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Influence of the mannoproteins of different strains of *Starmenella bacillaris* used in single and sequential fermentations on foamability, tartaric and protein stabilities of wines

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ABSTRACT

Aim: In this work, seven strains of *Starmerella bacillaris* were analysed for their ability to release polysaccharides during alcoholic fermentation (AF), both in single-strain and in sequential AF together with *Saccharomyces cerevisiae*.
Methods and results: A synthetic polysaccharide-free must was used to characterise the mannoproteins (MPs) released. The MPs were quantified, characterised in terms of carbohydrate composition, and tested to assess their ability to reduce protein and tartrate instabilities and their ability to affect the foaming properties of wine.
Conclusions: All the tested strains in sequential AF increased the total MPs production. Moreover, the strains affected the MPs properties in different ways regarding tartaric and protein stabilities. The MPs released in sequential AF by some *S. bacillaris* strains showed a significant effect on protein stabilisation and tartaric stability. An effect on the foamability was found for MPs obtained in single-strain AFs of *S. bacillaris*.
Significance and impact of the study: An improvement in wine stability can be achieved using the sequential AF.

KEYWORDS

polysaccharides, non-*Saccharomyces*, fermentation, wine quality, mannoproteins

Supplementary data can be downloaded through: https://oeno-one.eu/article/view/2948

INTRODUCTION

Alcoholic fermentation (AF) is one of the most important processes during winemaking and the management of this is key to obtaining a good quality product.

Mannoproteins (MPs) are macromolecules released by yeasts during AF and during the autolysis of cells in wine ageing on lees (Dupin *et al.*, 2000a; Chalier *et al.*, 2007). The release of MPs depends on yeast strain (González-Ramos *et al.*, 2008), must turbidity (Boivin *et al.*, 1998), temperature (Llaubères *et al.*, 1987), nutritional conditions (sugar, nitrogen) (Ribéreau-Gayon *et al.*, 2006) and the chemical composition of the wine (pH). These molecules are glycoproteins with a high sugar content, mainly mannose (Quirós *et al.*, 2010), and are, together with fibrous β-1,3-glucan, one of the major components of the yeast cell wall, representing an important part of total polysaccharides in wine (Vidal *et al.*, 2003).

In recent years, MPs have become one of the most interesting yeast molecules for the improvement of wine quality, due to their oenological properties such as the enhancement of mouthfeel and the improvement of wine protein and tartaric stabilities (Escot *et al.*, 2001; Moine-Ledoux and Dubourdieu, 2002; Caridi, 2006; González-Ramos *et al.*, 2008; Comuzzo *et al.*, 2011). In 2005, the European Community authorised the addition to wine of MPs obtained from the hydrolysis of yeast cell walls, for improving tartaric and protein stabilisation (EU Regulation 2165/2005).

One of the first oenological properties described for yeast MPs is the partial protection against protein precipitation in white wines. Ledoux *et al.* (1992) showed that a Sauvignon blanc wine aged on yeast lees has lower haze potential and lower bentonite requirements for stability than wine aged without lees. The compound responsible for improving protein stability in white wine aged on its lees has been identified as a 31.8 kDa mannoprotein (MP32), which was a yeast invertase fragment released from yeast cell walls (Moine-Ledoux and Dubourdieu, 1999).

Waters *et al.* (1993) isolated from Muscat of Alexandria wine a high molecular weight mannoprotein that is able to prevent visible wine protein haze formation. This molecule, called haze protective factor (HPF), was characterised and a putative structural gene was identified in the *Saccharomyces cerevisiae* genome (Dupin *et al.*, 2000b).

Another property attributed to MPs is the protection against tartaric instability. MPs inhibit the crystallisation of tartrate salts by lowering the crystallisation temperature (Gerbaud *et al.*, 1996; Moine-Ledoux and Dubourdieu, 2002), preventing thus the occurrence of precipitates in wine. Furthermore, MPs improve the foam of sparkling wines. The hydrophobicity, high glycosylation and high molecular mass of MPs allow them to surround, and thus stabilise, the gas bubbles of the foam. It has been demonstrated that both the production and the stabilisation of foam also depend on other proteins; however, the main contributors to the foam formation are MPs (Coelho *et al.*, 2011; Vincenzi *et al.*, 2014).

Some authors have proposed recombinant *S. cerevisiae* wine yeasts engineered to overproduce MPs (Gonzalez-Ramos and Gonzalez, 2006; Brown *et al.*, 2007; Gonzales-Ramos *et al.*, 2008). To bypass various countries' regulations on the use of GMOs in food, Quirós *et al.* (2010) developed a non-recombinant method to select wine yeasts overproducing MPs, consisting in a random mutagenesis using UV light as a physical agent, followed by a direct selection on YPD (yeast extract-peptone-dextrose medium) plates containing killer toxin from *Williopsis saturnus.*

To avoid the use of these microorganisms, likely to be viewed as releasing components not normally present in wine, Domizio *et al.* (2014) proposed the use of non-*Saccharomyces* wine yeasts, found in grape and winemaking environments, as novel sources of MPs in wine. Giovani *et al.* (2012) firstly demonstrated that non-*Saccharomyces* yeasts can release polysaccharides from their cell wall during AF, as with *S. cerevisiae.* They found out that the amount released depends on yeast species and cell vitality. Non-*Saccharomyces* yeasts, once defined as a spoilage microorganism, predominate in the early stages of must AF and recently their role has been reconsidered as some were found to enhance the analytical composition and aroma profile of the wine (Jolly *et al.*, 2014; Ciani and Comitini, 2015; Padilla *et al.*, 2016; Vilela, 2019). Several studies proposed the use of controlled mixed fermentations combining the inoculation of non-*Saccharomyces* yeasts contemporarily or before

Saccharomyces cerevisiae to improve wine complexity and quality (Ciani and Comitini, 2011; Jolly *et al.*, 2003). Mixed fermentations with *Metschnikowia pulcherrima* and *S. cerevisiae* significantly decreased volatile acidity and total acidity of the final wines and showed a positive correlation with medium-chain fatty acids, 2-phenyl ethanol and isoamyl acetate production (Comitini *et al.* 2011). Mixed and sequential fermentations (first the non-*Saccharomyces* yeast then *S. cerevisiae*) carried out with *Lachancea thermotolerans* and an *S. cerevisiae* starter showed a reduction in the volatile acidity and an increase in total acidity, glycerol, 2-phenyl ethanol, ethyl lactate, diacetyl and polysaccharides content (Comitini *et al.*, 2011; Gobbi *et al.*, 2013; Del Fresno *et al.*, 2017). The use of *Torulaspora delbrueckii* in mixed fermentations with *S. cerevisiae* resulted in an enhancement of the 2-phenyl ethanol and polysaccharides content and a significant reduction in the volatile acidity (Comitini *et al.* 2011). Other authors found that sequential fermentations with *T. delbrueckii* and *S. cerevisiae* increase the complexity of wine. In fact, the presence of *T. delbrueckii* increased the concentration of some positive esters, such as ethyl lactate or 2-phenylethyl acetate, and of ethyl acetate, diacetyl and isoamyl acetate in wines, keeping volatile acidity and acetaldehyde at suitable levels (Loira *et al.*, 2014; Taillandier *et al.*, 2014; González-Royo *et al.*, 2015; Del Fresno *et al.*, 2017). Moreover, the effect of non-*Saccharomyces* yeasts on wine foam has been studied, showing that sequential fermentations with *S. cerevisiae* may be a useful tool to improve wine characteristics and quality. Sequential inoculation with *T. delbrueckii* and *S. cerevisiae* improved wine foamability and foam persistence, while sequential inoculation with *M. pulcherrima* and *S. cerevisiae* increased both the foam persistence and the smoky and flowery notes in the wine aromatic profile (González-Royo *et al.*, 2015; Medina-Trujillo *et al.*, 2017).

