Food Bioscience 62 (2024) 105282

Contents lists available at ScienceDirect

Food Bioscience

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

Screening and characterization of indigenous *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts isolated from Sicilian vineyards

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ARTICLE INFO

Keywords:
Wine
Etna
Fermentation
Ethanol reduction
Wine aroma
Starter culture

ABSTRACT

The use of *Saccharomyces cerevisiae* as starter cultures in winemaking is widespread in all over the world. However, a growing interest in exploring native non-*Saccharomyces* yeast species as co-starters has emerged, driven by their potential to enhance wine complexity and regional identity. The aim of the present study was to investigate indigenous yeasts from Sicily, to select novel strains with promising technological traits. A comprehensive screening of indigenous *S. cerevisiae* and non-*Saccharomyces* yeasts was conducted across seven locations in Sicily, including the unique volcanic zone of Mount Etna. A total of 95 yeast isolates were obtained and genetically characterized by RFLP-PCR and sequencing of 5.8S-ITS rDNA. Isolates were screened for oenological traits and the most promising, belonging to *Starmerella bacillaris*, *Hanseniaspora uvarum*, and *Metschnikowia pulcherrima* species, were used for single and sequential fermentations in synthetic must. Results revealed a remarkable diversity of physiological traits within and among the species, regarding stress tolerance, enzymatic activity and growth at different temperatures. Notably, isolates of *H. uvarum* and *St. bacillaris* exhibited great ethanol tolerance, and the latter also strong glycerol production. Furthermore, *M. pulcherrima* exhibited a wide range of aroma-related enzymatic activities, particularly beta-glucosidase. The remarkable diversity, the phenotypic traits of the indigenous yeasts, represents a promise for the improvement of innovative fermentation strategies and for the development of wine indigenous starters related with the region's terroir.

1. Introduction

Over the last decades, wine production is facing new approaches due to the different and specific consumers' demands. Starters based on *Saccharomyces cerevisiae* are widely used to properly ferment grape musts in a safe and stable way, lowering the risk of spoilage (Pretorius, 2000). However, recently the use of indigenous non-*Saccharomyces* strains, naturally occurring in vineyards and cellar environments, and in turns in spontaneous fermentations, is amply recognized to generate new and interesting traits to wine complexity (Borren & Tian, 2020; Fazio et al., 2023). The selection of these yeasts, directly from the geographical area of interest, is closely linked to the concept of terroir, i.e., intended as a microbiological imprint that distinguishes the final product giving undeniable sensorial traits (Di Maio et al., 2012; Mas & Portillo, 2022; Torija et al., 2001). The exploitation of native strains in wine-fermentation is expected to contribute to the distinctiveness of final product giving a strong territorial identity, which correspond to the oenological heritage of a certain region. Nowadays, an increasing number of wineries are adopting indigenous non-*Saccharomyces* yeasts in mixed or, more frequently, in sequential fermentation, together with *S. cerevisiae* in alcoholic fermentation (AF), and some yeasts are already sold as commercial starters (Padilla et al., 2017; Vaudano et al., 2019; Vejarano & Gil-Calderón, 2021). For selection procedure, the screening within the non-*Saccharomyces* yeasts must be performed taking into account the presence or activities of specific enzymes, such as β-glucosidase, protease, and esterase, that contribute positively to the overall flavour profile (Padilla et al., 2016). Furthermore, additional technological traits, mainly related to the operational parameters adopted

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https://doi.org/10.1016/j.fbio.2024.105282
Received 27 July 2024; Received in revised form 9 October 2024; Accepted 11 October 2024
Available online 16 October 2024

during the winemaking process, as tolerance to ethanol or to SO_2, ability to grow at different temperatures, in acidic or overly sugary environments (truth in wine yeast) must be considered (Gonzalez & Morales, 2022). The selection based on these characteristics is of paramount importance being many of these traits species-specific or even strain specific. Moreover, the interest in indigenous strains has recently grown as they can contribute to boost wine characteristics in line with current market needs, such as reducing ethanol, SO_2 and amine content, increasing malic acid or bioactive compounds (Balmaseda et al., 2021; García et al., 2020; Ruiz-de-Villa et al., 2023).

Among non-*Saccharomyces* yeasts, *Torulaspora delbrueckii, Lachancea thermotolerans, Hanseniaspora uvarum, Starmerella bacillaris* and *Metschnikowia pulcherrima* appear as the most promising species and strains of these species are commercially available for use in both mixed and sequential fermentations thanks to their ability to improve flavour profile, glycerol content or lowering ethanol yield (Fazio et al., 2023; Lai et al., 2022; Perpetuini et al., 2023).

According to recent estimates, Italy is the second largest wine producers in the world and the first exporter country (International Organisation of Vine and Wine: OIV, accessed on July 15, 2024) with an estimate cultivated area of 690 962 ha (ha) and a production of 42 499 47 hL in 2023 (Istituto Nazionale di Statistica: ISTAT, accessed on July 15, 2024).

In Italy, several types of wines, mainly linked to territoriality, are produced, such as IGT (typical geographic indication), DOC (appellation of controlled origin) and DOCG (denomination of controlled and guaranteed origin). In particular, DOC and DOCG wines must meet stricter criteria to guarantee the geographical area they belong to, the production methods and consequently underline the importance of "terroir" concept (Bellia et al., 2022).

In this frame, Sicily presents an enormous territorial variability (due to different altitudes, different soil compositions, and so on), which results in diversified wine heritage. More specifically, one area of greatest interest is located around Etna volcano, the highest in Europe, and especially around the municipality of Castiglione di Sicilia, Milo, Randazzo, Viagrande and Linguaglossa in the province of Catania (CT). Here, with the unique volcanic soil composition and the wide temperature range, most of the vineyards are devoted to the cultivation of Nerello Mascalese and Carricante cultivars, used for production of Red Etna DOC and White Etna DOC wines, respectively (Di Bella et al., 2019; Oliveri et al., 2017).

Considering the very few reports on the use of native yeasts in Sicily, especially in Etna area, the aim of this study was to isolate and explore indigenous yeasts from different geographic areas of the DOC districts useful for winemaking purpose. For this reason, yeasts from different wineries were isolated and identified. The main oenological features, such as specific enzyme's activities, stress tolerance and sugar metabolism, were investigated. Finally, lab-scale in single and sequential fermentations with *S. cerevisiae* were performed in synthetic must and fermentation kinetic, and chemical profile of experimental wines were evaluated.

2. Materials and methods

2.1. Sample collection

Samples of musts and grapes used in the present study were collected from wineries located in different areas of Sicily (Fig. 1), during the 2019–2022 period. In detail, aliquots of spontaneous fermenting musts were kindly provided by seven wineries belonging to the DOC consortium, as Principe di Butera, located in Caltanissetta (CL); Patria, Gambino, Barone di Villagrande, Cottanera and a winery in Maletto, all located in Catania (CT), and Feudo Arancio located in Acate (RG). Samples belonging to different grape varieties (*Vitis vinifera* L.) were collected at different fermentation stages (Table 1) and kept at 4 °C until analyses. Furthermore, sampling was conducted in Barone di Villagrande vineyard, where two Carricante grape samples, in early ripening stage, were collected in two different sites. All samples were aseptically transferred to the Agri-food Microbiology Laboratory at the University of Catania for immediate processing.

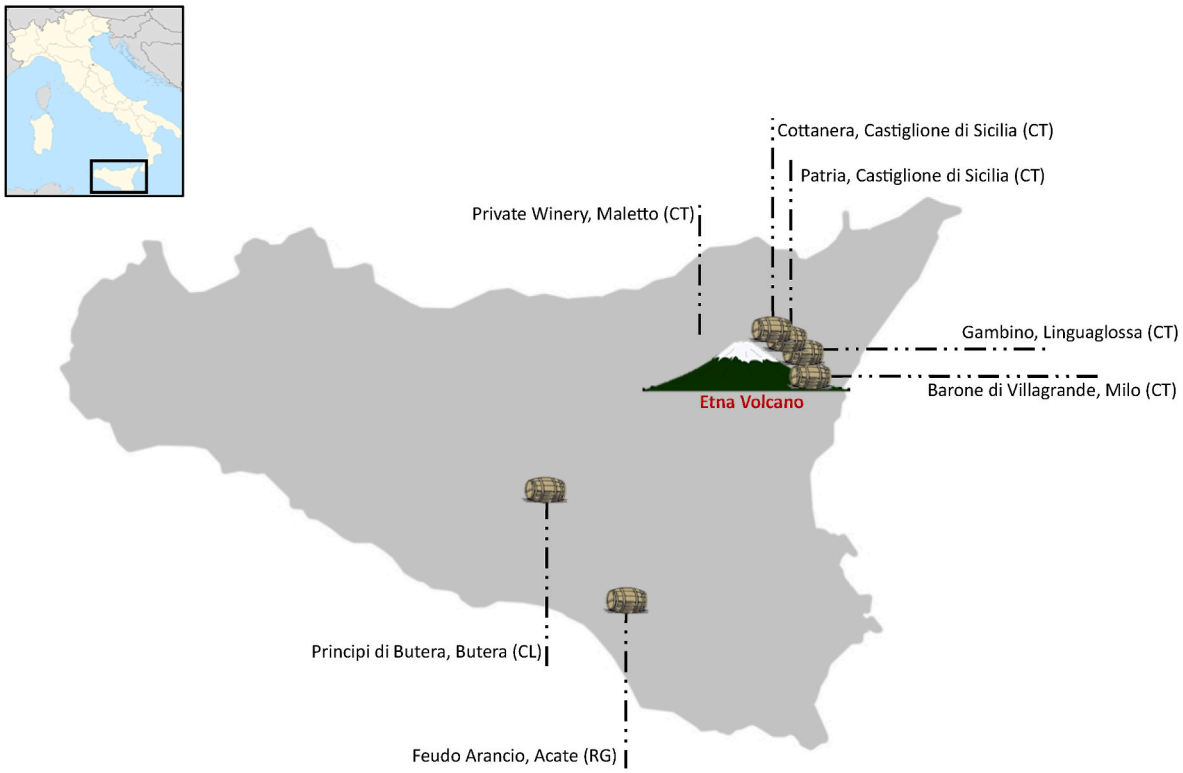

Fig. 1. Sampling sites: Principi di Butera, Patria, Gambino, Barone di Villagrande, Cottanera, Private winery, Feudo Arancio.

Table 1
Collected samples.

Winery	Location (Province)	Grape variety	Status of fermentation
Principi di Butera	Butera (CL)	Grillo	End
Patria	Castiglione di Sicilia (CT)	Nerello Mascalese	End
Gambino	Linguaglossa (CT)	Nerello Rosato	End
Barone di Villagrande	Milo (CT)	Carricante Nerello Mascalese	Early ripening grapes Start/End fermentation
Cottanera	Castiglione di Sicilia (CT)	Carricante Nerello Mascalese	End
Private winery	Maletto (CT)	Nerello Mascalese	End
Feudo Arancio	Acate (RG)	Frappato	After racking

2.2. Yeast isolation

Microbiological analyses on grape and must samples were performed. In detail, 10 g of each grape sample were mixed with 90 mL of sodium chloride sterile solution (Sigma Aldrich, Darmstadt, Germany) and homogenized for 3–5 min in a stomacher (Lab-Blender, Seward, London). From each spontaneous fermenting must and crushed grape samples, aliquots of 0.1 mL from serially diluted suspension were spread on Wallerstein Nutrient (WL) agar medium (Oxoid, ThermoFisher Scientific, USA) and incubated at 25 °C for 2–3 days; colonies showing distinctive morphologies, including differences in color, size, and shape, were selected and subsequentially picked up, streaked and incubated at 25 °C for 48 h. Preliminary discrimination among different indigenous yeast species was conducted based on typical morphologies described previously (Feng et al., 2020; Pallmann et al., 2001; Zhang, Li, et al., 2020). These studies provide detailed descriptions of the characteristic morphologies of the main oenological yeast species After incubation the isolates were maintained on YPD agar (Yeast extract 1% (w/v), Peptone 2% (w/v), Dextrose 2% (w/v), agar 1.5% (w/v)). A total of 95 isolates were collected and subsequentially stocked at −20 °C in a 20% glycerol medium solution and stored in YPD slope tubes.

