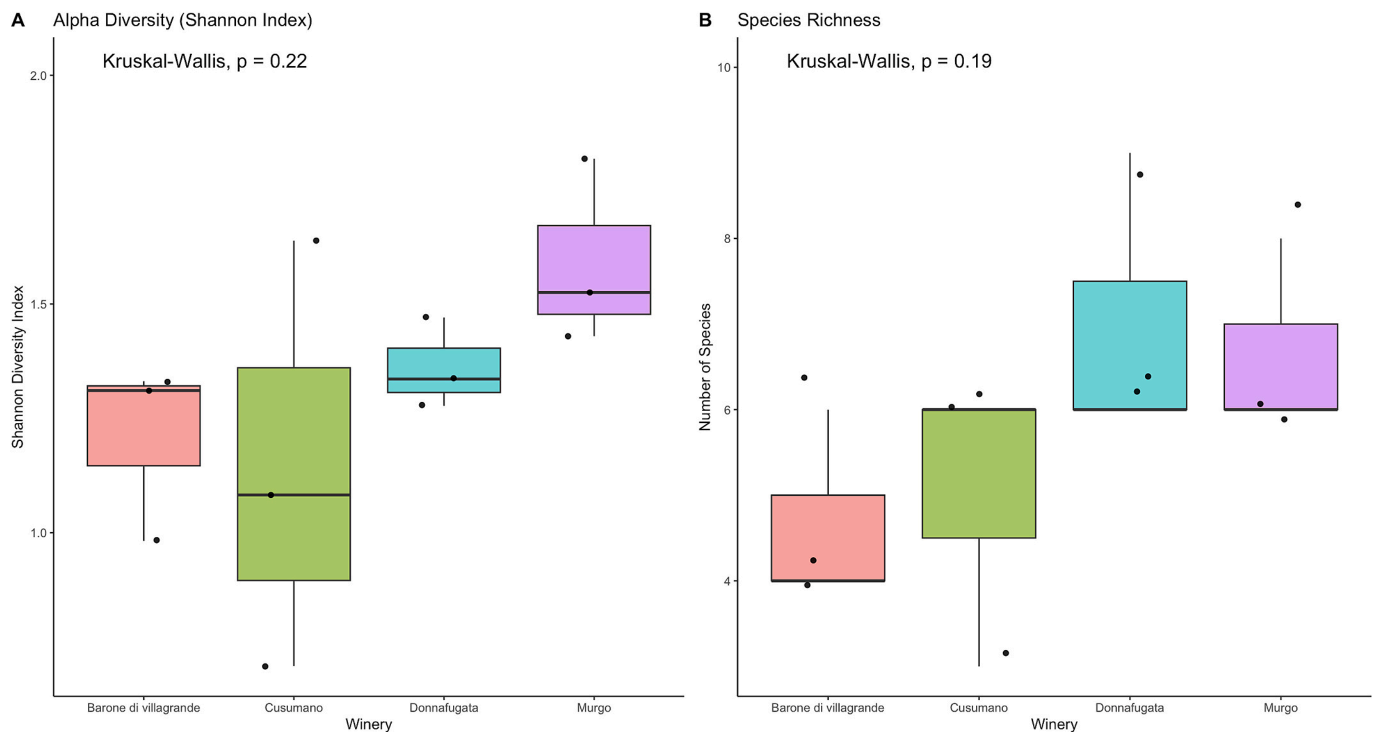

Fig. 3. Alpha diversity (Shannon index) (A) and species richness (B). The boxplots represent the median and interquartile range, while points show individual observations. Kruskal-Wallis tests indicate that observed differences among wineries were not statistically significant ($p = 0.218$ for Shannon; $p = 0.189$ for Richness).

Table 4
Microbial richness, abundance, and diversity (Shannon index) in wineries at different isolation times. Values are reported for each winery (Barone di Villagrande, Cusumano, Murgo, and Donnafugata) at three distinct times: T0 (initial time), T3 (after 3 days of isolation), and T10 (after 10 days of isolation). "Richness" represents the number of identified microbial species, "Abundance" indicates the total quantity of each species, and the "Shannon index" measures the diversity of the microbial community.

Winery	Isolation time	Richness	Abundance	Shannom index
	T0	6	45	1.33
Barone di Villagrande (BA)	T3	4	20	0.98
	T10	4	18	1.31
	T0	3	17	0.70
Cusumano (CU)	T3	6	42	1.08
	T10	6	20	1.63
	T0	8	61	1.81
Murgo (MU)	T3	6	30	1.52
	T10	6	22	1.42
	T0	9	84	1.47
Donnafugata (DF)	T3	6	57	1.33
	T10	6	38	1.27

diversity at mid-fermentation (T3), with some increasing by the late fermentation (T10), while CU samples undergo a contrasting trajectory, improving richness and diversity over time despite a drop in abundance by T10.

Zooming on beta-diversity evaluation, principal coordinate analysis (PCoA) showed the geographic patterns of yeast communities across the wineries, with 72.9 % of total variance explained by the first two principal coordinate (PC) axes (Fig. 4) with PC1 accounting for 53.5 % and PC2 for 19.4 %. The analysis revealed clear clustering patterns corresponding to wineries (BA, CU, DF, MU) and temporal points (T0, T3, T10).

Distinct clustering by winery indicates that location seems to influence the community composition, while temporal variation is secondary but noticeable in some wineries (e.g., MU and CU). BA exhibits high community stability over time, while MU shows greater variability. PERMANOVA confirmed these trends, showing a significant effect of Winery ($p = 0.006$, $R^2 = 52$ %) and a marginal effect of Time ($p = 0.079$, $R^2 = 21$ %). These results highlight the potential dominant role of geography in shaping yeast communities, with some influence from temporal factors during fermentation.

3.3. Microsatellite genotyping

The 29 Sicilian *S. cerevisiae* strains were further analyzed through 15 microsatellite loci and compared with a dataset of wine-related strains from other wineries (Table S1), specifically: 29 from Italy, 58 from France, 12 from Greece, and 7 from Spain. In addition, 37 commercial strains were added to the dataset for a total of 172 samples, which include the starters commonly used in the Etna wineries from which the samples were collected (QA23 and RB2). A total of **29 genotypes** were identified among Sicily strains, indicating that most samples exhibit distinct allelic configurations. All loci displayed polymorphism, with heterozygosity detected in at least one sample for each locus. The C4 locus emerged as the most heterozygous region, with heterozygosity detected in 22 samples, highlighting its high level of polymorphism suggesting its contribution to genetic differentiation among samples. On the other hand, the loci with the lowest levels of heterozygosity were C11 and C5, each exhibiting fewer samples with heterozygous configurations. The clustering of all the analyzed isolates is shown in Fig. 5 and underline that the strains primarily exhibited genetic differentiation based on their country of origin. In detail, the genetic analysis of yeast strains revealed that most isolates from wineries on Mount Etna cluster together or closely with other Italian strains previously analyzed, indicating a strong genetic relationship with the broader Italian yeast population. This pattern highlights the predominance of indigenous strains, which may reflect their phenotypic adaptability to the specific environmental and ecological conditions of the Etna region. Only a small subset of Etna isolates displayed genetic proximity to commercial strains commonly utilized in the area. Specifically, strains BA71, BA74, and

