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Survey of the yeast ecology of dehydrated grapes and strain selection for wine fermentation

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ABSTRACT
In this study we investigated the yeast population present on partially dehydrated Nebbiolo grapes destined for 'Sforzato di Valtellina', with the aim to select indigenous starters suitable for the production of this wine. Yeasts were enumerated, isolated, and identified by molecular methods (5.8S-ITS-RFLP and D1/D2 domain sequencing). A genetic, physiological (ethanol and sulphur dioxide tolerance, potentially useful enzymatic activities, hydrogen sulphide production, adhesive properties, and killer activity) and oenological (laboratory pure micro-fermentations) characterization was also carried out. Based on relevant physiological features, seven non-*Saccharomyces* strains were chosen for laboratory-scale fermentations, either in pure or in mixed-culture (simultaneous and sequential inoculum) with a commercial *S. cerevisiae* strain. Finally, the best couples and inoculation strategy were further tested in mixed fermentations in winery. In both laboratory and winery, microbiological and chemical analyses were conducted during fermentation. The most abundant species on grapes were *Hanseniaspora uvarum* (27.4% of the isolates), followed by *Metschnikowia* spp. (21.0%) and *Starmerella bacillaris* (12.9%). Technological characterization highlighted several inter and intra-species differences. The best oenological aptitude was highlighted for species *Starm. bacillaris*, *Metschnikowia* spp., *Pichia kluyveri* and *Zygosaccharomyces bailli*. The best fermentation performances in laboratory-scale fermentations were found for *Starm. bacillaris* and *P. kluyveri*, due to their ability to reduce ethanol (-0.34% v/v) and enhance glycerol production (+0.46). This behavior was further confirmed in winery. Results of this study contribute to the knowledge of yeast communities associated with a specific environment, like those of Valtellina wine region.

Keywords: Non-*Saccharomyces* yeasts, Indigenous yeasts, Nebbiolo dehydrated grapes, strain characterization, wine fermentations, mixed fermentations, *Sfursat*

1. Introduction

'Sforzato di Valtellina' (or 'Sfursat di Valtellina') is a traditional dry red wine obtained from *Vitis vinifera* L. cv. Nebbiolo grapes (locally called 'Chiavennasca') harvested in the mountain area of Valtellina, in Lombardy region (northern Italy). Particularly, 'Nebbiolo' grapes destined to produce this wine are subjected to a postharvest partial dehydration (about the 20% of water loss) in naturally ventilated rooms called '*fruttaio*'. Starting from 2003 the 'Sforzato di Valtellina' attained the DOCG (Controlled and Guaranteed Denomination of Origin) product denomination of origin (PDO), used for the highest quality wines in Italy and, consequently, its production is subjected to a strict regulation (Pomarici, & Vecchio, 2019). Different aspects determine the peculiar characteristics of this wine, starting from the vineyards' location at 350-800 m.a.s.l. (meters above sea level) to the aging for a minimum period of twenty months, both in oak casks (minimum 12 months) and bottle.

During grape dehydration, a plethora of chemical–physical modifications are induced, depending on grape features (size of the berry, compactness of the bunch, alteration of berry skin surface e.g., micropores and cracks, etc.), environmental conditions (ventilation, relative humidity of the air, temperature, air flow, sunlight) or the length of the process (Sanmartin et al., 2021). In general, this practice determines concentration of sugars and, consequently, the moderate-high alcohol content of the resulting wines. Furthermore, the total soluble solids and phenolic compounds increase, while the aromas associated with the withering process develop in parallel (Zenoni et al., 2016). Wines obtained from partially dehydrated grapes may contain high initial concentrations of undesirable oxidation compounds (e.g., acetic acid, acetaldehyde, and ethyl acetate) often associated with low wine quality (Kelly, Inglis, & Pickering, 2020).

The selection of indigenous strains, associated to a certain territory, appellation, or vineyard, with specific phenotypes, could be a valuable tool to maintain the 'characteristics', the complexity and typicality of wines. Various studies have been conducted to select strains that possess technological traits suitable for the fermentation of musts obtained from dehydrated grapes. A large part of them was focused on *Saccharomyces cerevisiae* (Azzolini et al., 2013; Aponte & Blaiotta, 2016), due to the ability of this species to colonize rapidly the grape must and be present until the late stages of the alcoholic fermentation (Tronchoni, Curiel, Morales, Torres-Pérez & Gonzalez, 2017; Alonso-del-Real, Pérez-Torrado, Querol, & Barrio, 2019). Consequently, the metabolic activity of *S. cerevisiae* could

influence the chemical composition and the fermentative aromas of the final product (Parapouli, Sfakianaki, Monokrousos, Perisynakis, & Hatziloukas, 2019). Nonetheless, several studies have also demonstrated the oenological potential of non-*Saccharomyces* yeasts in pure and mixed fermentations (Benito, 2018; Roudil et al., 2020; Morata et al., 2020), due to their ability to achieve goals that cannot be reached by *S. cerevisiae*.

The climatic conditions and vineyards' location of Valtellina wine area may contribute to particular yeast diversity, opening the possibility of isolating new yeast strains that could ferment Nebbiolo grape must with peculiar characteristics, such as the high sugar content caused by grape postharvest dehydration. Indigenous yeasts could improve wine quality by modulating specific wine attributes and complete the fermentation process, without the risk of stuck fermentation. With this in mind, the objective of the present study was to isolate, identify, and characterize, from physiological point of view, the indigenous yeasts from this peculiar ecosystem and to find strains suitable as starter cultures for the production of premium quality 'Sforzato di Valtellina' wines. In order to comprise a wider isolate diversity, yeasts were isolated from grapes grown from two different vineyards, and three sequential harvests each were performed. The experiment is articulated in three main phases: (i) isolation, genetical and phenotypic characterization of indigenous yeasts from cv. Nebbiolo partially dehydrated grapes, carried out in 2020 vintage; (ii) lab-scale pure and mixed-culture fermentations using the 7 most promising yeast strains isolated from the previous phase; and (iii) winery trials (2021 vintage) with the two best couple of strains obtained from the previous phases.

2. Materials and Methods

2.1. Sample collection and isolation of indigenous yeasts

Following the traditional procedures for the production of 'Sforzato di Valtellina' wines, *Vitis vinifera* L. cv. Nebbiolo grapes were harvested in 2020 vintage, from two vineyards located in Villa di Tirano (vineyard code X) and Berbenno di Valtellina (vineyard code Y) towns in Lombardia region (northern Italy). The vineyards are placed in the north side of Valtellina valley and cultivated with terraces, with South and South-East exposure, at an altitude comprised in the range 350-400 m a.s.l. For each vineyard, three sequential harvest times were selected: the first harvest date was at about 21.5 Brix grape soluble solids, and then at about 10 and 20 days after the first one, respectively. During harvest, grapes were placed in a single layer in perforated plastic boxes and then transferred for the dehydration in a 'fruttaio', a typical Valtellina dehydration room without temperature or relative humidity control, as imposed by the wine designation regulations. The grape postharvest dehydration process was carried out until December 1st (as prescribed by the regulations) reaching an average grape soluble solids degree of 26.7 Brix. Afterwards, six samples (200 grape berries each were chosen from different grape bunches) of partially dehydrated Nebbiolo grapes (three samples from Villa di Tirano and three from Berbenno di Valtellina vineyards) were aseptically collected manually and placed directly into sealed sterile bags. Refrigerated samples were then transferred in the laboratory for further analyses.

Nebbiolo grapes were manually crushed and processed for microbiological analysis. Appropriate decimal dilutions on sterile Ringers' solution (Biogenetics, Milan, Italy) were seeded onto Wallerstein laboratory nutrient agar (WLN) (Biogenetics). Plates were aerobically incubated at 25 ± 2 °C for 5 days and observed for differential cell count. According to their colony morphotype and cell morphology (Pallmann et al., 2001; Wang et al., 2019), representative isolates were selected and purified by successive streaking on WLN. The isolates were stored at –80 °C in Yeast Extract Peptone Dextrose (YPD) broth (1% w/v yeast extract, 2% w/v bacteriological peptone, 2% w/v glucose, all provided by Biogenetics) supplemented with glycerol (20% v/v final concentration; Sigma-Aldrich, Milan, Italy) or on YPD agar at 4 °C for short-term storage.

2.2. Yeasts identification and typing

From each yeast isolate, DNA was extracted as previously reported by Mills, Johannsen, & Cocolin (2002). Preliminary molecular identification of yeasts was achieved using a "clustering and sequencing" approach. Firstly, the 5.8S-ITS region was amplified using primers ITS1 and ITS4 and the restriction

endonucleases *Hae III*, *Hinf I* and *Cfo I* were used as described by Esteve-Zarzoso, Belloch, Uruburu, & Querol (1999). The restriction fragments were separated by gel electrophoresis on 2.5 % (w/v) agarose gel (Bio-Rad, Laboratories, Inc., Hercules, USA) with TAE buffer (0.8 mM Tris base and 0.02 mM EDTA, pH 8.0, adjusted with glacial acetic acid) at 120 V for 90 min. A 100 bp ladder (Promega Corporation, Madison, Wisconsin, USA) was used to estimate band sizes. Gels were visualized under ultraviolet light using Gel Doc XR+ (Bio-Rad, Hercules, USA). Isolates showing the same ITS and restriction fragments' profile were grouped together and confirmation of the identification of each group was achieved by sequencing the D1-D2 loop of the 26S rRNA gene (Kurtzman & Robnett, 1997) by the company GENEWIZ Germany GmbH. The identification of each isolate was achieved by comparing the obtained sequences in silico with those available at the National Center for Biotechnology Information (NCBI) using BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/).

