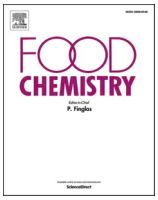
Food Chemistry 440 (2024) 138311

Contents lists available at ScienceDirect

Food Chemistry

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



Interactions between *Starmerella bacillaris* and *Saccharomyces cerevisiae* during sequential fermentations influence the release of yeast mannoproteins and impact the protein stability of an unstable wine

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ARTICLE INFO

Keywords:
Haze formation
Non-*Saccharomyces* yeasts
Glucanase
Scw4p
Mannoproteins
Proteomics

ABSTRACT

Wine protein haze formation is a problem due to grape proteins aggregation during wine storage. The cell wall components of wine yeasts, particularly high molecular weight mannoproteins, have a protective effect against haze formation, although their involvement remains poorly understood. This study aimed at characterizing glycosylated proteins released by *Starmerella bacillaris* and *Saccharomyces cerevisiae* during single and sequential fermentations in a synthetic must, and testing their impact on wine protein stability. Mannoproteins-rich extracts from sequential fermentations showed an increase in the low MW polysaccharide fraction and, when added to an unstable wine, had a greater effect on protein stability than *S. cerevisiae* extracts. Shotgun proteomics approaches revealed that the identified cell wall proteins exclusively found in sequential fermentations were produced by both *S. bacillaris* (MKC7, ENG1) and *S. cerevisiae* (Bgl2p). Moreover, sequential fermentations significantly increased the expression of Scw4p and 1,3-beta-glucanosyltransferase (GAS5), produced by *S. cerevisiae*. Finally, some of the key proteins identified might play a positive role in increasing wine protein stability.

1. Introduction

The presence of heat-unstable proteins can lead to haze formation in white wines, a visual defect mainly driven by unfolding, self-aggregation, flocculation, and combined cross-linking of grape pathogenesis-related (PR) proteins, such as thaumatin-like proteins (TLPs) and chitinases (CHIs) during the wine storage process (Van Sluyter et al., 2015). The most used method to prevent haze formation is the addition of bentonite, a negatively charged and insoluble clay that binds to proteins through electrostatic interactions. However, using this non-renewable compound has numerous disadvantages for the final wine and can negatively affect its quality (Silva-Barbieri et al., 2022).

Numerous alternatives to replace bentonite have been proposed in recent years, with additives consisting of compounds, such as yeast cell wall extracts, able to prevent or delay the destabilization process of grape proteins, or subtractive approaches aiming at removing the unstable proteins from the wine, such as fining with chitin, chitosan, grape seeds extracts, carrageenan, zeolites and magnetic nanoparticles or by enzymatic degradation and ultrafiltration (Silva-Barbieri et al., 2022). Specifically, yeast cell wall compounds, mainly high molecular weight mannoproteins have been demonstrated to be protective colloids, reducing the particle size of the aggregated proteins, and to have stabilizing effects on protein aggregation (Dufrechou, Doco, Poncet-Legrand, Sauvage, & Vernhet, 2015). The first evidence of mannoproteins protection ability against haze was demonstrated by Waters and colleagues that identified a high molecular weight mannoprotein fraction derived from yeast cell wall in wine, referred to as haze protective factor (HPF) (Waters, Wallace, Tate, & Williams, 1993; Waters, Pellerin, & Brillouet, 1994). In *S. cerevisiae*, two distinct haze protective mannoproteins, named Hpf1p and Hpf2p (Pst1p), have been characterized (Brown et al., 2007). Additionally, an invertase fragment from *S. cerevisiae*, isolated from the yeast cell wall, demonstrated to have the same protective effect (Ledoux, Dulau, & Dubourdieu, 1992; Moine-Ledoux & Dubourdieu, 1999).

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https://doi.org/10.1016/j.foodchem.2023.138311
Received 3 April 2023; Received in revised form 1 November 2023; Accepted 26 December 2023
Available online 28 December 2023

Non-*Saccharomyces* oenological yeasts, such as *Schizosaccharomyces japonicus*, *Starmerella bacillaris* (formerly *Candida zemplinina*), *Pichia fermentans*, *Metschnikowia pulcherrima*, *Saccharomycodes ludwigii*, *Lachancea thermotolerans* and *Torulaspora delbrueckii* have received attention due to their ability to produce and release mannoproteins during alcoholic fermentation (Domizio, Liu, Bisson, & Barile, 2014; Giovani, Rosi, & Bertuccioli, 2012; Millarini et al., 2020; Snyman, Mekoue Nguela, Sieczkowski, Marangon, & Divol, 2021). Studies have demonstrated that the combined use of *S. cerevisiae* and non-*Saccharomyces* yeasts during wine alcoholic fermentation can also enhance mannoproteins secretion into wine and consequently improve protein stability and prevent haze formation (González-Royo et al., 2015; Moreira et al., 2022).

S. bacillaris is a non-*Saccharomyces* yeast commonly found on the grape surface and in oenological environments (Csoma & Sipiczki, 2008; Drumonde-Neves, Franco-Duarte, Lima, Schuller, & Pais, 2017; Varela & Borneman, 2017). This species tolerates low temperatures, can grow at high sugar concentrations, shows a strong fructophilic character (it preferably consumes fructose than glucose), and produces high concentrations of glycerol, low ethanol, and moderate volatile acidity (Nadai, Fernandes Lemos, Favaron, Giacomini, & Corich, 2018). In recent years, several genomes of *S. bacillaris* strains have been sequenced, and a whole genome comparison of two *S. bacillaris* strains with other yeasts identified genes responsible for technologically relevant properties of yeasts in winemaking (Lemos Junior et al., 2018).

In our previous research, we demonstrated that the presence of *S. bacillaris* in a sequential alcoholic fermentation with *S. cerevisiae*, compared to *S. cerevisiae* single strain fermentation, reduced the turbidity in three white wines with different levels of protein instability (Moreira et al., 2022).

However, the interaction of *S. bacillaris* with *S. cerevisiae,* as well as the proteome of glycosylated proteins from yeast cell wall components, especially mannoproteins released by yeasts into the wine and their impact on wine protein stability, are still unclear. Therefore, in this study, glycosylated proteins released by yeasts after *S. bacillaris* – *S. cerevisiae* sequential fermentations were isolated, purified and molecular weights (MW) of their polysaccharides were characterized. These extracts were tested to evaluate their effects on the protein stability of an unstable white wine, together with those from single-strain fermentations. To further investigate *S. bacillaris-S. cerevisiae* interactions, proteomic analysis on purified extracts was performed and the released glycosylated proteins were identified.

2. Material and methods

2.1. Yeast strains

The non-*Saccharomyces* yeast strains *Starmerella bacillaris* FRI751 and PAS13 were isolated from fermenting must obtained from dried Raboso Piave grape cultivated in the Bagnoli PDO (Protected Denomination of Origin) area in Northeast Italy (Lemos Junior et al., 2017a, Lemos Junior et al., 2017b). *Saccharomyces cerevisiae* EC1118 strain was obtained from a commercial yeast supplier (Lallemand Inc., Montreal, Canada).

2.2. Pilot-scale fermentation trials

Precultures of each strain were prepared as follow: each yeast colony was isolated in a YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) and it was pre-inoculated in 10 mL of YPD broth. After 30 h of incubation at 30 °C, the cell concentration was determined by flow cytometry count using a CyFlow SL flow cytometer (Partec, Münster, Germany), following the manufacturer's instructions. Pilot-scale fermentations were performed as follow: an aliquot of each yeast culture was used to inoculate 2-liter capacity bottles, fitted with closures that enabled the CO_2 to escape and the sampling procedures, filled with 2 L of modified MS300, a synthetic polysaccharide-free must, that contained 110 g/L of both glucose and fructose (Bely, Sablayrolles, & Barre, 1990), at pH 3.2. A total of five pilot-scale fermentation trials were run: three single-strain fermentations (one *S. cerevisiae* EC1118, as starter, and two *S. bacillaris* FRI751 and PAS13), and two sequential fermentations (*S. bacillaris* strains and *S. cerevisiae,* SEQ-FRI751 and SEQ-PAS13) **(Supplementary Table 1)**. The inoculum concentration of each yeast was 2×10^6 cells/mL. In sequential fermentations, *S. cerevisiae* EC1118 was added 48 h after the inoculum of *S. bacillaris*. The fermentations were kept at 20 °C and shaken daily with a magnetic stirrer, for 2 min. Production of CO_2 was monitored by weighing the bottles twice a day and calculating the weight loss for each culture (**Supplementary** Fig. 1). EC1118, SEQ-FRI751 and SEQ-PAS13 fermentations were stopped when the weight loss was lower than 1 g in the last 24 h. PAS13 and FRI751 fermentations were stopped at the same time of the corresponding sequential fermentation (SEQ-PAS13 and SEQ-FRI751).

The concentrations of glucose, fructose, glycerol and ethanol were determined by HPLC as described by Lemos Junior (Lemos Junior et al., 2019). The concentrations, expressed as g/L, were calculated by using calibration curves of the individual compounds, and peak areas were determined by the Waters Breeze 2 software (Waters, Milford, MA, USA).