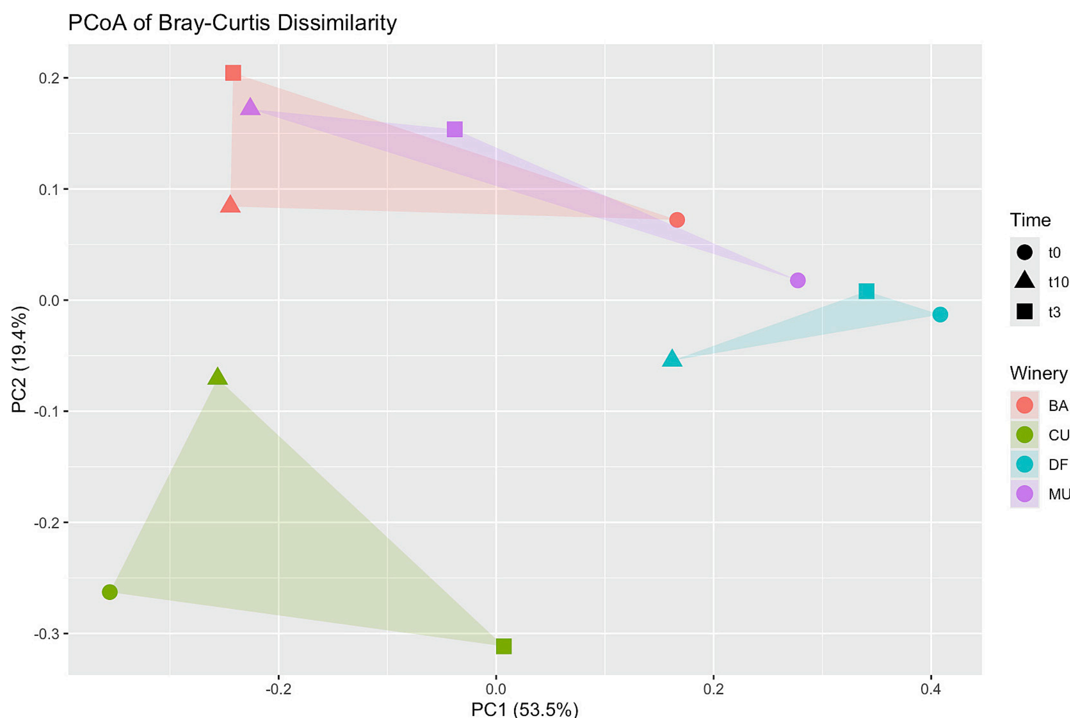

Fig. 4. Principal Coordinates Analysis (PCoA) of Bray-Curtis dissimilarity showing the distribution of samples based on microbial community composition. The first two principal coordinates (PC1 and PC2) explain 53.5 % and 19.4 % of the total variation, respectively. Samples are colored by Winery (BA, CU, DF, MU) and shaped according to Fermentation Time (T0, T3, T10). Convex hulls enclose points from the same winery, illustrating microbial composition clustering. The observed grouping suggests winery has a significant effect on microbial diversity than fermentation time, consistent with PERMANOVA results (p = 0.006 for Winery, p = 0.079 for Time).

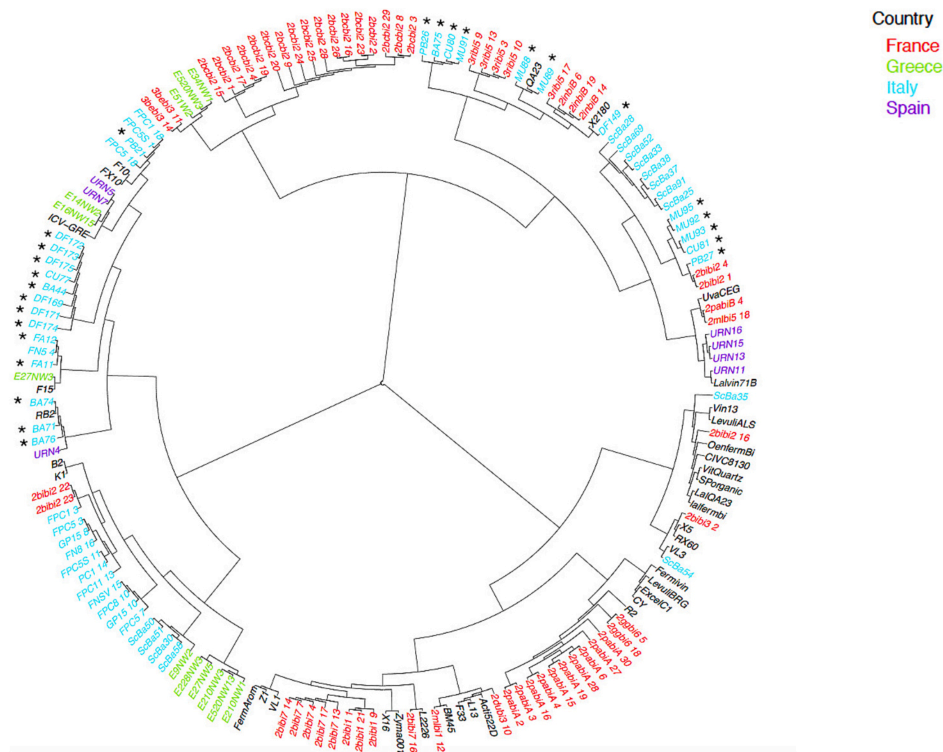

Fig. 5. Dendrogram trees of *S. cerevisiae* from microsatellite dataset using Bruvo's distance and Neighbour Joining (NJ) clustering. Commercial strains are in black, colors are according to the country of origin. Sicilian strains are highlighted with "*".

BA76 clustered with the commercial strain RB2, while MU88 and MU89 were closely related to QA23, frequently employed in Etna winemaking. Additionally, strains FA11 and FA12 showed genetic similarity to commercial F15, and PB21 grouped with the commercial strains F10 and FX10. Interestingly, some strains showed genetic differences within the same winery and even within the same grape variety. For example, strains MU 92, MU 95 and MU 93, isolated at the from Nerello Mascalese grape must of Murgo winery, showed some differences with other strains isolated in the same winery but from different variety (MU 88, MU 89 and MU 91 from Catarratto var.). Moreover, strains CU 81 and CU 80, both isolated at the 10th day of fermentation of Nerello Cappuccio musts from the Cusumano winery, showed different genetic distances between them. Similarly, strains MU 88 and MU 89, isolated from Catarratto musts from Murgo winery, were genetically distant from strain MU 91 isolated from the same variety.

