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ORIGINAL RESEARCH ARTICLE

Inoculation with a selected strain of *Metschnikowia pulcherrima* as a bioprotective alternative to sulphites for preventing browning of white grape must

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ABSTRACT

In this study, the effectiveness of a selected strain of *Metschnikowia pulcherrima* (MP1) was compared to that of sulphur dioxide for preventing white grape must from browning. Sulphur dioxide was found to drastically reduce oxygen consumption rate, protect hydroxycinnamic acids against oxidation and prevent grape must from browning, even in the presence of laccase. By contrast, supplementation with the selected strain of *Metschnikowia pulcherrima* (MP1) drastically increased oxygen consumption rate, thus reducing the amount of oxygen available for polyphenol oxidases. In the absence of laccase, this resulted in a decrease in browning and a certain degree of protection of the hydroxycinnamic acids, but in the presence of laccase it was not effective enough. Consequently, the selected strain of *Metschnikowia pulcherrima* (MP1) can be considered an interesting alternative to sulphur dioxide for preventing browning in white grape must, but only in conditions of healthy grapes.

KEYWORDS: grape must, *Metschnikowia pulcherrima*, bioprotection, browning, laccase

INTRODUCTION

The enzymatic browning of grape must is still a major problem in oenology today (Li *et al*., 2008), being particularly serious when the grapes have been infected by grey rot (Ky *et al*., 2012). Browning is an oxidation process that causes certain foods to turn brown, which often leads to them being rejected by consumers (Friedman, 1996). This is a particular problem in the case of wine, because grape must is very vulnerable to enzymatic browning (Oliveira *et al*., 2011).

Enzymatic browning is caused by the polyphenol oxidases tyrosinase and laccase. Tyrosinase (EC 1.14.18.1) is naturally present in grape berries (Fronk *et al*., 2015), whereas laccase (EC 1.10.3.2) is only present in grapes infected with *Botrytis cinerea* (Claus *et al.*, 2014). These two polyphenol oxidases can both oxidise certain phenolic compounds, such as hydroxycinnamic acids and other diphenols (Giménez *et al*., 2022), forming ortho-quinones; these later polymerise to form melanins (Friedman, 1996; Oliveira *et al*., 2011), which are responsible for enzymatic browning in white wines and for oxidasic haze in red wines (Ribéreau-Gayon *et al*., 2006a)

When grapes are infected by *Botrytis cinerea,* laccase activity is much higher than tyrosinase activity in healthy grapes (Steel *et al*., 2013), and therefore there is a much higher possibility that enzymatic browning will occur. In addition, laccase can oxidize a larger variety of substrates than tyrosinase (Oliveira *et al*., 2011; Claus *et al*., 2014).

In wineries, the addition of sulphur dioxide to grape must is the most common tool for protecting it against enzymatic browning. This additive is a potent inhibitor of tyrosinase and laccase (Ribéreau-Gayon *et al*., 2006a; Giménez *et al*., 2022) and also has antimicrobial properties (Ough and Crowell, 1987). Tyrosinase is more sensitive to sulphur dioxide than laccase, and it is usually inactivated once alcoholic fermentation has ended. In contrast, laccase can be active in wine after alcoholic fermentation (Du Toit *et al*., 2006; Ribéreau-Gayon *et al*., 2006a)