2.3. Purification of yeast polysaccharides released in the medium

At the end of the fermentation, each synthetic wine was centrifuged at 6000 x g, 20 min (Avanti JXN-26– Beckman Coulter), and the volume of the wine supernatant (approximately 1.9 L) was reduced by freeze drying (Freeze Dryer: Coolsafe 95/55-80LaboGene, Allerød, Denmark) for 48 h. The freeze-dried supernatant yield was 10 %. The dried supernatant was resuspended in 100–150 mL of Milli-Q water. Then, the samples were dialyzed with a 3.5 kDa MWCO cellulose tubular membrane (Cellu Step T1) against Milli-Q water at 4 ℃ for 48 h to obtain protein concentration and remove sugars and salts. Afterward, the dialyzed samples were freeze-dried again. Thirty mg of powder were dissolved into 50 mL Milli-Q water, vortexed, and filtered (0.45 μm acetate cellulose membranes) in order to clean the samples, that were loaded into a Concanavalin A Sepharose™ 4B column (Cytiva, Sweden; Lot 10299547) fitted on a Fast Protein Liquid Chromatography (FPLC, AKTA purifier, UPC10, serial no: 1505367, Sweden). Briefly, the column was regenerated for re-use between each sample by washing the medium intercalary with two regeneration buffers: 20 mM Tris-HCl (pH 8.5) and 0.1 M acetate buffer (pH 4.5), both containing 0.5 M NaCl, with 180 mL to each buffer, this cycle was repeated three times at a flowrate of 0.5 mL/min. Then, the column re-equilibration was performed with binding buffer (20 mM Tris-HCl buffer at pH 7.4 containing 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$). Then, 50 mL of resuspended samples were injected into the column, and the extracts were recovered by elution buffer (Tris-HCl pH 7.4, 0.2 M of methyl α-D-mannopyranoside). The eluted fractions were collected according to the corresponding peaks of elution by UV detection. Subsequently, the samples were dialyzed by a cellulose tubular membrane 3.5 kDa MWCO, to remove salts present in the elution buffer, and freeze-dried (**Supplementary** Fig. 2). The final weight of the freeze-dried samples (mannoprotein-rich extracts) was approximately 40 mg and 30 mg for the single fermentations (FRI751 and PAS13, respectively) and 80 mg for EC1118, and sequential fermentations (SEQ-FRI751 and SEQ-PAS13). The samples were stored at −80 °C until further use.

2.4. Polysaccharides quantification and MW distribution

High-resolution size-exclusion chromatography by (HRSEC) was used to quantify and identify the molecular weight distribution of the polysaccharides in the extracts using a previously described method (De Iseppi et al., 2021). Briefly, 10 μg of the freeze-dried extracts was resuspended with 1 mL of 50 mM ammonium formate buffer (Merk,

Darmstadt, Germany), vortexed, and centrifugation at 14,000 x g for 10 min (Mikro 200/R, Hettich) was performed to clean the samples before HPLC injection. Ten μL of the supernatant were injected into an HPLC Agilent 1260 series II quaternary pump LC (Agilent Technologies) equipped with both Diode Array Detector (DAD) and Refractive Index Detection (RID) detectors. The separation was made in isocratic mode using a PL-Aquagel-OH 40 column (Agilent), with a constant flow of 0.6 mL/min for 35 min in the mobile phase (ammonium formate buffer 50 mM). The temperature of the RID cell was kept at 35 °C. The molecular weight (MW) distribution of the extracts was determined by an analytical standard set with 10 Pullulan standards (SIGMA-ALDRICH) at MW ranging between 342 and 805,000 Da, and a quantitative calibration curve starting with 2000 mg/L of dextrans and pectins (Da).

To determine the total polysaccharide concentration in the extracts, the sum of peak fractions areas according to MW was considered. High MW fraction ranged between 1110 KDa and 180 kDa (Retention Time 11.243 min–13.393 min), Medium MW from 180 KDa to 40 kDa (13.393 min–15.179 min) and Low MW comprised between 40 kDa (15.179 min) and 7.5 kDa (17.167 min). The sum of High, Medium, and Low MW fractions was expressed as mg/g (**Supplementary** Fig. 3).

2.5. Wine heat-stability test

The effects of the mannoprotein-rich extracts from each fermentation trial (EC1118, FRI751, PAS13, SEQ-FRI751, and SEQ-PAS13) as well as the mixture of mannoprotein extracts from single fermentation of *S. bacillaris* (FRI751 or PAS13) plus *S. cerevisiae* (EC1118) were evaluated on wine protein stability. Each extract was added at a final concentration of 50 mg/L to an unstable ($\Delta Absorbance_{540}$ of 0.12, after a heat test, Supplementary **Table 2**) white wine (Vernaccia di San Gimignano, Tuscany, 2019, 12 % ethanol, 3.28 pH, 0.86 g/L residual sugar), and their impact on protein stability was evaluated by heat test. To reach the 50 mg/L final concentration, the total polysaccharides quantification by means of HRSEC analysis was used. The heat stability of the samples was determined on a 96-well microplate using 200 μL of Vernaccia wine, each added with 12.5 μL of a stock solution from each yeast extract. The absorbance at 540 nm was measured before samples were heated at 80 °C for 2 h, cooled at 4 °C for 1 h, and kept at room temperature for 1 h before the final measuring of the Abs at 540 nm to calculate the difference between the final and the initial absorbance values (Pocock & Waters, 2006).

2.6. Proteomic analyses

2.6.1. Protein extraction for LC-MS/MS

The proteomic samples were performed in triplicate. Lyophilized mannoproteins-rich extracts were resuspended into a 100 μL buffer (100 mM Tris–HCl, pH 7.5, 4 % SDS, 10 mM DTT). The samples were sonicated at 240 W, without heating, for 10 min, and the protein concentrations were measured and adjusted to 0.3 mg/L of protein (NanoDrop One – Thermo Scientific, Madison, WI, USA). The protein extraction was performed according to the suspension trapping (STrap) protocol with some modifications (Zougman, Selby, & Banks, 2014). Briefly, 18 μL suspension (30 μg of protein into lysis buffer) of each sample was incubated at 95 °C for 10 min (SimpliAmpTM Thermal Cycler, Applied Biosystems, Life Technologies). Two μL Iodoacetamide (50 mM) was added, and the samples were incubated for 20 min at room temperature in the dark, with subsequent acidification with 2.2 μL phosphoric acid (12.15 %). Then, 140 μL STrap solution (1:10 methanol, 100 mM Tris-HCl, pH 7.1) was added to the pipette-tip columns (200 μL tips, Eppendorf Research plus G-3-pack) of 2 x C18 resin (2215 – C18, Octadecyl, 47 mm, Empore, CDS, Oxford, PA, USA), plus 12 x micro-quartz fibre paper (Art. No: 420050, grade: MK 360, size: 30 mm, Lot: 3717, Ahlstrom Munksjö, Helsinki, Finland). The acidified samples were transferred into the C18-quartz tips column. All centrifugations were performed at 4000 x g for 15 min (VWR, Micro Star 17, Germany), and for each round of centrifugation the tips were rotated at 180°. This process was repeated until the samples or added reagents were loaded. The column was washed with 50 μL STrap solution, with the subsequent addition of 100 μL (NH4) HCO3 (500 mM). After that, 13 μL trypsin (proteomics grade, REF 03708985001, SigmaAldrich) was used for the digestion, and the column was centrifuged at 4000 x g, for 10 *sec*, with subsequent incubation at 47 °C for 1 h (Grant-bio, PHMT, Serial NO: B10 08 B0087, England, UK). The digestion was stopped with 50 μL TFA 0.5 %, with subsequent 100 μL TFA 0.1 %. Finally, the samples were eluted with 50 μL 80 % CAN/0.1 % TFA, and the peptides were dried in a SpeedVac concentrator (Model no. SVC-100H, Savant, Farmingdale, NY, USA) at 30 °C, 40 min. The samples were stored at −20 °C until LC-MS/MS analysis.

2.6.2. Label-free LC-MS/MS shotgun proteomics

The dried peptides were resuspended into a loading buffer (2 % ACN, 0.05 % TFA), and sonicated at 240 W, without heating, for 10 min (Bransonic 2510E, VWR, Norway). Then, the concentration was adjusted to 0.2 mg/mL before injection into a coupling a nano ultra-performance liquid chromatography (UPLC, nanoElute, Bruker Daltonics, Bremen, Germany) to a trapped ion mobility spectrometry/quadrupole time of flight mass spectrometer (timsTOF Pro, Bruker). The peptides were separated by a PepSep Reprosil C18 reverse-phase (1.5 μm, 100 Å) 25 cm X 75 μm analytical column coupled to a ZDV Sprayer (Bruker). The column temperature was kept at 50 °C using the integrated oven. Equilibration of the column was performed before the samples were loaded (equilibration pressure 800 bar). The flow rate was set to 300 nL/min, and the samples were separated using a solvent gradient from 2 % to 25 % solvent B over 70 min, and to 37 % over 9 min. The solvent composition was increased to 95 % solvent B over 10 min and maintained at that level for an additional 10 min. In total, a run time of 99 min was used to separate the peptides. Solvent A consisted of 0.1 % formic acid diluted in MilliQ water. Solvent B was 99.9 % acetonitrile and 0.1 % formic acid. The timsTOF Pro was run in positive ion data-dependent acquisition PASEF mode with the control software Compass Hystar version 5.1.8.1 and timsControl version 1.1.19 68. The acquisition mass range was set to 100–1700 *m/z*. The TIMS settings were: 1/K0 Start 0.85 V·s/cm2 and 1/K0 End 1.4 V·s/cm2, Ramp time 100 ms, Ramp rate 9.42 Hz, and Duty cycle 100 %. The Capillary Voltage was set at 1400 V, Dry Gas at 3.0 L/min, and Dry Temp at 180 ℃. The MS/MS settings were the following: number of PASEF ramps 10, total cycle time 0.53 *sec*, charge range 0–5, Scheduling Target Intensity 20000, Intensity Threshold 2500, active exclusion release after 0.4 min, and CID collision energy ranging from 27 to 45 eV.

2.6.3. Downstream proteomics data processing, analysis, and visualization

The raw LC-MS/MS data were processed using MaxQuant software 2.0.3.1 with the label-free quantification (LFQ) algorithm (false discovery rates were set to 0.01 at peptide and protein levels) and additional results filtering (minimally two peptides were necessary for protein identification of which at least one is unique, and at least one is unmodified were performed as described previously (Cox et al., 2014). A global proteome from *S. cerevisiae* EC1118 proteome (UniProt) and *S. bacillaris* FRI751 and *S. bacillaris* PAS13 were obtained following gene annotation combining two complementary strategies, namely, Blast-KOALA (Kanehisa, Sato, & Morishima, 2016) and RPS BLAST (Lemos Junior et al., 2017a). The samples were run against a global proteome database (EC1118, FRI751, and PAS13), and the output "ProteinGroups" was used for the analyses. The samples were filtered, and the proteins identified as contaminants or reverse were excluded. The data were analyzed considering the LFQ-intensity parameter using Log2 transformed values in at least three replicas for each extract (n = 3). Perseus software (Version 2.0.3.0) was used to build the heat map according to the LFQ intensity. The analyzed proteins were, at least, present in two out of three replicates. The identification of biological processes was carried out with the UniProt database and gene ontology. Putative

proteins from *S. bacillaris* were identified running a BLAST sequence similarity search by UniProt ID (UniProt database), and the highest similarity or a reviewed protein (Swiss-Prot) was annotated. The set of shared and unique proteins among all extracts was identified by Venn Diagram. The graphics were created by Perseus (2.0.3.0) and GraphPad (version 7.0) software.