Molecular strain characterization was performed applying rep-PCR fingerprinting as reported by Englezos et al. (2015) using the microsatellite oligonucleotide sequence (GTG)5 (Lederer et al.,2013). The obtained products were subjected to electrophoresis as above described. The resulting fingerprints were analyzed with the BioNumerics 4.6 software package (Applied Maths, Kortrijk, Belgium). The similarity among profiles was calculated using the Pearson correlation and an average linkage (UPGMA) dendrogram was derived from the profiles.

2.3. Isolates technological characterization

Preparation for the physiological characterization was performed by inoculating a single colony of each yeast isolate in 3 mL of sterile YPD broth and following incubation for 24 hours at 25 °C. Three biological replicates were performed for each isolate.

2.3.1 Physiological characterization

The H_2S production from each isolate was evaluated by using the BiGGY agar medium (Oxoid, Milan, Italy). The medium was spot inoculated with the yeast isolates and then incubated at 25 °C for 2 days. Based on the colony colour, H_2S production of isolates was identified using the following scale: 0 white (no production), 1 cream, 2 light brown, 3 brown, 4 dark brown, and 5 black (high production). Trials were performed in triplicate. The evaluation of the yeasts ability to produce the following extracellular enzymes (i.e., esterase, β-glucosidase, pectinase, and protease) was evaluated by plate assays on Petri dishes filled with appropriate media, as previously reported by Englezos et al. (2015).

Biofilm formation capacity of each yeast isolate was evaluated by using Yeast Nitrogen Base with amino acids (YNB) containing 4% (v/v) ethanol as a sole carbon source as reported by Zara et al. (2005). To generate the floral morphology (mats), isolates were grown in YPD for 48 h at 25 ± 2 °C. Five µL of the culture were used to inoculate the centre of a 90 mm Petri dish previously filled with YPD solidified with 0.3% agar as described by Reynolds & Fink, (2001). Plates were then incubated at 25 ± 2 °C for 15 days. At least two replicate mats of each isolate were prepared.

Ethanol and SO_2 tolerance were evaluated in microplates as previously described by (Englezos et al., 2015). Briefly, YNB (6.7 g/L, Remel, Lenexa, KS, USA), pH 5.5, was supplemented with 20 g/L of glucose and sterile filtered using a 0.45 µm membrane filter (VWR, Milan, Italy). The medium was then supplemented with ethanol (Sigma-Aldrich) to reach final concentrations of 0, 4, 6, 8, 10, 12, 14, and 16 % (v/v). In a similar way to test the SO_2 tolerance, the above-mentioned medium was supplemented with different amounts of total SO_2 to obtain final concentrations of 0, 25, 50, 100, and 150 mg/L, after adjustment of pH to 3.0. SO_2 was added as potassium metabisulphite (Sigma-Aldrich). The microplates were incubated at 25 ± 2 °C and the optical density was measured using a microplate reader (Savatec instruments, Torino, Italy) at 630 nm after 48 h of incubation. The yeast inoculum, as well as cell growth were determined according to Englezos et al. (2015). Trials were performed in triplicate, while the commercial *S. cerevisiae* EC1118 (Lallemand, Verona, Italy) was used as a reference strain.

2.3.2. Killer activity screening by qualitative method (QLM)

Each isolate was tested for its ability to kill two selected target yeasts belonging to spoilage species: *Hanseniaspora uvarum* Y1 and *Brettanomyces bruxellensis* DSM 7001. The first strain belongs to the DISAFA (Dipartimento di Scienze Agrarie, Forestali e Alimentari, University of Torino, Italy),

while the second was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Pre-cultures of all the yeasts were grown in 5 mL YPD broth medium at 30 ± 2 °C for 48 h.

Killer activity screening was performed using the seeded agar method on YPD-methylene blue (MB) medium (yeast extract 1% w/v, peptone 2% w/v, glucose 2% w/v, agar 2% w/v, 0.003% (w/v) methylene blue) buffered at pH 4.6 with 0.1 M citrate phosphate buffer as reported by Lopes & Sangorrín (2010). Approximately 2×10^6 cells/mL of each of the two target strains were uniformly seeded on YPD-MB plates and dried for 30 minutes. Consequently, 5 µL of isolates cultures was spot inoculated on plates. The plates were incubated at 20 ± 2 °C until a well-developed lawn of the potentially sensitive yeast strain was observed. Killer activity was scored as positive when the killer isolate was surrounded by a region of bluish-stained cells and the reaction was recorded as '1' (weak killer reaction). When a clear zone of growth inhibition (≥1mm) bounded by stained cells was observed, it was designated as '2' (strong killer reaction). Trials were performed in triplicate.

2.3.3. Pure culture micro-fermentation tests

Pure culture fermentations of each yeast isolate were performed in triplicate in 15 mL sterile falcon tubes, filled with 10 mL of pasteurized grape must (Englezos et al., 2019), obtained from partially dehydrated Nebbiolo grapes destined for the production of 'Sforzato di Valtellina'. A pre-adaptation was conducted by inoculating a single colony of each isolate in 3 mL of pasteurized must and incubated at 25 ± 2 °C for 48 h. Subsequently, these cultures were used to inoculate the pasteurized must with 2×10^6 cells/mL. The chemical composition of the grape must was the following: 274.27 g/L of sugars, 1.79 g/L of glycerol, pH 3.5 and titratable acidity 8.0 g/L (as tartaric acid). A commercial strain of *S. cerevisiae* (EC1118; Lallemand) was used as a control. Fermentations were performed in triplicate in a temperature-controlled chamber at 25 ± 2 °C for 15 days. At the end of the incubation the chemical composition of each fermentation was evaluated by HPLC as described in the next sections.

2.4. Pure and Mixed-Culture Fermentations

Based on technological properties and fermentation performance of each yeast isolate in micro-fermentation trials, seven non-*Saccharomyces* yeasts were chosen: 3 *Starm. bacillaris* (Sb X2-10, Sb Y1-2 and Sb Y2-9), *Zygosaccharomyces bailii* (Zb X2-6), *Metschnikowia pulcherrima* (Mp Y2-7), *Pichia kluyveri* (Pk X3-5), and *Candida apicola* (Ca Y2-13). These yeasts were used to inoculate the above-mentioned grape must alone (pure fermentations) and together with *S. cerevisiae* EC1118 (here referred as Sc; mixed culture fermentations), using the co-inoculations and sequential inoculation (2-day delay) approaches. In total, twenty-two further micro-fermentation trials were carried out in laboratory (8 pure, 7 mixed co-inoculum, and 7 sequential inoculum). Fermentations were performed in triplicate at 25 ± 2 °C in 100 mL flasks with 70 mL of pasteurized must previously described. These yeasts were pre-adapted at 25 ± 2 °C as described in Section 2.3.3. Yeast dynamics and metabolites evolution during and at the end of fermentation was monitored by plate counts on WLN medium and by HPLC analysis, respectively.

2.5. Fermentations at winery scale

Based on the laboratory scale fermentations, two non-*Saccharomyces* yeasts were chosen as starter cultures for fermentations in winery: *Starm. bacillaris* Sb X2-10 and *P. kluyveri* Pk X3-5. In winery, fermentations were carried out in triplicate by co-inoculation of non-*Saccharomyces* with *S. cerevisiae* EC1118. Pure fermentation with EC1118 strain was conducted as control. All strains were inoculated with an initial cell population of 1.0×10^6 cells/mL, as described above for laboratory scale trials. *Vitis vinifera* L. cv. Nebbiolo grapes were collected in vintage 2021 and subjected to withering as previously described (Section 2.1). One-hundred-and-ten (110) kg of dehydrated grapes were destemmed and crushed, and then the grape mash obtained (liquid and solid parts) was homogenously distributed in nine containers (15 L volume each). The initial must composition was: 256 g/L of reducing sugars (glucose and fructose), 1.13 g/L of glycerol, while pH and total acidity (expressed as g/L of tartaric acid) were respectively 3.12 and 5.24 g/L. The containers were inoculated with the respective yeast combination (control, *Starm. bacillaris* X2-10 and *S. cerevisiae* EC1118, *P. kluyveri*

X3-5 and *S. cerevisiae* EC1118, all in three replicates), and the grape must was then fermented at 25 ± 2 °C. To ensure the management of pomace cap, the liquid was pumped up twice a day, and the devatting was carried out when sugars were less than 2 g/L. Vinification process was carried out in the Bonafous experimental wine cellar of the University of Torino (Chieri, Italy).

2.6. Microbiological and molecular analyses

Samples during laboratory and winery scale fermentations were collected, serially diluted with sterile Ringer's solutions (Biogenetics) and plated on WLN medium. Plates were incubated at 28 ± 2 °C for 5 days, differentiated visually and counted. During wine fermentation five putative colonies of *Starm. bacillaris* and *P. kluyveri* from each sampling point were isolated and further characterized at strain level through Rep-PCR (Lederer, Nielsen, Toldam-Andersen, Herrmann, & Arneborg, 2013). Presumptive colonies of *S. cerevisiae* were typed by PCR interdelta analysis (δ-PCR) (Legras, & Karst, 2003) to understand strain dynamics over the fermentation process.