Starmerella bacillaris (synonym *Candida zemplinina*) is a non-*Saccharomyces* yeast commonly found in oenological environments (Bovo *et al.*, 2011). Recently, it has shown to be of oenological interest in mixed AF with *S. cerevisiae*, improving the content of compounds related with wine quality. Many studies indicate that the use of *S. bacillaris*, together with *S. cerevisiae*, enhance the glycerol content of wines and cider, with moderate volatile acidity production, and, due to the low ethanol yield, reduce ethanol content (Rantsiou *et al.*, 2012; Bely *et al.*, 2013; Wang *et al.*, 2014; Lemos Junior *et al.*, 2016; Lemos Junior *et al.*, 2017a; Lemos Junior *et al.*, 2017b; Nadai *et al.*, 2018). Moreover, Lemos Junior *et al.* (2019) proposed the potential use of *S. bacillaris* as a fermentation starter for the production of low-alcohol beverages obtained from unripe grapes.

In this study, seven strains of the non-conventional yeast *S. bacillaris* were investigated for their ability to release MPs during AF in synthetic must, both in single-strain and in sequential AF together with *S. cerevisiae*. These molecules were quantified, characterised in terms of carbohydrate composition, and tested to assess their ability to reduce protein and tartrate instabilities and to affect the foaming properties of wine.

MATERIALS AND METHODS

1. Yeast strain and fermentation trials in synthetic must

The yeasts strains used in this work (Table 1) were isolated from fermenting must obtained from dried grape of Raboso Piave variety, as described by Lemos Junior *et al.* (2016). A loopful of a 3-day-old culture of each yeast strain from a YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) was used to inoculate 10 mL of YPD broth in 50 mL tubes.

A stationary phase culture with approximately 10^7–10^8 cells/mL, determined by optical density (OD) measurements and confirmed by means of plate counts analysis (CFU/mL), was obtained after 24 h of incubation at 30 °C. In single-strain AF, the inoculum concentration was $1–1.5\times10^6$ cells/mL. The strain *S. cerevisiae* EC1118 (Lallemand Inc., Montreal, Canada) was used as control. In sequential AF the same inoculum size was used for the *S. bacillaris* strain and *S. cerevisiae* EC1118 (1×10^6 cells/mL). EC1118 was added 48 h after the inoculum of *S. bacillaris*.

Fermentations were run in synthetic must MS300, prepared as described by Bely *et al.* (1990), with 100 g/L of glucose, 100 g/L of fructose and 6 g/L of DL-malic acid, pH 3.3. Fermentation trials were performed in triplicate in 120 mL bottles as described by Bovo *et al.* (2016). After yeast inoculation, the bottles were

TABLE 1. Yeast strains used in work.

Strain	Species	Origin
FRI719	*S. bacillaris*	Winery
FRI728	*S. bacillaris*	Winery
FRI729	*S. bacillaris*	Winery
FRI751	*S. bacillaris*	Winery
FRI754	*S. bacillaris*	Winery
FRI779	*S. bacillaris*	Winery
FRI7100	*S. bacillaris*	Winery
EC1118	*S. cerevisiae*	Industrial strain (Lallemand Inc., Montreal, Canada)

incubated at 20°C. CO_2 production was followed by measuring the weight loss of each culture twice a day. All fermentations trials were stopped after 624 h, when the AF of *S. cerevisiae* EC1118 was completed.

2. Macromolecules isolation

The fermentation broth was filtered at 0.45 µm (cellulose acetate filter) (Sartorius, Goettingen, Germany) and successively ultrafiltered using an Amicon 8400 apparatus (3000 Da cutoff, regenerated cellulose) (Millipore, Burlington, MA) under a constant nitrogen pressure of 3.5 bars. The total macromolecules obtained from the culture filtrates (i.e. mainly MPs) were carefully recovered, dialysed against water using a regenerated cellulose membrane (3500 Da, Fisherbrand) (Millipore, Consett, Durham, UK), then freeze-dried. The obtained powder was weighted and resuspended in water at 10 mg/mL.

3. Monosaccharide composition

The hydrolysis of MPs for the determination of the monosaccharide composition was determined according to the method reported by Dai *et al.* (2010). 4M trifluoroacetic acid (500 µL) and water (300 µL) were added into the polysaccharide solution (200 µL, 10 mg/mL) in a vacuum hydrolysis tube (Pierce, Thermo Scientific, Waltham, MA). The tube was sealed under vacuum and kept for 2 h at 110 °C. After the tube was cooled to room temperature, 800 µL of hydrolysate were mixed with 800 µL of water and freeze-dried. The residue was resuspended in 200 µL of water, filtered and injected in HPLC.

4. HPLC analysis

HPLC analysis was performed to determine the concentration of glucose, fructose, mannose, acetic acid, glycerol, and ethanol as described by Nadai *et al.* (2016): 10 µl samples were analysed using a Waters 1525 HPLC binary pump (Waters, Milford, MA) equipped with a 300×7.8 mm stainless steel column packed with Aminex HPX_87H HPLC column (Bio-Rad, Hercules, CA). A Waters 2414 Refractive Index Detector (Waters, Milford, MA) was used to determine the mannose, glucose, fructose, acetic acid, glycerol, and ethanol. The concentrations, expressed as g/L, were calculated by using calibration curves of the individual compounds, and peaks area was determined by the Breeze (Waters, Milford, MA) programme.

5. Tartaric stability of white wine

The wine tartaric stability was measured using the mini contact test and was carried out by a Tartar Check (Ing. C. Bullio, San Prospero, Italy). The variation of electric conductivity (Δx), expressed in µs/cm, indicated the level of stability. Briefly, 20 mL of wine were brought to 0°C, then a dose of 300 mg micronised potassium hydrogen tartrate was added (corresponding to a final concentration of 15 g/L) and the conductivity decrease due to tartaric acid precipitation was tracked for 10 min. A very unstable wine (Glera wine from Conegliano area, vintage 2015, pH 3.13, titratable acidity 6.7 g/L, alcohol content 11.3%, 267µS drop) was used to test the effect of yeast macromolecules, added to the wine at a final concentration of 200 mg/L before the mini contact test.