2.7. Statistical analyses

The normal distribution of polysaccharides and heat test data was tested by the Shapiro-Wilk test. The proteomic analyses carried out using LFQ-intensity data (Log2) were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The pairwise comparisons were performed by unpaired *t*-test using the GraphPad Prism software (Version 7.0). Results were expressed as mean ± standard deviation. Different letters indicate a statistical difference with a significance level of 5 %.

3. Results and discussion

3.1. Fermentation performances in synthetic must

The two *S. bacillaris* strains were used in single (FRI751 and PAS13) and sequential fermentation with *S. cerevisiae* EC1118 (SEQ-FRI751 and SEQ-PAS13). *S. cerevisiae* EC1118 fermentation was performed as a control. A synthetic polysaccharide-free must was used to avoid possible interferences from grape juice components, such as plant proteins and polysaccharides. The *S. bacillaris* single-strain fermentations were stopped when the corresponding sequential fermentations were completed. This was necessary as, due to the low fermentative capability and ethanol resistance, *S. bacillaris* was not able to transform all the sugars present in the must.

At 48 h (immediately before the addition of *S. cerevisiae* in sequential fermentations) a limited decrease in fructose concentration (around 20 % of the total) was measured in all musts inoculated with *S. bacillaris* (91.67 ± 2.06 g/L in FRI751, 94.66 ± 7.46 g/L in PAS13, 90.46 ± 0.58 g/L in SEQ-FRI751 and 88.37 ± 0.80 g/L in SEQ-PAS13), whereas glucose concentration was almost unchanged. This result was expected, and confirmed the fructophilic behavior and the limited fermentation abilities of this species. *S. cerevisiae* in single fermentation consumed both sugars, with a preference for glucose (81.50 ± 0.37 g/L glucose and 91.82 ± 0.42 g/L fructose).

SEQ-PAS13 and PAS13 fermentations lasted 17 days, while EC1118, SEQ-FRI751 and FRI751 19 days (**Supplementary** Fig. 1). At the end of the process, the sugar consumption in the sequential fermentations was like *S. cerevisiae* single-strain fermentation (Table 1). Glucose was almost completely consumed, while a limited residual fructose was measured, evidencing a slight preference towards glucose due to the presence of *S. cerevisiae*. As expected, *S. bacillaris* single fermentations showed only a partial sugar consumption, with a total residual sugar of 132.2 ± 1.3 g/L in FRI751 after 19 days and 109.8 ± 2.8 g/L in PAS13 after 17 days, with a strong preference for fructose. Because of the reduced sugar consumption, in single *S. bacillaris* fermentations the production of ethanol was low (5.39 % v/v in FRI751 and 6.10 % v/v in PAS13), while in *S. cerevisiae* single-strain fermentation and in sequential fermentations the concentrations produced were similar (12.75 % v/v in EC1118, 12.91 % v/v in SEQ-FRI751 and 12.92 % v/v in SEQ-PAS13). Regarding glycerol production, the concentration produced by *S. bacillaris*, in single and sequential fermentations (4.46 g/L in FRI751, 6.48 g/L in PAS13, 4.05 g/L in SEQ-FRI751 and 4.48 g/L in SEQ-PAS13), was significantly higher than that produced by *S. cerevisiae* single-strain fermentation (2.49 g/L).

Table 1
Glucose, fructose, glycerol, and ethanol concentrations at the end of fermentations.

Samples	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (% v/v)
EC1118	0.09 ± 0.04^{b}	4.57 ± 0.32^{bc}	2.49 ± 0.13^{c}	12.75 ± 0.02^{b}
FRI751	108.28 ± 1.75^{a}	23.95 ± 0.48^{a}	4.46 ± 0.03^{b}	5.39 ± 0.03^{c}
PAS13	108.99 ± 2.37^{a}	0.08 ± 0.03^{d}	6.48 ± 0.11^{a}	6.10 ± 0.01^{c}
SEQ-FRI751	0.11 ± 0.04^{b}	5.21 ± 0.24^{b}	4.05 ± 0.23^{b}	12.91 ± 0.02^{a}
SEQ-PAS13	0.09 ± 0.03^{b}	3.92 ± 0.30^{c}	4.48 ± 0.29^{b}	12.92 ± 0.02^{a}

Data are expressed as the average of the replicates ± standard deviations. Different letters in each column indicate significant differences between values ($p < 0.05$).

3.2. Polysaccharides' quantification and molecular weight distribution

At the end of the alcoholic fermentation, the obtained synthetic wines were purified to obtain extracts rich in polysaccharides and glycosylated proteins from the yeast cell walls without the interferences of natural grape juice. The utilization of synthetic must enabled the isolation of polysaccharides and mannoproteins that yeast released during fermentation while excluding proteins and polysaccharides originating from the grape. The polysaccharides were extracted from the synthetic wines by a purification step in Concanavalin A-Sepharose 4B column, a metalloprotein isolated from jack bean (*Canavalia ensiformis*) that binds specifically to molecules containing α-D-mannose to the sterically related residues with available C-3, C-4, or C-5 hydroxyl groups in the presence of ions Ca^{2+} and Mn^{2+}. Therefore, the target molecules for this purification step were yeast mannoproteins, even if possible small contaminations due to the potential affinity to the ConA resin of oligosaccharides fragments from yeast cell wall cannot be excluded (Wu, Lisowska, Duk, & Yang, 2009). The so obtained mannoproteins-rich extracts were subsequently characterized by HR-*SEC* to obtain information on their total polysaccharides amount and on the molecular weight distribution of polysaccharides.

The extract PAS13 showed the highest total polysaccharide content (636.4 mg/g), while SEQ-FRI751 showed the lowest (385.4 mg/g). EC1118 single fermentation (539.4 mg/g), SEQ-PAS13 (546.3 mg/g)

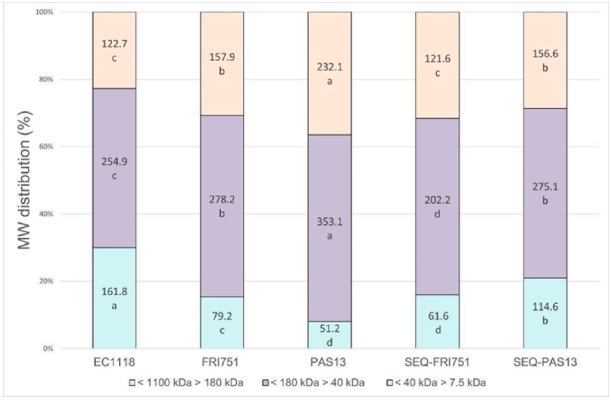

Fig. 1. Polysaccharides' quantification on the extract according to molecular weight (MW) distribution. High MW fraction (light blue bar) ranged between 1100 kDa and 180 kDa with retention time 11.243 min–13.393 min; Medium MW (purple bar): < 180 kDa (13.393 min) > 40 kDa (15.179 min); Low MW (pink bar): < 40 kDa (15.179 min) > 7.5 kDa (17.167 min). Values, expressed as mg/g, represent the average polysaccharide concentrations of each MW fraction. Different letters indicate significant differences ($p < 0.05$) within each MW fraction tested by one-way analysis of variance (ANOVA) followed by the Tukey *post-hoc* test. The largest concentration starts with the letter 'a' in the respective MW fractions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and FRI751 (515.3 mg/g) contain similar amounts of polysaccharides (Fig. 1).

Differences among the extracts were noticeable with respect to the molecular weight distribution (%). The medium MW fraction (40 kDa – 180 kDa) was the most abundant in all the extracts. EC1118 showed the highest concentration of the High MW (1110 kDa–180 kDa) fraction, corresponding to 30 % of the total, compared to the other extracts, whereas in the SEQ extracts this fraction was significantly reduced. In general, the MW fractions distribution (%) of *S. bacillaris* extracts was more similar to SEQ extracts than EC1118. On the contrary, *S. bacillaris* MW distribution (%) remained relatively constant, particularly in FRI751, suggesting a strain-related effect. Results suggested that the presence of *S. bacillaris* in sequential fermentations reduced the High MW fraction in favor to the Low MW one. This could be due to an altered mannoproteins biosynthesis caused by a direct interaction between the two strains or to a modification in the composition of the medium (the nitrogen availability is different between single and sequential fermentations). In fact, it is known that the metabolites composition of wine from co-cultures of *Saccharomyces* and non-*Saccharomyces* species did not match the assembly of two wines resulting from single yeast fermentation (Howell, Cozzolino, Bartowsky, Fleet, & Henschke, 2006; King, Swiegers, Travis, Francis, Bastian, & Pretorius, 2008). Alternatively, the modified percentage between High MW and Low MW fractions could be caused by the presence of *S. bacillaris* mannosidase or, generally, enzymes, able to hydrolyze mannose, reducing the sugar moiety length. Although no information is available for *S. bacillaris* species, genes coding for GPI-anchored membrane proteins (*DCW1* and *DFG5*) that showed putative mannosidase activity required for cell wall biosynthesis in bud formation were identified in *S. cerevisiae* (Kitagaki, Wu, Shimoi, & Ito, 2002) and cell wall mannosidase were isolated in non-*Saccharomyces* yeast such as *Candida albicans* (Mancuso et al., 2018). In general, these results demonstrated that interactions between the two yeasts species occurred during sequential fermentation and induced *S. cerevisiae* to modify the ratio among polysaccharides MW fractions.