Nevertheless, the wine industry is currently trying to reduce and even eliminate the use of sulphur dioxide due to its negative effects on the environment (D'Amico *et al*., 2016) and human health (Lester, 1995). Hence, some alternatives to sulphur dioxide additives have been proposed over recent years to prevent browning; for example, inert gases (Martinez and Whitaker, 1995), oenological tannins (Vignault *et al*., 2019; Vignault *et al*., 2020), ascorbic acid (Ribéreau-Gayon *et al*., 2006b), reduced glutathione (Kritzinger *et al*., 2013; Zimdars, 2020; Giménez *et al*., 2022) and inactivated dry yeasts rich in glutathione (Bahut *et al*., 2020; Gabrielli *et al*., 2017; Giménez *et al*., 2023). More recently, bioprotection has also been proposed as an alternative to sulphur dioxide (Rubio-Bretón *et al*., 2018; Simonin *et al*., 2020; Morata *et al*., 2021; Di Gianvito *et al*., 2022), not only to avoid microbiological deviations occuring (Simonin *et al*., 2018; Windholtz *et al*., 2021; Englezos *et al*., 2022; Escribano-Viana *et al*., 2022; Windholtz *et al*., 2023) but also to protect against enzymatic browning (Chacón-Rodríguez *et al*., 2020; Simonin *et al*., 2022; Windholtz *et al*., 2022; Giménez *et al*., 2023).

Different microorganisms have been proposed for bioprotection, such as *Torulaspora delbrueckii* (Simonin *et al*., 2018; Chacón-Rodríguez *et al*., 2020; Windholtz *et al*., 2021; Windholtz *et al*., 2023), *Lachancea thermotolerans* (Rubio-Bretón *et al*., 2018; Morata *et al*., 2021; Escribano-Viana *et al*., 2022; Windholtz *et al*., 2023) and even *Lactobacillus plantarum* (Rubio-Bretón *et al*., 2018). However, according to recent literature, *Metschnikowia pulcherrima* seems to be the most promising microorganism for wine bioprotection (Simonin *et al*., 2020; Puyo *et al*., 2023; Yao *et al*., 2023; Giménez *et al*., 2023; Canonico *et al*., 2023; Agarbati *et al*., 2023). It seems that *Metschnikowia pulcherrima* has a large capacity for consuming oxygen (Canonico *et al*., 2019; Chacón-Rodríguez *et al*., 2020). A reduction in oxygen in the must will hinder the development of other microorganisms (Di Gianvito *et al*., 2022; Windholtz *et al*., 2023) and significantly decrease the necessary substrate for the oxidative action of polyphenol oxidases (Giménez *et al*., 2023). In addition, the use of *M. pulcherrima* in winemaking is becoming more and more common for different reasons (Morata *et al*., 2019). Specifically, *M. pulcherrima* can improve the sensory aromatic profile of wine (Ruiz *et al*., 2018), can reduce the wine ethanol strength (Contreras *et al*., 2015) and has a bioprotective effect (Sipiczki, 2006).

The aim of this study was therefore to investigate the bioprotective effect of a selected strain of *Metschnikowia pulcherrima* (MP1) on enzymatic browning caused by polyphenol oxidases.

MATERIALS AND METHODS

1. Chemicals and equipment

Syringaldazine (purity ≥ 98 %), and $FeSO_4 \cdot 7H_2O$ (purity ≥ 99%) were purchased from Sigma-Aldrich (Madrid, Spain). L-(+)-Tartaric acid (purity ≥ 99.5 %), sodium hydroxide (purity ≥ 98 %), sodium acetate (purity ≥ 99 %), acetonitrile (purity ≥ 99 %), Methanol (purity ≥ 99 %), and $CuSO_4 \cdot 5H_2O$ (purity ≥ 99 %) were purchased from Panreac (Barcelona, Spain). Ethanol (96 % vol.) was supplied by Fisher Scientific (Madrid, Spain). Cellulose membranes of 3.5 KDa (6.4 mL/cm) were supplied by Spectrum Laboratories, Inc (Rancho Dominguez, USA). We used the following equipment: an Hpand an Entris II Series Analytical Balance (Sartorius, Goettingen, Germany); a spectrophotometer UV–Vis Helios Alpha™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); a centrifuge Heraeus™ Primo™ (Thermo Fisher Scientific Inc., Waltham, MA, USA); a liquid chromatograph Agilent Series 1200 (Agilent, Germany) equipped with a photodiode array detector (G1315D); and a Zorbax Eclipse XDB C18 column (4.6 x 150mm).