6. Protein stability of white wine

The protein stability was evaluated by measuring the turbidity of wine after a treatment at 80°C for 6 h followed by 4°C for 12 h (Pocock and Rankine, 1973). The turbidity was measured by the HI83749 Nephelometer (Hanna Instruments, Ronchi di Campanile, Italy) and the difference between the value before and after the treatment was used for calculations. A very unstable wine (Traminer wine, from Tezze sul Brenta, vintage 2015, pH 3.32, titratable acidity 5.6 g/L, alcohol content 12.4%, 225 NTU after heating) was used to test the effect of yeast macromolecules, added to the wine at a final concentration of 200 mg/L before the heating.

7. Evaluation of foam measurement on white wine

Foam parameters were measured with a classical Rudin tube (400×24 mm i.d.) (Rudin, 1957), closed at the bottom with a sintered glass plate (pore size 40–60 µm). MPs isolated from the different strains of *S. bacillaris* in single and sequential inoculum were all resuspended at the same concentration (10 mg/mL) in water and added to a Prosecco base wine at a concentration of 50 mg/L.

Before each analysis, the tube was cleaned with ethanol, and then rinsed three times with deionised water and three times with the sample to be analysed. Then, 50 mL filtered samples were placed in the tube and CO_2 was sparged at a constant flow rate (110 mL/min) and pressure (100 kPa) from the bottom. The foam height (FH) was measured every 15 s for 15 min, the gas flow was stopped, and then the foam decay was monitored. Each sample was analysed in triplicate.

8. Statistical analysis

Principal component analysis (PCA) and one-way analysis of variance (ANOVA) at a 95 % accuracy level were performed using XLSTAT software, vers.2016.02 (Addinsoft, Paris, France).

Results and discussion

1. MPs release during fermentation

Fermentation performances of seven strains of *S. bacillaris*, both in single-strain and in sequential AF together with *S. cerevisiae* EC1118, were evaluated in synthetic must. All the fermentations trials were stopped after 624 h, when the AF of *S. cerevisiae* EC1118 was completed. Fermentation vigour, in terms of CO_2 production after 48 h of incubation, CO_2 production after 312 h and when AF was stopped (624 h), together with sugars consumption and secondary metabolite production, were considered to assess AF performances (Table A.1 and Table A.2). *S. bacillaris* single-strain fermentation evidenced a high glycerol production and a fermentation rate slower than that of *S. cerevisiae* EC1118. When sequential fermentations were performed in synthetic must the *S. bacillaris* strains significantly increased glycerol content and reduced ethanol concentration, while no significant differences were found in acetic acid concentrations that were comparable to the *S. cerevisae* EC1118 tested strain.

The AF was conducted in MS300 synthetic must (i.e. without protein or polysaccharide), so every macromolecule present at the end of the AF was exclusively produced and released by yeast cells (Figure 1). *S. cerevisiae* strain produced a significantly ($p<0.001$) higher content of

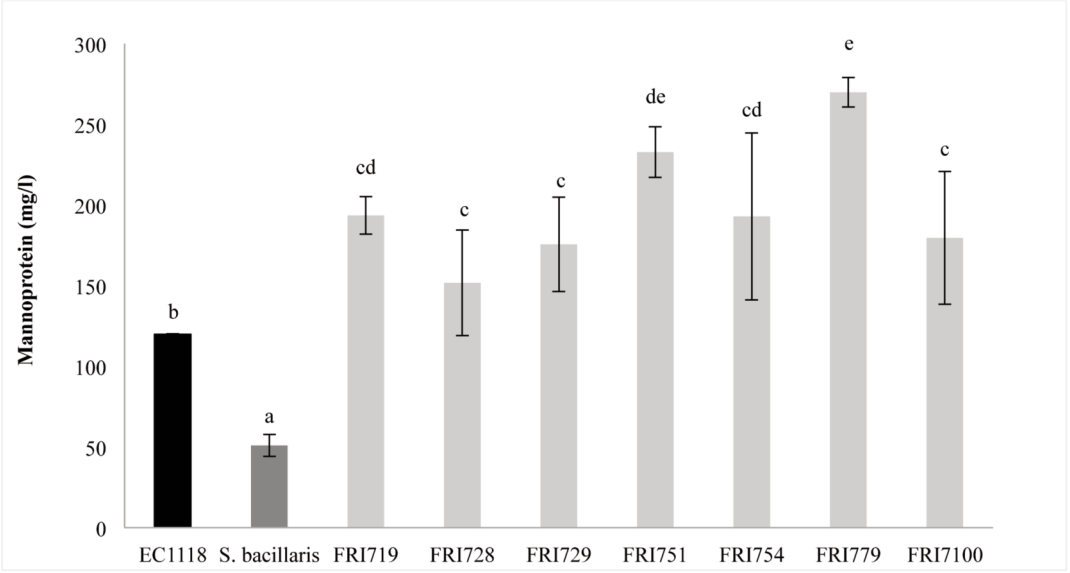

FIGURE 1. Mannoproteins content in *S. cerevisiae* (EC1118) and *S. bacillaris* single-strain fermentation (mean of all strains) and sequential fermentation.

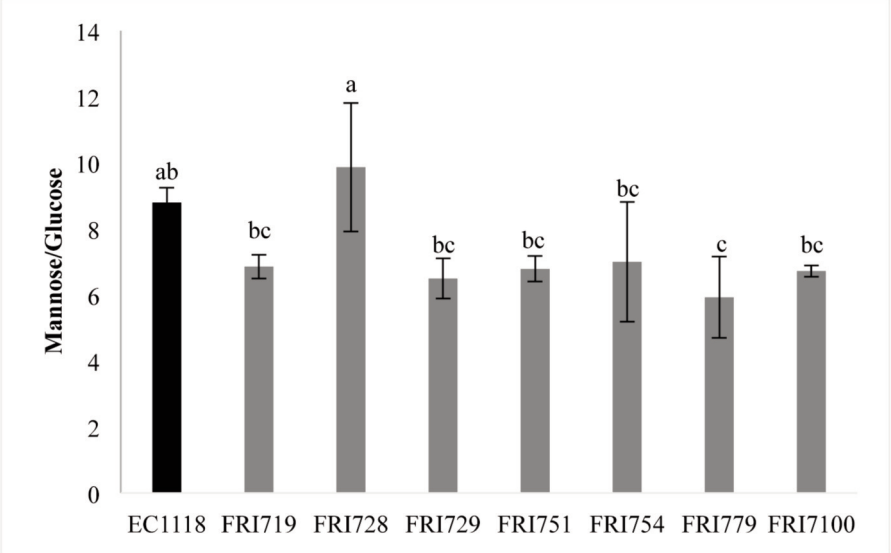

FIGURE 2. The composition with regards to sugars represented by a coefficient (mannose/glucose) of the polysaccharides released by each yeast strain at the end of alcoholic fermentation.

macromolecules (119 mg/L) than *S. bacillaris* single-strain AFs (from 40.51 to 57.56 mg/L).