3.2.1. *Effect of mannoproteins-rich extracts on wine stability*

Mannoproteins extracts are added to the wine to improve its quality and stability, and several commercial products have been available since their authorization (European Union Council Regulation (EC) No. 2165/2005). In this study, the mannoproteins-rich extracts were tested as an additive to improve the heat stability of a Vernaccia white wine. The extracts were added at 5 g/hL concentration, in line with the suggested addition rates of commercial mannoproteins formulations. In addition, the heat test was performed using mixed mannoproteins extracts from single strain fermentations of *S. bacillaris* and *S. cerevisiae* (**Supplementary Table 2**).

Results showed that all mannoproteins-rich extracts, alone or in combination, led to significant reductions in wine turbidity after heat tests (Fig. 2**A and 2B**), except when 30 mg/L of PAS13 extract was mixed with EC1118 at 20 mg/L (Fig. 2**B**). EC1118 extract strongly reduced wine turbidity (–32 %), while the effect of PAS13 (-16 %), and FRI751 (-10 %) was lower and comparable to those obtained using the different mixes. The extracts from *S. bacillaris* FRI751 (Fig. 2**A**) and PAS13 (Fig. 2**B**), as well as the mixed extracts, although significantly lowering the turbidity compared to the control wine, were not as efficient as EC1118 extract in reducing the wine turbidity.

When mannoproteins-rich extracts from sequential fermentations were compared with those from single fermentations, both extracts (SEQ-FRI751 and SEQ-PAS13) led to the highest reduction of turbidity (-52 % and −47 %, respectively). This result supported the findings of our previous work where *S. bacillaris* FRI751 and PAS13 were used in sequential fermentations with several grape varieties and *S. cerevisiae* EC1118 single strain fermentation was run as control (Moreira et al., 2022). The protein stability of the experimental wines was evaluated. Results indicated that the presence of *S. bacillaris* in sequential fermentations significantly increased the wine protein stability, especially in Manzoni Bianco wines where the reduction of turbidity was significantly higher than EC1118 control wine (-50 % in FRI751 wine and −45 % in PAS13 wine). With respect to other non-*Saccharomyces* yeasts, Millarini and colleagues evaluated the impact of *Schizosaccharomyces japonicus* polysaccharides on wine stability, evidencing that the addition of 600 mg/L of polysaccharides extracts was necessary to reached a 50 % total turbidity reduction in an unstable wine (Millarini et al., 2020). Domizio and colleagues assessed that *S. japonicas* released 10 to 20 times more polysaccharides than *S. cerevisiae* during single strain fermentation (Domizio, Lencioni, Calamai, Portaro, & Bisson, 2018). When *S. japonicus* was used in sequential fermentation, the amount of released polysaccharides was at an intermediate level. When the stability of the experimental wines was tested, the reduction of induced wine protein haze was correlated with the concentration of polysaccharides. In this case, the presence of *S. japonicus* produced an additive effect resulting in greater haze protection, contrary to what was observed in this study where *S. bacillaris*, increased haze protection only when employed in sequential fermentation.

Subjecting a wine to a heat test causes the denaturation and subsequent aggregation of the heat unstable proteins occurs, with the consequent appearance of visible haze. Yeast polysaccharides, mannoproteins in particular, have been reported to improve wine stability through interactions with grape proteins (Van Sluyter et al., 2015).

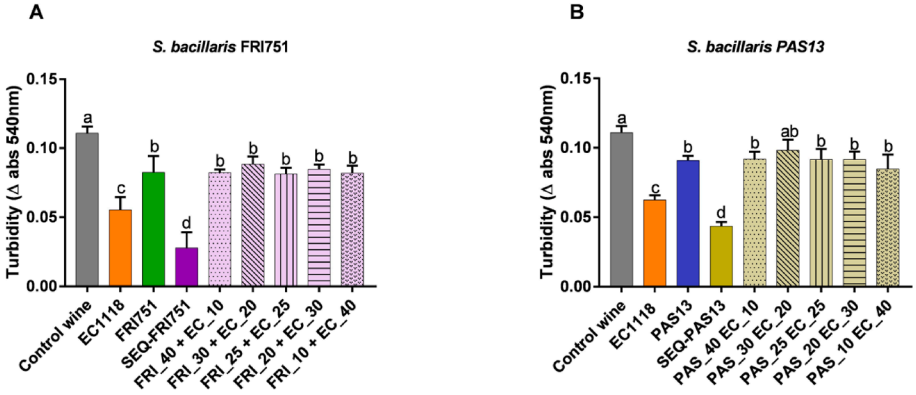

Fig. 2. Heat stability (expressed as ΔAbs) of an unstable Vernaccia wine after addition of 50 mg/L of mannoproteins-rich extracts. Extracts from single strains and sequential fermentations of *S. bacillaris* and *S. cerevisiae*, together with mixed extracts at different concentrations of *S. bacillaris* (40, 30, 25, 30 and 10 mg/L) and *S. cerevisiae* (10, 20, 25, 30, 40 mg/L) single fermentations, were tested. All the extracts were used at a final concentration of 50 mg/L. Data are reported for *S. bacillaris* FRI751 (**A**) and PAS13 (**B**), separately. Values are represented by the average (bars) and standard deviation (error bar) ($n = 3$). The difference between the averages was analyzed by one-way ANOVA and Tukey post hoc tests. Different letters indicate significant statistical differences ($p < 0.05$).

Recently, it has been suggested that yeast chitin could also be involved binding to grape chitinase and, thus, assisting in reducing the turbidity (Ndlovu, Divol, & Bauer, 2018).

Results of Fig. 2 showed that using mannoproteins-rich extracts as an additive always attenuated the haze formation compared to the control Vernaccia wine. Interestingly, the greatest stabilizing effect was achieved using SEQ extracts, alongside the limited effect of mixes and *S. bacillaris* extracts alone. This strongly indicates that yeast-to-yeast interactions occurred during sequential fermentation, and these interactions influenced SEQ extracts' polysaccharide compositions (as demonstrated by MW fraction analysis) and wine stability. Moreover, the increase of protein stability observed with the addition of SEQ extracts compared to EC1118 was not due to additive effects (higher polysaccharide concentration), as the final polysaccharides concentration, calculated on the basis of the total polysaccharide content of each extract (as measured by HRSEC analysis), was always 50 mg/L. Therefore, our finding revealed that the explanation is more complex, suggesting the role of the Low MW polysaccharide fraction (increased in sequential fermentations) and the specific characteristics of the released mannoproteins in the reduction of wine turbidity.

3.3. Shotgun proteomics of mannoproteins-rich extracts

3.3.1. Proteome profile and protein quantification

The five mannoproteins-rich extracts (after ConA purification) were submitted to shotgun proteomics for the determination of their protein composition and to demonstrate possible differences in their chemical composition. A total of 130 different proteins were detected in at least one replica sample from at least one of the five extracts. After using contaminants and reverse filters, 44 contaminants, two identified by site and one reverse sequence, were excluded, and the final number of proteins was 83. In the following proteome analysis, we considered proteins detected in three replicas (n = 3) by the LFQ-intensity parameter. By adopting this cut-off, 23 different proteins were identified at least in one of the five extracts and examined across all extracts (Table 2; **Supplementary** Fig. 4; Fig. 3**A**). According to this approach, 13 proteins were identified in FRI751 (Fig. 3**B**), 7 in PAS13 (Fig. 3**C**), 12 in EC1118 (Fig. 3**D**), 14 in SEQ-FRI751 (Fig. 3**E**), and 17 in SEQ-PAS13 (Fig. 3**F**).

The proteins' biological processes identified were mainly involved in carbohydrate metabolism and fungal-type cell wall organization (Table 1), due to the cell wall structure composed primarily of different polysaccharides requiring these proteins for self-regulation (assembly, maintenance, and secretion). The molecular functions of the proteins found in this study were hydrolase activity, hydrolyzing O-glycosyl compound, glucan *endo*-1,3-beta-D-betaglicosidase activity, and 1,3-betaglucosiltransferase activity. Yeast cell wall proteins are generally glycosylated through *N*- or O- linked glycans.

The heat-map in Supplementary Fig. 4 represents the similarity between the protein profiles obtained from the extracts. Interestingly, the SEQ profiles showed higher similarity to EC1118 than FRI751 and PAS13. These results demonstrated that the presence of *S. bacillaris* strains in the SEQ fermentation modified the proteins released during sequential fermentation (exoproteome), thus confirming the occurrence of yeast-to-yeast interactions.

Among the 23 proteins considered, three (Pst1p, Ygp1p and purine nucleoside permease) were present in all extracts. The cell wall mannoprotein Pst1p (UniProt ID: C8Z4X4) was the most abundant protein present in EC1118 and SEQ-FRI751, and the second most abundant in FRI751. On the contrary, its concentration in PAS13 was not significantly different compared to the others, and it was intermediate in SEQ-PAS13. When the Pst1p concentration was compared among the extracts, it was the highest in EC1118, and SEQ-FRI751, followed by FRI751. The lowest Pst1p concentration was observed in PAS13, and SEQ-PAS13 had an intermediate value between EC1118 and PAS13 (**Supplementary** Fig. 5**A**). Pst1p is extensively *N*- and *O*- mannosylated and belongs to the SPS2 proteins family. It is a GPI-anchored protein attached to beta-1,3-glucan through glycosylphosphatidylinositol (GPI) anchors and beta-1–6-glucan, located at the buds' surface and secreted by regenerating protoplasts. Besides, it is required during the active construction process of the cell wall but also is upregulated upon cell wall damage (Pardo et al., 2004). Interestingly, a study showed that Pst1p has haze-protective activity against the grape proteins in white wine (Brown et al., 2007). This finding strongly indicates that Pst1p contributed to reducing haze formation in this study, although it could not be responsible for improving protein stability during sequential fermentation, since the presence of *S. bacillaris* seemed to reduce its concentration (mainly when PAS13 is used).