2.7. Analytical determinations

Concentrations of reducing sugars (glucose and fructose, g/L), acetic acid (g/L), glycerol (g/L) and ethanol (% v/v) were evaluated throughout HPLC analysis, using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a UV detector set to 210 nm and a refractive index detector. The chromatographic conditions were previously described by Rolle et al. (2018). Total acidity (expressed as g/L of tartaric acid) was determined according to the official method OIV-MA-AS313-01 (OIV, 2020), while pH was measured using an InoLab 730 pH meter (WTW, Weilheim, DE). Volatile organic compounds (VOCs) of the wines produced in winery were determined according to the protocol reported by Englezos et al. (2018) with few modifications in the preparative phase. Three g of sodium chloride (Sigma-Aldrich, Milan, Italy) were weighed in 20 mL glass headspace (HS) vials and united with 5 mL of wine sample aliquots and 5 mL of Milli-Q grade water. Finally, vials were spiked with 50 µL of 2-octanol (Sigma-Aldrich) as an internal standard (IS, 58.56 mg/L in 10% v/v ethanol) and sealed hermetically with silicon septa magnetic screw caps. VOCs were subsequently extracted by headspace solid-phase microextraction (HS-SPME) for 60 min at 40 °C and determined using Agilent 7890C gas chromatograph (GC) coupled to Agilent 5975 mass selective detector (MSD). Volatile compounds were identified according to: retention indices, the mass spectra of pure standards (where available), and the NIST database (http://webbook.nist.gov/chemistry/) considering a good or excellent mass spectra match (at least 80% similarity). The results were expressed as a semi-quantitative data as µg/L equivalents of the IS 2-octanol, assuming a response factor equal to one.

2.8. Data analyses

Statistical analyses were performed using IBM SPSS Statistics software (version 25.0, IBM Corp., Armonk, NY, USA). The data obtained from the different trials were subjected to one-way analysis of variance (ANOVA) and coupled by the Tukey-HSD test ($p < 0.05$), to get significant differences between the data obtained. Physiological and oenological analyses data were plotted by Heatmapper software (Babicki, et al., 2016), and hierarchical clustering was performed with Euclidean distance metrics. A Principal Component Analysis (PCA) multivariate approach was performed on the volatile compounds data of wines produced in winery trials: for this aim, the R software (version 4.2.3; R Foundation for Statistical Computing, Vienna, Austria) with package "factoextra" was used (Kassambara & Mundt, 2020).

3. Results

3.1. Isolation and identification of indigenous yeasts

The selection of autochthonous yeasts started with the isolation of 62 yeast isolates from Nebbiolo dehydrated grapes, previously harvested from two vineyards (three from Villa di Tirano-X

and three from Berbenno di Valtellina-Y vineyards). The total count of samples coming from the two different vineyards was very close with values of 5.63 ± 0.36 Log CFU/mL (samples from Villa di Tirano-X) and 5.69 ± 0.39 Log CFU/mL (samples from Berbenno di Valtellina-Y). Colonies with different morphologies were isolated and identified by amplification and restriction analysis of the internal transcribed spacer ITS1/ITS2-5.8S rRNA region. DNA sequence analyses revealed 13 different yeast species, as can be seen in Table 1. The species most isolated was *H. uvarum* with 17 isolates, followed by *Metschnikowia fructicola* with 9 isolates and *Starm. bacillaris* with 8 isolates. The other identified isolates belonged to: *Metschnikowia pulcherrima*, *Candida californica*, *Candida apicola*, *Pichia kluyveri*, *Rhodotorula graminis*, *Rhodotorula nothofagi*, *Zygosaccharomyces bailii*, *Debaryomyces carsonii*, *Zygoascus hellenicus,* and *Zygoascus meyerae*.

All the isolates were then subjected to molecular characterization to define their intra-specific variability by rep-PCR. For all the considered species a similarity coefficient of 85% was selected and results are showed in Fig. S1. Seven clusters were identified for *H. uvarum* isolates, 3 for *Starm. bacillaris*, 2 for *P. kluyveri*, 2 for *Z. bailii*, 2 for *C. californica,* 3 for *C. apicola*, 2 for *M. fructicola* and only 1 for *M. pulcherrima* spp. Interestingly, no correlation with origin vineyard was found.

3.2. Physiological characterization and oenological properties

The results of the physiological and oenological properties investigated were represented as heatmap in Fig. 1., Table S1, Table S2 and Table S3 in supplementary material.

3.2.1. Ethanol tolerance

The ability of the isolates to grow at different concentrations of ethanol (4, 6, 8, 10, 12, 14 and 16 % v/v) was investigated (Fig. 1, Table S1). Five isolates showed a strong reduction of their viability at 4 % (v/v) of ethanol. On the other hand, *Starm. bacillaris*, *Z. bailii* and *P. kluyveri* were more resistant to high ethanol concentrations than *Candida* and *Metschnikowia* spp. All isolates of this last genera were able to grow up to 6% (v/v) of ethanol, except *M. fructicola* X2-11. Regarding *Candida* genera, all isolates were able to grow at 10% (v/v) of ethanol, but only two *C. apicola* isolates grew until 14% (v/v) of ethanol (Y2-11 and Y2-13).

3.2.2. SO_2 tolerance

Regarding SO_2 tolerance (from 10 to 150 mg/L), about the 55 % of the isolates were not able to grow at amounts of 50 mg/L (Fig. 1, Table S2). The reference strain *S. cerevisiae* EC1118, together with few non-*Saccharomyces* isolates grew at the highest tested concentration (150 mg/L): *Starm. bacillaris* (X1-5, X1-6 and Y1-2), three *Z. bailii* isolates (X2-7, X2-8 and Y3-7) and a *P. kluyveri* (X3-5).

3.2.3. Enzymatic activities

Fourteen isolates were found to possess a protease activity (Fig. 1, Table S3), among them *H. uvarum* Y2-1, *Starm. bacillaris* X1-5, and all *Metschnikowia* spp. isolates except X2-11 and Y2-6. Regarding *β*-glucosidase activity, only four isolates presented positive results (*M. fructicola* X2-11, *D. carsonii* X3-8, and the two *Rhodotorula* spp. isolates X2-12 and X3-9). Eight isolates possessed pectinase activity (*Starm. bacillaris* X1-5, X2-9, X2-10, and Y1-1, *M. fructicola* Y1-5 and Y1-6, *C. californica* X1-12 and *H. uvarum* Y2-2) and *R. graminis* X3-9 was the only isolate to show positive result for the esterase activity.

3.2.4. H_2S production

The semi-quantitative test on the H_2S development on BiGGY agar revealed (Fig. 1, Table S3) that all *C. californica* and the two *Zygoascus* spp. isolates were high producers of H_2S, while all *Z. bailii* isolates as well as *C. apicola*, *D. carsonii* and *Rhodotorula* spp. either did not produce or they produced relatively low amounts of this compound. *M. pulcherrima* and *M. fructicola* isolates demonstrated a

medium ability to produce H_2S. Great variability was observed for *Starm. bacillaris* and *H. uvarum* isolates and an opposite behavior was found for *P. kluyveri* isolates; in fact, three of them resulted as a high producer, while the other two low producers.

3.2.5. Biofilm formation

A total of 29 isolates of different species proved to be able to form biofilm (Fig. 1, Table S3) demonstrating that this is a strain-specific character (Cordero-Bueso et al., 2017). All *P. kluyveri* and all *C. californica*, *R. graminis* X3-9, *Z. hellenicus* Y3-6, two *Starm. bacillaris* (Y1-2 and Y1-3), nine *H. uvarum* (X3-1, X3-2, X3-3, X3-4, X2-4, Y2-2, Y2-3, Y2-4 and Y3-4), three *C. apicola* (Y2-10, Y2-11, Y2-13), and three *Z. bailii* (X2-5, X2-6, X2-7 and Y3-7) showed this ability. A further type of biofilm was also investigated: the ability to grow on wet and semi-solid agar media (mats formation). The capacity to form a mature mat was observed for some isolates of *Starm. bacillaris*, *H. uvarum*, *P. kluyveri*, *Z. bailii*, *C. californica*, and *C. apicola. M. pulcherrima* and *M. fructicola* demonstrated a lower ability to form mats giving rise to colonies with a lower number of radial spokes and a minor diameter. Divergently, *R. graminis*, *R. nothofagi*, *D. carsonii, Z. meyerae,* and *Z. hellenicus* did not show this ability. Interestingly, among isolates of the same species, differences in the morphology of the mats were found (dimensions, shape of the rim and the body). For *Starm. bacillaris,* three different phenotypes were observed: one with an 72 mm petal-like colony with an irregular rim (14 mm) and a brunched body (29+29 mm), a second with an irregular little colony (31 mm) with an intense white rim (19 mm) surrounded by a thin smooth body (6+6 mm) and a third one with a little floral-like colony (33 mm) with an irregular rim (5 mm) surrounded by a wavy body (14+14 mm). Five different morphologies were found for *H. uvarum*: one with a 44 mm colony with an intense rough withe rim (6) and a floral thin body (19+19 mm), another one with a 52 mm floral-like colony with a ring of intense white cells (40 mm) surrounded by a thin body (6+6 mm), the third is a 87 mm colony with an irregular rim (15 mm) and a very thin radially body (36+36 mm), the fourth is a 74 mm colony characterized by an irregular ring rim (13 mm) and a brunched body (31+30 mm) and the last one is a 38 mm colony with a little rough irregular rim (7 mm) and a wavy body (15+16 mm). Regarding *P. kluyveri* isolates, two phenotypes were observed: one of 69 mm colony with a ring irregular rim (15 mm) and a brunched body (27+27 mm) and another one of a little 30 mm colony with a 7 mm rim and a radially body (11+12 mm). *Z. bailii*. presented three morphotypes: a colony of 72 mm with an irregular rim (10 mm) and a thin radially body (31+31 mm), another one 33 mm colony with 6 mm rim and wavy body (13+14 mm) and finally an 18 mm colony with a umbonate rim (3 mm) and an irregular radially body (7+8).