These results are different from those obtained by Domizio *et al.* (2014) who found that *S. bacillaris* #22 released a level of total polysaccharides similar to *S. cerevisiae* EC1118 (around 200 mg/L). However, this effect could be explained by the different AF conditions. In fact, Domizio *et al.* (2014) performed AFs in a "minimal must medium" at 28 °C under continuous agitation at 150 rpm, while in the present study the fermentation conditions are mimicking a must in static fermentation.

The sequential AFs released much higher quantities of macromolecules (151–270 mg/L) than the *S. cerevisiae* single-strain AF (119 mg/L). This higher macromolecules production in sequential AFs cannot be ascribed solely to the presence of two microorganisms in the same medium, as, in some cases (in particular FRI751 and FRI779), the total macromolecules content is higher than the sum of the macromolecules released by the two microorganisms individually cultured. This behaviour clearly indicates that the interaction existing between the two microorganisms during AF leads to an increase in the macromolecules release into the culture medium. The interaction between different yeasts in multi-starter wine AF has been reviewed by Ciani and Comitini (2015), and has been shown to have an effect on both metabolite production (glycerol, ethanol, esters, etc.) and microbial growth. Regarding cell-wall polysaccharides, even Comitini *et al.* (2011) found a higher release of polysaccharides by *S. bacillaris* in sequential AF together with *S. cerevisae* than *S. cerevisiae* single-strain AF in natural grape juice. This could be explained by a modulation of *S. bacillaris* death due to the direct interaction with *S. cerevisiae* cells, as recently suggested by Englezos *et al.* (2019).

2. Polysaccharides characterisation

The different macromolecules obtained from the filtered media were suspended in water at 10 mg/mL and then used for further characterisation.

The analysis of sugar composition confirmed that these polysaccharides released in the media are of cell-wall origin, and therefore essentially MPs. As a matter of fact, only mannose and glucose were found after acidic hydrolysis, with a predominance of mannose, which accounts for about 90% of the total sugar in the polysaccharides isolated from *S. cerevisiae* (Figure 2). This result is in agreement with data from the literature reporting similar values of mannose content (Escot *et al.*, 2001; Vidal *et al.*, 2003; Domizio *et al.*, 2014). Conversely, the mean content of mannose in MPs isolated from single-strain AFs of *S. bacillaris* is 82.23±1.43%, according to the data from Domizio *et al.* (2014). These data confirm the existence of a chemical difference between the MPs released by the two microorganisms, which

can influence their technological properties, such as the ability to decrease the protein instability in white wines (Ribeiro *et al.*, 2014). Finally, in sequential AFs the mannose percentage of MPs was intermediate (with the exception of sequential AF of FRI728 strain), but still significantly lower (from 85.28 to 87.43%) when compared to single-strain AF of EC1118 (Figure 2).

3. Tartaric stabilisation

First, the MPs were added to a very unstable wine at a final concentration of 200 mg/L to test for their ability to decrease the tartaric instability.

The tendency of tartaric acid to precipitate was determined by measuring the conductivity drop after addition of potassium hydrogen tartrate (crystallisation nuclei) at 0 °C. The effect of MPs on this parameter was measured by comparing the conductivity drop of treated samples with that of untreated wine (Figure 3). On average, the MPs produced by *S. bacillaris* showed low inhibition capacity (from 6.00 to 31.00 µS) when compared with those of EC1118 alone (41 µS) ($p<0.001$). However, the MPs recovered after the sequential AF showed, in some cases (FRI719, FRI751 and FRI779), a significant higher activity compared to single-strain AF of EC1118, indicating a better capacity to inhibit the salt crystallisation. This behaviour confirms an interaction between the two yeast species, as not only the quantity, but also the intrinsic characteristics of MPs were modified when recovered after a sequential AF. We can hypothesise that these MPs modifications could depend on the activity of hydrolytic enzymes released by one or both species able to specifically act on the MPs of the co-fermenting species.

4. Protein stabilisation

The MPs were also tested for their ability to affect the protein instability, by adding them to an unstable wine at a final concentration of 200 mg/L. Then the wines were heated at 80 °C in order to induce the aggregation and precipitation of grape unstable proteins (Pocock and Rankine, 1973).

The induced turbidity was measured by nephelometry and the difference between the initial turbidity and that obtained after heating was noted. The wine used for the experiment was very unstable, giving a value of 225 NTU, and all the results were expressed in comparison to this value. In all cases the addition of MPs caused a reduction of haze risk (Figure 4), confirming the ability of these molecules to decrease the protein precipitation (Waters *et al.*, 1993; Waters *et al.*, 1994).

Considering the MPs released by the *S. bacillaris* strains when cultured alone, the protective activity (obtained considering the mean value of all the single-strain *S. bacillaris*) was always significantly higher than the MPs produced by the EC1118 strain ($p<0.001$). This

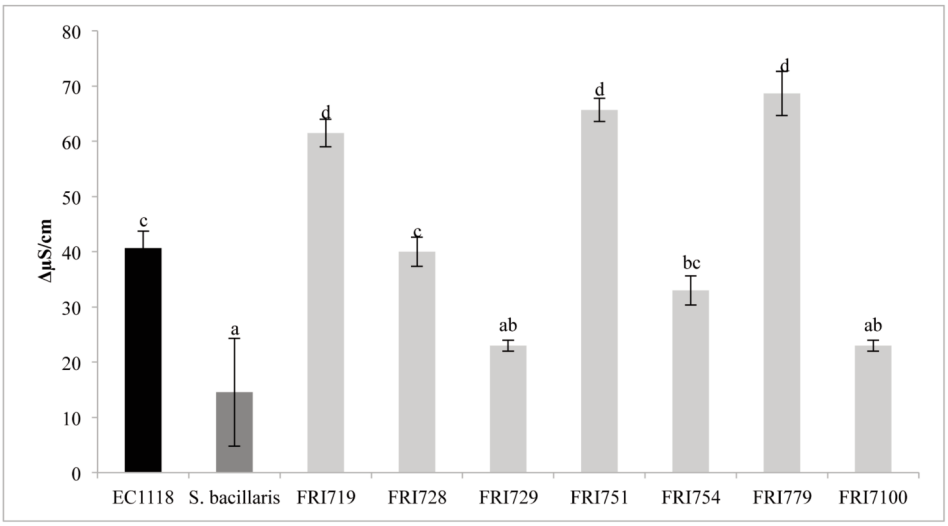

FIGURE 3. Tartaric stabilisation in *S. cerevisiae* (EC1118) and S. bacillaris single-strain fermentation (mean of all strains) and sequential fermentation.