Furthermore, the ancestry profiles of these individuals were then further inferred from the microsatellite dataset. Given the data set, the optimal number of ancestral populations inferred was K = 6 and the percentage of ancestry identified for yeast starters or grapes strains is presented in Fig. 6. Isolates from Etna wineries showed a high proportion of a unique ancestral population witch is different from the one of Italian isolates.

Zooming on *H. uvarum*, the 40 selected strains were compared with a dataset of samples already analyzed, including 101 samples from various wineries in France and South Africa (Table S2). To diversify the dataset, the study incorporated strains from winemaking contexts, including 5 strains sourced from New Zealand (NZ1, NZ5, NZ15, NZ148, and NZ234), CLIB 303 from Ukraine and the type strain Y-1614 from Russia. Furthermore, 6 strains from unrelated environments were included: YB-783 and YB-3199 (respectively associated to tree and fruit the USA), DSMZ 70285 (a soil-derived strain), Y-915 (associated with cider production in Germany), Y-1612 (isolated from soil in Indonesia), and 516,149 (isolated from maize in Japan), for a total of 154 samples in the dataset.

The analysis of the 10 microsatellite loci across the 40 samples revealed a certain genetic diversity, with a total of 32 identified genotypes. The locus HU440 showed the highest level of heterozygosity, with 33 samples displaying heterozygous profiles. In contrast, the loci HU409, HU467, HU620, HU68, and HU853 showed no heterozygosity, with all samples being homozygous at these regions. The clustering of all the analyzed isolates is shown in Fig. 7. The results revealed a clear genetic differentiation among strains from various geographical origins. In detail, Sicilian strains were dispersed across multiple clades, indicating substantial genetic diversity within this population. These strains did not cluster in a single group being distributed among groups associated with strains from other regions. This pattern suggests that Sicilian strains lack a distinct genetic grouping, instead sharing genetic similarities with strains from other geographic locations. In contrast, the French strains seem to form a well-defined and compact clade, reflecting high genetic homogeneity within this population. This genetic consistency indicates clear differentiation from strains of other regions and highlights a lower degree of diversification among French strains compared to those from other geographic areas.

Moreover, the ancestry profiles of these individuals were then inferred from the microsatellite dataset. Given the data set, the optimal number of ancestral populations inferred was K = 3 and the percentage of ancestry identified for yeast starters or grapes strains is presented in Fig. 8.

4. Discussion

The yeast populations driving spontaneous fermentation in Mount Etna wineries reveal a complex interplay of microbial diversity likely influenced by environmental, geographic, and fermentation dynamics. Results of the present study enhance our understanding of yeast ecology in such a volcanic terroir, emphasizing the importance of indigenous yeast communities in shaping wine profiles. These results fill a meaningful gap, as similar studies have been conducted to characterize yeast populations only in other Sicilian wineries (Di Maio et al., 2012b; Settanni et al., 2012). The dominance of non-*Saccharomyces* species in the early stages of fermentation, gradually supplanted by *S. cerevisiae*, is already well established (Conacher et al., 2021) so much so that non-*Saccharomyces* yeasts, as species belonging to *Hanseniaspora* genus, are usually dominant in the early fermentation stages, and may contribute to the development of fruity and floral sensory notes (Wang et al., 2023). However their presence has been related to high production of acetic acid during prefermentative stage (Albertin et al., 2014a), and Ciani et al. (2020), reported that even when present only in early-stage, non-*Saccharomyces* activity enhances glycerol production and modulates acetic acid levels. In the present study, *H. uvarum, H. guilliermondii, H. osmophila*, and *H. opuntiae* were the most abundant identified species in spontaneous must fermentation, as previously reported (Settanni et al., 2012; Padilla et al., 2016; Windholtz et al., 2021; Zhang et al., 2021). These findings may underscore how winemaking practices, such as the absence of SO_2 and the regulation of preferment temperatures, could significantly shape the microbial landscape of spontaneous fermentations in favor of *Hanseniaspora* species, as already reported by Albertin et al. (2014a). Notably, the persistence and dominance of *H. uvarum* in later fermentation stages, as recently stated (Regecová et al., 2024), may suggest its adaptability to nutrient-limiting conditions. Such resilience may indicate *Hanseniaspora* as a potential co-starter in fermentation to enhance aroma compounds, such as ethyl

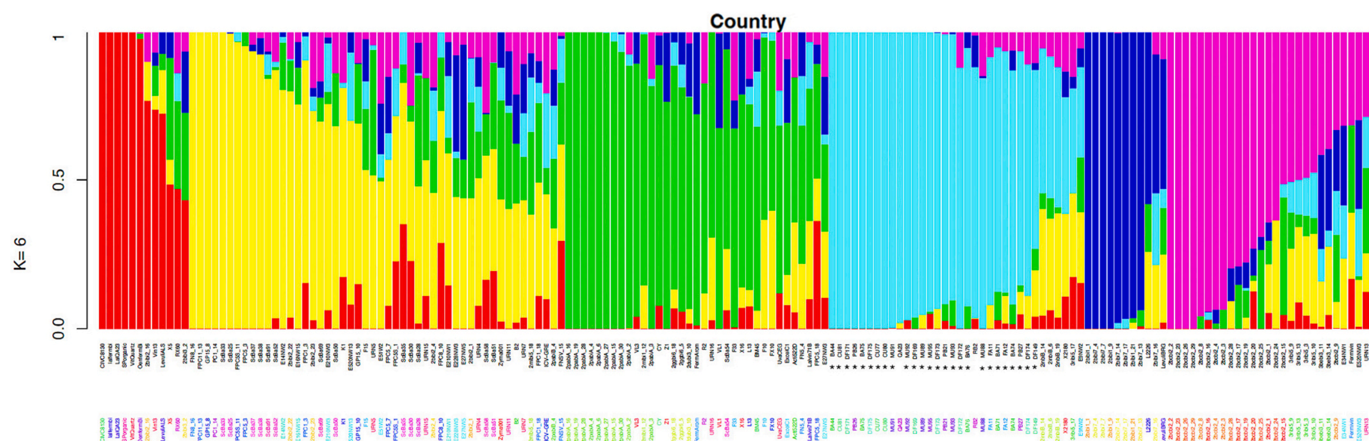

Fig. 6. Inference of population ancestry (optimal K = 6). Analyses were performed on a dataset containing 172 yeast strains. The Barplot presents the ancestry of all the *S. cerevisiae* isolates. Sicilian strains are highlighted with "*".