3.2.6. Killer activity

The antagonistic ability of the 62 indigenous isolates was evaluated by QLM technique against two wine spoilage yeast strains belonging to *B. bruxellensis* and *H. uvarum* (Fig.1, Table S3). No killer activity was observed against *H. uvarum* strain. Divergently, 36 isolates (58.06%) demonstrated an antagonistic behaviour against *B. bruxellensis*. In particular, for 17 isolates was observed a dark zone of stained blue cells, while 19 isolates were surrounded by a clear inhibition zone: *H. uvarum* X1-7*, Z. bailii* X2-8, four *Starm. bacillaris* (X1-5, X1-6, X2-9, X2-10) and for all the 13 *M. fructicola* and *M. pulcherrima* isolates.

3.2.7. Pure micro-fermentation trials in grape juice

None of the isolates was able to complete fermentation; residual sugars values ranged from 274 g/L (*C. californica* X1-10) to 102.62 g/L (*Starm. bacillaris* Y1-2). Conversely, only commercial *S. cerevisiae* EC1118 was able to consume all the sugars (<1.0 g/L residual sugars) from the medium (Table S5). *Starm. bacillaris* and *Z. bailii* isolates demonstrated the highest sugars consumption, with differences among strains. These two species showed the highest fructose consumption. The fructophilic character coupled with a high glycerol production were found in *C. apicola* isolates. The isolates of *Starm. bacillaris* and *Z. bailii* produced wines with the highest values of ethanol (ranging from 7.82% v/v for *Z. bailii* X2-5 to 10.49% v/v for *Starm. bacillaris* Y1-2 isolates). *Metschnikowia*

spp. and *Candida* spp. isolates showed the lowest acetic acid production (less than 0.20 g/L), while *H. uvarum* isolates exhibited the highest with values ranging from 0.43 g/L (X3-3) to 0.68 g/L (X1-8).

3.3. Pure and Mixed-Culture Fermentations in laboratory scale

Based on the results of the physiological characterization and on the fermentation performance of each yeast species in Nebbiolo must, seven non-*Saccharomyces* isolates were chosen to conduct mixed fermentations with the commercial strain *S. cerevisiae* EC1118, using the co-inoculation and sequential inoculation approach. To this aim, three *Starm. bacillaris* representing the three clusters found by typing analyses (X2-10, Y1-2, and Y2-9), *Z. bailii* X2-6, *M. pulcherrima* Y2-7, *P. kluyveri* X3-5, and *C. apicola* Y2-13 strains were selected. Pure fermentations with each strain were also conducted as controls. The growth dynamics during pure and mixed fermentations (both co-inoculation and sequential inoculation), are showed in Fig. 2 and 3. Regarding pure fermentations (Fig. 2 and 3, left panel), the highest population number was reached after 2 days by *S. cerevisiae* EC1118 (8.1±0.21 Log CFU/mL) and the three *Starm. bacillaris* (average value of 8.4±0.14 Log CFU/mL). *Z. bailii* X2-6 cell population achieved the stationary phase with values of 7.4±0.17 Log CFU/mL at day 4, followed by a decrease until the end of the monitored period (14th day), with values about 6.9±0.06 Log CFU/mL. *M. pulcherrima* Y2-7 decreased its population until 5.2±0.44 Log CFU/mL at day 14. Divergently, *P. kluyveri* X3-5 and *C. apicola* Y2-13 populations remained stable for the first 2 days and after decreased and were not detectable on WLN medium (< 10 CFU/mL) at day 7.

Regarding mixed fermentations, the three *Starm. bacillaris* in co-inoculum with EC1118 were able to grow until values of about 8.2±0.14 Log CFU/mL at day 2 and after decreased to 5.0±0.26 Log CFU/mL at day 14. In sequential inoculation, *Starm. bacillaris* strains were able to reach more than 8.0 Log CFU/mL, a decrease was then observed until 6.2±0.34 Log CFU/mL (Sc/Sb X2-10) and 6.6±0.28 Log CFU/mL (Sc/Sb Y1-2), while Sb Y2-9 was not detectable on WLN medium (< 10 CFU/mL) at the end of the monitored period. *Z. bailii* X2-6 grew until 6.6±0.25 Log CFU/mL at day 2 and 6.91±0.34 Log CFU/mL at day 4 in co-inoculum and sequential fermentations, respectively, and after decreased until about 5.51±0.24 Log CFU/mL in both cases. *M. pulcherrima* Y2-7 in mixed fermentations grew for the first 2 days and then a rapid decrease was observed and dropped to undetectable levels (< 10 CFU/mL) at day 7 and 14 for co-inoculum and sequential mixed fermentations, respectively. Finally, *P. kluyveri* X3-5 and *C. apicola* Y2-13 populations declined rapidly in all fermentations and were not detected at day 4, independently from inoculum protocol applied. In all co-inoculated fermentations, *S. cerevisiae* reached the same population levels with those registered in pure culture fermentations, on the other hand the early inoculation of the non-*Saccharomyces* yeasts during the sequential fermentations lead to a decrease of the first by reducing the cell number, with exception of the sequential fermentations performed with *C. apicola* and *P. kluyveri*.

The chemical composition of the wines produced in laboratory-scale fermentations are showed in Table 2. In pure fermentations all the non-*Saccharomyces* yeasts were not able to complete the fermentation, with residual sugars (glucose+fructose) concentrations after 14 days ranging from 237.71 g/L (*P. kluyveri* X3-5) to 106.21 g/L (*Starm. bacillaris* X2-10); the former strain consumed mostly glucose, while the latter depleted almost completely (0.76 g/L left) fructose. On the other hand, after 14 days of fermentation, all mixed fermentations had a residual sugars concentration lower than 2.0 g/L, except for the couple *Z. bailli* X2-6 and *S. cerevisiae* EC1118, that showed 2.22 and 11.26 g/L of residual sugars for co- and sequential inoculation, respectively. A low acetic acid production was observed in all fermentations, with the highest values found for mixed fermentations with *M. pulcherimma* Y2-7 (0.58 and 0.50 g/L for co- and sequential fermentation, respectively) and *P. kluyveri* X3-5 (0.59 and 0.47 g/L) with *S. cerevisiae* EC1118. *C. apicola* Y2-13 and *Starm. bacillaris* X2-10 evidenced the highest glycerol contents in pure fermentations. In sequential fermentations, the highest glycerol values were found for all *Starm. bacillaris* and *S. cerevisiae* EC1118 pairings (16.94-18.23 g/L), representing a 46% increase compared to *S. cerevisiae* in pure culture fermentation (11.93 g/L). Furthermore, an ethanol content reduction was observed in sequential mixed fermentations with all *Starm. bacillaris* strains, compared to the control fermentation with *S. cerevisiae* EC1118 in pure culture (16.27-16.31 % v/v versus 16.70 % v/v).

3.4. Fermentations at winery scale

To validate the results obtained in laboratory, the two best performing couples, using a co-inoculation strategy with *S. cerevisiae* EC1118, were selected to ferment in winery in a traditional fermentation-maceration red winemaking. Pure fermentation using the *S. cerevisiae* stain was performed as control. Fig. 4 shows the population dynamics during the three different trials. In pure fermentation, *S. cerevisiae* reached the maximum population number at day 7 (8.0±0.11 Log CFU/mL) and remained stable until the end of the monitored period. This yeast showed a similar behavior in mixed fermentation with *P. kluyveri* X3-5, while in couple with *Starm. bacillaris* X2-10 it reached the maximum population number at day 4 (8.2±0.04 Log CFU/mL). In mixed fermentations, after inoculation, *P. kluyveri* X3-5 population decreased, while *Starm. bacillaris* X2-10 grew until 7.5±0.33 log CFU/mL at day 4; afterwards a decrease was observed. Other non-*Saccharomyces* yeasts declined to undetectable levels at day 7 and day 4 in *P. kluyveri* and *Starm. bacillaris* trials, respectively.

A total of 60 colonies were isolated at different stages (D0, D2, D4, D7) and molecular fingerprinting analyses revealed that all *Starm. bacillaris* isolates showed the X2-10 profile (100%), while for *P. kluyveri*, the 96.7 % of the isolates showed the profile corresponding to X3-5 strain (data not shown). Regarding *S. cerevisiae*, 164 colonies with the morphology corresponding to this species were subjected to Interdelta-PCR typing. A total of 7 different profiles were found and, among them, the 61% showed the profile corresponding to *S. cerevisiae* EC1118. While, in mixed fermentations with *Starm. bacillaris* X2-10/*S. cerevisiae* EC1118 and *P. kluyveri* X3-5/*S. cerevisiae* EC1118, the highest part of the isolates showed the same interdelta-PCR profile with the strain used (66% of the total colonies analyzed).

Chemical analyses revealed that fermentation *Starm. bacillaris* X2-10 and *S. cerevisiae* EC1118 was characterized by a faster sugar consumption, however, all trials consumed all sugars (< 2.0 g/L) at the end of the monitored period (Table 3). Regarding acetic acid, no significant differences (p>0.05) were observed among the trials, with values at about 0.27 g/L. Wines produced by *Starm. bacillaris* X2-10 and *S. cerevisiae* EC1118 contained non-significantly higher glycerol levels (11.63 g/L) compared to the other trials, while both wines with mixed cultures showed a non-significant decreasing trend for ethanol content compared to the wine fermented only with *S. cerevisiae* (14.06 - 14.10 versus 14.40% v/v, respectively). Interestingly, *S. cerevisiae* EC1118 trial showed the highest titratable acidity, followed by *Starm. bacillaris* X2-10 / *S. cerevisiae* EC1118 and *P. kluyveri* X3-5 / *S. cerevisiae* EC1118 (p<0.001).