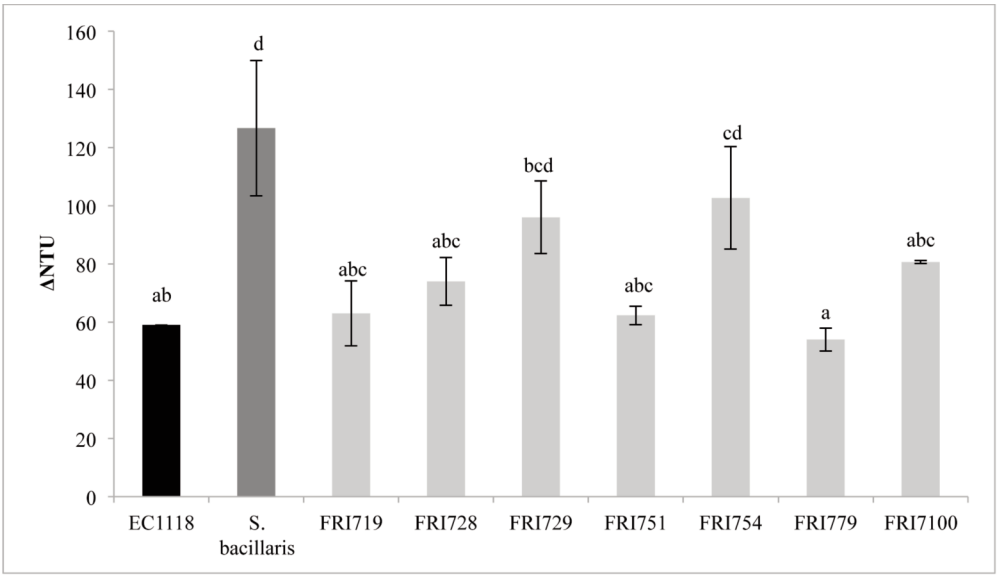

Figure 4. Protein stabilisation in *S. cerevisiae* (EC1118) and S. bacillaris single-strain fermentation (mean of all strains) and sequential fermentation. The difference in ΔNTU from the untreated wine (225 NTU) was reported.

is in disagreement with the data reported by Ribeiro *et al.* (2014), which showed a higher stabilisation effect for MPs with higher mannose to glucose ratio. These data suggest that mannose content is not the only factor affecting the stabilising effect of MPs, and that other factors such as the nature of glycosyl linkages among sugars or the aminoacidic composition of the peptidic fraction could have an important effect on the chemical-physical behaviour of these macromolecules. When the same *S. bacillaris* strains were cultured in sequential inoculum with EC1118, the protein stabilisation effect of the obtained MPs was reduced, but in general, the stabilising effect is comparable to that of the commercial strain alone, and only in one case (FRI754) significantly higher (Figure 4). This strain requires further study, especially in real grape juice, as its use in combination with *S. cerevisiae* should decrease the bentonite demand for protein stabilisation, with wine qualitative improvement, and this could be interesting for winemakers. In addition, the particular stabilising ability of *S. bacillaris* MPs could also be of great interest for the wine products industry, as the most interesting yeast strains could be used for the manufacturing of new oenological products.

5. Foamability

The effect on wine foamability of the collected MPs, isolated from *Saccharomyces* and *Starmerella* strains in single or in sequential AF, was studied by adding them to a Prosecco base wine.

When added to the wine all at the same concentrations (50 mg/L), the MPs obtained from *Saccharomyces* single-AF and sequential AFs increased the foamability (Figure 5), causing an average increase of the maximum height (HM) of about 40%. This is in agreement with the data reported by several studies (Martínez-Rodríguez *et al.*, 2001; Núñez *et al.*, 2006; Vincenzi *et al.*, 2014; González-Royo *et al.*, 2015) which showed that yeast MPs are important for the wine foamability. The foam volumes were significantly higher than that of the original wine (CTRL), but no significant differences were found among the MPs produced by the different strains when cultured in sequential AF, except for strain FRI729. Apparently, the modifications induced by *S. bacillaris* on MPs produced and released in sequential AF did not influence their effect on wine foamability, at least at the tested concentration (50 mg/L). Therefore, it could be hypothesised that the MPs released by *S. cerevisiae* in the presence or absence of *Starmerella* have a similar effect in terms of

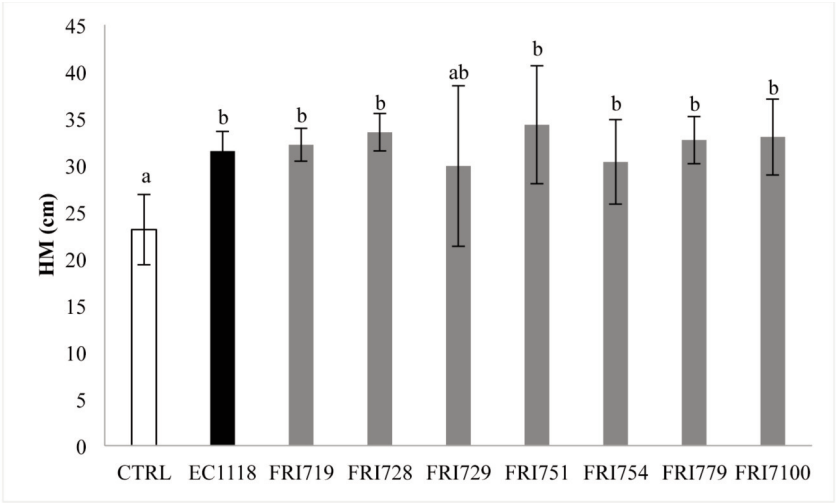

FIGURE 5. Maximum foam height (HM) obtained after the addition of 50 mg /L of mannoproteins isolated from single-strain fermentations of *Saccharomyces* and from sequential fermentations with the seven strains of *Starmerella bacillaris*. Samples with different letters are statistically different (p=0.05).

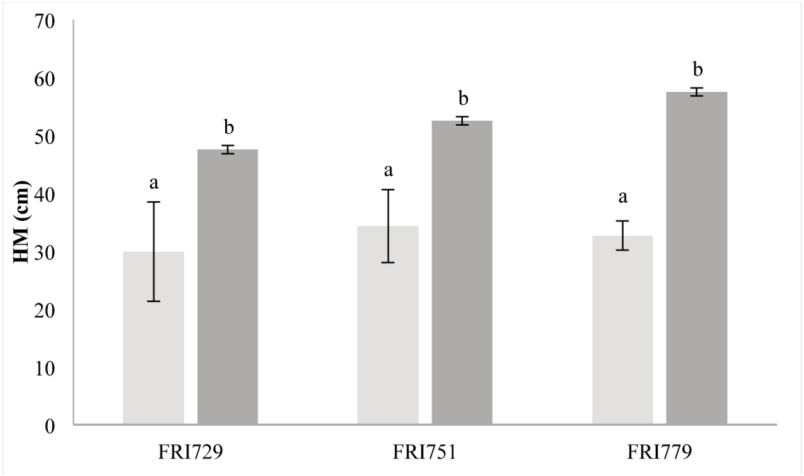

FIGURE 6. Maximum foam height (HM) obtained after the addition of 50 mg/L of mannoproteins isolated from different strains of Starmerella bacillaris in single-strain (■) and sequential (■) fermentation. For each strain, samples with different letters are statistically different (p<0.05).

wine foamability, and that the effect of the different strains of *Starmerella* is reflected in the quantity of released MPs. However, different concentrations of MPs, such as 100 mg/L, 200 mg/L or more, must be tested to verify the effect on wine foamability.