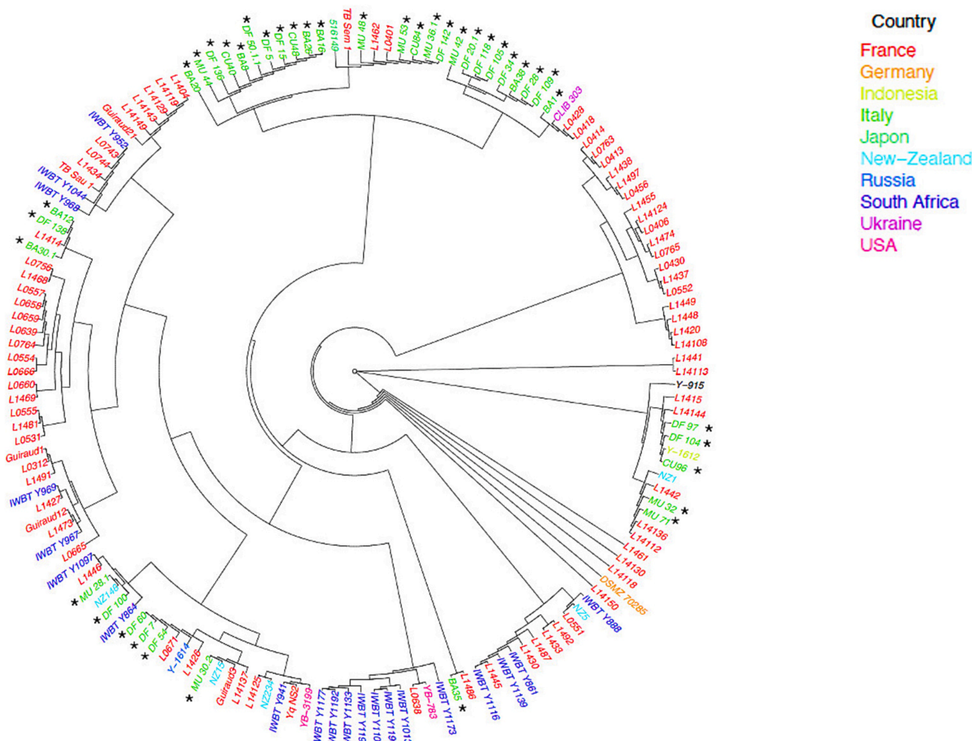

Fig. 7. Dendrogram trees of *H. uvarum* from microsatellite dataset using Bruvo's distance and Neighbour Joining (NJ) clustering. Colors are according to the country of origin. Sicilian strains are highlighted with "*".

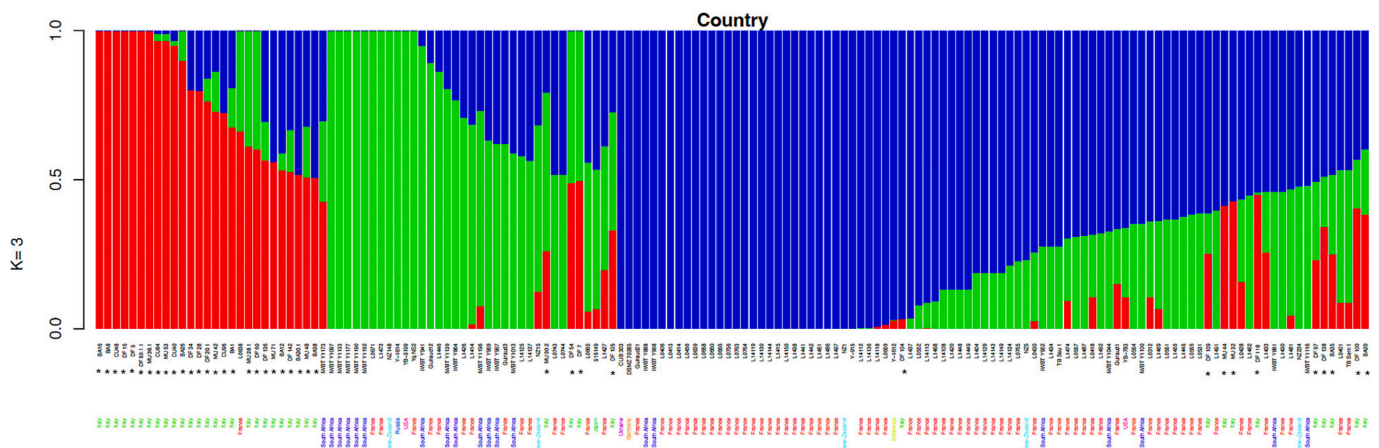

Fig. 8. Inference of population ancestry (optimal K = 3). Analyses were performed on a dataset containing 154 yeast strains. The Barplot presents the ancestry of the all the *H. uvarum* isolates. Sicilian strains are highlighted with "*".

ester, and reduce final acetic acid concentration (Zhang et al., 2023). However, it is still unclear if geographical differences in the distribution and abundance of *Hanseniaspora* species occur and whether such variations could play a role in shaping wine quality (Snyder et al., 2024). Together with the *Hansenisapora* genus *M. pulcherrima* has been frequently found in spontaneous fermentation (Chalvantzi et al., 2021) and in the present study, the dominance of *M. pulcherrima* was found in Donnafugata samples. Overall, the low abundance of *S. cerevisiae* here observed, even after 10 days of fermentation, contrasts with conventional fermentation dynamics, where its dominance is well-documented (Chen et al., 2022). One key factor contributing to this discrepancy is the controlled laboratory setting, which does not fully replicate the microbial ecology of winery environments.

In real winery conditions, ***S. cerevisiae*** populations are often maintained through residual cells persisting on **winery surfaces, barrels, presses, and other equipment** (Le Jeune et al., 2006; Bokulich et al., 2013). In detail, previous studies have demonstrated that these persistent populations create a "winery effect", leading to the rapid establishment of ***S. cerevisiae*** dominance even in uninoculated fermentations (Ayoub et al., 2021; Agarbati et al., 2024). The absence of such a **native yeast community** in our experimental conditions likely contributed to the delayed appearance of ***S. cerevisiae*** in our fermentations. Moreover, the specific experimental conditions applied in this study, particularly the absence of SO_2 treatment, may have allowed high concentrations of non-*Saccharomyces* species to persist, potentially inhibiting the growth and detection of *S. cerevisiae*, as previously reported (Albertin et al., 2014a; Windholtz et al., 2021). Overall, it seems that geographical and environmental factors, such as altitude, soil

composition, and microclimatic conditions, significantly influence microbial diversity. These findings align with previous studies that have demonstrated the interplay between environmental characteristics and microbial ecosystems, particularly in vineyard environments (Bokulich et al., 2014; Cheng et al., 2020; Chalvantzi et al., 2021). However, it is important to evaluate whether these factors alone are sufficient to explain the observed patterns, as recent studies suggest that human interventions, such as vineyard management practices and the use of fungicides, may also play a crucial role in shaping microbial communities (Bagheri et al., 2016; Setati et al., 2015; Morrison-Whittle and Goddard, 2018; Sumby et al., 2021; Englezos et al., 2022).