Ygp1p (UniProt ID: C8ZG69) was one of the most abundant proteins in all the extracts (Fig. 3**B, C, D, E, F**), showing the highest concentrations in SEQ-FRI751, SEQ-PAS13 and EC1118 (Supplementary Fig. 5**B**). A study using *Metschnikowia pulcherrima* and *Lachancea thermotolerans* as starters in single strain and sequential fermentations with *S. cerevisiae* also found Ygp1p at high concentrations in the obtained wines (Mostert & Divol, 2014). Ygp1p is a cell wall assembly protein and may be involved in cellular adaptations such as nutrient deprivation-associated growth arrest before the stationary phase, in preventing biofilm formation and in cellular amino acid metabolism (Destruelle, Holzer, & Klionsky, 1994). Its synthesis is induced in response to cell wall disruptions and nutrient limitation associated with a stress response. This can explain the presence at high levels in the medium after a complete fermentation that required a prolonged cell stationary phase.

The purine nucleoside permease protein was found in all extracts at similar concentrations (Supplementary Fig. 5C). It was identified as it matched to *S. bacillaris* proteome and had 34.7 % of similarity to *Candida albicans* (UniProt ID: Q5AGW8). This protein belongs to the NUP family and is a nucleoporin responsible for transmembrane transport of adenosine and guanosine (Detke, 1998).

Additionally, unique proteins were detected in each extract. Two proteins from *S. bacillaris* found in both SEQ extracts were identified after amino acid query sequence against the protein sequence database (UniProt) and had the same sequence similarity level to aspartic proteinase MKC7, (alternative name yapsin2) (UniProt ID: P53379) in *Saccharomyces cerevisiae* (ATCC 204,508/S288c) with 33.9 % and 35.3 %, respectively. This can be explained as in *S. bacillaris* this gene is duplicated. One of the two MKC7 was detected in SEQ-FRI751, and the other one in SEQ-FRI751 and SEQ-PAS13. MKC7 is a glycosylphosphatidylinositol-linked aspartyl protease, extensively *N*-glycosylated and is involved in the organization, activation, and integrity of several cell walls proteolytic processes, and signal transduction in filamentous growth. MKC7 has a paralog, yapsin1 (Yps1p), essential in maintaining cell wall glucan homeostasis. It regulates the activity of cell wall glucanases or other hydrolytic enzymes through degradation (Komano, Rockwell, Wang, Krafft, & Fuller, 1999). This could explain the high number of different proteins in SEQ-FRI751 and SEQ-PAS13 compared to EC1118, FRI751 and PAS13. In sequential fermentations, *S. bacillaris* was stimulated to release a higher level of MKC7 that could be responsible for releasing a greater number of proteins from the yeast cell wall into the medium. Moreover, studies have demonstrated that aspartic proteases can participate in degrading grape proteins by their enzymatic activity by hydrolyzing peptide bonds (Van Sluyter et al., 2015). In particular, an aspartic protease (BcAP8) from *Botrytis cinerea* can act on the degradation of chitinase during juice fermentation, reducing haze formation in the winemaking process (Van Sluyter et al., 2013). Therefore, *S. bacillaris-S. cerevisiae* interaction, increasing MKC7 level, may have contributed to degrading chitinase and reducing wine turbidity (Van Sluyter et al., 2015). From a practical point of view, sequential fermentations with *S. bacillaris - S. cerevisiae* could be used to achieve an increased release of endogenous yeast proteases into the medium. This could lead to partial degradation of heat-unstable proteins, such as chitinases, with a consequent reduction in the bentonite required to stabilize the wine, without the need to remove these enzymes from the wine.

Table 2
Proteins identified in the mannoproteins-rich extracts.

Protein name	UniProt ID	Biological process (GO)	Organism*	Extracts
Ygp1p	C8ZG69	Cell wall assembly (May be involved in cellular adaptations prior to stationary phase)	*S. cerevisiae* EC1118	EC1118, FRI751, PAS13, SEQ-FRI751, SEQ-PAS13
Pst1p	C8Z4X4	Fungal-type cell wall organization; prevent visible wine protein haze formation	*S. cerevisiae* EC1118	EC1118, FRI751, PAS13, SEQ-FRI751, SEQ-PAS13
Purine nucleoside permease	Q5AGW8	nucleoside metabolic process; nucleoside transport	*C. albicans* (34.7 %)	EC1118, FRI751, PAS13, SEQ-FRI751, SEQ-PAS13
Glycosidase	C8Z982	Carbohydrate metabolic process; fungal-type cell wall organization	*S. cerevisiae* EC1118	EC1118, FRI751, SEQ-FRI751, SEQ-PAS13
Kre9p	C8ZB45	(1->6)-beta-D-glucan biosynthetic process; (1->6)-beta-D-glucan metabolic process; cell wall biogenesis; fungal-type cell wall organization	*S. cerevisiae* EC1118	EC1118, FRI751, SEQ-FRI751, SEQ-PAS13
Bgl2p (Glucan 1,3-beta-glucosidase)	C8Z9H5	Carbohydrate metabolic process	*S. cerevisiae* EC1118	EC1118, FRI751, SEQ-FRI751, SEQ-PAS13
Extracellular *exo*-inulinase	A5DHM6	Polysaccharide catabolic process	*Meyerozyma guilliermondii* (36.7 %)	FRI751, PAS13, SEQ-FRI751, SEQ-PAS13
1,3-beta-glucanosyltransferase	C8ZHT4	Cell wall biogenesis/degradation: (1->3)-beta-D-glucan metabolic process and biosynthetic process; fungal-type cell wall (1->3)-beta-D-glucan biosynthetic process; fungal-type cell wall organization	*S. cerevisiae* EC1118	EC1118, FRI751, SEQ-PAS13
Cwp1p	C8ZC79	Structural constituent of cell wall	*S. cerevisiae* EC1118	EC1118, FRI751, SEQ-PAS13
Scw4p (Cell wall protein like glucanase)	C8Z9H2	Carbohydrate metabolic process	*S. cerevisiae* EC1118	EC1118, SEQ-FRI751, SEQ-PAS13
ECM33	A6ZL22	Cell wall organization	*S. cerevisiae* (YJM789) (37.0 %)	FRI751, PAS13, SEQ-PAS13
Hsp150p	C8ZB60	Fungal-type cell wall organization	*S. cerevisiae*(ATCC 204508/S288c) (90 %)	FRI751, SEQ-FRI751, SEQ-PAS13
Thioredoxin	C8Z9A2	Glycerol ether metabolic process	*S. cerevisiae* EC1118	EC1118, SEQ-FRI751
Invertase	Q6BJW6	Carbohydrate metabolic process	*Debaryomyces hansenii* (ATCC 36239) (39.60 %)	FRI751, SEQ-FRI751
Saccharase	C8ZAV0	Carbohydrate metabolic process	*S. cerevisiae* EC1118	FRI751, SEQ-PAS13
GAS1 (1,3-beta-glucanosyltransferase	P22146	Fungal-type cell wall organization; cell wall (1->3)-beta-D-glucan biosynthetic process; regulation of response to endoplasmic reticulum stress	*S. cerevisiae* (ATCC 204,508/S288c) (55 %)	PAS13, SEQ-PAS13
Glucan *endo*-1,3-beta-D-glucosidase 1	P53753	Metabolic process; septum digestion after cytokinesis	*S. cerevisiae* (ATCC 204,508/S288c) (42 %)	SEQ-FRI751, SEQ-PAS13
MKC7 (Aspartic proteinase)	P53379	Fungal-type cell wall organization; proteolysis; signal transduction involved in filamentous growth	*S. cerevisiae* (ATCC 204,508/S288c) (33.9 %)	SEQ-FRI751, SEQ-PAS13
YJL171C protein	P46992	Cell wall organization; induced in response to cell wall damage	*S. cerevisiae* (ATCC 204,508/S288c) (100 %)	EC1118
1,3-beta-glucanosyltransferase	C8ZF79	Cell wall organization, cell wall (1->3)-beta-D-glucan biosynthetic process	*S. cerevisiae* EC1118	EC1118
Cell surface mannoprotein MP65	Q59XX2	Cell wall biogenesis/degradation; Cell adhesion; Filamentous growth of a population of unicellular organisms in response to starvation; Cellular response to neutral pH; cell adhesion involved in single-species biofilm formation	*C. albicans* (strain SC5314/ATCC MYA-2876) (54.6 %)	PAS13
MKC7 (Aspartic proteinase)	P53379	Fungal-type cell wall organization; proteolysis; signal transduction involved in filamentous growth	*S. cerevisiae* (strain ATCC 204508/S288c) (Baker's yeast) (35.3 %)	SEQ-FRI751
Glucan 1,3-beta-glucosidase	P15703	Carbohydrate metabolic process; cell wall organization; fungal-type cell wall organization	*S. cerevisiae* (ATCC 204,508/S288c) (Baker's yeast) (63.30 %)	SEQ-PAS13

*Organisms were identified according to the *in-silico* analyses (Blast, UniProt) of proteins from *S. bacillaris* proteome when detected in the extracts FRI751, PAS13, SEQ-FRI751, and SEQ-PAS13.