However, among the MPs produced in single-strain AF by *Starmerella bacillaris*, three (FRI729, FRI751 and FRI779) showed a much higher foaming capacity compared to those produced by the same strains in sequential AFs (Figure 6). This is clearly linked to a different composition of the MPs produced by *Starmerella* in single-strain AF in respect to those produced in sequential AF with *S. cerevisiae*, as reported above.

A PCA analysis on all the collected data (MPs release, FH (HM), tartaric and protein stabilisation) for single-strain and sequential AFs (Figure 7) showed that two main factors (F1 and F2) account for 97.40% of total variance, with F1 and F2 accounting for 76.31 % and 21.09 %, respectively. The analysis confirmed the high level of differences between MPs of the single-strain AF of *S. bacillaris*, grouped on the right side of the graphic, and that of EC1118 (both in single-strain and in sequential AF), grouped on the left side.

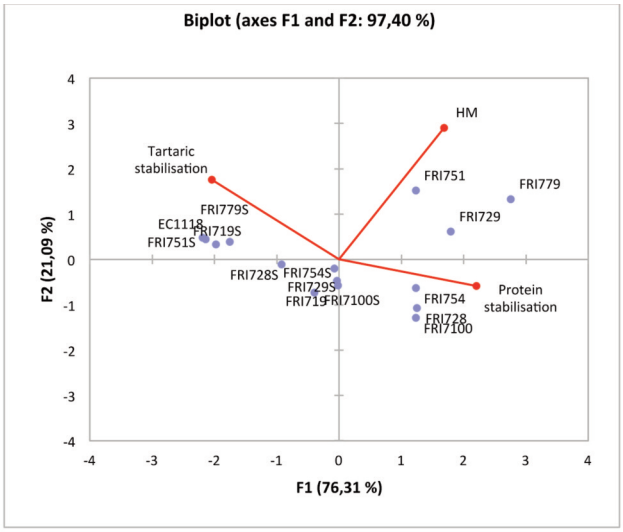

FIGURE 7. Principal component analysis (PCA) biplot showing fermentation performances (variables: mannoprotein production, foam height (HM), tartaric and protein stabilisation) of single-strain fermentations of *S. bacillaris* strains (observations from FRI719 to FRI7100) and strain EC1118 (observation EC1118) and of sequential fermentation of *S. bacillaris* strains together with EC1118 (observations from FRI719S to FRI7100S) in MS300. Only variables that showed significant correlations are reported.

It is interesting to note that the ability to decrease the tartaric instability in general does not coexist with the ability to improve the protein stability, in fact the two vectors are opposite, with a correlation coefficient of -0.887. This behaviour confirms that the tartaric and protein stabilisation are based on different mechanisms and, consequently, differently affected by the MPs structure.

The foaming properties of these MPs (HM), on the contrary, are not correlated to the other two parameters.

CONCLUSIONS

Even though they are not able to complete the AF, *S. bacillaris* strains can be used in sequential AF with *S. cerevisae* to improve some organoleptic properties. In addition, the data here reported demonstrate that the interaction between the two microorganisms affect differently the MPs production in terms of both quantity and quality. All the tested strains in sequential AF increased the total MPs production, but the strains affected in different ways the MPs properties in terms of tartaric and protein stabilities. This is interesting from the technological point of view, as it suggests a possibility to use different selected strains to obtain specific characteristics. It will be interesting to check the results in a real grape juice, as there may be differences in respect to synthetic must. In fact, a direct relationship between the degree of grape must clarification and the quantity of yeast macromolecules recovered in the wine has been described (Guilloux-Benatier *et al.*, 1995). Furthermore, Boivin *et al.* (1998) reported that yeast cell-wall porosity increased in clarified must, but that macromolecule production decreased. The most interesting aspect is that the improvement in stability can be achieved during the AF, reducing the extent of stabilising treatments at the end of winemaking process. This is converted in a greater sustainability as it leads to a reduction of costs and environmental pollution and in an improvement of organoleptic properties (i.e. a reduction of aroma loss associated with bentonite fining).

In addition, the particular ability of MPs released in single-strain AF by some *S. bacillaris* strains (i.e. a high effect on protein stabilisation or on foam volume) could be interesting as a source for the manufacturing of new oenological products.

Further studies in natural grape juice, aimed to verify the *S. bacillaris* MPs properties discovered in synthetic must, are needed, because in the latter medium yeasts could over-

release mannoproteins in comparison with fermentations in grape must.

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REFERENCES

Bely M., Sablayrolles J.M. and Barre P., 1990. Description of alcoholic fermentation kinetics: its variability and significance. *American Journal of Enology and Viticulture* 41(4), 319–324.

Bely M., Renault P., da Silva T., Masneuf-Pomarède I., Warren A., Moine V., Coulon J., Sicard D., de Vienne D. and Marullo P., 2013. Alcohol level reduction in wine. In P.L. Teissedre (Ed.), *Non-conventional yeasts and alcohol level reduction*. Bordeaux: Vigne et Vin Publications Internationales, pp. 33–37.

Boivin S., Feuillat M., Alexandre H. and Charpentier C., 1998. Effect of must turbidity on cell wall porosity and macromolecule excretion of *Saccharomyces cerevisiae* cultivated on grape juice. *American Journal of Enology and Viticulture* 49(3), 325–332.

Bovo B., Giacomini A. and Corich V., 2011. Effects of grape marcs acidification treatment on the evolution of indigenous yeast populations during the production of grappa. *Journal of Applied Microbiology* 111, 382–388. doi:10.1111/j.1365–2672.2011.05060.x

Bovo B., Nadai C., Vendramini C., Fernandes Lemos Junior W.J., Carlot M., Skelin A., Giacomini A. and Corich V., 2016. Aptitude of *Saccharomyces* yeasts to ferment unripe grapes harvested during cluster thinning for reducing alcohol content of wine. *International Journal of Food Microbiology* 236, 56–64. doi:10.1016/j.ijfoodmicro.2016.07.022

Brown S.L., Stockdale V.J., Pettolino F., Pocock K.F., de Barros Lopes M., Williams P.J., Bacic A., Fincher G.B., Høj P.B. and Waters E.J., 2007. Reducing haziness in white wine by overexpression of *Saccharomyces cerevisiae* genes YOL155c and YDR055w. *Applied Microbiology and Biotechnology* 73(6), 1363. doi:10.1007/s00253-006-0606-0

Caridi A., 2006. Enological functions of parietal yeast mannoproteins. *Antonie van Leeuwenhoek* 89, 417–422. doi:10.1007/s10482–005–9050-x

Chalier P., Angot B., Delteil D., Doco T. and Gunata Z., 2007. Interactions between aroma compounds and whole mannoprotein isolated from *Saccharomyces cerevisiae* strains. *Food Chemistry* 100(1), 22–30. doi:10.1016/j.foodchem.2005.09.004