2.3. Yeast identification

Yeast isolates were identified by molecular assay, as reported by Esteve-Zarzoso and co-workers (1999). In detail, cells were grown overnight on YPD and then smeared and incubated on YPDA at 25 °C for 48 h. Single colonies were mixed with 20 μL of sterile water into 1.5 mL Eppendorf tubes and used as DNA templates. Identification of all the isolates was performed by colony PCR amplifying the 5.8S Internal Transcribed Spacer (ITS) rRNA region. PCR was performed in a final volume of 50 μL using a Thermal Cycler 2720 (Applied Biosystems, Waltham, Massachusetts, USA); the reaction mix consisted of 25 μL of DreamTaq™ Green PCR Master Mix 2X (Thermo Fisher Scientific), 2 μL of primers ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3’), 16 μL of ultrapure water (DNAse/RNAse free) and 5 μL of previously obtained DNA template.

The amplification program was: initial denaturation at 95 °C for 15 min, 35 cycles of 1 min at 95 °C for denaturing, annealing at 54 °C for 2 min, extension for 2 min at 72 °C, and a final extension at 72 °C for 10 min. ITS-PCR products were visualized on 1.5% TBE 1X agarose gel (Lonza, Basel, Switzerland) stained with 3 μL of GelRed stain (Biotium, Fremont, CA, USA) under UV transilluminator (Axygen, Gel Documentation System).

Amplified products were subjected to Restriction Fragment Length Polymorphism (RFLP) using *Hae*III and *Hinf*I (Thermo Fisher Scientific, Waltham, MA, USA) enzymes. Restriction mixtures were incubated at 37 °C for 2 h and then analysed on 2.0% TBE 1X agarose gel at 100 V for 3 h. After comparing the RFLP profiles, a representative isolate for each cluster was selected for sequencing. In detail, amplified products were purified using the Qiaquick PCR purification kit (Qiagen Hilden, Germany) and sequenced using Sanger method performed by an external service (Eurofins Genomics, Vimodrone, Italy). Taxonomic identification was assessed using the basic local alignment search tool (BLASTn) software in the Standard databases, and those from non-*Saccharomyces* used for fermentation trials were deposited in GenBank.

2.4. Phenotypic traits

2.4.1. Sugar assimilation

All isolates were grown in WL agar for 48 h at 25 °C, each colony was observed under microscope (Olympus BX40, Tokyo, Japan). Assimilation tests were performed, as reported by Kurtzman et al. (2011); briefly, medium was prepared by mixing peptone (7.5 g/L), yeast extract (4.5 g/L), 1 mL/L of a stock solution of blue bromothymol (1.6 w/v) and sugars to be tested (glucose, fructose, sucrose, galactose, and lactose) were added at a final concentration of 2% w/v, after autoclaving. From each culture, previously incubated overnight at 27 °C in YPD broth, cells were collected by centrifugation at 4700 rpm (3200×*g*, 10 min, 4 °C), washed twice, re-suspended in sterile saline and diluted to an optical density at 600 nm (OD_{600}) absorbance value of 1.6, measured with a Model 680 microplate reader (Bio-Rad, iMark, USA), equivalent to around 10^7 cells/mL.

The ability to assimilate sugars was assessed into 96-well microtiter plates (Greiner Bio-One, Kremsmünster, Austria) containing 198 μL of the previously prepared medium and 2 μL of previously standardised culture. The analyses were performed in triplicate and the assimilation of sugars, after 48 h at 27 °C, was visually revealed by colour change, from blue (alkaline) to yellow (acidic) due to pH change.

2.4.2. Stress tolerance

The isolates were tested for tolerance to different stresses. Firstly, as reported by Binati and co-workers (2019), for each fresh culture, incubated overnight at 27 °C in YPD broth, the ability to grow at different temperatures, acidic pH, high sugar concentrations and different ethanol and SO_2 contents, was assessed after 48 h.

In details, cell suspensions were prepared as described before and then inoculate in YPD medium containing 4, 8, 12 and 15% (v/v) of ethanol, 300 g/L of glucose, 100, 200, and 300 mg/L of final SO_2, achieved by adjusting the amount of potassium metabisulphite ($K_2S_2O_5$) needed, and pH adjusted to 3. YPD without any addition was used as controls. In 198 μL of the respective medium, prepared in 96-well microtiter plates, 2 μL of standardized cell suspension were inoculated in triplicate and incubated for 48 h. Microplates were then incubated at 27 °C, while, for temperature stress assay, at 18 and 37 °C. After 48 h the OD_{600} was measured as previously described.

2.4.3. Enzymatic activities

The enzymatic activities of oenological interest were evaluated in different modified growth media. The inoculum was prepared as previously reported and each standardized cell culture was spotted (10 μL) in triplicate in respective modified media and results, ranging from 0 (no production), 50 (slight activity), 75 (moderate activity) to 100 (strong activity), were visually detected.

Beta-glucosidase activity was assessed in a pH 5-adjusted medium containing yeast extract (10 g/L), agar (20 g/L) and, after autoclaving at 121 °C for 5 min, 40 drops/100 mL of a 1% solution of ferric ammonium citrate and arbutin (5 g/L), previously filtered at 0.45 μm filter were added (Sidari et al., 2021). After incubation for 7 days at 25 °C, the intensity of enzymatic activity was assessed as colour change of the media from light brown (small production) to dark brown/black (intense production).

Protease activity was evaluated in medium containing malt extract (3 g/L), yeast extract (3 g/L), peptone (5 g/L), glucose (10 g/L), NaCl (5

g/L), agar (20 g/L); after autoclaving at 121 °C for 5 min, skim milk powder (10% w/v) in sterile water was separately added to medium before plating. Positive result was evaluated after 5 days of incubation at 25 °C through the appearing of a clear halo around the spotted colonies (Lin et al., 2020) and the intensity was assessed by measuring the halo diameter.

Esterase activity was screened on medium containing peptone (10 g/L), NaCl (5 g/L), $CaCl_2$ x $_2H_2O$ (0.1 g/L), tween 80 (10 g/L), agar (20 g/L) at pH 6.8. After incubation for 6 days at 25 °C the presence of precipitate halo around the colonies was evaluated as a positive response (Buzzini & Martini, 2002).

2.4.4. Organic acids and hydrogen sulfide production

Acid production was evaluated in chalk agar medium containing yeast extract (3 g/L), glucose (10 g/L), calcium carbonate (3 g/L), and agar (15 g/L). The presence of a halo around the colonies indicates the ability to produce organic acids (Ianieva & Podgorsky, 2021).

The hydrogen sulfide producing isolates were detected in BiGGY Nickerson Agar medium (Liofilchem, Italy), after incubation at 25 °C for 48 h, white to dark brown/black colour intensity indicated producing of H_2S (Comitini et al., 2011).

2.5. Laboratory-scale fermentation

Suitable isolates, according to preliminary results, were selected to carry out laboratory-scale fermentation trials both in single and sequential fermentations, on synthetic must. The must was prepared according to Riou et al. (1997) with some adjustments, as proposed by Beltran et al. (2004): 200 g/L of reducing sugars (100 g/L of fructose and glucose, respectively), and 300 mg/L of Yeast Assimilable Nitrogen Conent (YANC). The pH of must was adjusted to a final value of 3.3 and anaerobiotic factors were added. In a first screening, single fermentations with *H. uvarum* (GA112), *St. bacillaris* (BV13) plus *M. pulcherrima* (BVR3) and *S. cerevisiae* (BVR101) were conducted to evaluate the fermentation kinetics. Each isolate was overnight pre-grown in YPD at 28 °C and inoculated in triplicate at an initial concentration of 2×10^6 cells/mL into 250 mL glass bottles containing 200 mL of must. The bottles were closed with taps settled up with tubes connected with a 0.22 μm filter (Dominique Dutscher, Brumath, France) for gas flow and the others locked by an iron clip to allow sampling. Sequential fermentations were performed firstly adding a non-*Saccharomyces* species and after 48 h adding *S. cerevisiae* (Sc), at a final concentration of 2×10^6 cells/mL, to end the alcoholic fermentation. *S. cerevisiae* QA23® (Lallemand Inc, Canada) (QA23) was used as control for both single and sequential fermentation trials.

Fermentations were daily monitored by measuring must density with an electronic densitometer (Densito 30PX Portable Density Meter, Mettler Toledo, Hospitalet de Llobregat, Spain). Nitrogen content was measured at 24, 48, 72 and 96 h with Biosystem® Y15 autoanalyzer (Biosystems, Barcelona, Spain) and, during AF, yeast counting was assessed by plating 100 μL of serially diluted samples on WL agar, counting the specific non-*Saccharomyces* and *S. cerevisiae* yeast after 2–3 days of incubation at 28 °C. Fermentations were considered completed or stucked when the residual sugars were below 2 g/L, checked by Y15 auto-analyser (for sequential fermentations), or when the density value remained constant for longer than two days (for single culture fermentations). At the end of fermentations, experimental wines were centrifuged at 7000 rpm for 5 min, and the supernatants were frozen at −20 °C until chemical analyses.

2.6. Chemical analyses

D-glucose/D-fructose were quantified by enzymatic assay in Y15 auto-analyser to point out the end of fermentation. The main organic compounds (ethanol, glycerol, citric, malic, tartaric, acetic, lactic and succinic acids) were quantified by high-performance liquid chromatography (HPLC) using an Agilent 1100 (Agilent Technologies, Waldbronn, Germany) apparatus, as previously described by Zhu and co-workers (2020), equipped with a Hi-Plex H, 300 mm × 7.7 mm column inside a 1260 Multicolumn Thermostat (Infinity II MCT) and connected with two detectors, a multiwavelength detector (G1365B MWC) and a 1260 Infinity II refractive index detector (Agilent Technologies, Germany). The column was maintained at 60 °C, and 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min. Previously, samples were filtered through 0.22 μm pore size filters (Dominique Dutscher, Brumath, France). Chromatograms were analysed using the Agilent ChemStation Plus software (Agilent Technologies, Germany).

2.7. Statistical analyses

Heat maps analysis was performed on R (R 4.3.3) using RStudio (2024.04.1 + 748). Graph were generated with 'pheatmap' function from the 'pheatmap' package version 1.0.12 (Kolde, 2019) and dendrograms were built using Euclidean distance and Ward's clustering to see de differences on tested conditions among isolates. In addition, to further examine the relationships between the variables, a principal component analysis (PCA) was generated using 'FactoMineR" package (Lê S et al., 2008). Raw data can be found on supplementary data (Tables S3 and S4).

Fermentation kinetic curves were plotted using the GraphPad Prism 7 program (GraphPad Software, San Diego, California, USA). The SPSS software (version 21.0, IBM Statistics, Armonk, NY, USA) was used for HPLC data processing; organic compound data were expressed as mean ± standard deviation (SD). The values were considered as significantly different when $p \leq 0.05$. Statistical analysis was performed using one-way analysis of variance (ANOVA), and Tukey's HSD post-hoc test for means separation at a significance level of $p \leq 0.05$.

3. Results

3.1. Yeast screening

A total of 95 isolates were obtained, specifically 26 from grape and 69 from spontaneously fermenting musts. Preliminary results of the screening on WL medium showed that isolates from fresh grapes were mostly belonging to non-*Saccharomyces* species, while isolates from fermenting musts belonged mainly to *S. cerevisiae* species. This initial distinction was supported by differences observed in colony morphology and further investigated through microscopic examination of cell appearance. To confirm species identification, the RFLP (Restriction Fragment Length Polymorphism) profiles of the isolates were analysed, allowing us to group them into distinct clusters. A representative isolate from each RFLP cluster was then selected for sequencing of the ITS region. Sequencing results, as shown in Table 2, revealed the presence of nine species from six different genera: *Pichia manshurica, Pichia kudriavzeii, Pichia terricola, Candida californica, Hanseniaspora uvarum, Hanseniaspora opuntiae, Metschnikowia pulcherrima, Starmerella bacillaris, and Saccharomyces cerevisiae.*

The accession numbers of the three sequenced non-*Saccharomyces* isolates, used for laboratory fermentation trials, deposited in GenBank, are: PQ035970 (*St. bacillaris*), PQ035971 (*H. uvarum*), PQ035972 (*M. pulcherrima*).

