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Impact of mannoproteins from different yeast species on wine properties

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ABSTRACT

The extent to which the addition of extracted mannoproteins (MPs) improve wine properties such as mouthfeel, clarity and colour stability is a controversial topic, and conflicting results have been reported. One possible explanation for this is the diversity that exists between MPs, a prevalent cause for which is their yeast strain of origin. However, although wine yeast species other than *Saccharomyces cerevisiae* possibly present an untapped source of MPs, their influence on wine as extracted additives is still ill-characterised. This study sought to compare the impact of MPs extracted and purified from different yeast species, named *Saccharomyces boulardii*, *Saccharomyces cerevisiae*, *Metschnikowia fructicola* and *Torulaspora delbrueckii*, as well as a commercial control, on wine. MPs were applied to a red and a white wine at three different concentrations, and BSA-reactive tannins, polymeric pigments, colour characteristics, browning potential and protein haze-forming potential were measured over the course of six months. The most notable differences were observed for the commercial MP, which achieved lower BSA-reactive tannins, increased polymeric pigments and a greater reduction of browning potential. This could be due to the difference in preparation procedures compared to the MPs extracted and purified for this study, possibly leading to variations in the commercial MPs' structure and composition. However, some differences were also evident between species, with *M. fructicola* treatments achieving a 20 % reduction in browning compared to ~10 % for the other purified MPs and significantly increased colour intensity of red wine treated with low concentrations of *T. delbrueckii*. This study highlights alternative yeast species as a potential source of MPs with diverse benefits to wine and the need for further investigation into their diversity and properties to promote their eventual exploitation.

KEYWORDS: mannoproteins, yeasts, wine, colour, browning

INTRODUCTION

During wine alcoholic fermentation and ageing, mannoproteins are released into the wine matrix, where they have been shown to positively affect various technological and organoleptic properties such as protein and tartrate stability, mouthfeel, astringency, colour, and foamability in sparkling wines (Dupin *et al.*, 2000; Escot *et al.*, 2001). Although this activity is studied mostly in *Saccharomyces cerevisiae*, several other yeast species such as *Torulaspora delbrueckii*, *Schizosaccharomyces pombe* and *Saccharomycodes ludwigii* have been shown to release a greater quantity of polysaccharides, including mannoproteins, during alcoholic fermentation in comparison to *S. cerevisiae* (Belda *et al.*, 2016; Domizio *et al.*, 2014; Domizio *et al.*, 2017; Giese *et al.*, 2016; Giovani *et al.*, 2012). Furthermore, mannoproteins released from different yeasts have been shown to improve wine properties in diverse ways. For instance, whereas *S. pombe* and *Lachancea thermotolerans* MPs improved wine mouthfeel, those from *T. delbrueckii* provided haze protection and colour stabilisation (Belda *et al.*, 2016; Benito *et al.*, 2019).

Mouthfeel is an organoleptic property closely linked to the complex sensation of astringency, which has been described as a drying, roughing and puckering of the mouth surfaces (Gawel *et al.*, 2000). Although it is considered an important contributor to the quality of red wine, excessive or unbalanced astringency is often perceived as unpleasant by the consumer (Ramos-Pineda *et al.*, 2022). It is generally considered that astringency arises due to the binding of salivary proteins by wine tannins and their eventual precipitation as insoluble aggregates in the mouth, causing a loss of lubrication and increased friction (Ramos-Pineda *et al.*, 2022). Commercial yeast mannoproteins have previously been shown to modulate astringency by preventing or reducing insoluble polyphenol–protein precipitate formation (Ramos-Pineda *et al.*, 2018). However, important structural differences, such as the glycosidic content, may determine how mannoproteins interact with phenolic compounds and salivary proteins (Manjón *et al.*, 2021). Indeed, differences in the saccharide-to-protein ratio between mannoproteins from different yeast species have been evidenced in previous work (Giovani *et al.*, 2012).

Grape tannins are also involved in the modification and stabilisation of colour in red wines when they partake in condensation reactions with anthocyanins extracted from the berry skin, leading to the formation of new polymeric pigments (Somers, 1971; Cheynier *et al.*, 2006). As opposed to the fluctuations in absorbance at 520 nm observed for anthocyanins, which are largely responsible for the vibrant red colour of young wines, under pH changes and in the presence of bisulfite, absorbance due to polymeric pigments is mostly consistent under these conditions (Harbertson *et al.*, 2003; de Freitas *et al.*, 2017). An increase in polymeric pigments, therefore, represents an increase in the colour stability of red wine, often along with a deepening of wine colour and increased hue, and they have furthermore been shown to

contribute to a decrease in astringency (Cheynier *et al.*, 2006; Rinaldi and Moio, 2018). The contribution to polymeric pigment stability by the presence of mannoproteins has been observed in previous studies (Del Barrio-Galán *et al.*, 2012; Escot *et al.*, 2001; Rinaldi *et al.*, 2019). Alcalde-Eon *et al.* (2014) demonstrated the ability of a mannoprotein obtained through enzymatic extraction to stabilise some anthocyanin-derived pigments from a colloidal point of view, thus preventing their precipitation. Nevertheless, different mannoprotein preparations, even when derived from the same yeast species, do not necessarily display the same stabilising effect, and some have even been shown to exert no improvement in this regard at all (Guadalupe *et al.*, 2010; Guadalupe and Ayestarán, 2008; Rodrigues *et al.*, 2012). Some colour modifications in white wine are also linked to the formation of new polymeric pigments. However, these modifications are often unfavourable, such as the darkening, browning or yellowing of the wine that arises when phenolic compounds undergo oxidation reactions (Simpson, 1982; Zhao *et al.*, 2023). Different mannoproteins have been hypothesised to reduce white wine browning to various extents through interacting with these compounds, thus preventing their oxidation (Ribeiro *et al.*, 2014).

Haze formation is another quality defect found particularly in white wine. It is thought to arise primarily due to the unfolding and aggregation of certain pathogenesis-related grape proteins (Pocock and Waters, 2006; Van Sluyter *et al.*, 2015). However, many non-proteinaceous wine matrix components, such as phenolic compounds and polysaccharides, have also been shown to play a role in wine haze, although their interactions with each other and the consequent impact on the potential formation of haze have not been fully elucidated (Gazzola *et al.*, 2012; Lomolino and Curioni, 2007; McRae *et al.*, 2018). Previous studies have shown the ability of mannoproteins to stabilise the proteins responsible for haze formation by reducing their aggregate particle size (Waters *et al.*, 1993). It has also been suggested that the interaction of mannoproteins with phenolics may also play a role in the modification of wine haze, possibly through a competitive mechanism, as the interaction of some wine proteins with polyphenolics is likely involved in the formation of haze (Lomolino and Curioni, 2007).

Although it has been established that the mannoproteins released by some non-*Saccharomyces* yeasts during alcoholic fermentation and ageing on the lees may be involved in the modification of these wine properties to various extents, the evaluation of this impact has been limited to the participation of these strains in wine fermentations. The exogenous addition to the wine of mannoproteins extracted and isolated from the yeast cell walls could aid in unravelling their impact without the risk of microbial competition. Direct comparison of mannoproteins from different yeast species would furthermore be made possible without the interference of other metabolites released by the studied organisms that could also influence wine properties. Additionally, the dose-dependent impact of exogenous mannoproteins could be studied by

applying these compounds at different concentrations to wine. Lastly, the ability to study these compounds in isolation provides the opportunity for further investigations into their structure and composition, their behaviour in wine and their interaction with wine macromolecules, such as proteins and polyphenols, and the link to their impact on wine properties. In this context, this study aims to evaluate the impact of mannoproteins isolated from four different oenologically relevant yeast species as well as a commercial mannoprotein on red wine astringency and colour and white wine browning and haze-forming potential.