The higher alpha diversity indices observed in Murgo and Donnafugata compared to Barone di Villagrande and Cusumano may suggest that ecological richness could not be primarily driven by geographic location or microclimatic conditions, as these wineries are situated on opposite slopes of Mount Etna, with differing exposures and environmental factors. This observation challenges the assumption that geography alone dictates microbial composition and instead may point to the influence of vineyard-specific practices and other anthropogenic factors. For instance, agronomic practices such as organic farming, chemical inputs, and different oenological techniques may play a more decisive role in defining microbial diversity (Albertin et al., 2014a; Morrison-Whittle and Goddard, 2018). Additionally, natural barriers, such as variations in soil microbiota, vegetation surrounding the vineyards, insects, birds, and surrounding forests could potentially modulate yeast habitats by influencing nutrient availability and microbial interactions (Liu et al., 2019). These findings emphasize the need for a more detailed approach to understanding terroir, incorporating both ecological and management-related factors into microbial ecosystem studies (Setati et al., 2015; Zarraonaindia et al., 2015).

The interplay between these geographical and environmental factors underscores the concept of *microbial terroir*, where local yeast populations contribute to the distinct sensory characteristics of wines produced in specific regions. In such a frame, the preservation of indigenous yeast biodiversity is essential for enhancing the resilience of local winemaking practices against climate change and other anthropogenic pressures (Drumonde-Neves et al., 2016; Viel et al., 2017). Recent advancements in molecular techniques such as microsatellite analysis have provided deeper insights into the genetic diversity of yeast populations thanks to the high sensitivity and variability (Martínez-Estrada et al., 2023). These methods have revealed unique genetic markers associated with specific terroirs, highlighting how local environmental conditions shape microbial communities (Guillamón and Barrio, 2017; Cheng et al., 2020). Understanding these dynamics is vital for developing targeted strategies to harness native yeast strains for winemaking, potentially leading to the establishment of unique starter cultures that reflect the intrinsic qualities of Etna wines.

Microsatellite genotyping indicating the genetic diversity of *S. cerevisiae* and non-*Saccharomyces* isolates, here allowed the clustering of *S. cerevisiae* isolates with Italian populations underscoring the variability and the conservation of indigenous yeast biodiversity within the region. These findings resonate with studies emphasizing the role of microsatellite markers in elucidating genetic differentiation and regional specificity of *S. cerevisiae* populations (Capece et al., 2016; Börlin et al., 2020). In the present study, most clusters grouped isolates from the same country except for rare clusters where isolates from different European countries were mixed. Yeast could be disseminated over long distances through different countries and continents (Cibrario et al., 2019) due to dissemination phenomena by birds (Francesca et al., 2012; Alfonzo et al., 2013) and human activities (Gayevskiy et al., 2016) which could explain the mixed group of strains originating from different countries. The dissemination of industrial yeast strains into the environment poses a significant concern, as it can potentially impact the biodiversity of indigenous yeast populations in local wineries, thereby altering ecological balances, as previously stated (Cubillos et al., 2009; Viel et al., 2017). In our study, however, this aspect appears to be less pronounced. Genetic analyses revealed that most strains isolated from spontaneous fermentations showed no close genetic similarity to the industrial strains commonly used for winemaking in the Etna region, such as QA23 and RB2. Only a small subset exhibited genetic similarity to these commercial strains, suggesting that the use of commercial strains has a minimal effect on local yeast populations in Etna area.

Data on the genetic differentiation of non-*Saccharomyces* species, such as *H. uvarum,* could provide further highlights into its potential to respond to enological practices and environmental pressures. In our study, *H. uvarum* strains demonstrated variable genetic diversity, clustering differently across the examined groups. This finding suggests potential intra-species variability, even though *H. uvarum* exhibits a lower microsatellite polymorphism, compared to other yeast species (Albertin et al., 2014b, 2014c; Masneuf-Pomarede et al., 2015).

Interestingly, consistently with observations performed by Albertin et al. (2016), our study did not reveal strong genetic specificities associated with wineries or geographic regions, whereas clustering patterns appeared to be more significantly influenced by the harvest year.

To investigate the genetic variability of *H. uvarum* and to better understand factors driving population differentiation in this species future studies are necessary including a broader sampling from a more significant number of wineries and multiple vintages. Moreover, exploring the genetic variability within related species, such as *H. guilliermondii, H. opuntiae*, and *H. osmophila*—which were also abundant in the analyzed wineries—could provide comparative insights into the genomic and ecological diversity within the *Hanseniaspora* genus. Additionally, investigating the phenotypic diversity of these yeasts could help clarify their potential roles in fermentation and their adaptability to different enological conditions.

Although this study provides critical and new insights, certain limitations must be acknowledged. The restricted sampling scope and reliance on culturable species may underestimate microbial diversity.

Independent culture methods, such as high-throughput sequencing (e.g., ITS amplicon sequencing) or quantitative PCR-based approaches, have been increasingly applied to microbial ecology studies, allowing for a broader assessment of yeast diversity, including non-culturable and low-abundance species (Bokulich et al., 2014; Morrison-Whittle and Goddard, 2018). In this study, we relied on culture-based techniques coupled with MALDI-TOF MS for species identification, which enabled strain-level genetic characterization through microsatellites. However, integrating culture-independent methods in future studies would provide complementary insights into microbial community composition, offering a more comprehensive view of the yeast dynamics across different wineries and fermentation stages.

Additionally, longitudinal studies capturing many more wineries located in the Etna region and inter-annual variability would further elucidate the composition and stability of microbial *terroir* over time.

5. Conclusion

This study represents one of the first attempts aimed to characterize the yeast communities driving spontaneous fermentations in Mount Etna wineries. Through microbiological and genetic techniques, we revealed potential diversity across *S. cerevisiae* and non-*Saccharomyces* species. The predominance of non-*Saccharomyces* species, such as *Hanseniaspora* and *Metschnikowia*, during early and late fermentation stages could represent a key factor to their adaptability. Differences in yeast diversity among wineries may suggest an influence of local environmental and operational factors. These findings enhance our understanding of microbial ecosystems in the Etna wine region and provide a primary foundation for preserving and utilizing indigenous yeast biodiversity in local winemaking.

CRediT authorship contribution statement

Nunzio Alberto Fazio: Writing – original draft, Methodology,

Investigation. **Warren Albertin:** Methodology, Data curation. **Isabelle Masneuf-Pomarede:** Supervision, Resources, Conceptualization. **Cinzia L. Randazzo:** Supervision, Data curation. **Cinzia Caggia:** 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.

Acknowledgments

This study was conducted within a PhD research program in Biotecnologie (XXXVII cycle) by Nunzio Alberto Fazio who received a grant from INPS (AY 2021-2022) in the frame of "Dottorati innovativi - Intersettoriali, vertenti sulle tematiche dell'iniziativa "Industria 4.0", caratterizzati dal forte interesse industriale e dal coinvolgimento di imprese che svolgano attività industriali dirette alla produzione di beni o di servizi in coerenza con la "Strategia Nazionale di Specializzazione Intelligente 2014-2020" funded by European Commission, titled "Risorse microbiche per la valorizzazione delle produzioni enologiche dell'Etna" carried out at University of Catania, Department of Agriculture, Food and Environment (Scientific Tutors: Cinzia Caggia and Cinzia L. Randazzo) in collaboration with Barone di Villagrande winery (Milo, Italy), under the supervision of Marco Nicolosi.