3.2. Phenotypic traits

Growth of isolates on WL medium allowed a preliminary discrimination among species based on colony colour, morphology, and texture. Isolates belonging *S. cerevisiae* generally produced round, smooth, colonies, with creamy-white to light/green colors. On the other hand, isolates belonging to non-*Saccharomyces* species exhibited a wider variety of colony features. Their textures ranged from smooth (e.g., *C. californica, H. uvarum, H. opuntiae, M. pulcherrima, St. bacillaris*) to

Table 2
Species distribution across the 95 isolates. Each species is listed with the number of isolates (#) and their corresponding percentage (%) of the total isolates, and the number of isolates for each source of isolation.

Species	# of isolates (%)	Source of isolation (#)
Pichia manshurica	8 (8.4)	Must – end fermentation (8)
Pichia kudriavzeii	1 (1.1)	Early ripening grapes (1)
Pichia terricola	2 (2.1)	Early ripening grapes (1)
Candida californica	1 (1.1)	Must – end fermentation (1)
Hanseniaspora uvarum	16 (16.8)	Early ripening grapes (14), Must – end fermentation (2)
Hanseniaspora opuntiae	3 (3.1)	Early ripening grapes (1), Must – early fermentation (2)
Metschnikowia pulcherrima	7 (7.4)	Early ripening grapes (6), Must – early fermentation (1)
Starmerella bacillaris	2 (2.1)	Must – end fermentation (2)
Saccharomyces cerevisiae	55 (57.9)	Early ripening grapes (2); Must – early fermentation (23); Must – end fermentation (28); After racking (2)

rough (e.g., *P. manshurica, P. kudriavzeii*). Colony colors varied from creamy (e.g., *P. manshurica, H. opuntiae*), light green (e.g., *P. terricola, C. californica*), green (e.g., *H. uvarum*), dark green (e.g., *St. bacillaris*), to creamy with red hues (e.g., *M. pulcherrima*). Under the microscope, colonies of *S. cerevisiae* generally appeared larger than those of non-*Saccharomyces* species, with a typical oval shape. Among the non-*Saccharomyces* species, *M. pulcherrima* displayed a more rounded form; *H. opuntiae* and *H. uvarum* had lemon-shaped cells, while other species (*C. californica, St. bacillaris, P. manshurica, P. terricola,* and *P. kudriavzeii*) generally exhibited a rod-shaped appearance, with more pronounced filaments observed in *P. kudriavzeii*.

The data from sugar assimilation test reveals different sugar utilization patterns among the different yeast species which reflect their varied metabolic capabilities. *S. cerevisiae* and *P. kudriavzevii* generally exhibit a broad ability to assimilate multiple sugars. *S. cerevisiae* efficiently utilizes glucose and fructose across most isolates, while its capacity for galactose and sucrose shows some variability. Similarly, *P. kudriavzevii* shows robust utilization of glucose, fructose, galactose, and sucrose, though it does not metabolize lactose. In contrast, species such as *C. californica* and *H. opuntiae* exhibit more limited sugar utilization profiles; *C. californica* shows weak utilization of glucose and sucrose and cannot metabolize fructose, galactose, or lactose; *H. opuntiae* demonstrates strong glucose and fructose metabolism but lacks the ability to utilize galactose and shows variable sucrose utilization. Colony's traits of representative isolates are shown in Table S2 (supplementary). Microscope evaluation of representative yeast species is shown in Fig. S1 (supplementary) and sugar fermentations profile in Table S1 (supplementary)

3.3. Growth under stress conditions

Ethanol stress tolerance was evaluated measuring OD of cultures after 48 h in 96-well microplates on YPD supplemented with different percentage of ethanol. As reported in Fig. 2 panel a, among *S. cerevisiae* species, all isolates showed a good tolerance up to 15% (v/v) of ethanol, except the isolate BVC 27.1, which showed sensitivity at a 12% ethanol concentration. All non-*Saccharomyces* isolates were able to grow in presence of 5% of ethanol, while at concentrations of 8, 12 and 15%, *H. opuntiae, P. kudriavzeii, St. bacillaris, P. manshurica* and *C. californica* isolates showed higher growth than *H. uvarum*, *M. pulcherrima* and *P. terricola*, some of which were totally inhibited at concentrations of 12 and 15% of ethanol. Among non-*Saccharomyces* isolates, the *St. bacillaris* BV 11 and BV 13 strongly grow at the highest tested (15%) ethanol concentration.

Zooming on SO_2 tolerance (Fig. 2, panel b), most of the non-*Saccharomyces* isolates did not tolerate the presence of sulfur dioxide; specifically, among *H. uvarum*, only one isolate (GA112) was able to grow at concentration of 300 mg/L, and three (GA 111, BVC 4 and BVC 7) showed activity up to 200 mg/L. Among *M. pulcherrima* isolates the maximum tolerable concentration of SO_2 was 100 mg/L while all isolates of *P. manshurica* were able to grow at higher concentrations. Focusing on *S. cerevisiae*, huge variability was observed. In details, 12 isolates showed a sensitive behaviour to different concentrations of SO_2; with five isolates (BVC 3, BVC 27.1, BVR 105, BVR 106 and CO 14) that were not able to grow at 100 mg/L of SO_2; three (BVB 5, BVR 100 and NN 24) did not survive at 200 mg/L and four (BVB 3, BVB 4, FA 11, FA 12) did not survive at 300 mg/L while the remaining isolates grew at the highest tested levels.

Regarding the growth at different temperatures (Fig. 2, panel c), at 18 °C some isolates of *H. uvarum* (BVC 6, BVC 21, BVC 26, BVC 28), *H. opuntiae* (BVR 7) and *M. pulcherrima* (BVC 15, BVC 37, BVC 40) were inhibited, while other isolates were not affected. At 37 °C five isolates of *P. manshurica* (PB 22, PB 24, PB 29, PB 210, PB 212) and one isolate of *M. pulcherrima* (BVC 40) showed high sensitivity with poor growth, while other isolates did not show remarkable effect.

Zooming at acidic tolerance, at pH 3.5, all tested isolates showed good aptitude to grow. In details, *S. cerevisiae* (BVR 101), *H. uvarum* (BVC 4, BVC 6), *M. pulcherrima* (BVC 10), *P. manshurica* (PB 23, PB 24, PB 29, PB 210, PB 212) and *C. californica* (BV 12, BV14) showed the highest growth.

Focusing on osmotic stress (300 g/L of glucose), high growth variability was found, from partial inhibition to strong growth. In detail, strong cell growth was observed for *S. cerevisiae*, *St. bacillaris*, *P. manshurica* and *C. californica* isolates, while most *H. uvarum*, *H. opuntiae*, *P. terricola* and *M. pulcherrima* exhibited slow growth, and in some cases growth inhibition.

3.4. Technological traits

Enzymes play an important role on the aroma profile, structure, and on the overall quality of wine, therefore any selection procedure must take into account beta-glucosidase, protease, and esterase activities as well as the aptitude to produce compounds, as H_2S and acetic acid, that may adversely affect wine. In the present study, results regarding enzyme activity are shown in Fig. 2, panel d.

In details, the esterase activity was found with the highest frequency within the isolates, with 41% (39/95) of them showing precipitate halo around colonies. In details, among the non-*Saccharomyces*, 60% (24/40) showed esterase activity and *M. pulcherrima* was the species that exhibited the highest overall enzymatic activity in 6 out of 7 isolates (BVC 10, BVC 11, BVC 14, BVC 15, BVC 37, BVR 3); esterase activity was also detected in *H. uvarum* isolates (BVC 6.1, BVC 32, BVC 1, BVC 2, BVC 3.1, BVC 4, BVC 5, BVC 6, BVC 7, BVC 8, BVC 21, BVC 26, BVC 28 and BVC 29), *H. opuntiae*, (BVC 31), P. *kudriavzeii* (BVC 27) and *P. terricola* (BVC 22 and BVC 25). Furthermore, enzymatic activity was absent in *P. manshurica*, *C. californica*, and *St. bacillaris*. Among *S. cerevisiae* isolates, the 27.3% (15/55) showed enzymatic activity.

Protease activity was significantly detected in the 40% (38/95) of isolates; in details, within non-*Saccharomyces*, 57.5% (23/40) of isolates showed protease activity, with the strongest activity found in *M. pulcherrima* (BVC 10, BVC 11, BVC 14, BVC 15, BVC 37, BVR 3) followed by *H. uvarum* (BVC 6.1, BVC 32, BVC 1, BVC 2, BVC 3.1, BVC 4, BVC 5, BVC 6, BVC 7, BVC 8, BVC 21, BVC 28 and BVC 29) and by *H. opuntiae* (BVC 31), *P. kudriavzeii* (BVC 27) and P. *terricola* (BVC 22 and BVC 25), while it was absent in *P manshurica*, *C. californica* and *St. bacillaris*. Among the *S. cerevisiae* isolates the 27.3% (15/55) exhibited proteolytic activity.

Beta-glucosidase activity was detected in 12.4% of isolates, specifically among the 30% (12/40) of non-*Saccharomyces* and the 9.1% (5/55) of *S. cerevisiae*. Among non-*Saccharomyces, H. uvarum* (BVC 6.1, BVC 32, GA112), *H. opuntiae* (BVC 31), and *M. pulcherrima* (BVC 11, BVC 15,

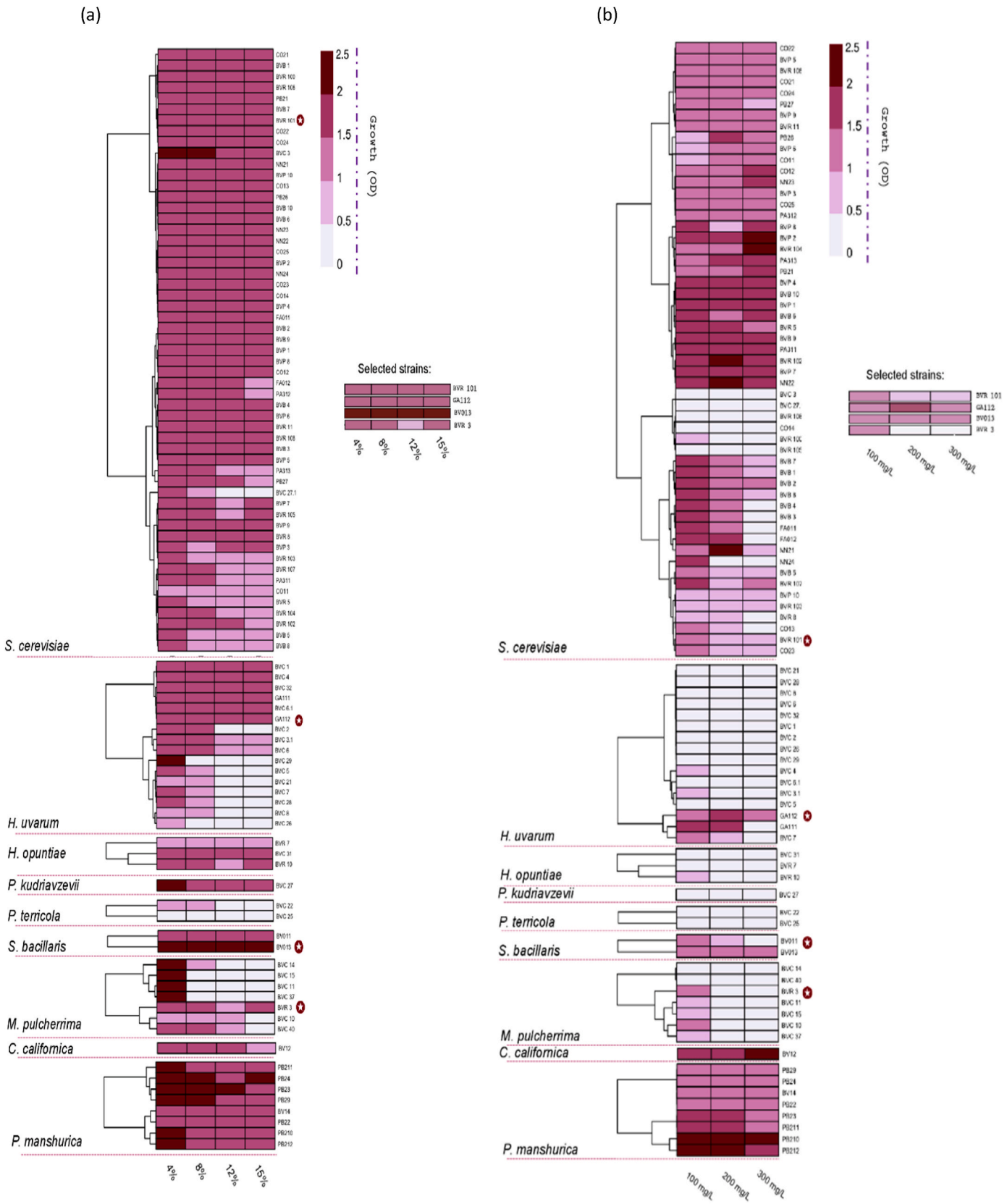

Fig. 2. Heatmaps and clustering per species of isolates generated by growth at different ethanol (a) and SO_2 concentrations (b); growth at different temperatures, low pH and osmotic stresses (c); enzyme production (d).