MATERIALS AND METHODS

1. Wines

The white (Chardonnay) and red (Cabernet-Sauvignon) wines used in this study were previously produced at the Stellenbosch University experimental cellar from grape berries harvested at Groenhof farm (Stellenbosch, South Africa) in 2020 and Bellevue vineyards (Stellenbosch) in 2021, respectively. After crushing and destemming, Chardonnay berries were pressed immediately using a pneumatic press, whereas Cabernet-Sauvignon berries and juice underwent pre-fermentative maceration for 4 days at 4 °C and the completion of alcoholic fermentation before pressing. Fermentation progression was followed through weight loss measurements as an indication of CO₂ release, and the completion of fermentation was confirmed with residual sugar analyses. Both were fermented with *Saccharomyces cerevisiae* Lalvin QA23™ (Lallemand Inc., Montreal, QC, Canada) at 25 °C, and after alcoholic fermentation, Cabernet-Sauvignon wine underwent malolactic fermentation following the inoculation of *Oenococcus oeni* Lalvin VP41™ (Lallemand) and incubation at 20 °C. Wines were subsequently moved to -4 °C for clarification and cold stabilisation, and 50 ppm SO₂ was added. After two weeks, wines were racked off fermentation lees and stored at 4 °C until use. Before use, wines were filtered through 0.45 µm syringe filters (FilterBio®, Nantong City, China) and analysed for pH, ethanol, titratable and volatile acidity using Fourier-transform mid-infrared spectroscopy (FOSS WineScan, Hillerød, Denmark) as described by Nieuwoudt *et al.* (2006). Protein haze potential was determined using a predictive heat assay as described by Pocock and Waters (2006), and turbidity was measured using a nephelometer (HI88703 Turbidimeter, Hanna Instruments®, Woonsocket, RI, USA). Total polyphenolic index (TPI) was calculated as the absorbance

at 280 nm of wine diluted in a synthetic wine-like medium (12 % ethanol (v/v), 4 g/L tartaric acid, pH 3.3) multiplied by the dilution factor (Aleixandre-Tudo *et al.*, 2017). These parameters are summarised in Table 1.

2. Treatments and sampling

Mannoproteins (MPs) had previously been extracted according to the enzymatic and ultrasound method described by Snyman *et al.* (2021) from the yeast species *Saccharomyces boulardii* (SB62), *Saccharomyces cerevisiae* (SC01), *Metschnikowia fructicola* (MF77) and *Torulaspora delbrueckii* (TD70), all obtained from the Lallemand yeast culture collection. The extracts were furthermore purified through fast protein liquid chromatography (FPLC) on a concanavalin A-sepharose affinity chromatography column using an ÄKTA purifier system (GE Healthcare) according to Li and Karboune (2019) and freeze-dried. MPs were dissolved in 1.75 mL aliquots of red wine in 2-mL microcentrifuge tubes and in 10 mL aliquots of white wine in 15-mL conical centrifuge tubes to reach final concentrations of 0.5, 0.125 and 0.05 mg dry weight/mL in triplicate. A commercial mannoprotein product supplied by Lallemand Oenology (Blagnac, France) was also included at the same concentrations (hereinafter referred to as LMP), as well as a control treatment with no added mannoproteins. Wines were stored at 15 °C between sampling points. At the time of MPs addition (T0), one (T1), three (T3) and six (T6) months after addition, 350 µL of the red wine was sampled for the analysis of colour characteristics, polymeric pigments, and BSA-reactive tannins. At the same time points, 2 mL of the white wine was sampled to analyse the browning and haze-forming potential.

3. Red wine evaluation

3.1. BSA-reactive tannins

The microplate tannin assay optimised by Heredia *et al.* (2006), which is based on the assay developed by Harbertson *et al.* (2003), was adapted for use in this study. This assay makes use of the ability of tannins to precipitate through interactions with proteins and the colour reaction with ferric chloride, which may be measured at a wavelength of 510 nm. Red wine samples from each treatment were diluted 2X in a synthetic wine-like medium (12 % ethanol (v/v), 4 g/L tartaric acid, pH 3.3), and 60 µL aliquots of this dilution were transferred in duplicate to 1.5-mL microcentrifuge tubes. These tubes each received 30 µL of an acetic acid/NaCl buffer (200 mM acetic acid,

TABLE 1. Oenological parameters of the wines used in this study. Data shown are the means for two independent experiments ± standard deviation.

Wine	pH	Ethanol ^a	TA ^b	VA ^c	ΔNTU ^d	TPI ^e
Chardonnay	3.97 ± 0.01	14.9 ± 0.01	4.63 ± 0.00	0.61 ± 0.02	21.4 ± 0.33	13.1 ± 0.78
Cabernet-Sauvignon	3.78 ± 0.00	12.4 ± 0.01	5.10 ± 0.00	0.47 ± 0.01	33.0 ± 1.34	38.5 ± 0.92

^a Millilitre of ethanol for 100 mL of wines at 20 °C. ^b Titratable acidity in g tartaric acid per litre. ^c Volatile acidity in g of acetic acid per litre. ^d Wine turbidity as a measure of the change in nephelometric turbidity units (ΔNTU) obtained after the predictive heat assay. ^e Total phenolic index.

170 mM NaCl, pH 4.9) containing 1 mg/mL BSA (Bovine Serum Albumin, Sigma-Aldrich, Johannesburg, South Africa) and the mixture was allowed to incubate at room temperature for 15 min. Samples were then centrifuged at $13,500 \times g$, and the supernatant was discarded. The pellet was washed with 250 μ L of the acetic acid/NaCl buffer, and the mixture was centrifuged again at $13,500 \times g$ for 1 min. After discarding the supernatant, the pellet was redissolved in 220 μ L of a TEA/SDS buffer (5 % triethanolamine (v/v), 5 % sodium dodecyl sulphate (w/v), Sigma-Aldrich). The sample was allowed to incubate at room temperature for 10 min, vortexed, and left for a further 10 min. Fifty μ L aliquots were then transferred in duplicate to a 96-well microplate (Greiner F-bottom, Sigma-Aldrich) before reading the background absorbance at 510 nm using a Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer with SkanIt™ software, with 50 μ L aliquots of TEA/SDS buffer as a blank. Subsequently, 20 μ L of ferric chloride reagent (10 mM FeCl₃, 10 mM HCl, Sigma-Aldrich) was added to the remaining 120 μ L sample and vortexed. After a 10-minute incubation period at room temperature, the final absorbance at 510 nm of 50 μ L aliquots was measured using TEA/SDS buffer as the blank.

A catechin standard curve was prepared by creating a serial dilution of (+)-catechin hydrate (Sigma-Aldrich) in TEA/SDS buffer at concentrations of 0, 5, 25, 50, 125 and 250 mg/mL. 220 μ L aliquots of each dilution were transferred to new 1.5-mL microcentrifuge tubes in duplicate, and background readings and final absorbance readings after FeCl₃ addition were performed as described in the previous paragraph. A graph was plotted in Microsoft Excel (version 2206; Microsoft, Redmond, WA), and linear regression analysis was used to determine the amount of tannin expressed as catechin equivalents (CE).

Absorbance due to tannin was calculated by subtracting the value obtained after the multiplication of the background reading of a sample before FeCl₃ addition with 0.875 from the value obtained after subtracting the reading obtained from TEA/SDS buffer containing no catechin from the final reading after FeCl₃ addition to the sample:

$$\text{Absorbance due to tannin} = [(A_{510\text{nm}} \text{ after FeCl}_3) - (A_{510\text{nm}} \text{ buffer})] - (A_{510\text{nm}} \text{ before FeCl}_3 \times 0.875)$$

The amount of tannin in the sample was calculated from the absorbance after FeCl₃ addition minus the background absorbance using the catechin standard curve:

$$\text{Concentration of tannin} = \left[\frac{\text{Absorbance due to tannin} - \text{intercept}}{\text{slope}} \right] \times 3$$

where the factor “3” accounts for the dilution of the sample and the reaction dilution.