Data availability

Data will be made available on request.

References

Agarbati, A., Comitini, F., Ciani, M., Canonico, L., 2024. Occurrence and persistence of *Saccharomyces cerevisiae* population in spontaneous fermentation and the relation with "winery effect". Microorganisms 12 (7), 1494. https://doi.org/10.3390/microorganisms12071494.

Albergaria, H., Arneborg, N., 2016. Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: role of physiological fitness and microbial interactions. Appl. Microbiol. Biotechnol. 100, 2035–2046. https://doi.org/10.1007/s00253-015-7255-0.

Albertin, W., Miot-Sertier, C., Bely, M., Marullo, P., Coulon, J., Moine, V., Colonna-Ceccaldi, B., Masneuf-Pomarede, I., 2014a. Oenological prefermentation practices strongly impact yeast population dynamics and alcoholic fermentation kinetics in chardonnay grape must. Int. J. Food Microbiol. 178, 87–97. https://doi.org/10.1016/j.ijfoodmicro.2014.03.009.

Albertin, W., Chasseriaud, L., Comte, G., Panfili, A., Delcamp, A., Salin, F., Marullo, P., Bely, M., 2014b. Winemaking and bioprocesses strongly shaped the genetic diversity of the ubiquitous yeast *Torulaspora delbrueckii*. PLoS One 9, e94246. https://doi.org/10.1371/journal.pone.0094246.

Albertin, W., Panfili, A., Miot-Sertier, C., Goulielmakis, A., Delcamp, A., Salin, F., Lonvaud-Funel, A., Curtin, C., Masneuf-Pomarede, I., 2014c. Development of microsatellite markers for the rapid and reliable genotyping of *Brettanomyces bruxellensis* at strain level. Food Microbiol. 42, 188–195. https://doi.org/10.1016/j.fm.2014.03.012.

Albertin, W., Setati, M.E., Miot-Sertier, C., Mostert, T.T., Colonna-Ceccaldi, B., Coulon, J., Girard, P., Moine, V., Pillet, M., Salin, F., Bely, M., Divol, B., Masneuf-Pomarede, I., 2016. *Hanseniaspora uvarum* from winemaking environments show spatial and temporal genetic clustering. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.01569.

Alfonzo, A., Francesca, N., Sannino, C., Settanni, L., Moschetti, G., 2013. Filamentous fungi transported by birds during migration across the Mediterranean Sea. Curr. Microbiol. 66, 236–242. https://doi.org/10.1007/s00284-012-0262-9.

Ayoub, M.J., Legras, J.L., Abi-Nakhoul, P., Nguyen, H.V., Saliba, R., Gaillardin, C., 2021. Lebanon's native oenological *Saccharomyces cerevisiae* flora: assessment of different aspects of genetic diversity and evaluation of winemaking potential. Journal of Fungi 7 (8), 678.

Bagheri, B., Bauer, F.F., Setati, M.E., 2016. The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. South African Journal of Enology and Viticulture 36. https://doi.org/10.21548/36-2-957.

Barata, A., Malfeito-Ferreira, M., Loureiro, V., 2012. The microbial ecology of wine grape berries. Int. J. Food Microbiol. 153, 243–259. https://doi.org/10.1016/j.ijfoodmicro.2011.11.025.

Bellia, C., Columba, P., Ingrassia, M., 2022. The brand–land identity of Etna volcano valley wines: a policy delphi study. Agriculture 12, 811. https://doi.org/10.3390/agriculture12060811.

Berbegal, C., Spano, G., Tristezza, M., Grieco, F., Capozzi, V., 2017. Microbial resources and innovation in the wine production sector. South African Journal of Enology & Viticulture 38. https://doi.org/10.21548/38-2-1333.

Bokulich, N.A., Ohta, M., Richardson, P.M., Mills, D.A., 2013. Monitoring seasonal changes in winery-resident microbiota. PLoS One 8 (6), e66437. https://doi.org/10.1371/journal.pone.0066437.

Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A., 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc. Natl. Acad. Sci. 111. https://doi.org/10.1073/pnas.1317377110.

Börlin, M., Claisse, O., Albertin, W., Salin, F., Legras, J.-L., Masneuf-Pomarede, I., 2020. Quantifying the effect of human practices on *S. Cerevisiae* vineyard metapopulation diversity. Sci. Rep. 10, 16214. https://doi.org/10.1038/s41598-020-73279-7.

Capece, A., Granchi, L., Guerrini, S., Mangani, S., Romaniello, R., Vincenzini, M., Romano, P., 2016. Diversity of *Saccharomyces cerevisiae* strains isolated from two italian wine-producing regions. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01018.

Chalvantzi, I., Banilas, G., Tassou, C., Nisiotou, A., 2021. Biogeographical regionalization of wine yeast communities in Greece and environmental drivers of species distribution at a local scale. Front. Microbiol. 12. https://doi.org/10.3389/fmicb.2021.705001.

Chen, Y., Jiang, J., Song, Y., Zang, X., Wang, G., Pei, Y., Song, Y., Qin, Y., Liu, Y., 2022. Yeast diversity during spontaneous fermentations and oenological characterisation of indigenous *Saccharomyces cerevisiae* for potential as wine starter cultures. Microorganisms 10, 1455. https://doi.org/10.3390/microorganisms10071455.

Cheng, E., Martiniuk, J.T., Hamilton, J., McCarthy, G., Castellarin, S.D., Measday, V., 2020. Characterization of sub-regional variation in *saccharomyces* populations and grape phenolic composition in pinot noir vineyards of a Canadian wine region. Front. Genet. 11. https://doi.org/10.3389/fgene.2020.00908.

Ciani, M., Canonico, L., Oro, L., Comitini, F., 2020. Footprint of non conventional yeasts and their contribution in alcoholic fermentations. Biotechnological Progress and Beverage Consumption 435–465. https://doi.org/10.1016/B978-0-12-816678-9.00014-X.

Cibrario, A., Avramova, M., Dimopoulou, M., Magani, M., Miot-Sertier, C., Mas, A., Portillo, M.C., Ballestra, P., Albertin, W., Masneuf-Pomarede, I., Dols-Lafargue, M., 2019. *Brettanomyces bruxellensis* wine isolates show high geographical dispersal and long persistence in cellars. PLoS One 14 (12). https://doi.org/10.1371/journal.pone.0222749.

Conacher, C., Luyt, N., Naidoo-Blassoples, R., Rossouw, D., Setati, M., Bauer, F., 2021. The ecology of wine fermentation: a model for the study of complex microbial ecosystems. Appl. Microbiol. Biotechnol. 105, 3027–3043. https://doi.org/10.1007/s00253-021-11270-6.