A Principal Component Analysis (PCA) approach (Fig. 5) on volatile compounds data (Table S4), showed that the wines from *Starm. bacillaris* X2-10 / *S. cerevisiae* EC1118 fermentation strategy were well differentiated in terms of volatile characteristic from those with the pure *S. cerevisiae* EC1118 or from *P. kluyveri* X3-5 / *S. cerevisiae* EC1118 trial. The differentiation was mainly due to the principal component 1 axis (43.8% of variance explained), which is positively correlated to some higher alcohols, including isoamyl alcohol and isobutanol, despite other compounds of this class (i.e., phenylethyl alcohol) showed the opposite behavior. Regarding terpenes, geraniol and alpha-terpinolene were found in significantly highest quantities in *Starm. bacillaris* X2-10 / *S. cerevisiae* EC1118 wines. Conversely, (R)-(+)-beta-citronellol and another varietal-derived compound, namely beta-damascenone, were found with the lowest contents concentrations in *Starm. bacillaris* X2-10 / *S. cerevisiae* EC1118 with respect to *S. cerevisiae* EC1118 (-57% and -9%, respectively) or *P. kluyveri* X3-5 / *S. cerevisiae* EC1118 (-48% and -15%, respectively) wines. The fermentation trials were able to differentiate also fully significantly in terms of 2-phenylethyl acetate (highest contents for *P. kluyveri* X3-5 / *S. cerevisiae* EC1118), hexyl acetate and 2-ethyl-1-hexanol (highest contents in *S. cerevisiae* EC1118), and n-nonanoic acid and ethyl isopentyl succinate (highest contents in *Starm. bacillaris* X2-10 / *S. cerevisiae* EC1118). In addition, *S.*

cerevisiae EC1118 wines showed the highest amounts of ethyl acetate, while those produced with mixed cultures presented a significantly lower concentration.

4. Discussion

Several studies have reported the importance of grape yeast ecology and its influence on wine quality, given that grapes are considered as the main source of microorganisms that are able to inoculate the must and start the alcoholic fermentation. The aim of the present study was to explore the yeasts biodiversity of partially dehydrated Nebbiolo grapes and to select indigenous starter cultures able to modulate the chemical profile of wines destined to 'Sforzato di Valtellina'. In the grape material tested after dehydration, yeast grapes population was around 5.0 Log CFU/g. These values are higher than the yeast count usually detected in grape berries that range from 10^2 to 10^4 CFU/g (Barata, Malfeito-Ferreira, & Loureiro, 2012), but similar population numbers were previously found in withered grapes by other authors (Alessandria et al., 2013; Englezos et al., 2022). Yeast colonies were divided based on their morphotype on WLN medium, which allows a preliminary discrimination between yeast species by colony morphology and color (Pallmann et al. 2001; Wang et al., 2019), as well as by the cells' morphology using a microscope.

Molecular identification revealed that the highest part of isolated yeasts belong to the natural grapes' community, while others are more specifically found in high sugar musts, namely *C. apicola* (Tofalo et al., 2009; Perrusquıa-Luevano et al., 2019) or in grape juice concentrate or sweetened wines like *Z. bailii* (Zuehlke, Petrova, & Edwards, 2013). For most sequenced isolates an identity percentage > 98% was found. Unfortunately, isolate *M. fructicola* X2-11 showed a minor sequence identity of the D1/D2 domain to any of the type (identity percentage > 86.27%). The same problem in *Metschnikowia*-like strains was found by other authors (Cordero-Bueso et al., 2017; Binati et al., 2019), probably because some pulcherrimin-producing species including *M. fructicola* have a non-homogeneous rDNA repeat that make difficult the species assignment (Sipiczki, 2020).

With the purpose to select yeast strains able to conduct and tailor 'Sforzato di Valtellina' wine composition, isolated yeasts were subjected to a molecular typing and a physiological characterization. Furthermore, pure fermentations were conducted to select the strains with interesting oenological properties to be used as starter cultures in the next steps of the study.

Molecular typing and physiological characterization highlighted several inter- and intra-species differences (Fig. 1 and Fig S1). Regarding ethanol tolerance, the results are consistent with previous studies demonstrating that species like *Starm. bacillaris*, *Z. bailii* and *P. kluyveri* are more resistant to high ethanol concentration than *Candida* and *Metschnikowia* spp. (Binati et al., 2019; Jiang, Zhang, Feng, Ye, & Liu, 2020; Kuanyshev, Adamo, Porro, & Branduardi, 2017; Tofalo et al., 2009). Two *C. apicola* isolates were able to grow until 14% (v/v) of ethanol, as previously highlighted by Tsegaye, Tefera, Gizaw, & Abatenh (2018). A major part of the isolates was very sensitive to the presence of SO_2 but some isolates of *Starm. bacillaris*, *P. kluyveri*, and *Z. bailii* showed a high resistance to this antimicrobial compound until values as high as 150 mg/L. The selection of yeast species able to grow in grape must with medium-high concentration of ethanol and SO_2, considered as antimicrobial compounds, is of interest.

Although, several molecular technologies can be used to select strains for wine production, the use of classical methods results of fundamental importance, especially when some characteristics like enzymatic activities are mostly strain-dependent (Sidari et al., 2021). Generally, the findings of the present study are consistent with other studies conducted on wine yeast selection. The ability of wine yeasts to produce extracellular enzymes like proteases and pectinases was investigated because these enzymes are useful to prevent wine haze and facilitate wine clarification and filtration, allowing the release of the colour and more flavour-related compounds contained in the grape skin, as well as the liberation of phenolic compounds (Belda et al., 2016). As reported by other authors, pectinolytic activity is rarely found in wine-related yeasts (Sidari et al., 2021): indeed, only about the 13% of our isolates (i.e., *Starm. bacillaris* X1-5, X2-10, and Y1-1, *M. fructicola* Y1-5 and Y1-6, *C. californica* X1-12 and *H. uvarum* Y2-2) showed this ability. Proteolytic activity was mainly found among *Metschnikowia* spp. isolates, as previously reported by other studies (Binati et al., 2019; Barbosa et al., 2018). Only 6% of

the isolates (i.e., *M. fructicola* X2-11, *D. carsonii* X3-8 and *Rhodotorula* spp. X2-12 and X3-9) were found to possess *β*-glucosidase activity, in contrast to the data reported by other screening works (Ianieva & Podgorsky, 2021; Grazia et al., 2019; Belda et al., 2016; Comitini et al., 2011). Interestingly, none of the isolates of *Hanseniaspora* spp. showed this activity, even if it is considered widespread among strains belonging to this genus (Belda et al., 2016), demonstrating that this ability is mostly strain-dependent. Finally, only one isolate (*R. graminis*) was found positive for extracellular esterases. In literature it is possible to find some contradictory results, since Ianieva & Podgorsky (2021) found that a small portion of non-*Saccharomyces* yeasts possess this ability, whereas other authors (Comitini et al., 2011; Escribano et al., 2017) found this enzymatic activity to be present also in a major part of the wine strains of *P. kluyveri* and *M. pulcherrima*. Semi-quantitative tests on the H_2S production were in line with the findings of other authors for non-*Saccharomyces* yeasts (Englezos et al., 2015; Mendoza, Vega-Lopez, Fernández de Ullivarri, & Raya, 2019; Lin et al., 2020).

Recent studies highlighted various possibilities to exploit non-*Saccharomyces* yeasts as biocontrol agents, such as addition of live cells or their metabolites to inhibit or reduce the development of undesired microorganisms in vineyard and during vinification (Simonin, Alexandre, Nikolantonaki, Coelho, & Tourdot-Maréchal, 2018; Nardi, 2020; Di Gianvito, Englezos, Rantsiou, & Cocolin, 2022). With this purpose, yeasts were investigated for their ability to produce antimicrobial compounds (killer activity) or fast colonize the space (biofilm formation).. Twenty-nine isolates obtaining to different species and different clusters in the same species proved to be able to form biofilm, demonstrating that this is a strain-specific character (Cordero-Bueso et al., 2017).

A further type of biofilm was also investigated: the ability to grow on wet and semi-solid agar media, so-called mats formation. This ability was firstly observed in *S. cerevisiae* (Reynolds, 2018), but other non-*Saccharomyces* yeasts can form these flower-like growth pattern, such as *Pichia* genus (Perpetuini et al., 2018; Perpetuini, Rossetti, Battistelli, Arfelli, & Tofalo, 2021), *Kluyveromyces marxianus* (Perpetuini, Tittarelli, Suzzi, & Tofalo, 2019), *B. bruxellensis* (Dimopoulou et al., 2019), and *H. uvarum* (Coetzee, Malandra, Wolfaardt, & Vilijoen-Bloom, 2004). Our results confirmed that this phenotype is common in natural yeast populations and is a strain-specific feature (different morphologies for isolates found in different clusters of the same species) (Reynolds & Fink, 2001; Perpetuini et al., 2018; 2019).

The killer activity of *Metschinikowia* spp. isolates was already described against several wine spoilage microorganisms, including *B. bruxellensis* (Oro, Ciani, & Comitini, 2014). This activity was associated to the production of pulcherrimin, a brown-red pigment that causes the sequestration of iron ions by chelation (Sipiczki, 2020). In fact, the zone surrounding all the *Metschnikowia* isolates spots was characterized by the presence of a maroon-red halo. The killer activity was previously described for the *Candida* genus (Robledo-Leal et al., 2014), *Starm. bacillaris* (Kuchen et al., 2019; Morera, de Ovalle, & González-Pombo, 2022) as well as for *H. uvarum* and *Z. bailii* (Liu et al., 2015). For these species, this ability was associated to the presence in the cytoplasm of virus-like particles ((ds)RNA) (Schmitt & Neuhausen, 1994; Mehlomakulu, Setati & Divol, 2014; Crucitti et al., 2022).