3.2. Polymeric pigments

The measurement of total polymeric pigments (small polymeric pigments and large polymeric pigments; SPP and LPP) was achieved using a spectrophotometric method

adapted from Harbertson *et al.* (2003). This assay is based on the bisulfite bleaching of monomeric pigments as well as the minimal absorbance at 520 nm contributed by monomers at pH 4.9, thus allowing the determination of polymeric pigments with minimal interference from monomeric anthocyanins. Red wine samples of 130 μ L were diluted with 260 μ L acetic acid/NaCl buffer (pH 4.9) in 1.5-mL microcentrifuge tubes and vortexed. Fifty μ L aliquots were then transferred in duplicate to a 96-well microplate before reading the background absorbance at 520 nm, using acetic acid/NaCl as a blank. To the remaining 290 μ L mixture, 23.2 μ L of 0.36 M potassium metabisulfite (K₂S₂O₅) was added. After a 10-minute incubation period at room temperature, the absorbance at 520 nm of 50 μ L aliquots was again determined. The absorbance obtained after K₂S₂O₅ addition was attributed to polymeric pigments (SPP + LPP), and the percentage contribution of polymeric pigments (PP) to wine colour at pH 4.9 was calculated as

$$\% \text{ Polymeric pigments} = \left(\frac{A_{520\text{nm}} \text{ after K}_2\text{S}_2\text{O}_5}{A_{520\text{nm}} \text{ before K}_2\text{S}_2\text{O}_5} \right) \times 100$$

3.3. Colour characteristics

The chromatic characteristics of red wine samples using the CIELab colour space were determined as proposed by the *Commission Internationale de l'Éclairage* (CIE, 1977) and as recommended for the application of wine by the Office International de la Vigne et du Vin (OIV, 2006). The CIE tristimulus values (X, Y, Z) and CIELab parameters of lightness (L*: L* = 0, black, and L* = 100, colourless), green/red colour component (a*: a* > 0, red, and a* < 0, green), blue/yellow colour component (b*: b* > 0, yellow, and b* < 0 blue) and total colour difference (ΔE^* : $\Delta E^* \geq 3$, colour difference between two red wine samples that is perceptible by the human eye) were determined according to Pérez-Caballero *et al.* (2003). Red wine samples were centrifuged at $13,500 \times g$ for 5 min, and the supernatants were transferred to a 96-well microplate in 50 μ L aliquots in duplicate, and water was used as a blank. Transmittance measurements were taken every 10 nm in the range of 380 nm to 780 nm on a Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer with SkanIt™ software, and illuminant D65 and 10° observer conditions were applied to the calculation of the colour coordinates.

Colour intensity (CI) was calculated using the absorbance values obtained at 420 nm, 520 nm and 620 nm according to the method of Glories (1984):

$$\text{CI} = A_{420\text{nm}} + A_{520\text{nm}} + A_{620\text{nm}}$$

4. White wine evaluation

4.1 Accelerated browning test

An accelerated test for the browning capacity of white wine samples was adapted from Singleton and Kramling (1976). Quadruplicate aliquots of 250 μ L white wine samples were transferred into 1.5-mL microcentrifuge tubes. Samples were saturated with air by shaking the tubes vigorously for 10 s and subsequently opening the cap for 5 s.

This process was repeated three times for each tube (Deshaies *et al.*, 2020). Duplicate aliquots of 100 µL from two of the tubes were transferred to a 96-well microplate, and the absorbance at 420 nm was measured (t0). The remaining two tubes were incubated in the dark at 55 °C for 10 days before measuring their absorbance in the same way (t10). A synthetic wine-like medium was used as a blank. Browning potential is calculated as the change in absorbance between samples before and after incubation at 55 °C. The percentage reduction of browning potential in treatment samples compared to control samples was obtained as follows:

$$\% \text{ browning reduction} = 100 - \left(\frac{t_{10\text{treatment}}A_{420\text{nm}} - t_{0\text{treatment}}A_{420\text{nm}}}{t_{10\text{control}}A_{420\text{nm}} - t_{0\text{control}}A_{420\text{nm}}} \times 100 \right)$$

4.2 Protein haze assay

The haze-forming potential of white wine samples was measured according to Pocock and Waters (2006). Samples were centrifuged at $6000 \times g$ for 5 min, and duplicate aliquots of 500 µL supernatants were transferred into 1.5-mL microcentrifuge tubes. From each tube, duplicate aliquots of 100 µL were transferred to a 96-well microplate, and the absorbance was measured at 520 nm. The remaining 300 µL of the sample was then incubated at 80 °C for 2 h and thereafter left at room temperature overnight (16 h) before again measuring absorbance at 520 nm in the same way. A synthetic wine-like medium was used as a blank. Haze-forming potential is depicted as the change in absorbance between heated and non-heated samples. Samples were considered unstable or prone to haze when this difference was greater than an absorbance unit of 0.02.

5. Statistical analysis

Analysis of variance (ANOVA) and multiple comparisons were performed using the computer software GraphPad Prism v. 9.1.2 (GraphPad, San Diego, CA, USA) to determine significant differences between treatments. The significance level was fixed at $p < 0.05$. Principal component analysis was performed using XLSTAT® (2022.3.2, Addinsoft SAS, New York, NY, USA).

RESULTS

1. Red wine evaluation

1.1. BSA-reactive tannins

The concentration of tannins that had precipitated with BSA was measured in red wine samples taken over the course of six months after MPs addition and is expressed in mg/mL of catechin equivalents (CE) (Figure 1). At T0 and T1, both the control wine and most wines treated with MPs yielded concentrations between 0.069–0.080 mg/mL CE without any significant differences. However, T0 samples of wine treated with 0.5 mg/mL LMP and T1 samples with 0.05, 0.125 and 0.5 mg/mL LMP all showed significantly lower concentrations of BSA-reactive tannins (0.005–0.008 mg/mL) compared to the controls sampled at the respective time-points. At T3, tannin concentrations had decreased to 0.021–0.028 mg/mL CE for most wine samples, with the exception of LMP-treated wines, which again showed significantly reduced concentrations (< 0.02 mg/mL) compared to the control. Tannin concentrations obtained at T6 ranged between 0.023–0.033 mg/mL CE for the control and all treatments, with no significant differences.

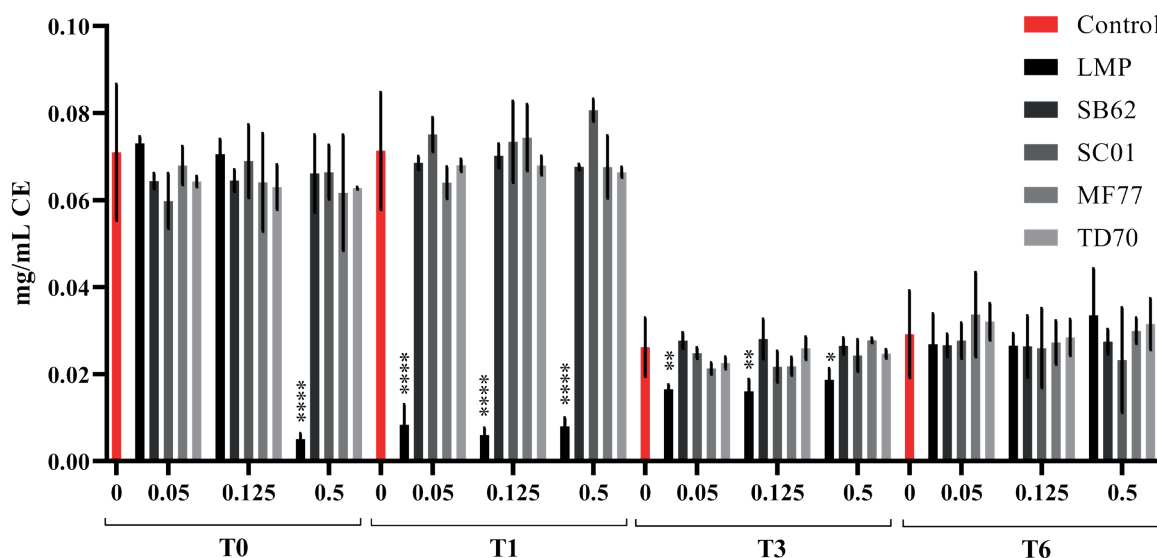


FIGURE 1. BSA-reactive tannins expressed in mg/mL catechin equivalents (CE) measured in red wine samples taken at T0, T1, T3 and T6 after MPs addition at concentrations of 0.05, 0.125 and 0.5 mg/mL from a commercial product (LMP), *S. boulardii* (SB62), *S. cerevisiae* (SC01), *M. fructicola* (MF77), *T. delbrueckii* (TD70), or no addition (control). The data points shown are the means for three independent experiments, and the error bars indicate the standard deviation between triplicates. Asterisks indicate significant differences between the treatment and the respective control within each time point as analysed by two-way ANOVA and Fisher's LSD test.