Condina, M.R., Dilmetz, B.A., Razavi Bazaz, S., Meneses, J., Ebrahimi Warkiani, M., Hoffmann, P., 2019. Rapid separation and identification of beer spoilage bacteria by inertial microfluidics and MALDI-TOF mass spectrometry. Lab Chip 19, 1961–1970. https://doi.org/10.1039/C9LC00152B.

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M.D., Valero, E., 2011. Influence of the farming system and vine variety on yeast communities associated with grape berries. Int. J. Food Microbiol. 145, 132–139. https://doi.org/10.1016/j.ijfoodmicro.2010.11.040.

Cubillos, F.A., VÃ¡squez, C., Faugeron, S., Ganga, A., MartÃnez, C., 2009. Self-fertilization is the main sexual reproduction mechanism in native wine yeast populations. FEMS Microbiol. Ecol. 67, 162–170. https://doi.org/10.1111/j.1574-6941.2008.00600.x.

Di Maio, S., Polizzotto, G., Di Gangi, E., Foresta, G., Genna, G., Verzera, A., Scacco, A., Amore, G., Oliva, D., 2012a. Biodiversity of indigenous *Saccharomyces* populations from old wineries of South-Eastern Sicily (Italy): preservation and economic potential. PLoS One 7, e30428. https://doi.org/10.1371/journal.pone.0030428.

Di Maio, S., Genna, G., Gandolfo, V., Amore, G., Ciaccio, M., Oliva, D., 2012b. Presence of *Candida zemplinina* in Sicilian musts and selection of a strain for wine mixed fermentations. South African Journal of Enology and Viticulture 33, 1. https://doi.org/10.21548/33-1-1309.

Drumonde-Neves, J., Franco-Duarte, R., Lima, T., Schuller, D., Pais, C., 2016. Yeast biodiversity in vineyard environments is increased by human intervention. PLoS One 11, e0160579. https://doi.org/10.1371/journal.pone.0160579.

Englezos, V., Mota-Gutierrez, J., Giacosa, S., Río Segade, S., Pollon, M., Gambino, G., Rolle, L., Ferrocino, I., Rantsiou, K., 2022. Effect of alternative fungicides and inoculation strategy on yeast biodiversity and dynamics from the vineyard to the winery. Food Res. Int. 162, 111935. https://doi.org/10.1016/j.foodres.2022.111935.

Fazio, N.A., Russo, N., Foti, P., Pino, A., Caggia, C., Randazzo, C.L., 2023. Inside current winemaking challenges: exploiting the potential of conventional and unconventional yeasts. Microorganisms 11, 1338. https://doi.org/10.3390/microorganisms11051338.

Feghali, N., Albertin, W., Tabet, E., Rizk, Z., Bianco, A., Zara, G., Masneuf-Pomarede, I., Budroni, M., 2019. Genetic and phenotypic characterisation of a *Saccharomyces cerevisiae* population of 'Merwah' white wine. Microorganisms 7, 492. https://doi.org/10.3390/microorganisms7110492.

Ferlito, F., Nicolosi, E., La Malfa, S., Cicala, A., Gentile, A., 2018. First characterisation of minor and neglected. Cultivars from Mount Etna. Hortic. Sci. 45, 37–46. https://doi.org/10.17221/198/2016-HORTSCI.

Francesca, N., Canale, D.E., Settanni, L., Moschetti, G., 2012. Dissemination of wine-related yeasts by migratory birds. Environ. Microbiol. Rep. 4 (1), 105–112. https://doi.org/10.1111/j.1758-2229.2011.00310.x.

Gayevskiy, V., Lee, S., Goddard, M.R., 2016. European derived *Saccharomyces cerevisiae* colonisation of New Zealand vineyards aided by humans. FEMS Yeast Res. 16 (7). https://doi.org/10.1093/femsyr/fow091.

Guillamón, J.M., Barrio, E., 2017. Genetic polymorphism in wine yeasts: mechanisms and methods for its detection. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.00806.

Kamvar, Z.N., Tabima, J.F., Grünwald, N.J., 2014. *Poppr* : an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2, e281. https://doi.org/10.7717/peerj.281.

Lappa, I.K., Kachrimanidou, V., Pateraki, C., Koulougliotis, D., Eriotou, E., Kopsahelis, N., 2020. Indigenous yeasts: emerging trends and challenges in winemaking. Curr. Opin. Food Sci. 32, 133–143. https://doi.org/10.1016/j.cofs.2020.04.004.

Le Jeune, C., Erny, C., Demuyter, C., Lollier, M., 2006. Evolution of the population of *Saccharomyces cerevisiae* from grape to wine in a spontaneous fermentation. Food Microbiol. 23 (8), 709–716. https://doi.org/10.1016/j.fm.2006.02.007.

Legras, J.L., Ruh, O., Merdinoglu, D., Karst, F., 2005. Selection of hypervariable microsatellite loci for the characterization of *Saccharomyces cerevisiae* strains. Int. J. Food Microbiol. 102, 73–83. https://doi.org/10.1016/j.ijfoodmicro.2004.12.007.

Liu, D., Zhang, P., Chen, D., Howell, K., 2019. From the vineyard to the winery: how microbial ecology drives regional distinctiveness of wine. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.02679.

Liu, Y., Rousseaux, S., Tourdot-Maréchal, R., Sadoudi, M., Gougeon, R., Schmitt-Kopplin, P., Alexandre, H., 2017. Wine microbiome: a dynamic world of microbial interactions. Crit. Rev. Food Sci. Nutr. 57, 856–873. https://doi.org/10.1080/10408398.2014.983591.

Martínez-Estrada, S. C., Soto-Cruz, N. Ó., Rojas-Contreras, J. A., López-Miranda, J., Rodríguez-Herrera, R., & Páez-Lerma, J. B. (2023). Phylogenetic analysis in yeast population using microsatellites and simple sequence tandem repeats. *Microbial biodiversity, biotechnology and ecosystem sustainability. 409–426*. Springer nature Singapore. https://doi.org/10.1007/978-981-19-4336-2_16.

Marullo, P., Claisse, O., Raymond Eder, M.L., Börlin, M., Feghali, N., Bernard, M., Legras, J.-L., Albertin, W., Rosa, A.L., Masneuf-Pomarede, I., 2020. SSU1 checkup, a rapid tool for detecting chromosomal rearrangements related to the SSU1 promoter in *Saccharomyces cerevisiae*: an ecological and technological study on wine yeast. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.01331.

Mas, A., Portillo, M.C., 2022. Strategies for microbiological control of the alcoholic fermentation in wines by exploiting the microbial terroir complexity: A mini-review. Int. J. Food Microbiol. 367, 109592. https://doi.org/10.1016/j.ijfoodmicro.2022.109592.