Pure micro-fermentation trials highlighted the best oenological attitude of species, namely *Starm. bacillaris* and *Z. bailii*, that showed the highest sugar consumption, the fructophilic character and an optimal fermentation purity (Englezos et al., 2015; Cabral, Prista, Loureiro-Dias, & Leandro, 2015; Saayman & Viljoen-Bloom, 2006; Rantsiou et al., 2017a). The fructophilic character coupled with a high glycerol production was also highlighted in *C. apicola* isolates as previously reported by Gangl et al. (2018). Based on the results of molecular typing and physiological characterization, seven isolates belonging to the main non-*Saccharomyces* species investigated for wine production were chosen to conduct mixed fermentations with the commercial strain *S. cerevisiae* EC1118 in laboratory scale, due to the inability of the first to complete the fermentation process. Two different types of yeast inoculation protocols were tested, since the inoculation time is generally recognized a crucial factor, able to impact the characteristics of the wine (Roulleir Gall, Bordet, David, Schmitt-Kopplin, & Alexander, 2022).

The couple *Z. baili* X2-6 and *S. cerevisiae* EC1118 was not able to complete fermentation in co- and sequential inoculation protocols, evidencing a negative interaction between the two strains as previously described in synthetic must by Zhu, Navarro, Mas, Torija, & Beltran (2020). In line with Capece et al. (2022), it has been observed that the presence of *Z. bailii* in mixed fermentations leads to a fermentation rate lower than other non-*Saccharomyces* species, namely *Starm. bacillaris* and *H.*

uvarum. As reported in other studies (Comitini et al., 2011), non-*Saccharomyces* strains in pure fermentations were not able to consume all the sugar available confirming their lower fermentation capacity, compared to *S. cerevisiae* control. *Starm. bacillaris*, independently by the inoculation protocol applied, reached a high cells density at day 2. The cells count was lower at the 14th day in case of co-inoculum, compared to the respective sequential trials. This trend could be associated to the quicker depletion of available nutrients from the medium by *S. cerevisiae*. *P. kluyveri* and *C. apicola* were not detected at day 4, confirming their low fermentative power. On the contrary, in fermentations conducted in musts rich of sugars, *C. apicola* was present during all the fermentation phases and suggested to be an osmotolerant species (Tofalo et al., 2009). Varela, & Borneman (2017) noted the weak fermentative aptitude of *Pichia* species due to the subtraction of oxygen and increased ethanol yet at the first steps.

Independently by the isolate used, *Starm. bacillaris* and *S. cerevisiae* mixed fermentations showed a reduced acetic acid and ethanol production and an increased glycerol concentration with respect to other mixed fermentations tested, particularly in case of sequential inoculum, as previously reported by Rantsiou et al. (2012). This couple demonstrated the highest potentiality to reduce the ethanol production, especially for *Starm. bacillaris* X2-10. This strain-specific ability was demonstrated by Englezos et al. (2016) and it was the main selective factor (combined with killer activity) that led to the selection of this strain for the winery scale fermentations. In addition, mixed fermentation with *P. kluyveri* was further tested in winery scale conditions due to its confirmed ability to potentially increase flavour-like compounds of wine, glycerol production, and capacity to rapidly colonize the grape must. Both couples were inoculated with *S. cerevisiae* EC1118 using a co-inoculation approach.

In all fermentations, the inoculated starter cultures conducted the fermentation as revealed by molecular fingerprinting analysis. To be noted a faster reduction of fructose in mixed fermentations with *Starm. bacillaris* and *S. cerevisiae* was registered as previously demonstrated by Englezos et al. (2015). The ethanol production in both mixed fermentations resulted similar and slightly (p>0.05) lower than control one with *S. cerevisiae* EC1118. The fermentation *Starm. bacillaris* and *S. cerevisiae* showed a slightly increased trend in the production of glycerol, presumably induced by the peculiar metabolism of *Starm. bacillaris*. This feature was found to be a species-specific characteristic of this yeast (Englezos, Giacosa, Rantsiou, Rolle, & Cocolin, 2017). The acetic acid content in winery fermentations was equal to those reached by *S. cerevisiae* EC1118 in pure culture. These low values reached in winery fermentations together with the low ethyl acetate values found in both mixed fermentations compared to *S. cerevisiae* EC1118 in pure culture were considered of interest confirming the optimal performance of these autochthonous yeasts.

Conclusion

In the present paper, an in-depth characterization of non-*Saccharomyces* yeasts found in partially dehydrated Nebbiolo grapes destined as starter culture for 'Sforzato di Valtellina' wine production. The findings reported in this study highlight the importance of the characterization of the autochthonous yeasts able to modulate the chemical profile of wines, and in particular the promising role of non-*Saccharomyces* yeasts, namely *Starm. bacillaris* and *P. kluyveri*, to tailor territorial wines composition, like 'Sforzato di Valtellina'. In the same context, the use of selected yeasts with biocontrol ability as those characterized in this study, could help to enhance wine quality, and control the presence of unwanted microorganisms. Therefore, the use of strains with the double role of starter and bioprotective agent could help to respond to current challenges for the winemaking industry as the demand for sustainable wines is in continuous increase. The results obtained will be useful in the development of starter cultures for partially dehydrated wines with specific characteristics.

CRediT authorship contribution

Gabriele Serafino: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. Paola Di Gianvito: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. Simone Giacosa: Formal analysis, Methodology, Project administration, Resources, Writing – review & editing. Domen Skrab: Investigation, Methodology, Writing – review & editing. Luca Cocolin: Conceptualization, Resources, Writing – review & editing. Vasileios Englezos: Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing. Kalliopi Rantsiou: Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

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Table 1
Distribution of indigenous yeast species in Nebbiolo partially dehydrated grapes (2020 vintage).

Species	No. of isolates	Proportion (%)
Metschnikowia fructicola	9	14.5
Metschnikowia pulcherrima	4	6.5
Starmerella bacillaris	8	12.9
Hanseniaspora uvarum	17	27.4
Candida californica	5	8.1
Candida apicola	4	6.5
Zygosaccharomyces bailii	5	8.1
Rhodotorula nothofagi	1	1.6
Rhodotorula graminis	1	1.6
Pichia kluyveri	5	8.1
Debaryomyces carsonii	1	1.6
Zygoascus meyerae	1	1.6
Zygoascus hellenicus	1	1.6

Table 2
Chemical profile of wines obtained from laboratory-scale fermentations

Yeast strain	Glucose (g/L)	Fructose (g/L)	Residual sugars (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Fermentation purity	Ygly	Yeth
S. cerevisiae EC1118	0.23 A	0.66 A	0.89 A	0.31 B	11.93 C	16.7 E	0.013 AB	0.037 A	0.061 C
Starm. bacillaris X2-10	106.21 C	0.76 A	106.97 B	0.54 D	17.33 DE	9.80 D	0.045 E	0.093 EF	0.058 B
Starm. bacillaris Y1-2	108.28 C	0.52 A	108.80 B	0.51 D	16.44 D	9.88 D	0.042 DE	0.088 DE	0.059 BC
Starm. bacillaris Y2-9	113.87 CD	0.48 A	114.35 BC	0.42 C	16.71 D	9.40 CD	0.035 CDE	0.093 EF	0.058 BC
Z. bailii X2-6	124.93 C	0.49 A	125.42 C	0.31 B	9.12 B	8.62 C	0.025 BC	0.049 B	0.057 B
C. apicola Y2-13	91.20 B	23.00 B	114.20 BC	0.38 BC	18.34 E	9.33 CD	0.031 CD	0.104 F	0.058 B
M. pulcherrima Y2-7	81.07 B	113.64 C	194.71 D	0.12 A	8.08 B	4.36 B	0.005 A	0.079 D	0.053 A
P. kluyveri X3-5	106.94 C	130.77 D	237.71 E	0.47 CD	4.2 A	2.26 A	0.172 F	0.066 C	0.059 BC
Sign.	***	***	***	***	***	***	***	***	***
Starm. bacillaris X2-10 and EC1118 (C)	0.80 A	0.88 A	1.68 A	0.30 A	11.97	16.50	0.014 A	0.038	0.061
Starm. bacillaris Y1-2 and EC1118 (C)	0.45 A	0.20 A	0.65 A	0.30 A	12.03	16.60	0.013 A	0.037	0.061
Starm. bacillaris Y2-9 and EC1118 (C)	0.35 A	0.16 A	0.51 A	0.30 A	12.13	16.70	0.013 A	0.037	0.061
Z. bailii X2-6 and EC1118 (C)	1.39 A	0.83 A	2.22 A	0.34 AB	11.87	16.58	0.015 A	0.037	0.061
C. apicola Y2-13 and EC1118 (C)	0.20 A	1.17 A	1.23 A	0.35 AB	12.22	16.58	0.015 A	0.038	0.061
M. pulcherrima Y2-7 and EC1118 (C)	0.20 A	0.66 A	0.90 A	0.58 C	11.75	16.62	0.029 A	0.036	0.061
P. kluyveri X3-5 and EC1118 (C)	0.45 A	0.63 A	1.08 A	0.59 C	12.28	16.58	0.030 A	0.038	0.061
Sign.	***	***	***	***	NS	NS	***	NS	NS
Starm. bacillaris X2-10 and EC1118 (S)	0.56 A	0.30 A	0.86 A	0.37 AB	16.94 C	16.29 CD	0.017 A	0.055 C	0.059 A
Starm. bacillaris Y1-2 and EC1118 (S)	0.74 A	0.28 A	1.02 A	0.35 A	18.23 D	16.31 CD	0.015 A	0.060 D	0.059 A