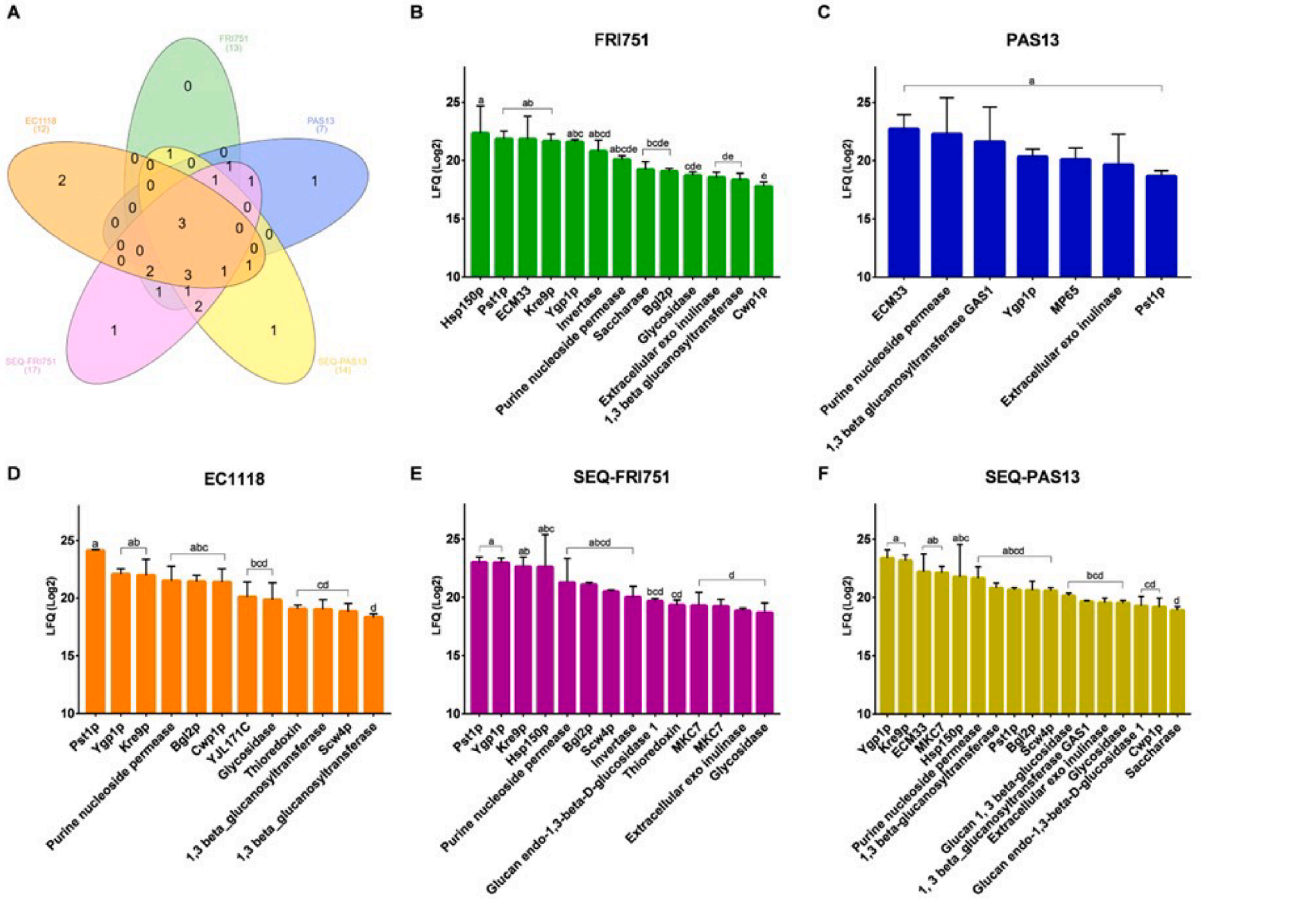

Fig. 3. Proteomic analysis of mannoproteins-rich extracts. Venn diagram of proteins present in each extract (**3A**). Proteins quantification: EC1118 (**3B**); FRI751 (**3C**); PAS13 (**3D**); SEQ-FRI751 (**3E**); SEQ-PAS13 (**3F**). Statistical analyses were performed among a set of proteins found in each extract. The values were represented by the average protein abundance (bar) and standard deviation (error bar) and expressed as LFQ (Log2). Different letters indicate significant statistical differences ($p < 0.05$) tested by one-way analysis of variance (ANOVA) followed by the Tukey *post-hoc* test.

Another protein was exclusively found in both SEQ extracts and matched to *S. bacillaris* proteome: glucan *endo*-1,3-beta-D-glucosidase 1 (ENG1) (UniProt ID: P53753), which had 42 % of similarity to *S. cerevisiae* (ATCC 204,508/S288c). This protein is involved in the dissolution of the mother-daughter septum during cell separation (Baladrón et al., 2002). This protein could contribute to cell wall degradation and, thus, release mannoproteins into the medium.

One glucan 1,3-beta-glucosidase (UniProt ID: P15703) was identified exclusively in SEQ-PAS13 and matched *BGL2* gene sequence. *BGL2* encodes an *endo*-beta-1,3-glucanase that is abundant in the yeast cell wall and is involved in cell wall organization; it introduces intrachain 1,6-beta linkages into 1,3-beta glucan, contributing to the rigid structure of the cell wall (Teparić & Mrša, 2013).

Cell surface mannoprotein MP65 (UniProt ID: Q59XX2) was identified in the PAS13 extract, with 54.6 % sequence similarity with *C. albicans* (strain SC5314/ATCC MYA-2876). MP65 is involved in the biogenesis, organization, adhesion and degradation processes of the cell wall, and in biofilm formation (Sandini et al., 2011).

The extract EC1118 had two unique proteins, YJL171C protein (P46992), a GPI-anchored cell wall protein of unknown function which belongs to the PGA52 family. This protein seems to have a role in response to cell wall damaging agents, and its abundance increases in response to DNA replication stress. The second is a 1,3-beta-glucanosyltransferase (UniProt ID: C8ZF79) with 90 % similarity to GAS3, and belongs to the GAS family that are involved in cell wall biosynthesis, specifically the elongation of the β-1,3-glucan branches (Rolli, Ragni, Rodriguez-Pena, Arroyo, & Popolo, 2010). *GAS3* is generally weakly expressed during the exponential growth phase (Rolli et al., 2010). The proteins of this family have the function of splitting internally a 1,3-beta-glucan molecule and transferring the newly generated reducing end (the donor) to the non-reducing end of another 1,3-beta-glucan molecule (the acceptor) forming a 1,3-beta linkage, resulting in the elongation of 1,3-beta-glucan chains in the cell wall. Interestingly, two other Gas proteins were identified in this study, which will be discussed below.

3.4. Comparison of proteomes according to the S. bacillaris strains

Common proteins were identified among the extracts obtained from single EC1118, *S. bacillaris* and the respective sequential fermentations. Therefore, the intersections EC1118 x FRI751 x SEQ-FRI751; and EC1118 x PAS13 x SEQ-PAS13 were investigated.

Some putative proteins were exclusively identified by proteome analyses in *S. bacillaris* FRI751 and/or PAS13, in both or one condition (single and sequential extracts), and they were not present in EC1118.

Glycosidase (UniProt ID: C8Z982) (Fig. 4**A**) and Kre9p (UniProt ID: C8ZB45) (Fig. 4**B**) were present in EC1118, FRI751, SEQ-FRI751 without statistical differences in their levels, while glucan 1,3-beta-glucosidase (Bgl2p) (UniProt ID: C8Z9H5) (Fig. 4**C**) had the lowest concentration in FRI751 ($p < 0.05$). Regarding the EC1118 x PAS13 x SEQ-PAS13 comparison, these proteins were also identified in EC1118 and SEQ-PAS13 extracts, but were not present in PAS13. No statistical differences were found among the extracts ($p < 0.05$) (Fig. 5**A, 5B, 5C**).

These results demonstrated that the presence of *S. bacillaris* in

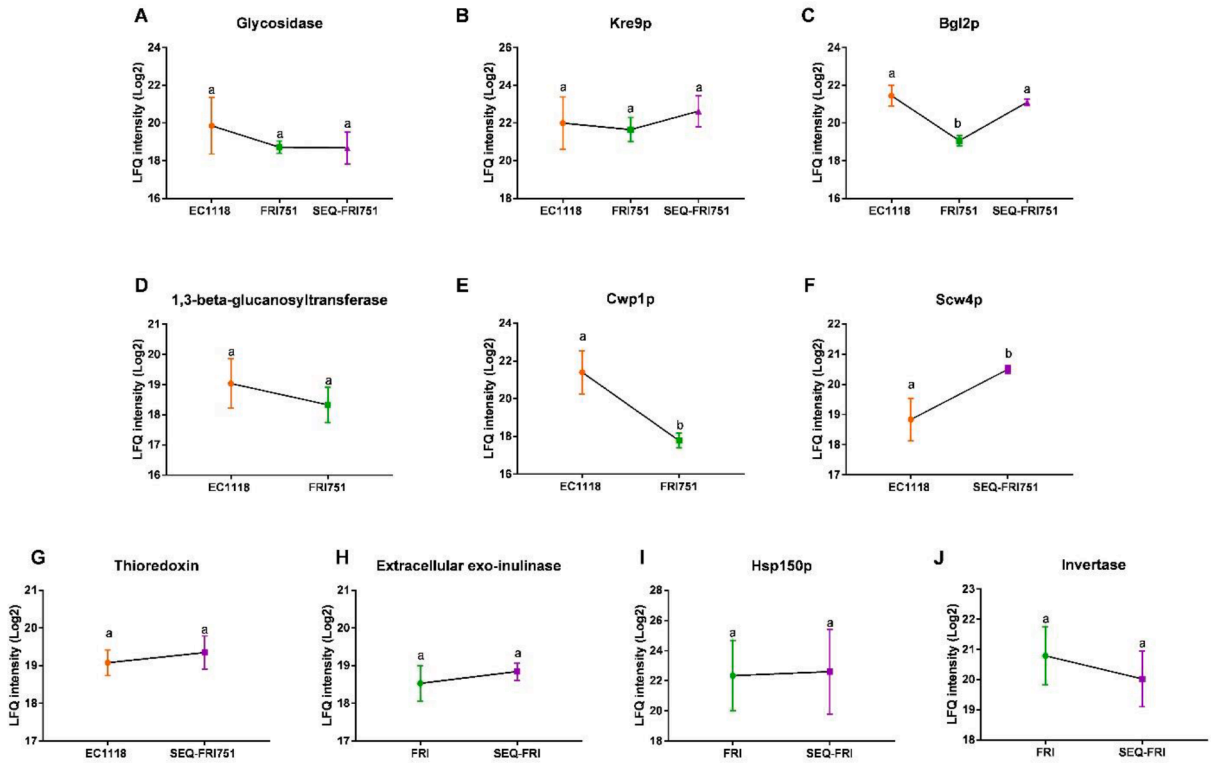

Fig. 4. EC1118 x FRI751 x SEQ-FRI751 intersection. Proteins found in the extracts EC1118, SEQ-FRI751, FRI751: Glycosidase (**A**), Kre9p (**B**), Bgl2p (**C**); proteins detected in the extracts EC1118 and FRI751, and not present in SEQ-FRI751: 1,3-beta glucosyltransferase (**D**), Cwp1p (**E**); proteins presents in EC1118 and SEQ-FRI, and not present in FRI751: cell wall protein like glucanase (Scw4p) (**F**), and thioredoxin (**G**); proteins present in the extracts FRI751 and SEQ-FRI751, and not detected in EC1118: extracellular *exo*-inulinase (**H**), Hsp150p (**I**), and invertase (**J**). The values were represented by average (circle) and standard deviation (error bar). The difference between the averages was analyzed by the one-way ANOVA test followed by the Tukey *post-hoc* test. Different letters indicate significant statistical differences among the extracts ($p < 0.05$).