2. Sample collection and preparation

Muscat of Alexandria grapes were harvested from the experimental vineyard of Rovira i Virgili University (Mas dels Frares, Constantí, Tarragona: 41°08'44.1''N, 1°11'51.0''E, height: 59 masl) during the 2022 vintage. The grapes were picked manually when they were considered sufficiently ripe (around 12.5 % of potential ethanol content and 6.0 g tartaric acid/L titratable acidity). The grapes were immediately pressed in an environment saturated with nitrogen with a vertical manual press until 60% of their juice had been extracted. The grape must was immediately transferred to a 750-mL bottle also saturated with nitrogen.

3. Synthetic buffer

A solution of 4 g/L of L-(+)-tartaric acid, 3 mg/L of iron (in the form of iron (III)), chloride hexahydrate, and 0.3 mg/L of copper (in the form of copper (II) sulphate pentahydrate) was adjusted to pH 3.5 using sodium hydroxide. This solution was used for all the experiments.

4. Laccase enzyme production and enzymatic activity measurement

Active laccase extracts were obtained from *Botrytis cinerea* isolate 213 strain following the methodology described by Vignault *et al*. (2020). The laccase extract was treated with 0.16 g/mL of PVPP for 10 min and centrifuged at 7500 rpm for 10 min. Subsequently, the supernatant was subsequently dialysed with a cellulose membrane (MW cutoff 3.5 KDa) for 2 days against a 0.3 M ammonium formate solution and for 2 more days against distilled water with continuous shaking. The laccase activity of the extract was determined using an adaptated syringaldazine test method (Grassin and Dubourdieu, 1986).

5. *Metschnikowia pulcherrima* strain

Metschnikowia pulcherrima strain (MP1) was selected for its high oxygen consumption capacity (Level2 Initia™, Lallemand Inc, Montreal, Canada). Ten min before beginning the measurements, the *Metschnikowia pulcherrima* yeasts were hydrated to ten times their weight with distilled water. The water temperature was 30 °C.

6. Reaction conditions for measuring oxygen consumption

The experimental conditions are described in Giménez *et al*. (2023). The assays were carried out in clear glass flasks (66 mL) with an oxygen sensor spot (PreSens Precision Sensing GmbH, order code: SP-PSt3-NAU-D5-CAF; batch number: 1203- 01_PSt3-0828-01, Regensburg, Germany) for measuring the dissolved oxygen noninvasively by luminescence (Nomasense TM O2 Trace Oxygen Analyzer by Nomacorc S.A., Thimister Clermont, Belgium).

The grape must was diluted (20 % (v/v)) with the above-mentionned synthetic buffer to reduce its oxygen consumption rate. This made it possible to monitor it with higher precision, and also to standardise the pH of the different measurements. More specifically, thirteen mL of grape must and 52 mL of buffer were added to each flask, to which the different chemical agents were added. The agents used were: sulphur dioxide (20 mg/L in the form of potassium disulphite), *Metschnikowia pulcherrima* strain MP1 (250 mg/L), and a combination of sulphur dioxide (20 mg/L) and *Metschnikowia pulcherrima* strain MP1 (250 mg/L). A control with no added agent was also prepared. These assays were also performed with and without the addition of 2 units/mL of laccase. All the assays were performed in triplicate.

The bottles were immediately saturated in oxygen (around 7–8 mg O_2/L) via vigorous manual stirring for a few seconds. The oxygen concentration was measured (Diéval *et al*., 2011) periodically until asymptotic behaviour was attained (around 3 h) to determine the oxygen consumption kinetics. All measurements were made in an air-conditioned laboratory at 22 ± 2 °C. The total oxygen consumption capacity (TOCC) was calculated using the mathematic model previously reported by Pons-Mercadé *et al*. (2021). Once the oxygen concentrations were below 1 mg/L, or the oxygen consumption showed asymptotic behaviour, the samples were supplemented with 50 mg/L of sulphur dioxide to stop the colour evolution. The samples were used for colour measurements and for HPLC analysis of hydroxycinnamic acids and GRP.