Starm. bacillaris Y2-9 and EC1118 (S)	0.69 A	0.14 A	0.83 A	0.34 A	17.23 CD	16.27 CD	0.015 A	0.056 C	0.059 A
Z. bailii X2-6 and EC1118 (S)	11.26 B	0.33 A	11.59 B	0.41 B	12.87 B	15.87 BC	0.020 BC	0.042 B	0.060 A
C. apicola Y2-13 and EC1118 (S)	0.33 A	0.17 A	0.50 A	0.41 B	13.53 B	14.22 A	0.022 C	0.043 B	0.051 A
M. pulcherrima Y2-7 and EC1118 (S)	0.89 A	0.69 B	1.58 A	0.50 C	11.38 A	15.64 B	0.026 D	0.035 A	0.057 B
P. kluyveri X3-5 and EC1118 (S)	0.70 A	0.21 B	0.91 A	0.47 C	11.05 A	16.53 D	0.023 CD	0.034 A	0.060 A
Sign.	***	***	***	***	***	***	***	***	***

Initial sugar composition: 274.27 g/L (glucose: 130.41 g/L and fructose: 143.86 g/L). The values are expressed as means. Different Upper Latin letters within the same column indicate significant differences (Sign.) between using the same inoculation protocol (Tukey-HSD test, $p < 0.05$). Sign: *** and NS indicate significance at $p < 0.001$ and no significant differences, respectively. Ygly (glycerol yield): glycerol/sugar consumption; Yeth (ethanol yield): ethanol/sugar consumption; Fermentation purity: acetic acid/ethanol.

Table 3
Chemical profile of wines obtained in winery.

Compound	***Saccharomyces cerevisiae* EC1118**	***Starmerella bacillaris* X2-10/ *Saccharomyces cerevisiae* EC1118**	***Pichia kluyveri* X3-5/ *Saccharomyces cerevisiae* EC1118**	**Sign**
Residual sugars (g/L)	< 2.0	< 2.0	< 2.0	-
Acetic acid (g/L)	0.27 ± 0.02	0.27 ± 0.01	0.27 ± 0.02	NS
Glycerol (g/L)	11.17 ± 0.30	11.63 ± 0.18	11.11 ± 0.30	NS
Ethanol (% v/v)	14.40 ± 0.07	14.06 ± 0.21	14.10 ± 0.13	NS
Fermentation purity	0.018 ± 0.002	0.019 ± 0.001	0.019 ± 0.001	NS
Ygly - Glycerol Yield	0.043 ± 0.001A	0.045 ± 0.003B	0.043 ± 0.001A	***
Yeth - Ethanol Yield	0.056 ± 0.002	0.054 ± 0.01	0.054 ± 0.001	NS
pH	3.51 ± 0.01A	3.54 ± 0.02B	3.51 ± 0.01A	*
Titratable acidity (g/L)	9.00 ± 0.07C	8.34 ± 0.14A	8.70 ± 0.09B	***

Initial sugar composition: 256 g/L (glucose: 125 g/L and fructose: 131 g/L). All data are expressed as an average value of three independent experiments ± standard deviation. Different Latin letters within the same row indicate significant differences among the applied inoculation protocols, according to the Tukey-HSD test ($p < 0.05$). nd: not determinable. Sign: *, *** and NS indicate significance at $p < 0.05$, $p < 0.001$ and not significant, respectively. Ygly (glycerol yield): glycerol/sugar consumption; Yeth (ethanol yield): ethanol/sugar consumption; Fermentation purity: acetic acid/ethanol.

Figure captions

Fig. 1. Heatmap of the physiological and technological results of yeast isolates. Each column of the heatmap represents each parameter taken in consideration in this study. For each parameter, data were normalized, and hierarchical clustering was done based on Euclidean distance. The color scheme from blue to red represents the normalized value from low to high, respectively.

Fig. 2. Population dynamics of laboratory-scale fermentations. Pure, co-inoculation and sequential inoculation of selected non-*Saccharomyces* (white circle) and commercial *Saccharomyces cerevisiae* EC1118 (black circle). Error bars at each point represent standard deviations.

Fig. 3. Population dynamics of laboratory scale fermentations. Pure, co-inoculation and sequential of selected non-*Saccharomyces* (white circle) and commercial *Saccharomyces cerevisiae* EC1118 (black circle). Error bars at each point represent standard deviations.

Fig. 4. Population dynamics during pure and co-inoculation of selected non-*Saccharomyces* (white circle) and commercial *Saccharomyces cerevisiae* EC1118 (black circle); autochthonous non-*Saccharomyces* (white rhombus)- and metabolites –glucose (black square), fructose (white square), glycerol (black triangle), ethanol (white triangle)- dynamics of winery fermentations. Error bars at each point represent standard deviations.

Fig. 5. Principal Component Analysis of volatile compounds data of winery fermentations: loadings plot of each variable (a) and individuals plot (b).

Figures

Fig. 1.

Fig. 2.

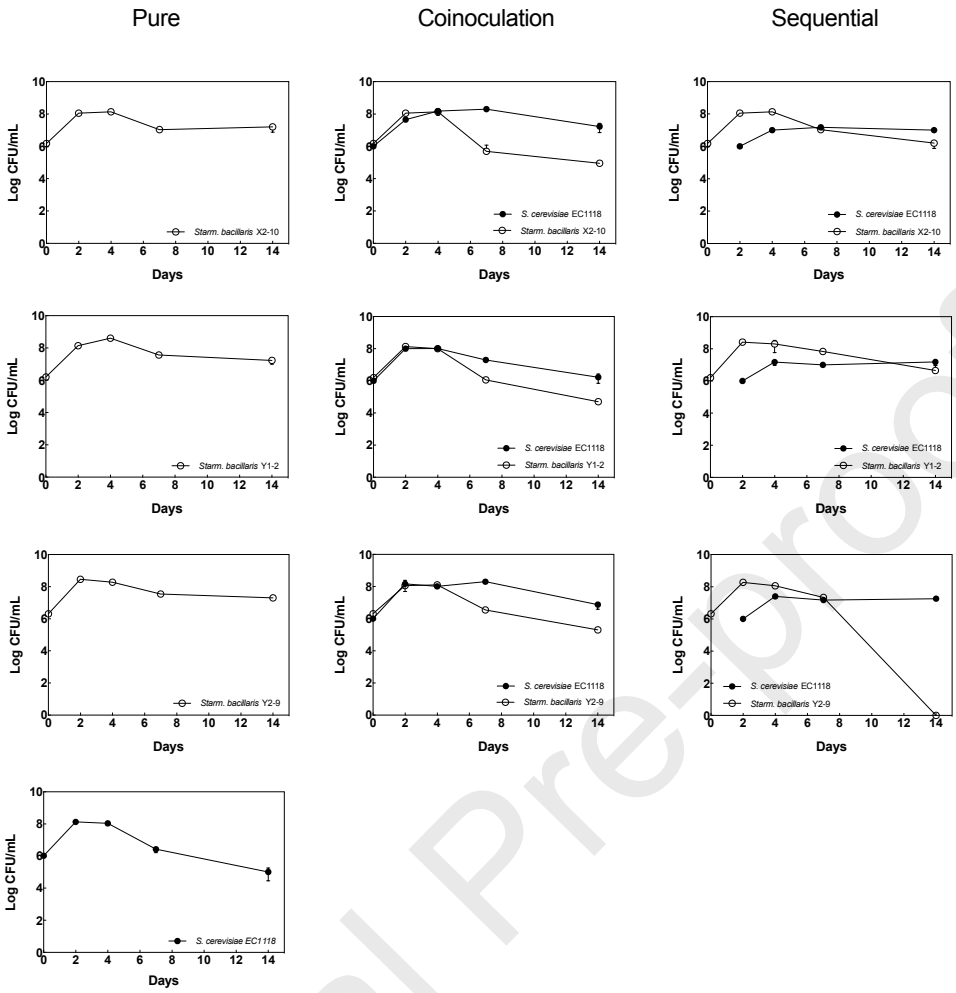


Fig. 3.