sequential fermentation did not affect the abundance of these proteins. Glycosidase is an enzyme non-covalently bound to β-1,3-glucan, responsible for building, maintaining, and remodeling cell wall polysaccharides, and Kre9p has an indirect but essential role in the formation of β-1,6-glucosidic bonds (Teparić & Mrša, 2013). Bgl2p is one of the most common cell wall proteins, as previously reported, is a glucan 1,3-beta-glucosidase, belongs to the GH17 family, and it is an *endo*-transglucosidase essential in forming the molecular ensemble of yeast cell walls. Therefore, it contributes to the structural rigidity and responds to the wall stress acting in its decomposition. This protein participates in incorporating the GPI-anchored cell-wall proteins, supports the chitin attachment site and increases the branching of β-1,3-glucan. In addition, this protein is also involved in assembling synthesized mannoproteins (Plotnikova, Selyakh, Kalebina, & Kulaev, 2006). A previous work demonstrated the presence of Bgl2p in unstable wine, suggesting that this glucan 1,3-beta-glucosidase promotes wine instability (Marangon et al., 2011). In contrast, commercial preparations containing yeast glucanases are commonly used for the release of mannoproteins in wine, and it has been shown that, in these cases, they did not affect the wine stability (Moriwaki, Matioli, Arévalo-Villena, Barbosa, & Briones, 2015). Our data suggests that *S. bacillaris* did not influenced the Bgl2p level in SEQ extracts, since FRI751 revealed significantly lower levels than EC1118 and SEQ-FRI751, while this protein was not detected in PAS13.

The protein 1,3 beta-glucanosyltransferase (UniProt ID: C8ZHT4) was detected in the extracts EC1118 and FRI751, with no statistical differences among the concentrations (Fig. 4**D**). This protein had 90 % similarity to 1,3-beta-glucanosyltransferase GAS5 from *S. cerevisiae* (UniProt ID: Q08193). Regarding EC1118 x SEQ-PAS13 comparison, this protein is present at higher levels in SEQ-PAS13 than in EC1118 ($p < 0.05$) (Fig. 5**D**). As it was not detected in PAS13, it can be assumed that the presence of strain PAS13 in sequential fermentation could be responsible for the increased protein level in SEQ-PAS13. The other Gas protein, 1,3-beta-glucanosyltransferase GAS1 (UniProt ID: P22146), with 1,3 beta-glucanosyltransferase activity, the same of GAS5, was found in PAS13 and SEQ-PAS13 without statistical differences in concentration (Fig. 5**I**). Proteome analysis confirmed that GAS1 was released exclusively by the *S. bacillaris* strain. The protein sequence showed 55 % of similarity with *GAS1* gene from *S. cerevisiae* (strain ATCC 204508/S288c). On the contrary, Mostert and Divol (2014), investigating all the proteins released in synthetic must fermentation, found these two proteins in *S. cerevisiae*, *Lachancea thermotolerans* and *Metschnikowia pulcherrima* single-strain fermentations and in most of the corresponding paired-fermentations. Transcriptional analyses in *S. cerevisiae* showed that *GAS1* and *GAS5* are expressed in vegetative growth and repressed in sporulation. *GAS1* is required to assemble the cell wall and probably also *GAS5*, which may play an ancillary function (Rolli et al., 2010).

Cwp1p, a GPI-anchor protein, showed statistically higher levels in EC1118 compared to FRI751 (Fig. 4**E**), whereas in EC1118 and in SEQ-PAS13 no statistical differences were observed between their concentrations (Fig. 5**E**). Scw4p, a cell wall protein with glucanase activity, was detected in EC1118, SEQ-FRI751 (Fig. 4**F**) and SEQ-PAS13 (Fig. 5**F**). Interestingly, Scw4p was at higher concentrations in both SEQ extracts than EC1118 ($p < 0.05$). The proteome analysis revealed that this protein is synthetized by *S. cerevisiae* EC1118, suggesting that *S. bacillaris* stimulated *S. cerevisiae* to increase its level in the medium. Scw4p is a protein that is bound to the cell wall non-covalently and partly covalently, is involved in cell-wall integrity and contains motifs that resemble Pir repetitive sequences. However, its incorporation into the cell wall remains unclear. It was demonstrated that proteins like Scw4p, Scw10p and Bgl2p are synergistically involved in building up the

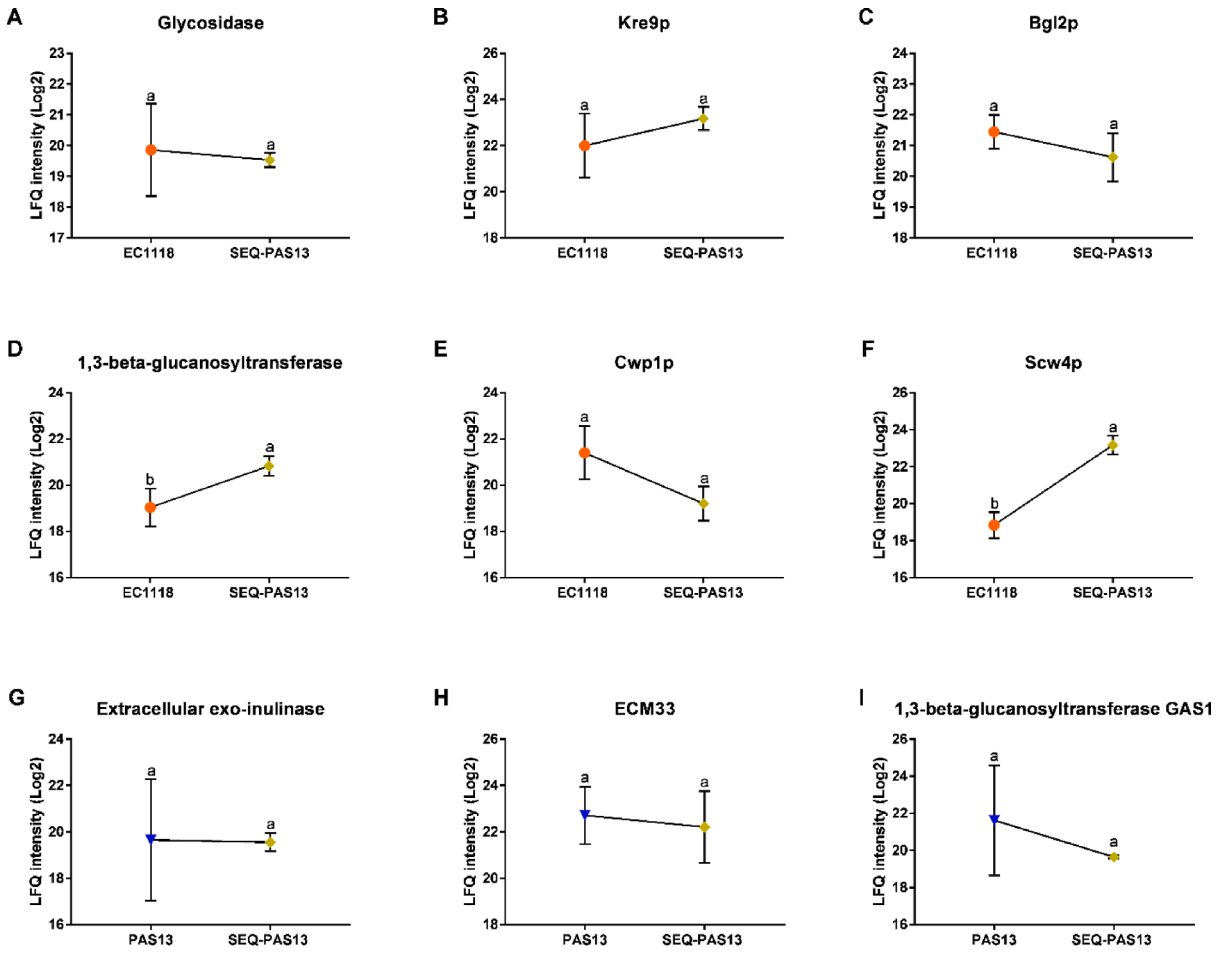

Fig. 5. Proteins found in the intersection EC1118 x PAS13 x SEQ-PAS13. Proteins present in the extracts EC1118 and SEQ-PAS13, and not detected in PAS13: Glycosidase (**A**), Kre9p (**B**), Bgl2p (**C**), 1,3-beta-glucanosyltransferase (**D**), cell wall protein Cwp1p (**E**), and cell wall protein like glucanase (Scw4p) (**F**); proteins detected in PAS13 and SEQ-PAS13 and not detected in EC1118: extracellular *exo*-inulinase (**G**), cell wall protein ECM33 (**H**), and 1,3-beta-glucanosyltransferase GAS1 (**I**). The values were represented by average (circle) and standard deviation (error bar). The difference between the averages was analyzed by unpaired *t*-test. Different letters indicate significant statistical differences among the groups ($p < 0.05$).

wall and that the lack of the non-covalently bound wall proteins Scw4p, Scw10p and Bgl2p increases the mortality of *S. cerevisiae* cells grown exponentially under standard laboratory conditions (Teparić, Stuparević, & Mrša, 2004). A possible explanation for the highest Scw4p levels in SEQ extracts compared to EC1118 could be the high concentration of MKC7 in the SEQ extracts, where these proteins can activate and increase Scw4p (Grbavac, Čanak, Stuparević, Teparić, & Mrša, 2017), with a consequent increase in the secretion of Scw4p by the *S. cerevisiae* into the medium during the sequential fermentations SEQ-FRI751 and SEQ-PAS13. Scw4p could be indirectly involved in improving the stability of wines obtained through SEQ fermentations. In fact, this glucanase, partially degrading the yeast cell wall, could be responsible for the increased release of mannoproteins from *S. cerevisiae* cell wall with potential positive effects on haze reduction.