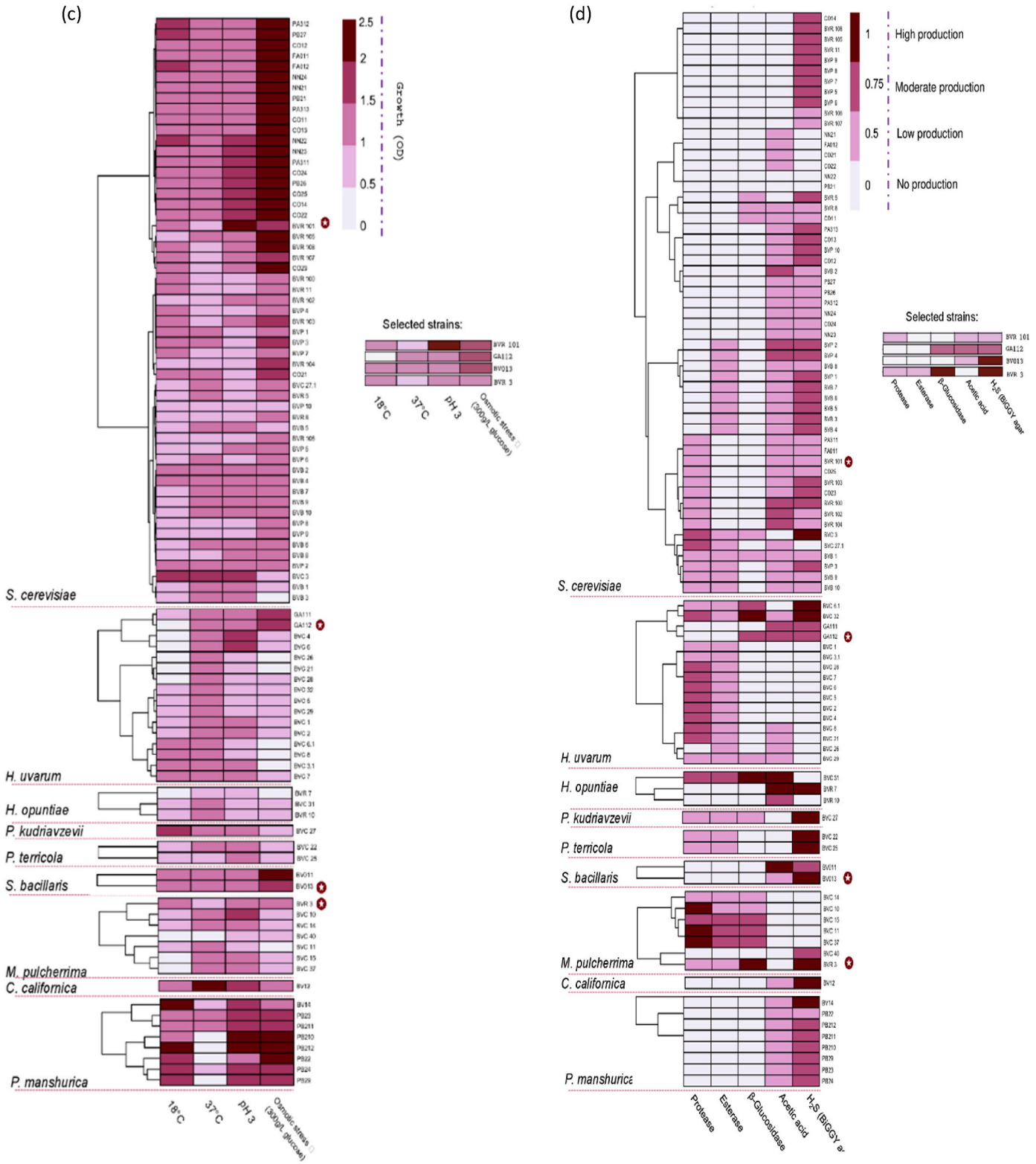

Fig. 2. (*continued*).

BVC 37, BVR 3) showed the highest beta-glucosidase activity.

Ability to produce organic acids was detected in the 64.2% of the isolates, with a lower frequency (43.7%) within *H. uvarum,* whereas it was completely absent in *M. pulcherrima*.

Regarding H_2S production, most of the samples (39%) showed a dark hazel colony, followed by hazel (23.1%) and black (10.5%). The 27.4% of the isolates showed no H_2S production.

A Principal Component Analysis (PCA) was performed to better explain the phenotypic variation profile based on the data generated from the screening approach, as illustrated in Fig. S2 (supplementary). The results show the separation of the 95 isolates (Fig. S2a) and the 15 phenotypic variables (Fig. S2b). The first two principal components (Dim1 and Dim2) explained 53.5% of the total variance, with Dim1 accounting for 41.9% and Dim2 for 12.6%. The analysis of the biplot

indicates a clear separation among the samples. The variables related to ethanol (EtOH 4%, EtOH 8%, EtOH 12%, and EtOH 15%) exhibited different correlations with the principal components. EtOH 4% was positively correlated with both Dim1 and Dim2, suggesting that resistance to low ethanol concentrations contributed significantly to the separation along these axes. In contrast, EtOH 8%, EtOH 12% and EtOH 15% showed negative correlations with Dim1, indicating that isolates positioned on the left side of the plot have higher tolerance to these higher ethanol concentrations. This separation reflects the ability of these strains - *P. manshurica*, *S. bacillaris*, *C. californica* and especially *S. cerevisiae* - to withstand elevated ethanol stress.

Enzymatic traits such as β-glucosidase, protease, and esterase were strongly positively correlated with Dim1, playing a crucial role in sample differentiation. Meanwhile, Dim2 was influenced by factors like osmotic stress, pH, and SO_2 resistance, contributing to the vertical separation of the isolates. A notable divergence was observed in two *H. uvarum* isolates (GA 112 and GA 111), which separated from the remaining isolates, including *M. pulcherrima* species, that were heterogeneously positioned on the right side of the plot, influenced by their enzymatic activity profiles.

3.5. Single and sequential fermentations

Based on the screening tests (Fig. 2) and according to literature, four indigenous isolates were chosen to carry out fermentation trials, both in single and in sequential fermentations (Table 3). Among the non-*Saccharomyces* species, *H. uvarum* GA112 (Hu) was selected for its resistance profiles and moderate production of β-glucosidase; *St. bacillaris* BV113 (Sb) was chosen for its good resistance to ethanol; *M. pulcherrima* BVR3 (Mp) was chosen for its strong beta-glucosidase activity. The indigenous *S. cerevisiae* BVR101 (Sc) was selected based on its resistance profile, particularly at acidic conditions, and for its protease activity. The commercial strain *S. cerevisiae* Lalvin QA23™ from Lallemand was used as control and its performance was compared to that of the indigenous *S. cerevisiae* strain (see Table 4).

Regarding the single fermentation trials (Fig. 3, panel a), as expected, none of the non-*Saccharomyces* species completed fermentation in synthetic must, being not able to deplete the fermentable sugars. In detail, *M. pulcherrima* was the least performing strain, stopping fermentation on the 8th day and stuck its activity when the must density was approximately 1062 g/L with a cell concentration reaching 1×10^7 CFU/mL after 48 h (Fig. 3, panel b), and then decreasing to approximately 7×10^6 CFU/mL to became undetectable (<10 CFU/mL) on WL plates approximately after 7 days.

H. uvarum presented a slightly better fermentation profile, reaching a cell concentration of about 5×10^7 CFU/mL, consuming more sugar and leading the end of fermentation after 14 days with a must density value of approximately 1024 g/L and a final cell concentration of 3×10^6 CFU/mL.

St. bacillaris consumed the largest amount of sugars although in a slower way, in fact, its activity lasted up to 22 days with a final must density value of approximately 1007 g/L and a viable cell count of almost 7×10^7 CFU/mL between the third and the fourth day, which slightly decreased at the end of fermentation (at about 2×10^7 CFU/mL). Among the two *S. cerevisiae* cultures, it is interesting to point out that the indigenous strain completed fermentation in 8 days, with a final sugar value of 0.70 g/L, while the commercial *S. cerevisiae* QA23 completed the fermentation in 9 days, with a residual sugar value of 0.60 g/L.

Sequential fermentations were carried out by inoculating the indigenous *S. cerevisiae* strain after 48 h from the inoculum of each single culture of the non-*Saccharomyces* yeast. Among the experimental trials, the sequential fermentation carried out together with *St. bacillaris* (SbSc) showed the fastest fermentation kinetic, ending the fermentation after 9 days (Fig. 4, panel a), with a final sugar concentration of 0.50 g/L. Zooming on the growth dynamics of each strain, *St. bacillaris* reached a concentration of about 10^7 and 10^8 CFU/mL after 2 and 4 days, respectively, to decline to about 10^6 CFU/mL on the 10th day; while *S. cerevisiae* reached a population of 10^7 CFU/mL after 2 days and remained quite constant until the end of fermentation (Fig. 4, panel d).

The experimental trial carried out with *M. pulcherrima* and *S. cerevisiae* (MpSc) showed a slower kinetic with the end of fermentation registered after approximately 11 days, with a residual sugar content of 0.40 g/L. Regarding the viable cell counting, *M. pulcherrima* grew up to 10^7 CFU/ml and declined after 48 h, just after the addition of *S. cerevisiae*, and reached a density of about 10^6 CFU/mL after 4 days, to become undetectable after 5 days; while *S. cerevisiae* remained stable at a concentration of 10^7 CFU/mL until the end of fermentation (Fig. 4, panel c).

Sequential fermentation trial that started with *H. uvarum* (HuSc) revealed the slowest kinetic, ending after approximately 14 days, with a residual sugars content of about 0.70 g/L, with a cell density remaining constant at about 10^7 CFU/mL until the 5th day, after which it decreased to 10^4 CFU/mL. Viable *H. uvarum* even became undetectable after 10 days after which *S. cerevisiae* maintained its concentration of about 10^7 CFU/mL until the end of fermentation (Fig. 4, panel d).

At the end of fermentations, wine samples were analysed for residual sugars (glucose and fructose), organic acids (malic, tartaric, succinic, and lactic acid) ethanol and glycerol through HPLC. None of the non-*Saccharomyces* yeast in single culture was able to end the AF, leaving high concentration of residual sugars, except *St. bacillaris* (Sb) that consumed all the fructose present in the synthetic must.

Both *S. cerevisiae* cultures, in single fermentations, were able to complete the AF consuming the glucose present in the synthetic must and leaving fructose, below 1.69 g/L; on the other hand, in sequential fermentations, the final fructose concentration was below 0.7 g/L.

Focusing on malic acid, its concentration was lower in sequential fermentations HuSc and MpSc than in SbSc.

Glycerol levels were significantly higher in fermentations with *St. bacillaris* in both single and sequential fermentations, with a concentration of about 10 and 8 g/L, respectively; HuSc also reported a significant increase in glycerol content, where it was detected at a concentration higher than 1 g/L compared to the control. Furthermore, the concentration of acetic acid in all samples remained within the established limits (International Organisation of Vine and Wine (OIV), accessed on July 15, 2024) with the lower value registered in the trial fermented with *M. pulcherrima* in single fermentation.

In addition, no significant differences among the fermentation trials were observed regarding the final ethanol concentration.

Table 3
Indigenous isolates used for fermentation trials. All isolates were obtained from spontaneous fermenting grape musts.