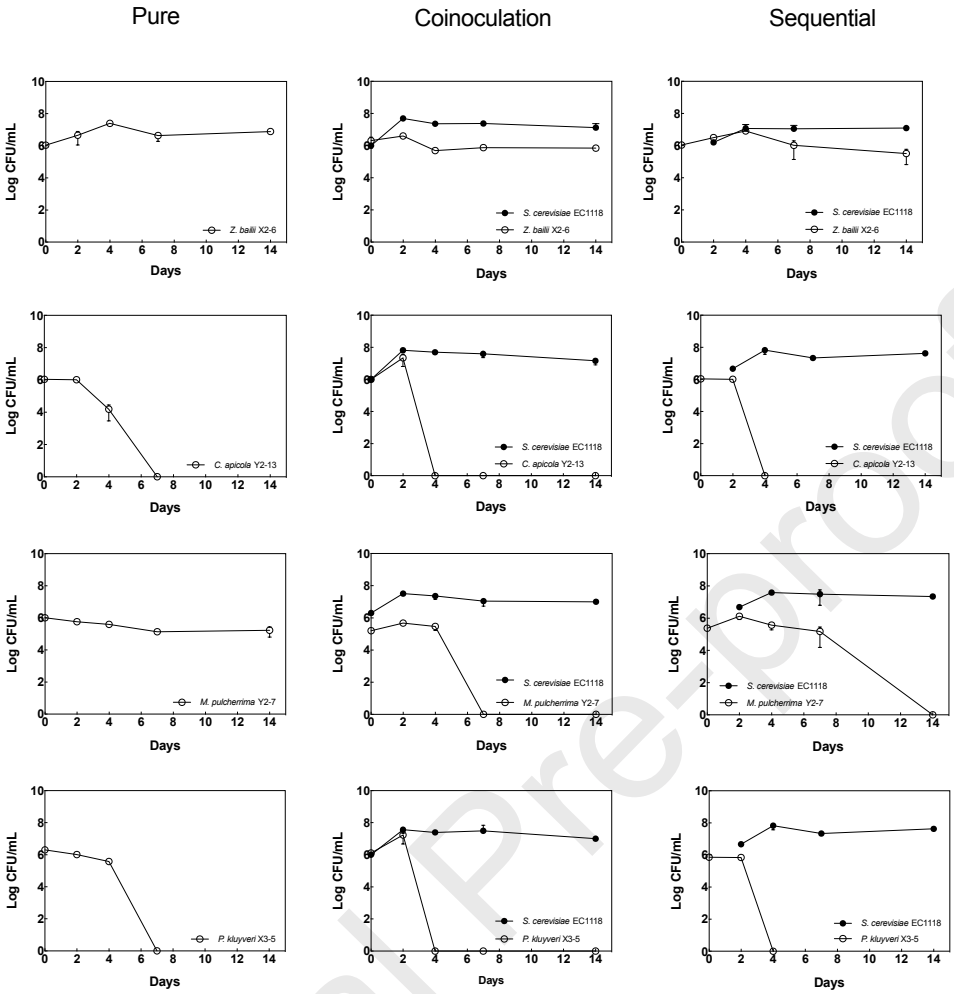


Fig. 4.

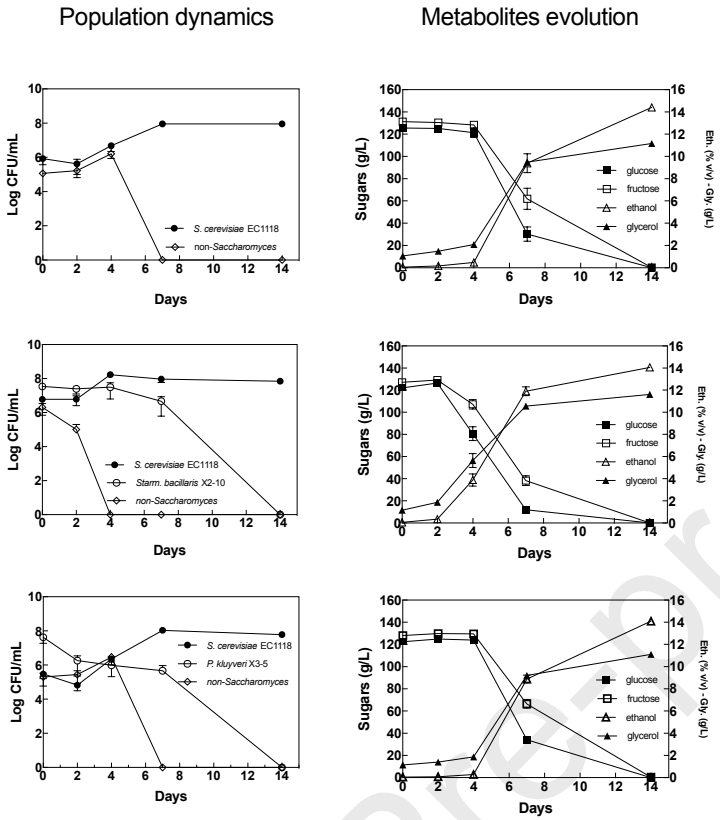
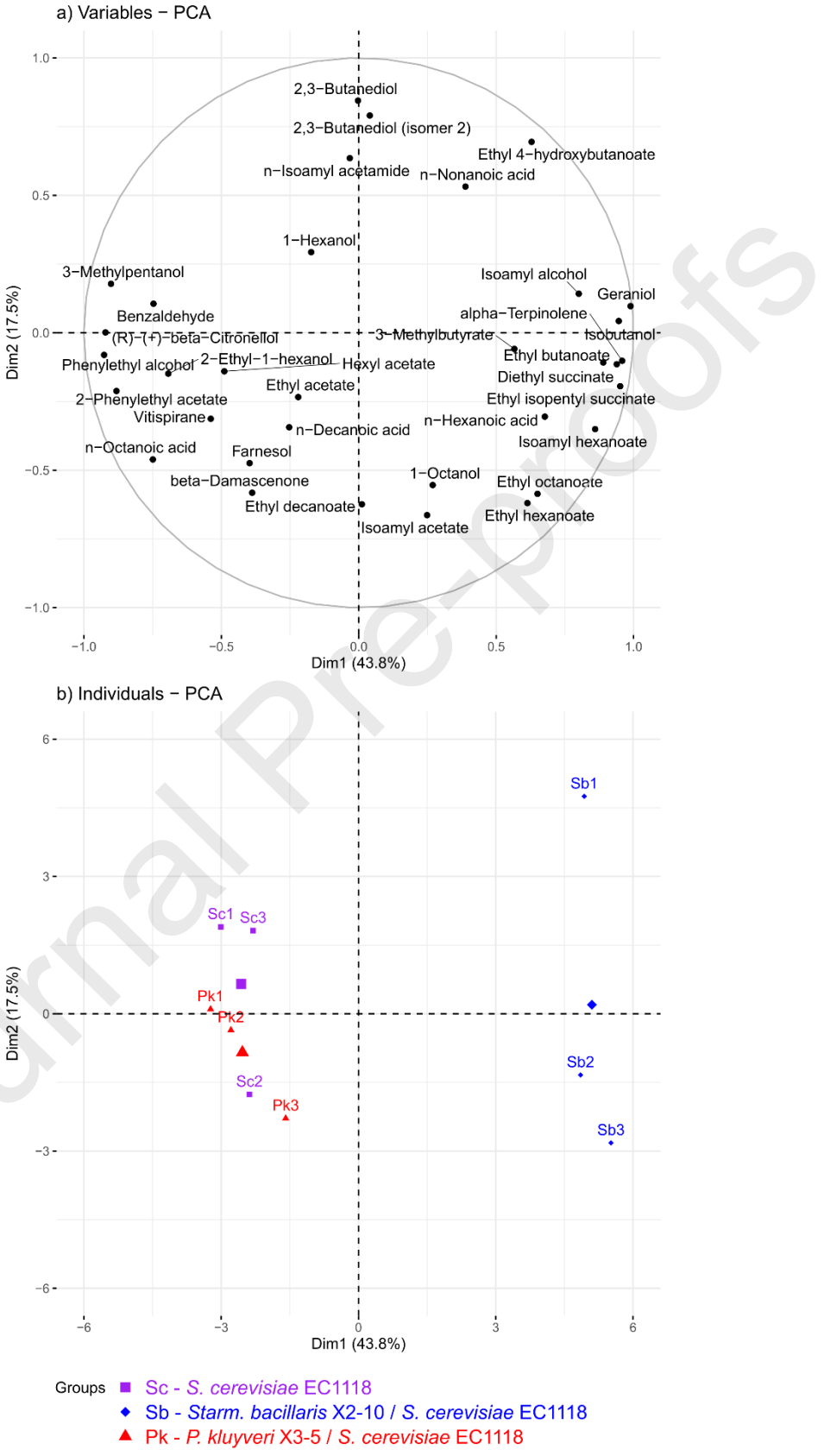


Fig. 5.



CRediT authorship contribution

Gabriele Serafino: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. Paola Di Gianvito: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. Simone Giacosa: Formal analysis, Methodology, Project administration, Resources, Writing – review & editing. Domen Skrab: Investigation, Methodology, Writing – review & editing. Luca Cocolin: Conceptualization, Resources, Writing – review & editing. Vasileios Englezos: Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing. Kalliopi Rantsiou: Conceptualization, Resources, Supervision, Writing – review & editing.

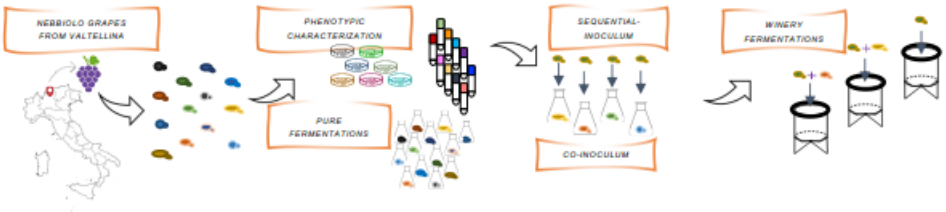
Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Sixty-two non-*Saccharomyces* yeasts were isolated from partially dehydrated grapes
- Great inter and intra-species variability was observed among isolates
- The potential use of *Starm. bacillaris* and *P. kluyveri* as starters was investigated
- Strains contribution on chemical and aroma profile of Sforzato wine was confirmed



YEAST ISOLATION

CHARACTERIZATION OF non-*Saccharomyces* YEASTS

SEQUENTIALLY- & CO-INOCULATED LAB-FERMENTATIONS

WINERY FERMENTATIONS

MICROBIOLOGICAL, CHEMICAL, AND VOLATILE COMPOUNDS ANALYSIS