Thioredoxin (C8Z9A2) was identified in EC1118 and SEQ-FRI751, without statistical differences in their levels (Fig. 4**G**). This protein matched the EC1118 proteome (UniProt ID: C8Z9A2) and had 100 % of similarity to thioredoxin-2 (P22803) (*S. cerevisiae*, strain ATCC 204508/S288c). It is involved in many cellular processes, such as protein folding and redox homeostasis (Grant, 2001).

Several studies demonstrated that *S. cerevisiae* fermentation rate is strongly reduced in sequential fermentations with *S. bacillaris* (Gobert et al., 2017; Nadai, Giacomini, & Corich, 2021), with respect to single-strain fermentation. Some of the proteins more abundant in SEQ fermentations with respect to EC1118 are expressed during *S. cerevisiae* exponential growth phase (GAS5, Scw4p and partially Bgl2p (UniProt ID: P15703)). These results suggested that the presence of *S. bacillaris* affected the correct cell wall assembly of *S. cerevisiae*, during the exponential growth phase, inducing the release of these proteins in the medium.

The following proteins were specific for *S. bacillaris* since they were not detected in the EC1118 extract. Moreover, the proteome analyses confirmed that these proteins were synthesized by *S. bacillaris* species and are involved in polysaccharide catabolic processes and cell wall organization. These proteins are not directly involved in the improvement of protein stability, as demonstrated in this study. In fact, FRI751 and PAS13 extracts were not more effective than EC1118 in Vernaccia wine haze protection, although their indirect effect cannot be excluded.

Extracellular *exo*-inulinase (UniProt ID: A5DHM6) was detected in the extracts from both *S. bacillaris* strains in single strain and sequential fermentations, without statistical difference in both pairwise comparisons (Fig. 4**H** and Fig. 5**G**). This protein had 37 % similarity to that found in *Meyerozyma guilliermondii*, and, interestingly, 36.6 % to an invertase (sucrose hydrolyzing enzyme) from *Torulaspora hansenii* (UniProt ID: Q6BJW6). Yeasts such as *Candida* sp., *Pichia* sp. and *Kluyveromyces* sp. can produce inulinases, responsible for the catalysis of the endohydrolysis of 2,1-beta-D-fructosidic linkages in inulin. An invertase (UniProt ID: Q6BJW6) was detected in FRI751 and SEQ-FRI751 (Fig. 4**J**). The proteome analysis revealed that this protein was exclusively synthesized by *S. bacillaris* and had a molecular weight of 57.28 kDa, with

39.5 % of similarity to that of *Torulaspora hansenii*. Moreover, another sucrose hydrolyzing enzyme identified as saccharase (UniProt ID: C8ZAV0) was found in FRI751 and SEQ-PAS13 with similarity to *S. cerevisiae* EC1118. From a technological point of view, a study demonstrated that an invertase fragment (31.8 kDa) from *S. cerevisiae* had a heat-stabilizing effect on the proteins in white wine (Moine-Ledoux & Dubourdieu, 1999). The whole invertase protein has also been shown to have an haze-protective activity in white wines (Dupin et al., 2000).

Hsp150p (C8ZB60) levels were present at the same concentrations in FRI751 and SEQ-FRI751 ($p < 0.05$) (Fig. 4**I**). In addition, this protein was detected in SEQ-PAS13. Hsp150p is an O-mannosylated heat shock protein, covalently attached to the cell wall, it belongs to the Pir family and is secreted into the medium. This protein is essential in cell wall integrity and cell wall biogenesis/degradation. It is induced by heat stress, nitrogen starvation, stress response, and by the presence of aluminum (Hsu, Chiang, Liu, Chang, & Jones, 2015). Although this protein was not detected in the extract EC1118, its sequence matched the *S. cerevisiae* EC1118 proteome.

The protein ECM33 was identified in the extracts PAS13 and SEQ-PAS13, without a statistical difference between their levels (Fig. 5**H**). Besides, it was found in FRI751. This *N*-glycosylated protein belongs to the SPS2 family, covalently linked to encoding cell surface GPI-anchored. It is also involved in cell wall integrity and the correct assembly of synthesized mannoproteins (Pardo et al., 2004). ECM33 is paralog to Pst1p, and its expression is upregulated to substitute the function of Pst1p (Pardo et al., 2004). Therefore, ECM33 could reduce haze formation since Pst1p was demonstrated to positively influence the haze formation (Brown et al., 2007), as already reported above.

In general, the proteome of the mannoproteins-rich extracts revealed key proteins which could potentially be linked to the observed reduction in wine turbidity increasing white wines' protein stability. In the quest for more sustainable and less impacting alternative to bentonite for wine protein stabilization, having yeasts releasing into the wine compounds able to naturally improve its stability would be welcome by winemakers. Hence, approaches including fermentations with strains overproducing and releasing yeast cell walls into the medium, especially mannoproteins, in synthetic must or growth medium (e.g., YPD) could represent an exciting alternative to improve wine stability in the wine industry. Moreover, performing the wine alcoholic fermentation directly using *S. bacillaris* in a sequential fermentation with *S. cerevisiae* can be a way to naturally release these cell walls polysaccharides and proteins into the matrix, thus reducing the wine turbidity. Besides, the degradation of grape proteins by yeast cell walls enzymes could possibly be used to naturally increase nitrogen availability, reducing the nitrogen addition during the winemaking process. However, the mechanisms by which yeast compounds contribute to wine protein stability are still unclear. Thus, applying *in situ* analyses to investigate the interactions between grape proteins, the responsible compounds to cause haze formation, and polysaccharides/glycosylated proteins present in mannoproteins-rich extracts can be an exciting tool to understand the haze protective mechanisms in wines.

4. Conclusions

The addition of mannoproteins-rich extracts to a heat unstable white wine led to a reduction in its hazing potential. In particular, extracts from *S. bacillaris* in sequential fermentations with *S. cerevisiae* (SEQ-FRI751 and SEQ-PAS13) were the most effective in improving wine protein stability. The presence of *S. bacillaris* in sequential fermentation modified the chemical composition of the extracts, increasing the low MW polysaccharide fraction. Proteome analysis revealed that no mannosidases or, generally, enzymes able to hydrolyze mannose were present in the extracts. Therefore, differences in the distribution of polysaccharides fractions could be related to not-released proteins acting on the cell wall or originated during the synthesis in the Golgi lumen.

Most of the molecular functions of the proteins found in this study were glucan hydrolysis (hydrolase activity, hydrolase of *O*-glycosyl compound, glucan *endo*-1,3-beta-D-glucosidase activity) that can be involved in the release of cell wall mannoproteins. The identified cell wall proteins exclusively found in the sequential fermentations were produced by both *S. bacillaris* (MKC7 and ENG1) and *S. cerevisiae* (Bgl2p). Moreover, Scw4p and 1,3 beta-glucanosyltransferase similar to GAS5, produced by *S. cerevisiae*, significantly increased in sequential fermentations. They were found to be expressed during *S. cerevisiae* exponential growth phase, suggesting that the presence of *S. bacillaris* affected the correct cell wall assembly of *S. cerevisiae*, during the exponential growth phase, slowing down fermentation activity.

All these findings demonstrated a strong interaction between *S. bacillaris* and *S. cerevisiae* that deeply influenced the mannoproteins-rich extracts composition.

Proteins glucan *endo*-1,3-beta-D-glucosidase 1 (ENG1) and aspartic proteinase (MKC7), present exclusively in both SEQs extracts, and glucan 1,3-beta-glucosidase, present in SEQ-PAS13, could play an essential role in enhancing wine protein stability, both increasing protein release and degrading grape proteins responsible for wine turbidity. Moreover, some of the proteins specifically found in *S. bacillaris* and not present in EC1118, such as extracellular *exo*-inulinase, cell wall protein ECM33, GAS1, Hsp150p, invertase, and saccharase, could be good candidates for further investigation to clarify their potential involvement in modulating wine turbidity and other interesting technological aspects. In general, fermentation type influences extracts' chemical composition and differently affects wine stability. The biotechnological overproduction of polysaccharides and glycosylated proteins from cell wall released by yeasts into the medium, or the use of *S. bacillaris* strains in sequential fermentation with *S. cerevisiae* as a co-starter culture, can be suggested as an exciting alternative to conventional treatments to increase wine protein stability thus reducing the need for bentonite fining. However, further studies are necessary to investigate the haze-protective mechanisms of extracts.

Funding

This work was supported by Università degli Studi di Padova, Italy [grant numbers DOR2233272/22, DOR1804247/18]. The HPLC Agilent 1260 series II quaternary pump LC was funded by Ministero dell'istruzione, dell'università e della ricerca (MIUR), Italy [grant number CUP C26C18000190001]. Part of the research was founded by iNEST-interconnected Nord-Est Innovation 476 Ecosystem - Affiliato Spock 7 - SMART AGRI-FOOD [project number C43C22000340006].

CRediT authorship contribution statement

Luiza de Paula Dias Moreira: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Davide Porcellato:** Project administration, Writing – review & editing, Formal analysis, Conceptualization. **Matteo Marangon:** Writing – review & editing, Formal analysis, Conceptualization. **Chiara Nadai:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Vinícius da Silva Duarte:** Investigation. **Tove Gulbrandsen Devold:** Project administration, Conceptualization. **Alessio Giacomini:** Project administration, Funding acquisition, Writing – review & editing, Resources, Conceptualization. **Viviana Corich:** Supervision, Writing – review & editing, Writing – original draft, Resources, Conceptualization, Funding acquisition, Project administration.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgment

The authors would like to thank the Fondazione CARIPARO (Cassa di Risparmio di Padova e Rovigo) for the scholarship of Luiza de Paula Dias Moreira. The topics covered in this work fall within the VITAE Project [CUP C23C23000240001].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.138311.

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