Yeast isolates (identification code)	Sampling	Grape variety	Abbreviation used in this study
Saccharomyces cerevisiae (BVR101)	End of fermentation	Nerello Mascalese	Sc
Hanseniaspora uvarum (GA112)	End of fermentation	Nerello Mascalese	Hu
Starmerella bacillaris (BV13)	End of fermentation	Carricante	Sb
Metschnikowia pulcherrima (BVR3)	Mid-fermentation	Nerello Mascalese	Mp
Saccharomyces cerevisiae (QA23)	Commercial starter		QA23

4. Discussion

Several studies have already demonstrated the efficacy of native yeasts, in mixed or sequential fermentations with *S. cerevisiae*, in enhancing both the complexity and the sensory profile of wines (Escribano-Viana et al., 2018; Fazio et al., 2023; Lee & Park, 2020;

Table 4
Compounds of oenological interest (g/L) detected by HPLC in wines.

Strain	Species	Malic acid (g/L)	Citric acid (g/L)	Tartaric acid (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (% v/v)
QA23	*S. cerevisiae* (QA23)	1.87 ± 0.06^{b}	0.36 ± 0.02^{b}	1.85 ± 0.08^{c}	0.23 ± 0.03^{c}	0.19 ± 0.06$^{a, b}$	0.88 ± 0.04^{Ns}	Nd	1.00 ± 0.14$^{c, d}$	5.20 ± 0.25^{d}	12.51 ± 0.60^{a}
Bvr101	*S. cerevisiae* (Sc)	2.14 ± 0.01^{a}	0.51 ± 0.00^{b}	2.03 ± 0.01^{c}	0.34 ± 0.00$^{b, c}$	0.18 ± 0.02$^{a, b}$	0.77 ± 0.16 N	Nd	1.69 ± 0.03^{c}	5.46 ± 0.12^{d}	12.83 ± 0.18^{a}
Ga112	*H. uvarum* (Hu)	2.29 ± 0.05^{a}	0.30 ± 0.01^{b}	3.77 ± 0.14^{a}	0.36 ± 0.00$^{b, c}$	Nd	0.70 ± 0.07 N	42.51 ± 0.4^{c}	36.92 ± 0.11^{b}	5.22 ± 0.00^{d}	7.77 ± 0.12^{c}
Bv13	*St. bacillaris* (Sb)	0.90 ± 0.09^{d}	1.16 ± 0.19$^{a, b}$	4.34 ± 0.37^{a}	0.23 ± 0.05^{c}	Nd	0.70 ± 0.00 N	57.33 ± 6.14^{b}	Nd	10.43 ± 0.22^{a}	10.04 ± 0.72^{b}
Bvr3	*M. pulcherrima* (Mp)	1.21 ± 0.00^{c}	0.95 ± 0.08$^{a, b}$	2.97 ± 0.010^{b}	Nd	Nd	0.06 ± 0.03 N	88.85 ± 1.51^{a}	82.65 ± 0.9^{a}	1.63 ± 0.25^{e}	1.42 ± 0.18^{d}
Ga112/ Bvr101	*H. uvarum/S. cerevisiae* (HuSc)	1.10 ± 0.01^{c}	0.66 ± 0.33$^{a, b}$	2.15 ± 0.11^{c}	0.45 ± 0.05^{b}	0.13 ± 0.01^{b}	0.50 ± 0.05 N	Nd	0.71 ± 0.19$^{c, d}$	6.51 ± 0.20^{c}	12.60 ± 0.06^{a}
Bv13/ Bvr101	*St. bacillaris/S. cerevisiae* (SbSc)	1.17 ± 0.01^{c}	1.70 ± 0.73^{a}	2.30 ± 0.03^{c}	0.36 ± 0.03$^{b, c}$	0.12 ± 0.00^{b}	0.50 ± 0.01 N	Nd	0.41 ± 0.01$^{c, d}$	8.10 ± 0.13^{b}	12.55 ± 0.15^{a}
Bvr3/ Bvr101	*M. pulcherrima/S. cerevisiae* (MpSc)	1.05 ± 0.01$^{c, d}$	0,42 ± 0.02^{b}	1.98 ± 0.03^{c}	0.67 ± 0.07^{a}	0.23 ± 0.02^{a}	0.53 ± 0.05 N	Nd	0.55 ± 0.07$^{c, d}$	5.23 ± 0.15^{d}	12.23 ± 0.16^{a}

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different at $p \leq 0.05$ according to a Tukey post-hoc comparison test; Nd: not detected. ^{Ns}: Not significant.

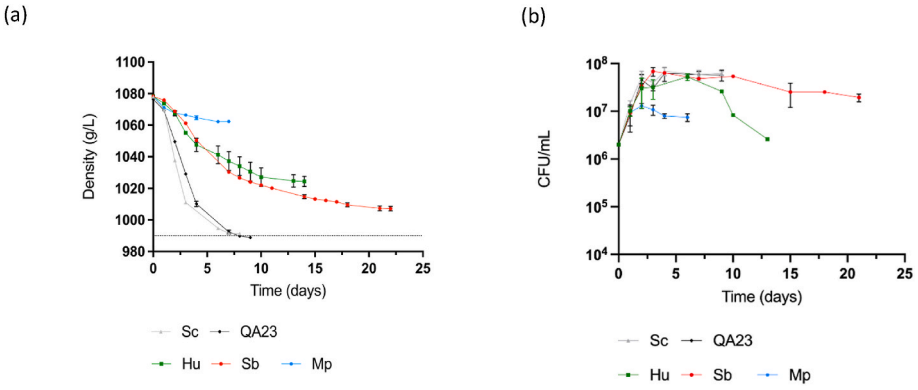

Fig. 3. Kinetics of alcoholic fermentations: evolution of density (a) and yeast viable population (b) in single fermentations, using the single fermentation with *S. cerevisiae* QA23 as a control.

Padilla et al., 2017; Tristezza et al., 2016). Furthermore, a growing interest in exploiting indigenous starters has been registered, taking into account that the geographical origin of fermenting yeasts correlates specifically with the volatile profile of resulting wines (Tufariello et al., 2014).

In the present study, 95 indigenous yeasts were isolated and identified as belonging to 6 genera (*Pichia*, *Candida*, *Hanseniaspora*, *Metschnikowia*, *Starmerella*, and *Saccharomyces*). As known, and recently reviewed by Albergaria et al. (2016), most of the yeasts isolated from grapes belong to apiculate group, mainly to *H. uvarum* species, while yeasts isolated from musts belong to *S. cerevisiae* species. However, non-*Saccharomyces* were also isolated from fermenting musts, showing good resistance profiles and, among them, *H. uvarum*, *P. manshurica*, and *M. pulcherrima* were the most detected species, according to previous reports (Brysch-Herzberg & Seidel, 2015; Feng et al., 2020; Zabukovec et al., 2020). Substantial number of isolates belonging to *P. manshurica* species, known as spoilage yeast able to colonize winery environments (Perpetuini et al., 2020) was found also in must samples.

In order to select the most promising yeasts to be used for winemaking purposes, preliminary trials were performed, testing in particular their capacity to thrive under challenging conditions.

The ethanol can be challenging for many species since it causes loss of yeast cell integrity and permeability (Navarro-Tapia et al., 2018) and, as expected, *S. cerevisiae* isolates were the most ethanol-tolerant yeasts able to grow significantly at 15% of ethanol. Among non-*Saccharomyces* species, differences were found also within the same species. *M. pulcherrima* generally showed the poorest tolerance to ethanol, confirming the findings of Binati et al. (2019) and the statement of Borren & Tian (2021), although few of them could easily survive at 15% of ethanol. Variability within *H. uvarum* was also detected, as some isolates showed tolerance at ethanol concentrations of 12 or 15%, in discordance with results reported by Mančić et al. (2022) who reported that ethanol concentrations up to 5% could reduce the growth of *H. uvarum* isolates by nearly 60%. Similar results were observed for *St. bacillaris,* which tolerated about 12%–15% of ethanol, slightly higher than value mentioned by Aponte and Blaiotta (2016) and Borren & Tian (2021) which indicated tolerance up to 10% of ethanol. Regarding the high tolerance of *P. manshurica* to ethanol, comparable to that of *S. cerevisiae*, our results are in line with those reported by Ruiz-Muñoz et al. (2022).

Sulfur dioxide is commonly used in winemaking to prevent both oxidation and growth of spoilage microorganisms, including lactic acid bacteria and undesirable non-*Saccharomyces* yeasts (Ribéreau-Gayon et al., 2005). In the present study, the non-*Saccharomyces* isolates were confirmed sensitive to sulfites (Morata et al., 2019), except for *P. manshurica* which showed strong resistance, in contrast to results reported by Perpetuini et al. (2018). Furthermore, variability among *S. cerevisiae* were detected, confirming that the resistance to SO_2 is a strain-dependent trait, as previously proposed (Divol et al., 2012).

It is known that temperatures higher than 30 °C can occur in the must during AF, with an effect on yeast viability and on production of undesirable compounds (Valentine et al., 2019). Results of the present study showed that at 37 °C only *P. manshurica* was negatively affected,

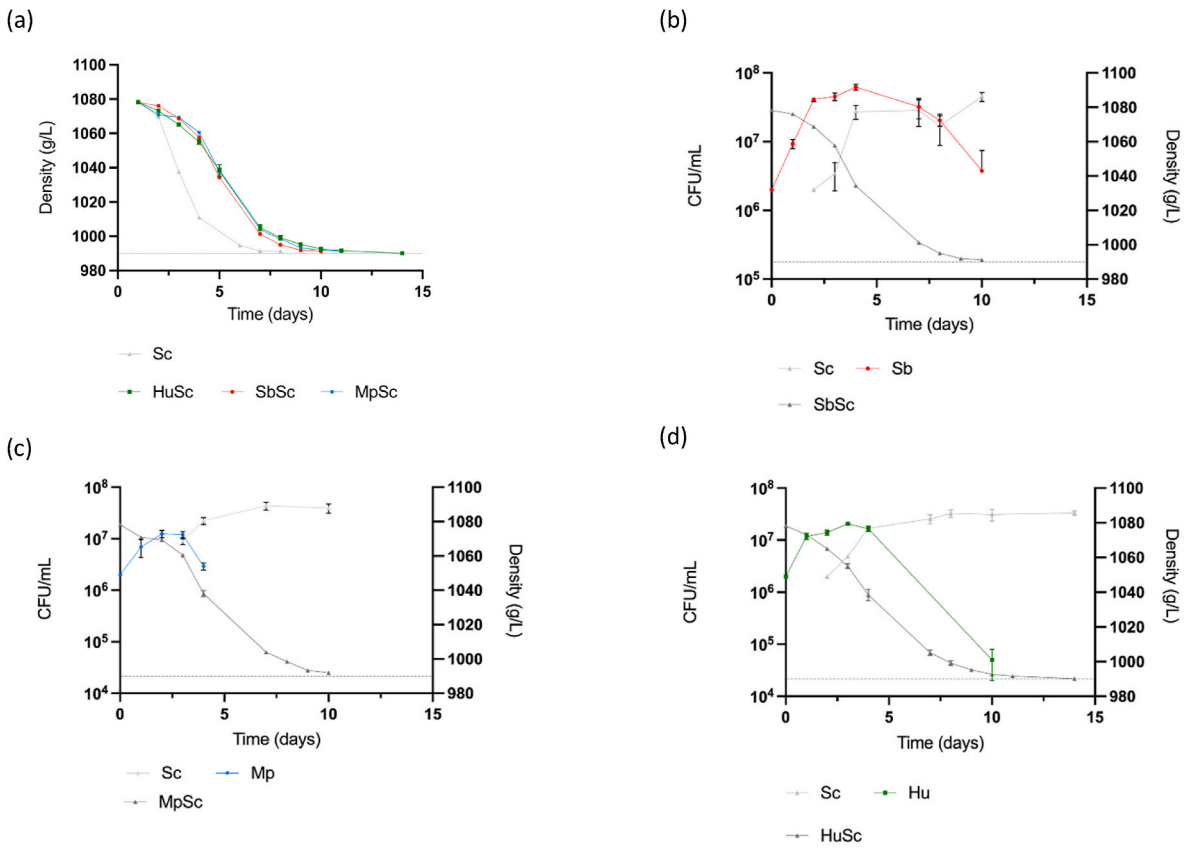

Fig. 4. Evolution of yeast density in sequential fermentations (a) carried out with one of the three non-*Saccharomyces* strains and the indigenous *S. cerevisiae* strain BVR10, using the single fermentation of this indigenous *S. cerevisiae* as a control.

thus refuting the previous findings of Pilap and co-workers (2022) which demonstrated that the species could grow up to 40 °C and performed poorly at 45 °C, suggesting that it is thermotolerant. Nevertheless, the other isolates were able to grow at 37 °C, despite results of Mateus and co-workers (2020) that reported the poor ability of *M. pulcherrima* to grow at high temperatures.