Masneuf-Pomarede, I., Juquin, E., Miot-Sertier, C., Renault, P., Laizet, Y., Salin, F., Alexandre, H., Capozzi, V., Cocolin, L., Colonna-Ceccaldi, B., Englezos, V., Girard, P., Gonzalez, B., Lucas, P., Mas, A., Nisiotou, A., Sipiczki, M., Spano, G., Tassou, C., Bely, M., Albertin, W., 2015. The yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) shows high genetic diversity in winemaking environments. FEMS Yeast Res. 15, fov045. https://doi.org/10.1093/femsyr/fov045.

Masneuf-Pomarede, I., Bely, M., Marullo, P., Albertin, W., 2016. The genetics of non-conventional wine yeasts: current knowledge and future challenges. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.01563.

Morrison-Whittle, P., Goddard, M.R., 2018. From vineyard to winery: a source map of microbial diversity driving wine fermentation. Environ. Microbiol. 20, 75–84. https://doi.org/10.1111/1462-2920.13960.

Nisiotou, A., Gyftogianni, E., Banilas, G., 2022. Evaluation of different molecular markers for genotyping non-*Saccharomyces* wine yeast species. Microbiol. Res. 13, 643–654. https://doi.org/10.3390/microbiolres13030046.

Oksanen, J., Kindt, R., O'Hara, B., 2013. Package vegan: community ecology package. 2, 1–295.

Onetto, C.A., Borneman, A.R., Schmidt, S.A., 2020. Investigating the effects of *Aureobasidium pullulans* on grape juice composition and fermentation. Food Microbiol. 90, 103451. https://doi.org/10.1016/j.fm.2020.103451.

Padilla, B., García-Fernández, D., González, B., Izidoro, I., Esteve-Zarzoso, B., Beltran, G., Mas, A., 2016. Yeast biodiversity from DOQ priorat uninoculated fermentations. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.00930.

Pallmann, C.L., Brown, J.A., Olineka, T.L., Cocolin, L., Mills, D.A., Bisson, L.F., 2001. Use of WL medium to profile native flora fermentations. Am. J. Enol. Vitic. 52, 198–203. https://doi.org/10.5344/ajev.2001.52.3.198.

Raymond Eder, M.L., Rosa, A.L., 2020. Non-tandem repeat polymorphisms at microsatellite loci in wine yeast species. Mol. Gen. Genomics. 295, 685–693. https://doi.org/10.1007/s00438-020-01652-2.

Regecová, I., Výrostková, J., Semjon, B., Lovayová, V., Jevinová, P., Megyesy Eftimová, Z., Bartkovský, M., Pipová, M., Marcinčák, S., 2024. The influence of maceration on the biodiversity of yeasts in the early winemaking stages of white wine from the slovak tokay wine region. Foods 13, 3792. https://doi.org/10.3390/foods13233792.

Schuller, D., Cardoso, F., Sousa, S., Gomes, P., Gomes, A.C., Santos, M.A.S., Casal, M., 2012. Genetic diversity and population structure of *Saccharomyces cerevisiae* strains isolated from different grape varieties and winemaking regions. PLoS One 7 (2), e32507. https://doi.org/10.1371/journal.pone.0032507.

Setati, M.E., Jacobson, D., Bauer, F.F., 2015. Sequence-based analysis of the *Vitis vinifera l.* cv cabernet sauvignon grape must mycobiome in three south African vineyards employing distinct agronomic systems. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.01358.

Settanni, L., Sannino, C., Francesca, N., Guarcello, R., Moschetti, G., 2012. Yeast ecology of vineyards within Marsala wine area (western Sicily) in two consecutive vintages and selection of autochthonous *Saccharomyces cerevisiae* strains. J. Biosci. Bioeng. 114, 606–614. https://doi.org/10.1016/j.jbiosc.2012.07.010.

Snyder, T., Osborne, J., Curtin, C., 2024. Contributions of *Hanseniaspora* species to pinot noir microbial terroir in Oregon's Willamette Valley wine region. Appl. Environ. Microbiol. 90 (9). https://doi.org/10.1128/aem.00810-24.

Sumby, K.M., Caliani, N.S., Jiranek, V., 2021. Yeast diversity in the vineyard: how it is defined, measured and influenced by fungicides. Aust. J. Grape Wine Res. 27, 169–193. https://doi.org/10.1111/ajgw.12479.

Vicente, J., Navascués, E., Benito, S., Marquina, D., Santos, A., 2023. Microsatellite typing of *Lachancea thermotolerans* for wine fermentation monitoring. Int. J. Food Microbiol. 394, 110186. https://doi.org/10.1016/j.ijfoodmicro.2023.110186.

Viel, A., Legras, J.-L., Nadai, C., Carlot, M., Lombardi, A., Crespan, M., Migliaro, D., Giacomini, A., Corich, V., 2017. The geographic distribution of *Saccharomyces cerevisiae* isolates within three italian neighboring winemaking regions reveals strong differences in yeast abundance, genetic diversity and industrial strain dissemination. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.01595.

Wang, X., Fan, G., Peng, Y., Xu, N., Xie, Y., Zhou, H., Liang, H., Zhan, J., Huang, W., You, Y., 2023. Mechanisms and effects of non-*Saccharomyces* yeast fermentation on the aromatic profile of wine. J. Food Compos. Anal. 124, 105660. https://doi.org/10.1016/j.jfca.2023.105660.

Windholtz, S., Dutilh, L., Lucas, M., Maupeu, J., Vallet-Courbin, A., Farris, L., Coulon, J., Masneuf-Pomarède, I., 2021. Population dynamics and yeast diversity in early winemaking stages without sulfites revealed by three complementary approaches. Appl. Sci. 11, 2494. https://doi.org/10.3390/app11062494.

Zabukovec, P., Čadež, N., Čuš, F., 2020. Isolation and identification of indigenous wine yeasts and their use in alcoholic fermentation. Food Technol. Biotechnol. 58, 337–347. https://doi.org/10.17113/ftb.58.03.20.6677.

Zarraonaindia, I., Owens, S.M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., Bokulich, N.A., Mills, D.A., Martin, G., Taghavi, S., van der Lelie, D., Gilbert, J.A., 2015. The soil microbiome influences grapevine-associated microbiota. MBio 6. https://doi.org/10.1128/mBio.02527-14.

Zhang, J., Plowman, J.E., Tian, B., Clerens, S., On, S.L.W., 2021. Application of MALDI-TOF analysis to reveal diversity and dynamics of winemaking yeast species in wild-fermented, organically produced, New Zealand pinot noir wine. Food Microbiol. 99, 103824. https://doi.org/10.1016/j.fm.2021.103824.

Zhang, Z., Wang, H., Xia, H., Sun, L., Zhang, Q., Yang, H., Zhang, J., 2023. Wine aroma modification by *Hanseniaspora uvarum*: A multiple-step strategy for screening potential mixed starters. Food Chemistry: X 20, 100930. https://doi.org/10.1016/j.fochx.2023.100930.