*: $p \leq 0.05$; **: $p \leq 0.01$; ****: $p \leq 0.0001$

1.2. Polymeric pigments

The percentage contribution of polymeric pigments (PP) to red wine colour at pH 4.9 was followed in the different MP treatments, and the results of this time-course experiment are depicted in Figure 2. At T0 and T1, the contribution of PP for the control and most treatments ranged between 34–39 %, with the exception of significantly increased contributions of PP for TD70 at T0 and for LMP at both time points compared to the respective controls. At T3, the contribution of PP to colour increased for all treatments to 50–55 %, and there were no significant differences from the control. At T6, PP colour contribution was significantly increased in 0.05 and 0.125 mg/mL LMP-treated wines, which reached 69 % and 71 %, respectively, compared to the control at 57 %. SB62-treated wines at 0.05 and 0.5 mg/mL, SC01-treated wines at 0.05 mg/mL, MF77-treated wines at 0.5 mg/mL and TD70-treated wines at 0.125 and 0.5 mg/mL also showed significant increases in %PP compared to the control.

1.3. Colour characteristics

Colour differences (due to L^* , a^* and b^* colour parameters) between MP-treated wines sampled at various time points over six months and their respective controls are represented by ΔE^* values in Figure 3. The individual L^* , a^* and b^* (L^* : lightness, a^* : green/red colour component, b^* : blue/yellow colour component) parameters derived from the CIE tristimulus values (X , Y , Z) are shown in Figure 4. For each MP, treatments with different concentrations showed mostly similar ΔE^* trends over time. This observation can also be made of the individual L^* , a^* and b^* parameters in Figure 4. However, some exceptions to this trend were evident, and differences between different MPs could also be found. While the ΔE^* values for 0.05 and 0.125 mg/mL LMP treatments had increased from 0.58 and 0.32 at T0 to 1.13 and 1.27 at T6, respectively, the same was not true for the 0.5 mg/mL treatment, which had decreased from 0.89 to 0.54. The 0.5 mg/mL SB62 treatment similarly decreased in colour difference over time.

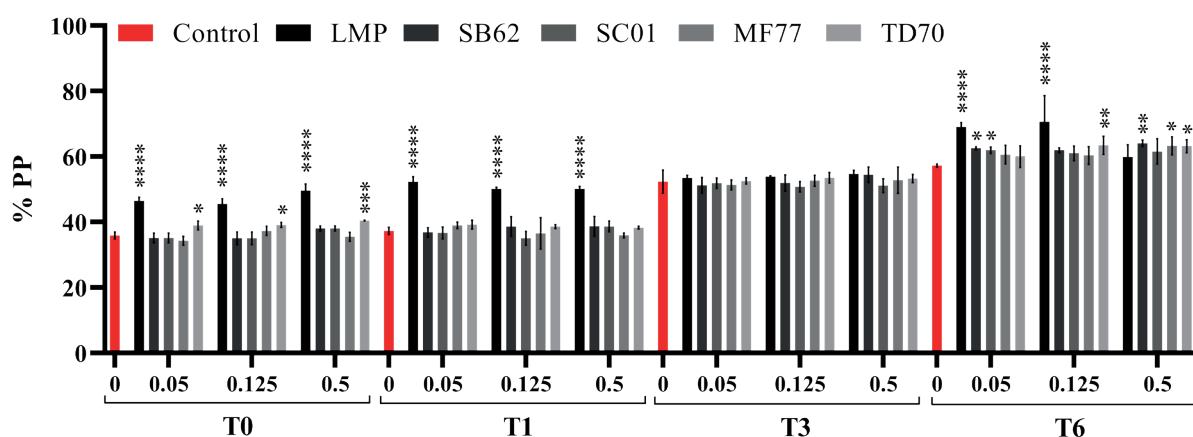


FIGURE 2. Percentage polymeric pigments (% PP) measured in red wine samples taken at T0, T1, T3 and T6 after MPs addition at concentrations of 0.05, 0.125 and 0.5 mg/mL from a commercial product (LMP), *S. boulardii* (SB62), *S. cerevisiae* (SC01), *M. fructicola* (MF77), *T. delbrueckii* (TD70), or no addition (control). The data points shown are the means for three independent experiments, and the error bars indicate the standard deviation between triplicates. Asterisks indicate significant differences between the treatment and the respective control within each time point as analysed by two-way ANOVA and Fisher's LSD test.

*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$

Although the initial ΔE^* value was 2.32, likely due to decreased lightness (L^*) and increased redness (a^*) compared to the control, after six months, it had decreased to 1.67. The 0.05 and 0.125 mg/mL SB62 treatments, however, showed greater colour differences at T6 (2.37 and 1.96, respectively) than both the 0.5 mg/mL treatment at this time and their initial T0 values. Colour in SC01-treated wines was mostly similar to the control at T6 for all MP concentrations after a decrease in initial colour differences due to increased lightness and decreased redness at T0 and T1. None of the LMP, SB62 or SC01 treatments reached ΔE^* values above 2.37. However, the 0.05 and 0.5 mg/mL treatments of both MF77 and TD70 showed colour differences at T6 that exceeded the threshold of $\Delta E^* \geq 3$, which indicates visibility to the human eye. Decreased lightness, increased redness and increased

yellowness are likely responsible for these differences. Indeed, the ΔE^* values for 0.05, 0.125 and 0.5 mg/mL MF77 treatments increased to 3.44, 3.07 and 2.89, respectively, whereas the 0.05 and 0.125 mg/mL TD70 treatments reached 5.53 and 5.21, respectively. However, the 0.5 mg/mL TD70-treated wine did not exceed 2.42.

Colour intensity (CI) calculated as the sum of the absorbances at 420 nm (Yellow), 520 nm (Red) and 620 nm (Blue) for the different MP treatments over time is shown in Figure 5. Differences in CI were revealed between wines treated with different MPs, although wines treated with different concentrations of the same MP showed similarities. Control wine CI gradually decreased from 0.47 at T0 to 0.43 at T6, and LMP-, SB62- and SC01-treated wines showed a similar CI decrease over time. However, CI in MF77-treated wine

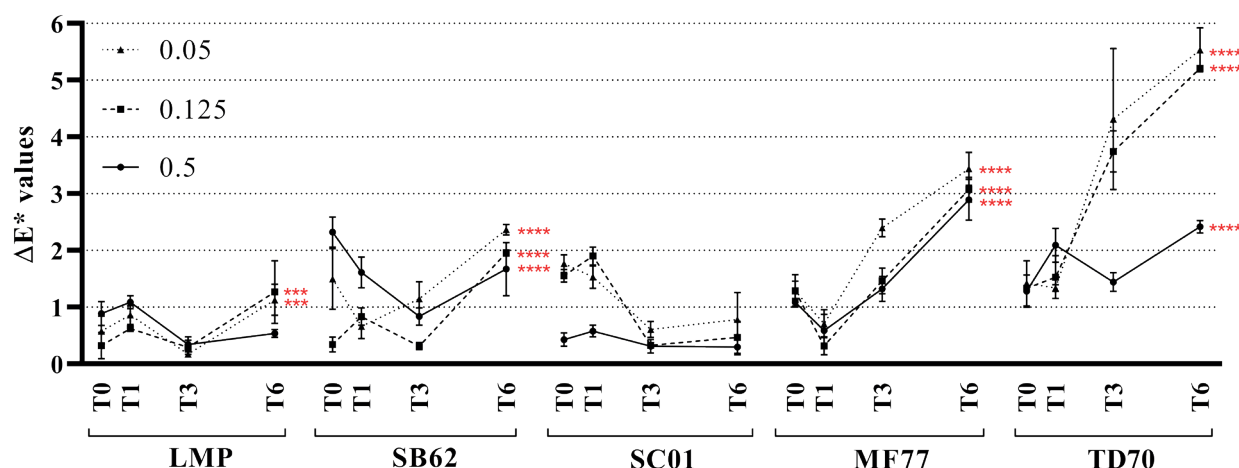


FIGURE 3. Colour differences (ΔE^*) between control wine and red wine samples taken at T0, T1, T3 and T6 after MPs addition at concentrations of 0.05, 0.125 and 0.5 mg/mL from a commercial product (LMP), *S. bouldarii* (SB62), *S. cerevisiae* (SC01), *M. fructicola* (MF77) or *T. delbrueckii* (TD70). The data points shown are the means for three independent experiments, and the error bars indicate the standard deviation between triplicates. Asterisks indicate significant differences between the treatment and control at the T6 time-point as analysed by one-way ANOVA and Dunnett's multiple comparisons test.