7. Colour measurements

The intensity of yellow colour (A420nm) was measured and the CieL*a*b* coordinates of the samples were determined according to Ayala *et al*. (1997). Data were processed using the MSCV software (MSCVes.zip., 2013). The total colour difference (ΔEab*) was calculated as the Euclidian distance between two points in the CieL*a*b* space using the following formula:

$$\Delta Eab^* = ((L^*_1-L^*_2)^2 + (a^*_1-a^*_2)^2 + (b^*_1-b^*_2)^2)^{1/2}$$

where L* is the lightness; a* is the green–red component of the colour, and b* is the blue–yellow component. The total colour difference (ΔEab*) was used to determine whether the difference between two samples could be detected visually by the human eye. Generally, the difference is considered to be visible to the human eye when $\Delta Eab^* > 3$ (Martínez *et al*., 2001).

8. Hydroxycinnamic acid and GRP analysis by HPLC-DAD

Hydroxycinnamic acids and grape reaction product (GRP) were analysed by the reversed-phase HPLC−DAD following the method described by Lago-Vanzela *et al*. (2011). This comprised an Agilent Series 1200 HPLC (Agilent, Waldbronn, Germany) equipped with a DAD (G1315D) coupled to an Agilent Chem Station (version B.01.03) data processing station. The samples were filtered (0.20 µm, polyester membrane, Chromafil PET 20/25, Machery-Nagel, Düren, Germany) and then injected (20 µL) into a Zorbax Eclipse XDB C18 column (4.6 x 150mm). The solvent was A [water/formic acid/acetonitrile (88.5:8.5:3, v/v/v)], B [water/formic acid/acetonitrile (41.5:8.5:50, v/v/v)] and C [water/formic acid/methanol (1.5:8.5:90, v/v/v)] and the flow rate 0.19 mL/ min. The gradient was: A = 96 %/B = 4 %

from 0 to 37 min; A = 70%/B = 17%/C = 13% from 37 to 51 min; A = 50%/B = 30%/C = 20% from 51 to 57 min; A = 0 %/B = 50%/C = 50% from 57 to 64 min. The compounds were quantified by measuring the absorbance at 320 nm with an external calibration curve of caftaric acid. The different compounds were identified according to the retention time that was previously determined by HPLC–DAD-ESI-MS/MS analysis using the same chromatographic conditions.

9. Statistics

All data are expressed as the arithmetic average ± standard deviation of three replicates. One-factor analysis of variance (ANOVA F test) was conducted using the SPSS 15.0 software (SPSS Inc., Chicago, IL). Different letters indicate the existence of statistical difference ($p < 0.05$).

RESULTS AND DISCUSSION

1. Oxygen consumption kinetics

Figure 1A shows the oxygen consumption kinetics of the diluted grape must without supplementation (the control), and supplemented with sulphur dioxide, with *Metschnikowia pulcherrima* strain MP1 and with a combination of both agents, in the absence or presence of 2 U/mL of laccase.