Non-*Saccharomyces* species can be used for fermentation at low temperatures since when inoculated during winemaking they release higher concentration of esters (García-Ríos et al., 2017; Samoticha et al., 2019). *H. uvarum* has been previously considered as thermotolerant able to grow at a wide range of temperatures (Mančić et al., 2022); however, in the present study results showed sluggish growth at 18 °C for few isolates.

To meet consumer demand, modern winemaking often uses commercial enzymes during fermentation to improve technological properties (such as adjustment of viscosity and turbidity), stabilize color, and enhance the aroma profile by producing volatile compounds. This also has positive economic implications through reduction of costs (Espejo, 2020). Yeasts of oenological interest can naturally produce different enzymes that might increase the overall quality of wine (Vejarano, 2020).

Beta-glucosidase has been widely recognized as a key enzyme for wine's aromatic complexity. The release of aromatic compounds is due to the hydrolyzes different types of glycosidic bonds (1,4-β and 1,6-α linkages) promoting, during the AF, the formation of free terpenes, phenylpropenes and other aliphatic esters (Gao et al., 2022; Zhang et al., 2021). For example, it was already stated how beta-glucosidase from *M. pulcherrima* can boost the production of esters, and fatty acids, improving fruity and floral flavors in wine (Zhang, Li, et al., 2020). Our results were consistently in line with those reported by other authors, finding a higher beta-glucosidase activity in *M. pulcherrima* (Aponte & Blaiotta, 2016; Binati et al., 2019; Comitini et al., 2011; Grazia et al., 2019; Ianieva & Podgorsky, 2021; Sidari et al., 2021) than in *S. cerevisiae* (Spagna et al., 2002*)*. Furthermore, our results on *H. uvarum* confirmed previously studies about the variability within the same species (Arevalo Villena et al., 2005; Capece et al., 2005; Ganga & Martinez, 2004), since 3 isolates out of 16 showed moderate activity.

The use of proteases enzymes, which can hydrolyze proteins into amino acids, can help reduce haze formation in wine, thereby aiding in its clarification (Maicas & Mateo, 2015; Espejo, 2020). Traditionally, winemaking has employed bentonite for clarification, but this method can lead to quality loss and environmental concerns (Cosme et al., 2020). In addition, it has also been reported that increasing levels of protease produced by yeast can enhance the concentration of ethyl esters and fatty acids, contributing to improved aroma and flavor profiles in wine (Maturano et al., 2015). In the present study a consistent number of isolates, mainly belonging to *M. pulcherrima* and *H. uvarum,* presents protease activity, in accordance with previous results (Binati et al., 2019; Escribano et al., 2017; Sidari et al., 2021) and in contrast with other results (Belda et al., 2016; Charoenchai et al., 2008; Comitini et al., 2011), which found no proteolytic activity in *M. pulcherrima* and *S. cerevisiae*. Furthermore, although the effect of *C. californica* in winemaking is still unclear, in our study all *C. californica* isolates showed all tested enzymatic activities, confirming the statement of Hesham and co-workers (2017), who proposed *C. californica* as a multi-starter inoculum together with *S. cerevisiae* to enrich aromatic complex of wine (Aplin et al., 2019).

Extracellular yeast esterases facilitate the breakdown of short, medium, and long-chain aliphatic esters into alcohols and acids. These byproducts play a role in improving the mouthfeel and aroma of fruit wines by interacting synergistically with other aromatic compounds. In detail, it was found that *IAH1*(isoamyl acetate hydrolyzing esterase 1) was the gene involved in coding this enzyme, which promote the high production of acetate esters (Borren & Tian, 2020). It is well-established

that extracellular esterases occur in *S. cerevisiae* (Iranzo et al., 1998), and our results partially confirm this, as only few isolates showed remarkable activity. Unlike previous reports (López et al., 2015), most of the *H. uvarum* isolates analysed in this study, showed moderate esterase production. This variation suggests that enzymatic activities can differ depending on different geographical areas. Similar results were found for *M. pulcherrima*, with isolates exhibiting strong esterase activity, as previously reported (Binati et al., 2019; Escribano-Viana et al., 2021; Ianieva & Podgorsky, 2021; Kręgiel et al., 2022; Sidari et al., 2021). Esterase was also observed for *P. kudriavzevii,* supporting the studies of Xiao and co-workers (2023), which revealed a higher production of this enzyme 3.5 times greater than that of *S. cerevisiae*.

Hydrogen sulfide negatively impacts sensory traits of wine due to the typical rotten eggs scent and in the present study both an inter- and intra-species variability was observed; specifically, *S. cerevisiae* showed the highest percentage of H_2S-producing isolates, whereas, within *H. uvarum* and *M. pulcherrima*, only few isolates present such an activity, partially confirming previous studies that found low/absent sulfite reductase activity within these species (Belda et al., 2016; Polizzotto et al., 2016).

Recent winemaking techniques involve multi-starter fermentation with non-*Saccharomyces* and *S. cerevisiae* yeasts. In detail, non-*Saccharomyces* strains are firstly added into the musts to start and perform the AF and *S. cerevisiae* strains are sequentially inoculated to complete the AF. Based on data reported in the literature and on the preliminary screening, as the presence of enzymes of enological interest, and the resistance to different stresses, four isolates were selected to perform laboratory scale fermentation trials.

None of the non-*Saccharomyces* was able to complete the AF, leaving residual carbon sources at the end of fermentation and confirming the poor fermentation ability of *H. uvarum* and *M. pulcherrima* (Mančić et al., 2022; Su et al., 2020). The detection of sugars confirmed the fructophilic behavior of *St. bacillaris* (Raymond Eder & Rosa, 2021). In contrast to the other non-*Saccharomyces*, *St. bacillaris* showed the highest sugar consumption, and in turn, a high percentage of ethanol. In addition, as previously reported by Rantsiou and co-workers (2017), during the AF its population remains at a cellular density of closer to 10^8 CFU/mL for 23–24 days, after when it became undetectable.

Moving on sequential fermentation trials, *St. bacillaris* showed good growth ability as it was able to maintain a high viable population (10^6 CFU/mL) together with *S. cerevisiae* until the end of fermentation. Such a co-existence of both species is attributed to a low competition for carbon source, as *St. bacillaris* prefers fructose and *S. cerevisiae* glucose (Englezos et al., 2019).

In wine the presence of glycerol, at a threshold of about 5.2 g/L, positively impacts the structure and the quality of final product, since improves its mouthfeel perception giving persistence and smoothness (Lubbers et al., 2001; Noble & Bursick, 1984). It has been observed how fermentations driven by non-*Saccharomyces* yeasts lead to a higher content of glycerol, compared to fermentation carried out by *S. cerevisiae* in single culture (Ivit et al., 2020). Accordingly, in the present study, the fermentation trials using *St. bacillaris*, both in single and in sequential fermentations, showed the highest amount of glycerol, 10.43 g/L and 8.10 g/L, respectively, confirming several previous studies (Lemos Junior et al., 2019; Li et al., 2023; Mestre Furlani et al., 2017; Nadai et al., 2021; Rantsiou et al., 2017). However, as already reported (Englezos et al., 2016, 2017), the glycerol production is related to several factors, as concentration of sugars in the grape must, and temperatures (Goold et al., 2017).

As already reported, the ideal concentration of acetic acid is between 0.2 and 0.7 g/L, being concentrations higher than 1.1 g/L considered detrimental (Padilla et al., 2016). However, it is important to emphasize that in synthetic must, the acetic acid content may be higher than that observed in natural must (Llauradó et al., 2002; Torija et al., 2003).

Our findings on volatile acidity seems to be not affected by *St. bacillaris*, as previously reported (Lencioniet al., 2018; Rantsiou et al., 2012) who suggested a decrease in acetic acid contents using *St. bacillaris*, and are in contrast with results reported by Moreira and co-workers (2022) who described an higher acetic acid production using *St. bacillaris* instead of *S. cerevisiae*.

In addition, recent studies (Hranilovic et al., 2020; Perpetuini et al., 2023) showed that when *M. pulcherrima* was used in sequential fermentation a higher decrease in acetic acid concentration than in single fermentation was detected. This result has been related to the competition for oxygen by the two species, that, in turn, affects the activity of pyruvate dehydrogenase (PDH) that regulates acetic acid production (Sadoudi et al., 2017; Zhu et al., 2020).

5. Conclusion

This study represents an interesting starting point for the selection of indigenous *S. cerevisiae* and non-*Saccharomyces* yeasts isolated from peculiar areas of Sicily and around Etna, a poorly investigated region. Molecular screening by RFLP-PCR of 5.8S-ITS rDNA allowed to distinguish different species isolated from spontaneous fermenting musts and fresh grape samples. The different levels of stress tolerance, the presence of interesting enzyme activities (beta-glucosidases, esterase, and proteases) confirmed the promising applicability of these isolates as starters cultures in specific winemaking processes.

Results of fermentation trials in synthetic must highlighted the potential use of non-*Saccharomyces* yeasts confirming that *St. bacillaris* showed a significant glycerol production, critical for both the structure and the aromatic profile of wines. Furthermore, fermentations performed with *H. uvarum* and *M. pulcherrima* resulted in low total acidity levels.

Further studies could be carried out using pasteurized or nonsterile local Sicilian musts to understand the behavior and the effect of these indigenous yeasts on the final aromatic profile of wine. Different combinations could be further investigated, through simultaneous co-inoculations with different commercial and indigenous *S. cerevisiae* strains, choosing key parameters for selection as large-scale starters.

CRediT authorship contribution statement

Nunzio Alberto Fazio: Writing – original draft, Investigation, Conceptualization. **Alessandra Pino:** Visualization, Investigation, Data curation. **Paola Foti:** Visualization, Investigation, Data curation. **Braulio Esteve-Zarzoso:** Writing – original draft, Investigation, Data curation. **Cinzia L. Randazzo:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **María-Jesús Torija:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Cinzia Caggia:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

Funding

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).

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.105282.

Data availability

Data will be made available on request.