*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$

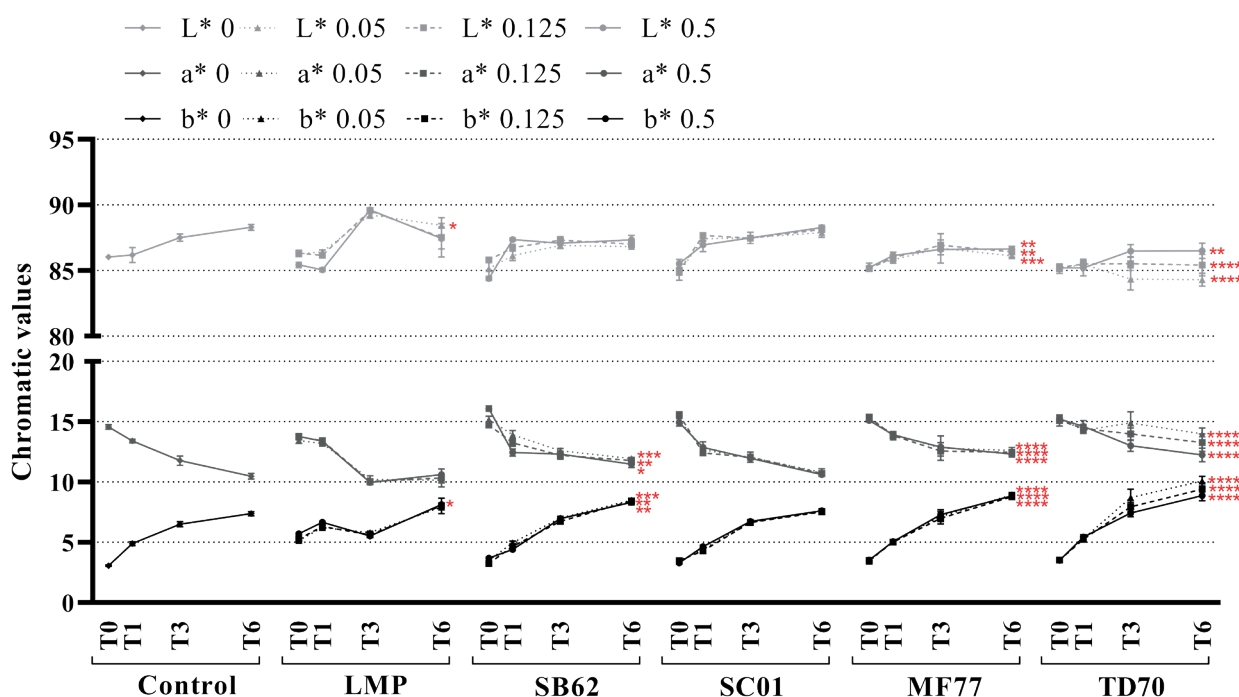


FIGURE 4. L^* a^* b^* colour coordinates measured in red wine samples taken at T0, T1, T3 and T6 after MPs addition at concentrations of 0.05, 0.125 and 0.5 mg/mL from a commercial product (LMP), *S. bouldarii* (SB62), *S. cerevisiae* (SC01), *M. fructicola* (MF77), *T. delbrueckii* (TD70), or no addition (control). The data points shown are the means for three independent experiments, and the error bars indicate the standard deviation between triplicates. Asterisks indicate significant differences between the treatment and control at the T6 time-point as analysed by one-way ANOVA and Dunnett's multiple comparisons test.

*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$

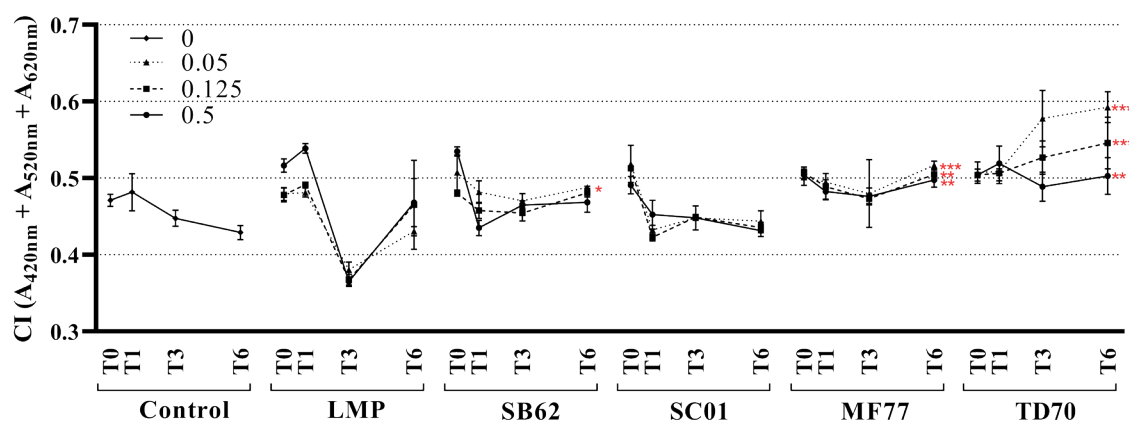


FIGURE 5. CI (colour intensity) measured in red wine samples taken at T0, T1, T3 and T6 after MPs addition at concentrations of 0.05, 0.125 and 0.5 mg/mL from a commercial product (LMP), *S. boulardii* (SB62), *S. cerevisiae* (SC01), *M. fructicola* (MF77), *T. delbrueckii* (TD70), or no addition (control). The data points shown are the means for three independent experiments, and the error bars indicate the standard deviation between triplicates. Asterisks indicate significant differences between the treatment and control at the T6 time-point as analysed by one-way ANOVA and Dunnett's multiple comparisons test.

*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$

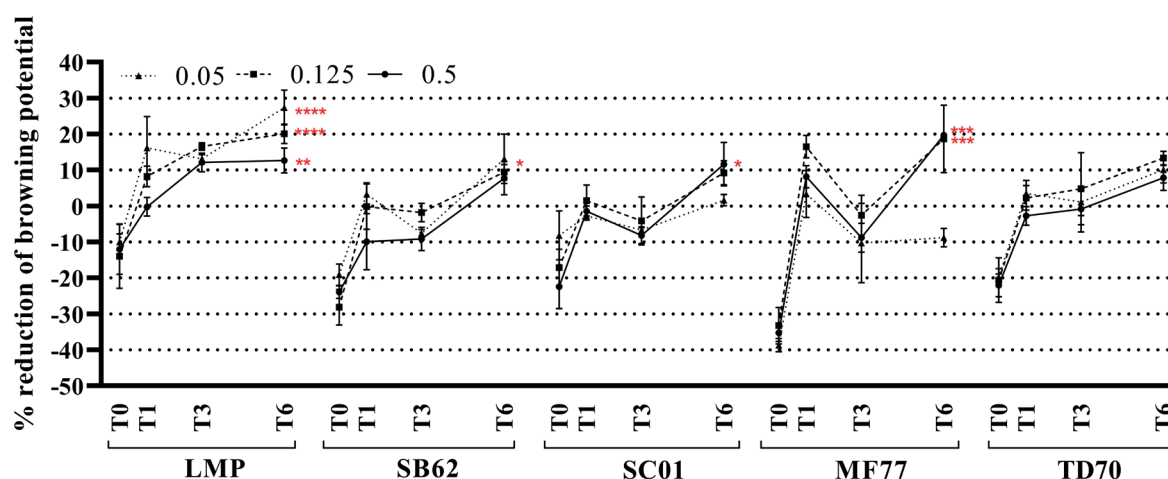


FIGURE 6. Percentage variation of browning potential compared to control samples for white wine samples taken at T0, T1, T3 and T6 after MPs addition at concentrations of 0.05, 0.125 and 0.5 mg/mL from a commercial product (LMP), *S. boulardii* (SB62), *S. cerevisiae* (SC01), *M. fructicola* (MF77) or *T. delbrueckii* (TD70). The data points shown are the means for three independent experiments, and the error bars indicate the standard deviation between triplicates. Asterisks indicate significant differences between the treatment and control at the T6 time-point as analysed by one-way ANOVA and Dunnett's multiple comparisons tests.