Ciani M. and Comitini F., 2011. Non-Saccharomyces wine yeasts have a promising role in biotechnological approaches to winemaking. *Annals of Microbiology* 61, 25–32. doi: 10.1007/s13213–010–0069–5

Ciani M. and Comitini F., 2015. Yeast interactions in multi-starter wine fermentation. *Current Opinion in Food Science* 1, 1–6. doi:10.1016/j.cofs.2014.07.001

Coelho E., Rocha, S.M. and Coimbra M.A., 2011. Foamability and foam stability of molecular reconstituted model sparkling wines. *Journal of Agricultural and Food Chemistry* 59(16), 8770–8778. doi:10.1021/jf2010657

Comitini F., Gobbi M., Domizio P., Romani C., Lencioni L., Mannazzu I. and Ciani M., 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiology* 28(5), 873–882. http://doi.org/10.1016/j.fm.2010.12.001

Comuzzo P., Tat L., Fenzi D., Brotto L., Battistutta F. and Zironi P., 2011. Interactions between yeast autolysates and volatile compounds in wine and model solution. *Food Chemistry* 127(2), 473–480. ttps://doi.org/10.1016/j.foodchem.2011.01.026

Dai J., Wu Y., Chen S.W., Zhu S., Yin H.P., Wang M. and Tang J., 2010. Sugar compositional determination of polysaccharides from *Dunaliella salina* by modified RP-HPLC method of precolumn derivatization with 1-phenyl-3-methyl-5-pyrazolone. *Carbohydrate Polymers* 82(3), 629–635. doi:10.1016/j.carbpol.2010.05.029

Del Fresno J.M., Morata A., Loira I., Bañuelos M.A., Escott C., Benito S., González Chamorro C. and Suárez-Lepe J.A., 2017. Use of non-*Saccharomyces* in single-culture, mixed and sequential fermentation to improve red wine quality. *European Food Research and Technology* 243(12), 2175–2185. doi: 10.1007/s00217–017–2920–4

Domizio P., Liu, Y., Bisson L.F. and Barile D., 2014. Use of non-*Saccharomyces* wine yeasts as novel sources of mannoproteins in wine. *Food Microbiology* 43, 5–15. doi: 10.1016/j.fm.2014.04.005

Dupin I.V., McKinnon B.M., Ryan C., Boulay M., Markides A.J., Jones G.P., Williams P.J. and Waters E.J., 2000a. *Saccharomyces cerevisiae* mannoproteins that protect wine from protein haze: their release during fermentation and lees contact and a proposal for their mechanism of action. *Journal of Agricultural and Food Chemistry* 48(8), 3098–3105. doi: 10.1021/jf0002443

Dupin I.V., Stockdale V.J., Williams P.J., Jones G.P., Markides A.J. and Waters E.J., 2000b. *Saccharomyces cerevisiae* mannoproteins that protect wine from protein haze: evaluation of extraction methods and immunolocalization. *Journal of Agricultural and Food Chemistry* 48(4), 1086–1095. doi:10.1021/jf9905020

Englezos V., Rantsiou K., Giacosa S., Segade S.R., Rolle L. and Cocolin L., 2019. Cell-to-cell contact

mechanism modulates *Starmerella bacillaris* death in mixed culture fermentations with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology* 289, 106–114. doi:10.1016/j.ijfood micro.2018.09.009

Escot S., Feuillat M., Dulau L. and Charpentier C., 2001. Release of polysaccharides by yeasts and the influence of released polysaccharides on colour stability and wine astringency. *Australian Journal of Grape and Wine Research* 7(3), 153–159.

Gerbaud V., Gabas, N., Laguerie C., Blouin J., Vidal S., Moutounet M. and Pellerin P., 1996. Effect of wine polysaccharides on the nucleation of potassium hydrogen tartrate in model solutions. *Chemical Engineering Research and Design* 74(7), 782–790.

Giovani G., Rosi I. and Bertuccioli M., 2012. Quantification and characterization of cell wall polysaccharides released by non-*Saccharomyces* yeast strains during alcoholic fermentation. *International Journal of Food Microbiology* 160(2), 113–118. doi: 10.1016/j.ijfoodmicro.2012.10.007.

Gobbi M., Comitini F., Domizio P., Romani C., Lencioni L., Mannazzu I. and Ciani M., 2013. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. *Food Microbiology* 33(2), 271–281. doi:10.1016/j.fm. 2012.10.004

Gonzalez-Ramos D. and Gonzalez P., 2006. Genetic determinants of the release of mannoproteins of enological interest by *Saccharomyces cerevisiae*. *Journal of Agricultural and Food Chemistry* 54(25), 9411–9416. doi:10.1021/jf062196d

Gonzales-Ramos D., Cebollero E. and Gonzalez P., 2008. A recombinant *Saccharomyces cerevisiae* strain overproducing mannoproteins stabilizes wine against protein haze. *Applied and Environmental Microbiology* 74(17), 5533–5540. doi: 10.1128/AEM .00302–08.

González-Royo, E., Pascual, O., Kontoudakis, N., Esteruelas M., Esteve-Zarzoso B., Mas A., Canals J.M. and Zamora F., 2015. Oenological consequences of sequential inoculation with non-*Saccharomyces* yeasts (*Torulaspora delbrueckii* or *Metschnikowia pulcherrima*) and *Saccharomyces cerevisiae* in base wine for sparkling wine production. *European Food Research and Technology* 240(5), 999–1012. doi:10.1007/s00217 –014–2404–8

Guilloux-Benatier M., Guerreau J. and Feuillat M., 1995. Influence of initial colloid content on yeast macromolecule production and on the metabolism of wine microorganisms. *American Journal of Enology and Viticulture* 46(4), 486–492.

Jolly N.P., Augustyn O.P.H. and Pretorius I.S., 2003. The use of Candida pulcherrima in combination with *Saccharomyces cerevisiae* for the production of Chenin blanc wine. *South African Journal of Enology and Viticulture* 24(2), 63–69. doi:10.21548/24-2-2641

Jolly N.P., Varela C. and Pretorius I.S., 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Research* 14(2), 215–237. doi:10.1111/1567-1364.12111

Ledoux V., Dulau L. and Dubourdieu D., 1992. Interprétation de l'amélioration de la stabilité protéique des vins au cours de l'élevage sur lies. *J. Int. Sci. Vigne Vin* 26(4), 239–251. doi:10.20870 /oeno-one.1992.26.4.1188

Lemos Junior W.J., Bovo B., Nadai C., Crosato G., Carlot M., Favaron F., Giacomini A. and Corich V., 2016. Biocontrol ability and action mechanism of *Starmerella bacillaris* (synonym *Candida zemplinina*) isolated from wine musts against gray mold disease agent *Botrytis cinerea* on grape and their effects on alcoholic fermentation. *Frontiers in Microbiology* 7, 1249. doi: 10.3389/fmicb.2016. 01249.