References

Aplin, J. J., White, K. P., & Edwards, C. G. (2019). Growth and metabolism of non-*Saccharomyces* yeasts isolated from Washington state vineyards in media and high sugar grape musts. *Food Microbiology, 77*, 158–165. https://doi.org/10.1016/j.fm.2018.09.004

Aponte, M., & Blaiotta, G. (2016). Potential role of yeast strains isolated from grapes in the production of taurasi DOCG. *Frontiers in Microbiology, 7*. https://doi.org/10.3389/fmicb.2016.00809

Arevalo Villena, M., Ubeda Iranzo, J. F., Cordero Otero, R. R., & Briones Perez, A. I. (2005). Optimization of a rapid method for studying the cellular location of beta-glucosidase activity in wine yeasts. *Journal of Applied Microbiology, 99*, 558–564. https://doi.org/10.1111/j.1365-2672.2005.02627.x

Balmaseda, A., Rozès, N., Leal, M.Á., Bordons, A., & Reguant, C. (2021). Impact of changes in wine composition produced by non-*Saccharomyces* on malolactic fermentation. *International Journal of Food Microbiology, 337*, Article 108954. https://doi.org/10.1016/j.ijfoodmicro.2020.108954

Belda, I., Ruiz, J., Alastruey-Izquierdo, A., Navascués, E., Marquina, D., & Santos, A. (2016). Unraveling the enzymatic basis of wine "flavorome": A phylo-functional study of wine related yeast species. *Frontiers in Microbiology, 7*. https://doi.org/10.3389/fmicb.2016.00012

Bellia, C., Columba, P., & Ingrassia, M. (2022). The brand–land identity of Etna volcano valley wines: A policy delphi study. *Agriculture, 12*(6), 811. https://doi.org/10.3390/agriculture12060811

Beltran, G., Novo, M., Rozes, N., Mas, A., & Guillamon, J. (2004). Nitrogen catabolite repression in during wine fermentations. *FEMS Yeast Research, 4*(6), 625–632. https://doi.org/10.1016/j.femsyr.2003.12.004

Binati, R. L., Innocente, G., Gatto, V., Celebrin, A., Polo, M., Felis, G. E., & Torriani, S. (2019). Exploring the diversity of a collection of native non-*Saccharomyces* yeasts to develop co-starter cultures for winemaking. *Food Research International, 122*, 432–442. https://doi.org/10.1016/j.foodres.2019.04.043

Borren, E., & Tian, B. (2020). The important contribution of non-*Saccharomyces* yeasts to the aroma complexity of wine: A review. *Foods, 10*, 13. https://doi.org/10.3390/foods10010013

Brysch-Herzberg, M., & Seidel, M. (2015). Yeast diversity on grapes in two German wine growing regions. *International Journal of Food Microbiology, 214*, 137–144. https://doi.org/10.1016/j.ijfoodmicro.2015.07.034

Buzzini, P., & Martini, A. (2002). Extracellular enzymatic activity profiles in yeast and yeast-like strains isolated from tropical environments. *Journal of Applied Microbiology, 93*, 1020–1025. https://doi.org/10.1046/j.1365-2672.2002.01783.x

Capece, A., Fiore, C., Maraz, A., & Romano, P. (2005). Molecular and technological approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine origin. *Journal of Applied Microbiology, 98*, 136–144. https://doi.org/10.1111/j.1365-2672.2004.02434.x

Charoenchai, C., Fleet, G. H., Henschke, P. A., & Todd, B. E. N. T. (2008). Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. *Australian Journal of Grape and Wine Research, 3*(1), 2–8. https://doi.org/10.1111/j.1755-0238.1997.tb00109.x

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., & Ciani, M. (2011). Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces* cerevisiae. *Food Microbiology, 28*, 873–882. https://doi.org/10.1016/j.fm.2010.12.001

Cosme, F., Fernandes, C., Ribeiro, T., Filipe-Ribeiro, L., & Nunes, F. M. (2020). White wine protein instability: Mechanism, quality control and technological alternatives for wine stabilisation—an overview. *Beverages, 6*(1), 19. https://doi.org/10.3390/beverages6010019

Di Bella, A., Petino, G., & Scrofani, L. (2019). The Etna macro-region between peripheralization and innovation: Towards a smart territorial system based on tourism. *Regional Science Policy & Practice, 11*, 493–508. https://doi.org/10.1111/rsp3.12176

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

Divol, B., du Toit, M., & Duckitt, E. (2012). Surviving in the presence of sulphur dioxide: Strategies developed by wine yeasts. *Applied Microbiology and Biotechnology, 95*, 601–613. https://doi.org/10.1007/s00253-012-4186-x

Englezos, V., Giacosa, S., Rantsiou, K., Rolle, L., & Cocolin, L. (2017). *Starmerella bacillaris* in winemaking: Opportunities and risks. *Current Opinion in Food Science, 17*, 30–35. https://doi.org/10.1016/j.cofs.2017.08.007

Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Ortiz-Julien, A., Gerbi, V., Rolle, L., & Cocolin, L. (2016). *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Applied Microbiology and Biotechnology, 100*, 5515–5526. https://doi.org/10.1007/s00253-016-7413-z

Englezos, V., Rantsiou, K., Giacosa, S., Río Segade, S., Rolle, L., & Cocolin, L. (2019). Cell-to-cell contact mechanism modulates *Starmerella bacillaris* death in mixed culture fermentations with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology, 289*, 106–114. https://doi.org/10.1016/j.ijfoodmicro.2018.09.009

Escribano, R., González-Arenzana, L., Garijo, P., Berlanas, C., López-Alfaro, I., López, R., Gutiérrez, A. R., & Santamaría, P. (2017). Screening of enzymatic activities within different enological non-*Saccharomyces* yeasts. *Journal of Food Science and Technology, 54*, 1555–1564. https://doi.org/10.1007/s13197-017-2587-7

Escribano-Viana, R., González-Arenzana, L., Garijo, P., López, R., Santamaría, P., & Gutiérrez, A. R. (2021). Selection process of a mixed inoculum of non-*Saccharomyces* yeasts isolated in the D.O.Ca. Rioja. *Fermentation, 7*, 148. https://doi.org/10.3390/fermentation7030148

Escribano-Viana, R., González-Arenzana, L., Portu, J., Garijo, P., López-Alfaro, I., López, R., Santamaría, P., & Gutiérrez, A. R. (2018). Wine aroma evolution throughout alcoholic fermentation sequentially inoculated with non- *Saccharomyces*/*Saccharomyces* yeasts. *Food Research International, 112*, 17–24. https://doi.org/10.1016/j.foodres.2018.06.018

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

Feng, L., Wang, J., Ye, D., Song, Y., Qin, Y., & Liu, Y. (2020). Yeast population dynamics during spontaneous fermentation of icewine and selection of indigenous *Saccharomyces cerevisiae* strains for the winemaking in Qilian, China. *Journal of the Science of Food and Agriculture, 100*, 5385–5394. https://doi.org/10.1002/jsfa.10588

Ganga, M. A., & Martinez, C. (2004). Effect of wine yeast monoculture practice on the biodiversity of non-*Saccharomyces* yeasts. *Journal of Applied Microbiology, 96*, 76–83. https://doi.org/10.1046/j.1365-2672.2003.02080.x

Gao, P., Peng, S., Sam, F. E., Zhu, Y., Liang, L., Li, M., & Wang, J. (2022). Indigenous non-*Saccharomyces* yeasts with β-glucosidase activity in sequential fermentation with *Saccharomyces cerevisiae*: A strategy to improve the volatile composition and sensory characteristics of wines. *Frontiers in Microbiology, 13*. https://doi.org/10.3389/fmicb.2022.845837

García, M., Esteve-Zarzoso, B., Cabellos, J. M., & Arroyo, T. (2020). Sequential non-*Saccharomyces* and *Saccharomyces cerevisiae* fermentations to reduce the alcohol content in wine. *Fermentation, 6*, 60. https://doi.org/10.3390/fermentation6020060

García-Ríos, E., Morard, M., Parts, L., Liti, G., & Guillamón, J. M. (2017). The genetic architecture of low-temperature adaptation in the wine yeast *Saccharomyces cerevisiae*. *BMC Genomics, 18*, 159. https://doi.org/10.1186/s12864-017-3572-2

Gonzalez, R., & Morales, P. (2022). Truth in wine yeast. *Microbial Biotechnology, 15*, 1339–1356. https://doi.org/10.1111/1751-7915.13848

Goold, H. D., Kroukamp, H., Williams, T. C., Paulsen, I. T., Varela, C., & Pretorius, I. S. (2017). Yeast's balancing act between ethanol and glycerol production in low-alcohol wines. *Microbial Biotechnology, 10*, 264–278. https://doi.org/10.1111/1751-7915.12488

Grazia, A., Pietrafesa, A., Capece, A., Pietrafesa, R., Siesto, G., & Romano, P. (2019). Exploitation of technological variability among wild non- *Saccharomyces* yeasts to select mixed starters for the production of low alcohol wines. *BIO Web of Conferences, 15*, Article 02031. https://doi.org/10.1051/bioconf/20191502031

Hranilovic, A., Gambetta, J. M., Jeffery, D. W., Grbin, P. R., & Jiranek, V. (2020). Lower-alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co-fermentations: The effect of sequential inoculation timing. *International Journal of Food Microbiology, 329*, Article 108651. https://doi.org/10.1016/j.ijfoodmicro.2020.108651

Ianieva, O., & Podgorsky, V. (2021). Enological potential of non- *Saccharomyces* yeast strains of enological and brewery origin from Ukrainian Collection of Microorganisms. *Mycology, 12*, 203–215. https://doi.org/10.1080/21501203.2020.1837272

Iranzo, J. U., Perez, A. B., & Canas, P. I. (1998). Study of the oenological characteristics and enzymatic activities of wine yeasts. *Food microbiology, 15*, 399–406. https://doi.org/10.1006/fmic.1998.0183

Istituto Nazionale di Statistica (ISTAT). Average monthly household expenditure by type of goods and services. http://dati.istat.it/Index.aspx?QueryId=33706&lang=en#. (Accessed 15 July 2024).

Ivit, N. N., Longo, R., & Kemp, B. (2020). The effect of non-*Saccharomyces* and *Saccharomyces* non-*cerevisiae* yeasts on ethanol and glycerol levels in wine. *Fermentation, 6*, 3. https://doi.org/10.3390/FERMENTATION6030077. MDPI AG.

Kolde, R. (2019). Pheatmap: Pretty heatmaps. *R package version 1.0.10*. Https://CRAN.R-Project.Org/Package=pheatmap (2012).

Kręgiel, D., Pawlikowska, E., Antolak, H., Dziekońska-Kubczak, U., & Pielech-Przybylska, K. (2022). Exploring use of the *Metschnikowia pulcherrima* clade to improve properties of fruit wines. *Fermentation, 8*, 247. https://doi.org/10.3390/fermentation8060247

Kurtzman, C., Fell, J. W., & Boekhout, T. (2011). In *The yeasts: A taxonomic study*. San Diego (USA): Elsevier.

Lê, S., Josse, J., & Husson, F. (2008). FactoMineR: A package for multivariate analysis. *Journal of Statistical Software, 25*, 1–18. https://doi:10.18637/jss.v025.i01.

López, S., Mateo, J., & Maicas, S. (2015). Screening of *Hanseniaspora* strains for the production of enzymes with potential interest for winemaking. *Fermentation, 2*, 1. https://doi.org/10.3390/fermentation2010001

Lai, Y.-T., Hsieh, C.-W., Lo, Y.-C., Liou, B.-K., Lin, H.-W., Hou, C.-Y., & Cheng, K.-C. (2022). Isolation and identification of aroma-producing non-*Saccharomyces* yeast strains and the enological characteristic comparison in wine making. *LWT - Food Science and Technology, 154*, Article 112653. https://doi.org/10.1016/j.lwt.2021.112653

Lee, S.-B., & Park, H.-D. (2020). Isolation and investigation of potential non-*Saccharomyces* yeasts to improve the volatile terpene compounds in Korean muscat bailey a wine. *Microorganisms, 8*, 1552. https://doi.org/10.3390/microorganisms8101552

Lemos Junior, W. J. F., Nadai, C., Crepalde, L. T., de Oliveira, V. S., de Matos, A. D., Giacomini, A., & Corich, V. (2019). Potential use of *Starmerella bacillaris* as fermentation starter for the production of low-alcohol beverages obtained from unripe grapes. *International Journal of Food Microbiology, 303*, 1–8. https://doi.org/10.1016/j.ijfoodmicro.2019.05.006

Li, R., Liu, Y., Zheng, J., Xu, M., Wang, H., Sun, C., Cai, S., Guo, X., Wu, X., & Chen, Y. (2023). Oenological characteristics of two indigenous *Starmerella bacillaris* strains isolated from Chinese wine regions. *Applied Microbiology and Biotechnology, 107*, 3717–3727. https://doi.org/10.1007/s00253-023-12502-7

Lin, M. M. H., Boss, P. K., Walker, M. E., Sumby, K. M., Grbin, P. R., & Jiranek, V. (2020). Evaluation of indigenous non-*Saccharomyces* yeasts isolated from a South Australian vineyard for their potential as wine starter cultures. *International Journal of Food Microbiology, 312*. https://doi.org/10.1016/j.ijfoodmicro.2019.108373

Llauradó, J., Rozès, N., Bobet, R., Mas, A., & Constantí, M. (2002). Low temperature alcoholic fermentations in high sugar concentration grape musts. *Journal of Food Science, 67*, 268–273. https://doi.org/10.1111/j.1365-2621.2002.tb11396.x

Lubbers, S., Verret, C., & Voilley, A. (2001). The effect of glycerol on the perceived aroma of a model wine and a white wine. *LWT - Food Science and Technology, 34*, 262–265. https://doi.org/10.1006/fstl.2001.0766