*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$

was similar at T6 to the initial T0 value of 0.5, whereas 0.05 and 0.125 mg/mL TD70-treated wine increased in intensity to 0.58 and 0.53 at T3 and 0.59 and 0.55 at T6, respectively. 0.5 mg/mL TD70-treated wine retained a CI value of 0.5 at T6, similar to the MF77 samples.

2. White wine evaluation

2.1. Browning potential

The percentage reduction of browning potential compared to the control at each respective time-point was followed for white wine treated with different MPs (Figure 6).

All treated wines showed more browning than the control at T0, from 14 % increased browning for LMP to 38 % for MF77. However, by T1, most treatments did not show an impact on browning as measured by the accelerated browning test, except for a 10 % increase in the 0.5 mg/mL SB62 treatment and an 8 and 16 % decrease in the 0.125 and 0.05 mg/mL LMP treatments, respectively. Increases in browning potential were found in samples of SB62, SC01 and TD70 at T3. Nevertheless, all treatments showed reduced browning potential at T6 compared to the control, except for the 0.05 mg/mL treatments of SC01 and MF77, which showed no impact and a 10 % browning increase, respectively.

2.2. Protein haze-forming potential

The haze-forming potential of wine for the white wine treatments of this study is represented as the change in absorbance at 520 nm before and after heating at 80 °C for 2 h (Figure 7). Already at T0, LMP treatments showed significantly reduced haze potential compared to the control for which a $\Delta A_{520\text{nm}}$ value of 0.086 was recorded. However, the 0.125 mg/mL SB62-treated wine, as well as the 0.5 mg/mL MF77 and TD70 treatments, resulted in significantly increased haze at this time. After one month, treatments of 0.05 mg/mL SB62, SC01 and MF77 showed significantly reduced haze compared to the control. Additionally, 0.125

and 0.5 mg/mL LMP-treated wines were less hazy, as well as wine treated with 0.125 mg/mL SB62. However, at T3 and T6, no treatments had led to reduced haze compared to the control at each time-point, which obtained $\Delta A_{520\text{nm}}$ values of 0.048 and 0.065, respectively. In fact, all LMP- and TD70-treated wines, as well as the 0.05 mg/mL SB62-treated and 0.125 mg/mL SC01- and MF77-treated wines, demonstrated significantly increased haze at T3. Furthermore, at T6, wines treated with LMP, 0.05 and 0.125 mg/mL SC01, 0.125 mg/mL TD70 and 0.5 mg/mL SB62 were all significantly hazier than the control. No treatments or control at any time reached a $\Delta A_{520\text{nm}}$ value less than or equal to 0.02, which is the threshold above which a wine is considered heat unstable.

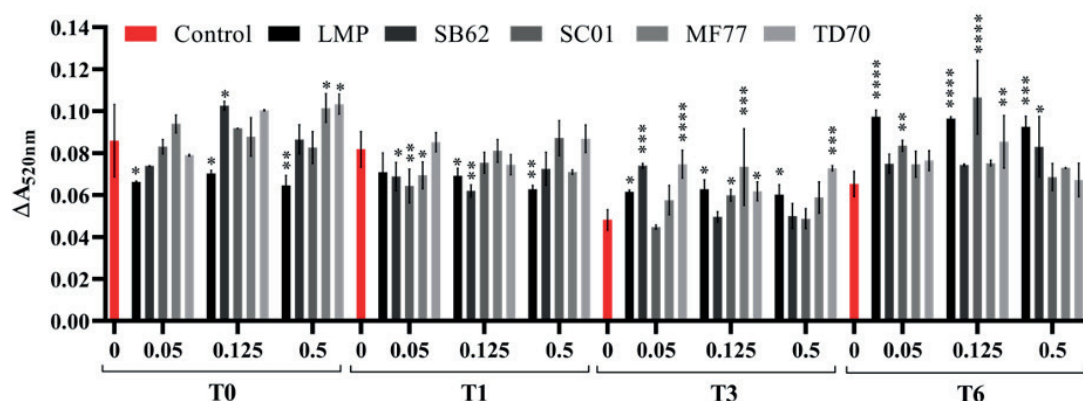


FIGURE 7. Heat stability represented the change in absorbance at 520 nm before and after heating at 80 °C for 2 h measured in white wine samples taken at T0, T1, T3 and T6 after MPs addition at concentrations of 0.05, 0.125 and 0.5 mg/mL from a commercial product (LMP), *S. boulardii* (SB62), *S. cerevisiae* (SC01), *M. fructicola* (MF77), *T. delbrueckii* (TD70) or no addition (control). The data points shown are the means for three independent experiments, and the error bars indicate the standard deviation between triplicates. Asterisks indicate significant differences between the treatment and the respective control within each time point as analysed by two-way ANOVA and Fisher's LSD test.

*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$

3. Relationships between wine properties and MP treatments

A principal component analysis (PCA) was carried out on the red wine data gathered at each sampling time-point to investigate the relationships between the MP treatments and the red wine properties evaluated in this study. A visual representation of the relationships between the wine properties and MP treatments with the first two selected principal components (PCs) is shown in Figure 8.

The relationships between wine properties were similar throughout the time-course experiment. Polymeric pigments and BSA-reactive tannins displayed a strong negative correlation with each other, whereas neither showed strong relationships with L^* (lightness) or colour intensity. The latter two, on the other hand, were consistently negatively correlated with each other, with a^* (green/red colour component) and b^* (blue/yellow colour component), furthermore demonstrating positive correlations with colour intensity at T3 and T6. The different concentrations of the

same MPs are furthermore mostly clustered together, with LMP in particular clustering separately from the other MP treatments at most time points. TD70 was positively correlated with the dimensions characterised by strong positive loadings of colour intensity, a^* and b^* at T3 and T6, as was MF77, although not as strongly.

The first dimension of the PCA at T0 was positively correlated with a^* and negatively correlated with polymeric pigments, L^* and b^* , whereas PC2 showed a positive correlation with colour intensity and a negative correlation with BSA-reactive tannins. Figure 8 shows that LMP treatments separated from the other MPs and showed strong negative loading on PC1. The different concentrations of the MF77 and TD70 treatments were clustered together, although these did not show a strong correlation with either of the first two dimensions. On the other hand, the different concentrations of SB62 and SC01 separated from each other. Negative correlations with PC2 were found for the SB62 0.125 mg/mL and SC01 0.5 mg/mL treatments similar to the control, whereas the 0.5 and 0.05 mg/mL treatments of SB62 and

SC01, respectively, showed positive correlations with this dimension. Furthermore, the 0.125 mg/mL treatment of SC01 was positively correlated with PC1.

When the data of T1 was subjected to PCA, it was clear that the SC01, MF77 and TD70 treatments clustered together and that the LMP treatments clustered separately with strong positive loadings on PC1 and negative loadings on PC2. The first dimension was characterised by a strong negative correlation of tannins and L^* and a positive correlation with colour intensity and b^* , whereas PC2 was positively related to tannins and a^* and negatively to polymeric pigments.

At T3, LMP and TD70 treatments were strongly negatively and positively correlated with PC1, respectively. PC1 is characterised by strong positive loadings of a^* , b^* and colour intensity, as well as a negative correlation with L^* .

LMP again clustered separately from the other treatments, and the different concentrations of SB62 and MF77 clustered together. Treatments mostly did not show strong correlations with PC2, which was associated with strong positive loadings of polymeric pigments.

The first dimension of the PCA at T6 showed strong positive correlations with L^* and was negatively associated with colour intensity, a^* and b^* . SB62, SC01 and MF77 treatments clustered together, with SB62 and MF77 showing positive and negative correlations with this dimension, respectively, although they were weakly associated with PC2. Strong positive loadings of polymeric pigments and negative loadings of tannins again characterised the second dimension. TD70 treatments were furthermore negatively associated with PC1, while the 0.05 and 0.125 mg/mL treatments of LMP were positively correlated with this dimension.