Lemos Junior W.J.F., Treu L., Duarte V.D.S., Campanaro S., Nadai C., Giacomini A. and Corich V., 2017a. Draft Genome Sequence of the Yeast *Starmerella bacillaris* (syn. *Candida zemplinina*) FRI751 Isolated from Fermenting Must of Dried Raboso Grapes. *Genome Announcements* 5(17), pii: e00224–17. doi: 10.1128/genome A.00224–17.

Lemos Junior W.J.F., Treu L., da Silva, Duarte V., Carlot M., Nadai C., Campanaro S., Giacomini A. and Corich V., 2017b. Whole-Genome Sequence of *Starmerella bacillaris* PAS13, a Non conventional Enological Yeast with Antifungal Activity. *Genome Announcements* 5(32), pii: e00788–17. doi: 10.1128/genomeA.00788–17.

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

Llaubères P.M., Dubourdieu D. and Villettaz J.C., 1987. Exocellular polysaccharides from *Saccharomyces* in wine. *Journal of the Science of Food and Agriculture* 41(3), 277–286. doi:10.1002/ jsfa.27404 10310.

Loira I., Vejarano P., Bañuelos M.A., Morata A., Tesfaye W., Uthurry C., Villa A., Cintora I. and Suárez-Lepe J.A., 2014. Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality. *LWT-Food Science and Technology* 59(2), 915–922. doi:10.1016/j.lwt.2014.06.019

Martínez-Rodríguez A., Carrascosa A.V., Barcenilla J.M., Pozo-Bayón M.A. and Polo M.C., 2001. Autolytic capacity and foam analysis as additional criteria for the selection of yeast strains for sparkling wine production. *Food Microbiology* 18(2), 183–191. doi:10.1006/fmic.2000.0390

Medina-Trujillo L., González-Royo E., Sieczkowski N., Heras J., Canals J.M. and Zamora F., 2017. Effect of sequential inoculation (*Torulaspora delbrueckii/Saccharomyces cerevisiae*) in the first fermentation on the foaming properties of sparkling wine. *European Food Research and Technology* 243(4), 681–688. doi:10.1007/s00217–016–2781–2

Moine-Ledoux V. and Dubourdieu D., 1999. An invertase fragment responsible for improving the protein stability of dry white wines. *Journal of the Science of Food and Agriculture* 79(4), 537–543. http://doi.org/10.1002/(SICI)1097–0010(19990315)79:4<537::AID-JSFA214>3.0.CO;2-B

Moine-Ledoux V. and Dubourdieu D., 2002. Rôle des mannoprotéines de levures vis-à-vis de la stabilisation tartrique des vins. *Bulletin de l'OIV* 75(857–58), 471–482.

Nadai C., Treu L., Campanaro S., Giacomini A. and Corich V., 2016. Different mechanisms of resistance modulate sulfite tolerance in wine yeasts. *Applied Microbiology and Biotechnology* 100(2), 797–813. doi:10.1007/s00253-015-7169-x

Nadai C., Junior W. J.F.L., Favaron F., Giacomini A. and Corich V., 2018. Biocontrol activity of *Starmerella bacillaris* yeast against blue mold disease on apple fruit and its effect on cider fermentation. *PloS One* 13(9), e0204350. doi:10.1371/journal.pone.0204350

Núñez Y.P., Carrascosa A.V., González P., Polo M.C. and Martínez-Rodríguez A., 2006. Isolation and characterization of a thermally extracted yeast cell wall fraction potentially useful for improving the foaming properties of sparkling wines. *Journal of Agricultural and Food Chemistry* 54(20), 7898–7903. doi:10.1021/jf0615496

Padilla B., Gil J.V. and Manzanares P., 2016. Past and future of non-*Saccharomyces* yeasts: from spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology* 7, 411. doi: 10.3389/fmicb.2016.00411.

Pocock K.F. and Rankine B.C., 1973. Heat test for detecting protein instability in wine. *Australian Wine, Brewing and Spirit Review* 91(5), 42–43.

Quirós M., Gonzalez-Ramos D., Tabera L. and Gonzalez P., 2010. A new methodology to obtain wine yeast strains overproducing mannoproteins. *International Journal of Food Microbiology 139(1)*, 9–14. doi: 10.1016/j.ijfoodmicro.2010.02.014.

Rantsiou K., Dolci P., Giacosa S., Torchio F., Tofalo P., Torriani S., Suzzi G., Rolle L. and Cocolin L., 2012. *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. *Applied and Environmental Microbiology* 78(6), 1987–1994. doi:10.1128/AEM. 06768–11.

Ribeiro T., Fernandes C., Nunes F.M., Filipe-Ribeiro L. and Cosme F., 2014. Influence of the structural features of commercial mannoproteins in white wine protein stabilization and chemical and sensory properties. *Food Chemistry* 159, 47–54. doi:10.1016/ j.foodchem.2014.02.149

Ribéreau-Gayon P., Dubourdieu D., Donèche B. and Lonvaud A., 2006. *Handbook of enology: The microbiology of wine and vinifications*. Chichester: Wiley and Sons Ltd., Vol. 1). doi:10.1002/04700 10363

Rudin A.D., 1957. Measurement of the foam stability of beers. *Journal of Institute of Brewing* 63, 506e509. doi:10.1002/j.2050-0416.1957.tb06291.x

Taillandier P., Lai Q.P., Julien-Ortiz A. and Brandam C., 2014. Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: influence of inoculation and nitrogen content. *World Journal of Microbiology and Biotechnology* 30(7), 1959–1967. doi:10.1007/s11274-014-1618-z

Vidal S., Williams P., Doco T., Moutounet M. and Pellerin P., 2003. The polysaccharides of red wine: total fractionation and characterization. *Carbohydrate Polymers* 54(4), 439–447. doi:10.1016/S0144–8617(03)00152–8.

Vilela A., 2019. Use of nonconventional yeasts for modulating wine acidity. *Fermentation* 5(1), 27. doi:10.3390/fermentation5010027

Vincenzi S., Crapisi A. and Curioni A., 2014. Foamability of Prosecco wine: cooperative effects of high molecular weight glycocompounds and wine PR-proteins. *Food Hydrocolloids* 34, 202–207. doi:10.1016/j.foodhyd.2012.09.016

Wang C., Esteve-Zarzoso B. and Mas A., 2014. Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* (synonym *Candida zemplinina*). populations during alcoholic fermentation by fluorescence in situ hybridization. *International Journal of Food Microbiology* 191, 1–9. doi: 10.1016/j.ijfoodmicro.2014.08.014.

Waters E.J., Wallace, W., Tate M.E. and Williams P.J., 1993. Isolation and partial characterization of a natural haze protective factor from wine. *Journal of Agricultural and Food Chemistry* 41(5), 724–730. doi:10.1021/jf00029a009

Waters E.J., Pellerin P. and Brillouet J.M., 1994. A *Saccharomyces* mannoprotein that protects wine from protein haze. *Carbohydrate Polymers* 23(3), 185–191. doi:10.1016/0144-8617(94)90101-5