Maicas, S., & Mateo, J. J. (2015). Enzyme contribution of non-*Saccharomyces* yeasts to wine production. *Universal Journal of Microbiology Research, 3*, 17–25. https://doi.org/10.13189/ujmr.2015.030202

Mančić, S., Stamenković Stojanović, S., Danilović, B., Djordjević, N., Malićanin, M., Lazić, M., & Karabegović, I. (2022). Oenological characterization of native *Hanseniaspora uvarum* strains. *Fermentation, 8*, 92. https://doi.org/10.3390/fermentation8030092

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. *International Journal of Food Microbiology, 367*, Article 109592. https://doi.org/10.1016/j.ijfoodmicro.2022.109592

Maturano, Y. P., Assof, M., Fabani, M. P., Nally, M. C., Jofré, V., Rodríguez Assaf, L. A., Toro, M. E., Castellanos de Figueroa, L. I., & Vazquez, F. (2015). Enzymatic activities produced by mixed *Saccharomyces* and non-*Saccharomyces* cultures: Relationship with wine volatile composition. *Antonie Van Leeuwenhoek, 108*, 1239–1256. https://doi.org/10.1007/s10482-015-0578-0

Mestre Furlani, M. V., Maturano, Y. P., Combina, M., Mercado, L. A., Toro, M. E., & Vazquez, F. (2017). Selection of non-*Saccharomyces* yeasts to be used in grape musts with high alcoholic potential: A strategy to obtain wines with reduced ethanol content. *FEMS Yeast Research, 17*. https://doi.org/10.1093/femsyr/fox010

Morata, A., Escott, C., Bañuelos, M., Loira, I., del Fresno, J., González, C., & Suárez-Lepe, J. (2019). Contribution of non-*Saccharomyces* yeasts to wine freshness. A review. *Biomolecules, 10*, 34. https://doi.org/10.3390/biom10010034

Nadai, C., Giacomini, A., & Corich, V. (2021). The addition of wine yeast *Starmerella bacillaris* to grape skin surface influences must fermentation and glycerol production. *OENO One, 55*, 47–55. https://doi.org/10.20870/oeno-one.2021.55.2.4556

Navarro-Tapia, E., Querol, A., & Pérez-Torrado, R. (2018). Membrane fluidification by ethanol stress activates unfolded protein response in yeasts. *Microbial Biotechnology, 11*, 465–475. https://doi.org/10.1111/1751-7915.13032

Noble, A. C., & Bursick, G. F. (1984). The contribution of glycerol to perceived viscosity and sweetness in white wine. *American Journal of Enology and Viticulture, 35*, 110–112. https://doi.org/10.5344/ajev.1984.35.2.110

Oliveri, C., Bella, P., Tessitori, M., Catara, V., & La Rosa, R. (2017). Grape and environmental mycoflora monitoring in old, traditionally cultivated vineyards on Mount Etna, southern Italy. *Journal of the Science of Food and Agriculture, 97*(1), 65–73. https://doi.org/10.1002/jsfa.7683

Padilla, B., Gil, J. V., & Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology, 7*. https://doi.org/10.3389/fmicb.2016.00411

Padilla, B., Zulian, L., Ferreres, À., Pastor, R., Esteve-Zarzoso, B., Beltran, G., & Mas, A. (2017). Sequential inoculation of native non-*Saccharomyces* and *Saccharomyces cerevisiae* strains for wine making. *Frontiers in Microbiology, 8*. https://doi.org/10.3389/fmicb.2017.01293

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. *American Journal of Enology and Viticulture, 52*, 198–203. https://doi.org/10.5344/ajev.2001.52.3.198

Perpetuini, G., Rossetti, A. P., Quadrani, L., Arfelli, G., Piva, A., Suzzi, G., & Tofalo, R. (2023). Sequential inoculation of *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* as a biotechnological tool to increase the terpenes content of pecorino white wines. *Fermentation, 9*, 785. https://doi.org/10.3390/fermentation9090785

Perpetuini, G., Tittarelli, F., Battistelli, N., Suzzi, G., & Tofalo, R. (2020). Contribution of *Pichia manshurica* strains to aroma profile of organic wines. *European Food Research and Technology, 246*, 1405–1417. https://doi.org/10.1007/s00217-020-03499-8

Perpetuini, G., Tittarelli, F., Schirone, M., Di Gianvito, P., Corsetti, A., Arfelli, G., Suzzi, G., & Tofalo, R. (2018). Adhesion properties and surface hydrophobicity of *Pichia manshurica* strains isolated from organic wines. *LWT, 87*, 385–392. https://doi.org/10.1016/j.lwt.2017.09.011

Polizzotto, G., Barone, E., Ponticello, G., Fasciana, T., Barbera, D., Corona, O., Amore, G., Giammanco, A., & Oliva, D. (2016). Isolation, identification and oenological characterization of non -*Saccharomyces* yeasts in a Mediterranean island. *Letters in Applied Microbiology, 63*, 131–138. https://doi.org/10.1111/lam.12599

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. *Yeast, 16*, 675–729. https://doi.org/10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., Suzzi, G., Rolle, L., & Cocolin, L. (2012). *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. *Applied and Environmental Microbiology, 78*, 1987–1994. https://doi.org/10.1128/AEM.06768-11

Rantsiou, K., Englezos, V., Torchio, F., Risse, P.-A., Cravero, F., Gerbi, V., Rolle, L., & Cocolin, L. (2017). Modeling of the fermentation behavior of *Starmerella bacillaris*. *American Journal of Enology and Viticulture, 68*, 378–385. https://doi.org/10.5344/ajev.2017.16108

Raymond Eder, M. L., & Rosa, A. L. (2021). Genetic, physiological, and industrial aspects of the fructophilic non-*Saccharomyces* yeast species. *Starmerella bacillaris. Fermentation, 7*, 87. https://doi.org/10.3390/fermentation7020087

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., & Lonvaud, A. (2005). In *Handbook of enology: The chemistry of wine stabilization and treatments* (2nd ed.). Ltd. Chichester, England: John Wiley and Sons. https://doi.org/10.1002/0470010363.

Riou, C., Nicaud, J.-M., Barre, P., & Gaillardin, C. (1997). Stationary-phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. *Yeast, 13*, 903–915. https://doi.org/10.1002/(SICI)1097-0061(199708)13:10<903:AID-YEA145>3.0.CO;2-1

Ruiz-de-Villa, C., Poblet, M., Bordons, A., Reguant, C., & Rozès, N. (2023). Comparative study of inoculation strategies of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on the performance of alcoholic and malolactic fermentations in an optimized synthetic grape must. *International Journal of Food Microbiology, 404*, Article 110367. https://doi.org/10.1016/j.ijfoodmicro.2023.110367

Ruiz-Muñoz, M., Hernández-Fernández, M., Cordero-Bueso, G., Martínez-Verdugo, S., Pérez, F., & Cantoral, J. M. (2022). Non-*Saccharomyces* are also forming the veil of flor in sherry wines. *Fermentation, 8*, 456. https://doi.org/10.3390/fermentation8090456

Sadoudi, M., Rousseaux, S., David, V., Alexandre, H., & Tourdot-Maréchal, R. (2017). *Metschnikowia pulcherrima* influences the expression of genes involved in PDH bypass and glyceropyruvic fermentation in *Saccharomyces cerevisiae*. *Frontiers in Microbiology, 8*. https://doi.org/10.3389/fmicb.2017.01137

Samoticha, J., Wojdyło, A., Chmielewska, J., & Nofer, J. (2019). Effect of different yeast strains and temperature of fermentation on basic enological parameters, polyphenols and volatile compounds of aurore white wine. *Foods, 8*, 599. https://doi.org/10.3390/foods8120599

Sidari, R., Ženišová, K., Tobolková, B., Belajová, E., Cabicarová, T., Bučková, M., Puškárová, A., Planý, M., Kuchta, T., & Pangallo, D. (2021). Wine yeasts selection: Laboratory characterization and protocol review. *Microorganisms, 9*, 2223. https://doi.org/10.3390/microorganisms9112223

Spagna, G., Barbagallo, R. N., Palmeri, R., Restuccia, C., & Giudici, P. (2002). Properties of endogenous β-glucosidase of a *Saccharomyces cerevisiae* strain isolated from Sicilian musts and wines. *Enzyme and Microbial Technology, 31*, 1030–1035. https://doi.org/10.1016/S0141-0229(02)00239-9

Su, Y., Seguinot, P., Sanchez, I., Ortiz-Julien, A., Heras, J. M., Querol, A., Camarasa, C., & Guillamón, J. M. (2020). Nitrogen sources preferences of non-*Saccharomyces* yeasts to sustain growth and fermentation under winemaking conditions. *Food Microbiology, 85*, Article 103287. https://doi.org/10.1016/j.fm.2019.103287

Torija, M. (2003). Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *International Journal of Food Microbiology, 80*, 47–53. https://doi.org/10.1016/S0168-1605(02)00144-7

Torija, M. J., Rozès, N., Poblet, M., Guillamón, J. M., & Mas, A. (2001). Yeast population dynamics in spontaneous fermentations: Comparison between two different wine-producing areas over a period of three years. *Antonie Van Leeuwenhoek, 79*, 345–352. https://doi.org/10.1023/A:1012027718701

Tristezza, M., Tufariello, M., Capozzi, V., Spano, G., Mita, G., & Grieco, F. (2016). The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Frontiers in Microbiology, 7*. https://doi.org/10.3389/fmicb.2016.00670

Tufariello, M., Chiriatti, M. A., Grieco, F., Perrotta, C., Capone, S., Rampino, P., Tristezza, M., Mita, G., & Grieco, F. (2014). Influence of autochthonous *Saccharomyces cerevisiae* strains on volatile profile of Negroamaro wines. *LWT - Food Science and Technology, 58*, 35–48. https://doi.org/10.1016/j.lwt.2014.03.016

Valentine, G. D. S., Walker, M. E., Gardner, J. M., Schmid, F., & Jiranek, V. (2019). Brief temperature extremes during wine fermentation: Effect on yeast viability and fermentation progress. *Australian Journal of Grape and Wine Research, 25*, 62–69. https://doi.org/10.1111/ajgw.12365

Vaudano, E., Quinterno, G., Costantini, A., Pulcini, L., Pessione, E., & Garcia-Moruno, E. (2019). Yeast distribution in Grignolino grapes growing in a new vineyard in Piedmont and the technological characterization of indigenous *Saccharomyces* spp. strains. *International Journal of Food Microbiology, 289*, 154–161. https://doi.org/10.1016/j.ijfoodmicro.2018.09.016

Vejarano, R. (2020). Non-*Saccharomyces* in winemaking: Source of mannoproteins, nitrogen, enzymes, and antimicrobial compounds. *Fermentation, 6*, 3. https://doi.org/10.3390/fermentation6030076

Vejarano, R., & Gil-Calderón, A. (2021). Commercially available non-*Saccharomyces* yeasts for winemaking: Current market, advantages over *Saccharomyces*, biocompatibility, and safety. *Fermentation, 7*, 171. https://doi.org/10.3390/fermentation7030171

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

Zhang, Q., Li, H., Sun, X., Huang, W., & Zhan, J. (2020). Exploitation of indigenous wine yeasts from spontaneously fermenting grape must and vineyard soil in Beijing, China. *eFood, 1*, 316–325. https://doi.org/10.2991/efood.k.200731.001

Zhang, P., Zhang, R., Sirisena, S., Gan, R., & Fang, Z. (2021). Beta-glucosidase activity of wine yeasts and its impacts on wine volatiles and phenolics: A mini-review. *Food Microbiology, 100*, Article 103859. https://doi.org/10.1016/j.fm.2021.103859

Zhang, W., Zhuo, X., Hu, L., & Zhang, X. (2020). Effects of crude β-glucosidases from *Issatchenkia terricola, Pichia kudriavzevii, Metschnikowia pulcherrima* on the flavor complexity and characteristics of wines. *Microorganisms, 8*, 953. https://doi.org/10.3390/microorganisms8060953

Zhu, X., Navarro, Y., Mas, A., Torija, M. J., & Beltran, G. (2020). A rapid method for selecting non-*Saccharomyces* strains with a low ethanol yield. *Microorganisms, 8*. https://doi.org/10.3390/microorganisms8050658