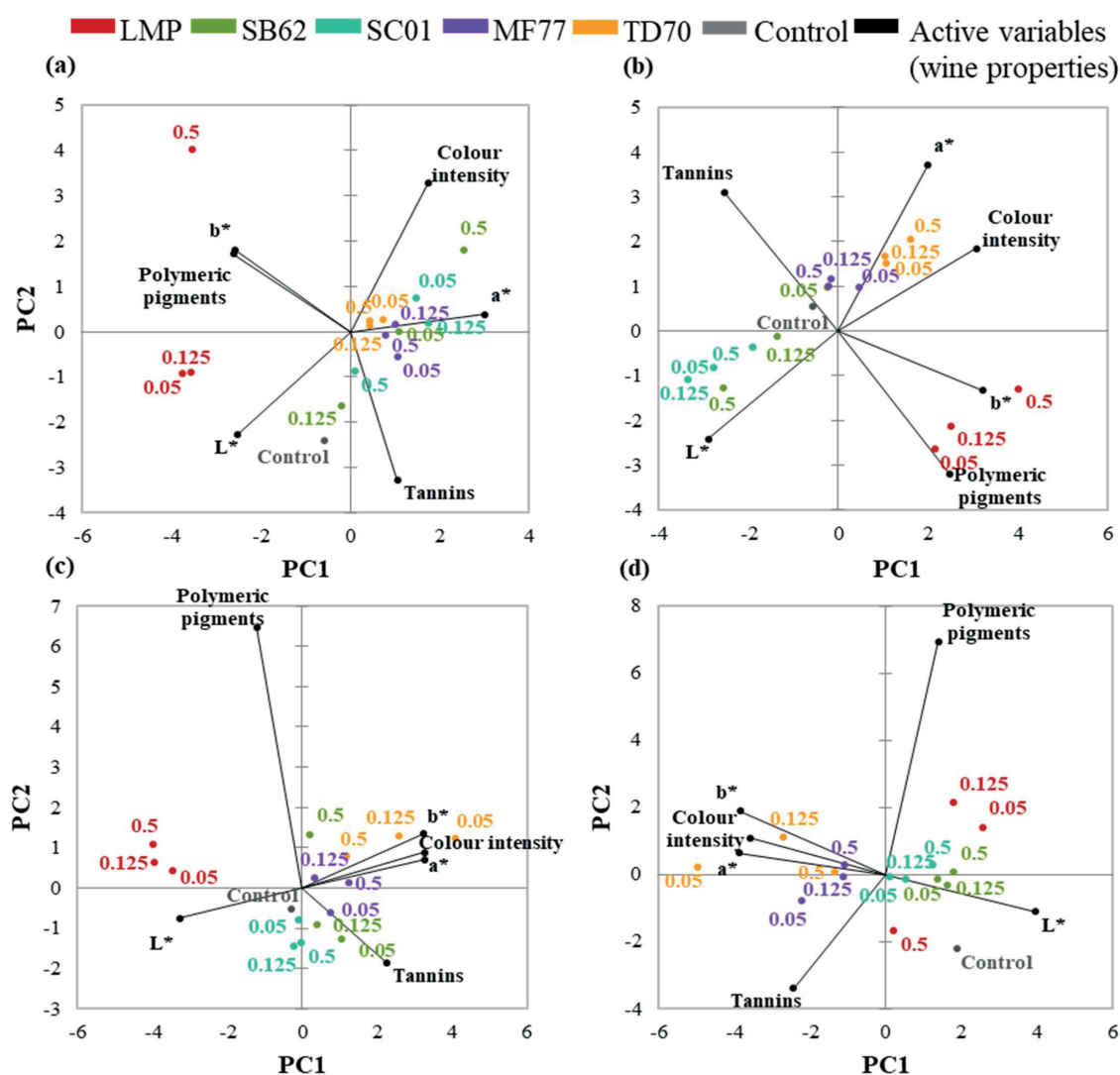


FIGURE 8. Biplots representing the relationship of red wine properties (BSA-reactive tannins, polymeric pigments, colour intensity and the colour coordinates L^* , a^* and b^*) measured at (a) T0, (b) T1, (c) T3 and (d) T6, and of the MP treatments at concentrations of 0.05, 0.125 and 0.05 mg/mL from a commercial product (LMP), *S. boulardii* (SB62), *S. cerevisiae* (SC01), *M. fructicola* (MF77), *T. delbrueckii* (TD70) or no addition (control), with the first two principle components (PC1 and PC2) selected after subjecting this data to principal component analysis (PCA).

DISCUSSION

Non-*Saccharomyces* yeasts present a possible source of novel mannoproteins for improving wine properties such as mouthfeel and colour stability (Belda *et al.*, 2016; Benito *et al.*, 2019; Domizio *et al.*, 2014). In this study, mannoproteins purified from four different yeast species and a commercial mannoprotein formulation from *S. cerevisiae* were added exogenously to a red and a white wine and their impact was followed over the course of six months. The yeasts used in this study, selected from a pool of species previously evaluated using fluorescence microscopy, have been chosen for their distinct capacity to produce varying levels of mannoproteins (Snyman *et al.*, 2021). Analysis of *S. boulardii*, in particular, showed promise for relatively higher levels of cell wall mannoprotein content, which motivated its use in this study.

Mannoprotein interaction with wine polyphenols and its influence on astringency is a complex phenomenon which has received much attention in recent years (García-Estévez *et al.*, 2018; Manjón *et al.*, 2020; Ramos-Pineda *et al.*, 2018; Ramos-Pineda *et al.*, 2022). In this study, significant reductions in tannin precipitation with BSA compared to the control were only seen in LMP treatments (Figure 1). Structural differences, such as the glycosidic content, may determine the mechanism by which mannoproteins interact with phenolic compounds and salivary proteins (Manjón *et al.*, 2021). Indeed, differences in the saccharide-to-protein ratio between mannoproteins from different yeast species have been evidenced in previous work (Giovani *et al.*, 2012). It is furthermore likely that highly glycosylated MPs were enriched during the purification step of affinity chromatography with concanavalin A undergone by the SB62, SC01, MF77 and TD70 extracts but not by LMP, which had undergone size fractionation. The drop in BSA-reactive tannins for all treatments at T3 could be attributed to structural changes in tannin molecules over time, such as the incorporation of anthocyanins to form polymeric pigments, leading to weaker interactions with protein (He *et al.*, 2008; McRae *et al.*, 2010; Smith *et al.*, 2015). Indeed, a negative correlation between BSA-reactive tannins and polymeric pigments throughout the time-course experiment was evident from the principle component analyses (Figure 8). Nevertheless, judging by the total phenolic index (TPI) of the red wine used in this study and the low BSA-reactive tannin concentration in the control at T0, this Cabernet-Sauvignon was not representative of an astringent wine and assays in this regard may not have yielded representative results (Aleixandre-Tudo *et al.*, 2017). This highlights the importance of not only the source and purification methods of the MPs used to their impact on wine but possibly also the nature and composition of the matrix itself.

Along with a decrease in BSA-reactive tannin concentration, treatments with LMP also led to increased polymeric pigments, which were significantly higher than the control at T0, T1 and T6 (Figure 2). The contribution to polymeric pigment stability by the presence of mannoproteins

has been observed in previous studies (Del Barrio-Galán *et al.*, 2012; Escot *et al.*, 2001; Rinaldi *et al.*, 2019). Alcalde-Eon *et al.* (2014) demonstrated the ability of a mannoprotein obtained through enzymatic extraction to stabilise some anthocyanin-derived pigments from a colloidal point of view, thus preventing their precipitation. Nevertheless, different mannoprotein preparations, even when derived from the same yeast species, do not necessarily display the same stabilising effect, and some have even been shown to exert no improvement in this regard at all (Guadalupe *et al.*, 2010; Guadalupe and Ayestarán, 2008; Rodrigues *et al.*, 2012). Del Barrio-Galán *et al.* (2012) evaluated the impact of different mannoproteins from *S. cerevisiae* on red wine properties and found that only some of the preparations showed improved colour stability due to polymeric pigments. The authors concluded that this was probably due to the different compositions and levels of purity of the MPs used. Indeed, of the six preparations that they studied, it was only the two with polysaccharide purity above 80 % that did not show higher values of polymeric anthocyanin content. Once again, it is possible that the differences in impact on wine observed between the commercial LMP and the MPs purified with concanavalin A in this study are due to their different preparation procedures.

Although increased polymeric pigments are linked with improved colour stability, in this study, there does not seem to be a strong relationship with red wine colour intensity (CI) according to the PC analyses of data gathered at the different time points (Figure 8). Indeed, polymeric pigment stability and colour are not always related (Cheynier *et al.* 2006). Nevertheless, Timberlake and Bridle (1976) showed that significant correlations exist between increased red wine colour intensity and the copolymerisation of anthocyanins and phenolic compounds, the extent of which is variable depending on the type of phenolic compounds involved. This effect could be the same observed for the initially elevated colour intensity in treatments with the highest concentration of LMP, which also showed significantly increased levels of polymeric pigments at T0 and T1 (Figure 5). The higher hue of LMP treatments at these times indicates a larger proportion of yellow to red colour as further shown by decreased a^* (red/green colour components) and increased b^* (blue/yellow colour components) values (Figure 4), is furthermore a typical expression of the formation of polymeric pigments (Oliveira *et al.*, 2019). However, at T3, the LMP treatments experienced a drop in colour intensity, along with increased lightness (L^*) (Figures 4 and 5). A similar observation was made by Ribéreau-Gayon *et al.* (1983) when a decrease in colour intensity was observed after the proportion of polymeric pigments to monomeric anthocyanins exceeded a certain threshold at which point the authors concluded that precipitation occurs, leading to decreased colour intensity. Nevertheless, changes in LMP colour characteristics were not perceptible to the human eye at any time point ($\Delta E^* < 3$) (Figure 3).

Important differences in red wine colour characteristics were furthermore evident between the different MP treatments

of this study. SB62 and SC01 treatments both showed a sharp decrease in colour intensity and redness from T0 to T1, accompanied by a sharp increase in lightness, while these changes were not as pronounced in MF77 and TD70 (Figures 4 and 5). Previous studies have found similar reductions in colour intensity and increased lightness upon the addition of some mannoproteins to red wine (Guadalupe and Ayestarán, 2008). This impact has been attributed to the precipitation of monomeric anthocyanins and anthocyanin derivatives by some mannoproteins (Alcalde-Eon *et al.*, 2019b; Del Barrio-Galán *et al.*, 2012). However, this interaction depends on mannoprotein structural differences, such as the degree of phosphorylation, which have been shown to differ between yeast strains (Jigami and Odani, 1999; Mekoue Nguela *et al.*, 2023). Whereas SC01 did not differ from the control after T1, SB62 showed slightly increased colour intensity, redness and yellowness, and decreased lightness (Figures 4 and 5). These changes were pronounced in MF77 and even more so in TD70. Indeed, according to the PCA, colour intensity correlated positively with a^* and b^* and negatively with L^* for MF77 and TD70 at T3 and T6 (Figure 8). The same effects of mannoproteins on wine colour have been reported elsewhere (Alcalde-Eon *et al.*, 2014; Del Barrio-Galán *et al.*, 2012). Alcalde-Eon *et al.* (2014) found that mannoprotein-treated wines, which were associated with decreased lightness and increased a^* and b^* coordinates, were positively correlated with increased pigment content, particularly flavanol-anthocyanin direct condensation products and A-type vitisins. The authors hypothesised that the mannoproteins could have acted as steric stabilisers, protecting these anthocyanin-derived pigments from aggregation and precipitation through colloidal interactions, thus preventing loss of colour. Furthermore, for the first time, a dose-dependent effect was apparent in this study, where wine colour became darker and more intense with decreasing concentrations of TD70 (Figures 4 and 5). Indeed, these differences translated into changes perceptible to the human eye ($\Delta E^* > 3$), particularly for the lower two concentrations of TD70, but also for MF77 (Figure 3) (Martínez *et al.*, 2001). The relevance of mannoprotein dosage impact on wine colour has been noted in previous studies and shown to influence their action as either stabilisers or flocculating polymers (Alcalde-Eon *et al.*, 2019a; Alcalde-Eon *et al.*, 2019b; Guadalupe *et al.*, 2010).

Colour modification in white wine, specifically the potential of browning, was also measured in this study (Figure 6). All treatments initially increased browning potential, but after six months, most wines showed at least a 10 % reduction of browning potential, with the greatest reductions seen in MF77 and LMP treatments. Ribeiro *et al.* (2014) produced similar findings in white wines treated with different mannoprotein additives and hypothesised that the phenolic compounds usually responsible for the formation of browning interacted with the mannoprotein, thus preventing their oxidation. Nevertheless, differences in how different commercial mannoproteins protected wine from browning have been observed (Ribeiro *et al.*, 2014). Similarly, variable capacities for browning reduction were observed in a study

using lees from different yeast strains (Márquez *et al.*, 2009). Ribeiro *et al.* (2014) concluded that the protective effect against browning seemed dependent on mannoprotein composition, which in turn could have influenced their interaction with the phenolic compounds in question. It is also possible that compounds other than MPs present in the extracts, such as membrane lipids and sterols and cell wall β -glucans, could be responsible for variations in oxygen-consuming capacity and browning formation (Comuzzo *et al.*, 2015).

The impact of the different mannoproteins on white wine haze formation was also evaluated in this study (Figure 7). Statistically significant reductions in haze were observed for LMP at T0 and for different concentrations of all MPs except TD70 at T1. However, none of these reductions were sufficient to achieve heat stability ($\Delta A_{520nm} < 0.02$) and from T3 onwards, either no impact or increased haze formation was observed. Previous studies have shown the ability of mannoproteins to stabilise the proteins responsible for haze formation by reducing their aggregate particle size, although these studies have not found much confirmation in the following 30 years (Waters *et al.*, 1993). It is possible that at T0 in this study, a temporary protective effect on wine haze formation occurred due to mannoprotein interaction with wine proteins and/or phenolic compounds (Lomolino and Curioni, 2007). The increased haze formation from T3 onwards could be due to heat instability of the protein moiety of the MP itself, the effect of which was higher than its potential stabilising influence (Lomolino and Curioni, 2007). The extent of MP contribution to heat stability may be informed by its structure and composition (Lomolino and Curioni, 2007; Ribeiro *et al.*, 2014; Schmidt *et al.*, 2009). Strain-dependent impacts on wine haze formation have furthermore been observed (Giese *et al.*, 2016). Nevertheless, haze formation is a complex phenomenon in which not only protein, phenolic compounds and polysaccharides such as mannoproteins play a role but also other wine molecules and conditions such as sulphate, organic acids, pH, ionic strength and temperature (Van Sluyter *et al.*, 2015). As wine is a dynamic environment in which changes and interactions, such as those between tannins and anthocyanins to form new phenolic compounds, occur over time, it is likely that the effects observed as a result of mannoprotein addition will change after one, three and six months.

CONCLUSION

In this study, differences in the impact on wine properties, such as colour characteristics, could be observed between mannoproteins isolated from different yeast species and, often, most noticeably between the commercial MP and the MPs extracted from SB62, SC01, MF77 and TD70. These differences could be due to variations in the structure and composition between different yeast species. Furthermore, this study confirmed that mannoprotein purity, which likely differs between LMP and the other MPs due to their different preparation procedures, plays a role in its behaviour and impact on wine, together with the wine matrix. Future work may, therefore, benefit from the application of a commercial

MP that has been prepared similarly to the other MPs, either with or without purification. Further characterisation of the non-MP components contained in the extracts could also contribute towards understanding the differences in the impact of the different treatments on wine. Furthermore, using a red wine with a higher phenolic content, as well as other white wines with different protein stabilities, could provide more comprehensive information regarding the impact of mannoproteins, which are thought to interact with these compounds. Lastly, further investigations regarding the nature of these interactions could provide the necessary context for the findings of this study, which, although highlighting some differences in the impact of MPs from different yeast species, lacks the information required to explain them fully.

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