The oxygen consumption of the diluted control grape must without additives was initially very fast, later becoming moderate until around 2 mg/L of O_2 was reached after 3 hours. This behaviour was probably due to a reduction in the amount of substrates available for polyphenol oxidase, which slows down the reaction rate. Meanwhile, the oxygen consumption rate (OCR) decreased drastically when the sample was supplemented with 20 mg/L of sulphur dioxide. Other assays with higher doses of this additive showed similar results (data not shown). These results confirm that this additive is a good inhibitor of the polyphenol oxidase present in healthy grape juice (tyrosinase) (Ribéreau-Gayon *et al*., 2006a). In contrast, supplementation with *Metschnikowia pulcherrima* strain MP1 drastically increased the OCR, which confirms that this non-*Saccharomyces* yeast strain has a high capacity for consuming oxygen. The combined supplementation of sulphur dioxide and *Metschnikowia pulcherrima* strain MP1 resulted in an intermediate OCR; i.e., at levels between those of the control conditions and those of sulphur dioxide supplementation. It should be noted that in terms of OCR increase, the difference between the control and the *Metschnikowia pulcherrima* strain MP1-supplemented must was higher than that of sulphur dioxide and sulphur dioxide + *Metschnikowia pulcherrima* strain MP1. This indicates that sulphur dioxide inhibited not only the polyphenol oxidases activity but also the metabolism of this *Metschnikowia pulcherrima* strain.

Figure 1B shows the OCR of all these samples in the presence of laccase. In general, laccase supplementation accelerated the OCR of the control and of the sample supplemented with sulphur dioxide and *Metschnikowia pulcherrima* strain MP1. By contrast, the samples supplemented with sulphur dioxide alone or with just *Metschnikowia pulcherrima* strain MP1 showed similar behaviour to the corresponding musts without laccase. This confirms that sulphur dioxide is a very effective inhibitor, of not only tyrosinase but also laccase (Ough and Crowell, 1987; Giménez *et al*., 2023).

Although Figure 1 is very useful for visualising OCR, it cannot be used to determine whether there are statistically significant differences within the different experimental groups. Therefore, a previously used kinetic model (Pons-Mercadé *et al*., 2021) was applied to statistically compare the results of this graph and calculate the total oxygen consumption (TOC) in the different experimental conditions. This model shows the inverse of consumed oxygen versus the inverse of elapsed time. Using this mathematical model, the following equation can be established: $1/[O_2] = A/t + B$. After clearing up the oxygen in the equation, TOC can be calculated as the limit when time tends towards infinity.

Figure 2 shows the calculated TOC of the diluted grape must without supplementation (the control), and supplemented with sulphur dioxide, with the *Metschnikowia pulcherrima* strain MP1 and with the combination of the two agents in the absence or presence of 2 U/mL of laccase.

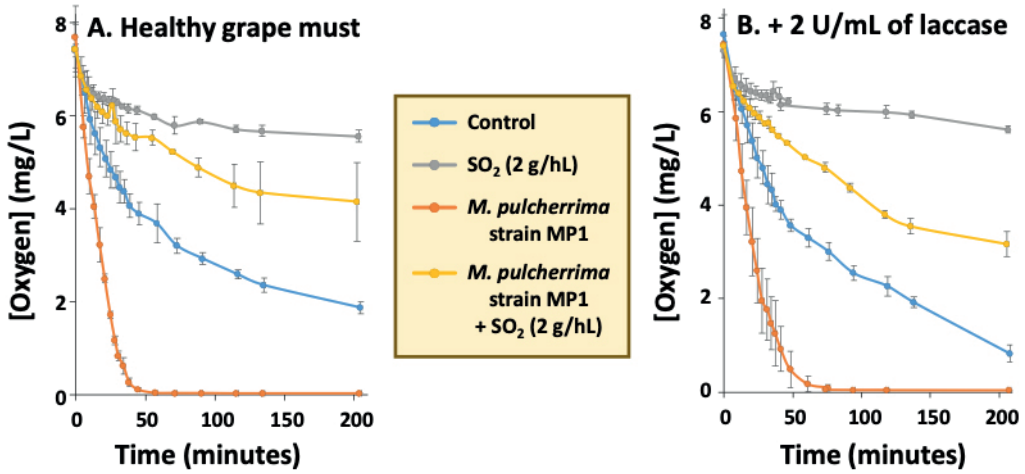

FIGURE 1. Influence of inoculation of *M. pulcherrima* strain MP1 on the grape must oxygen consumption kinetics.

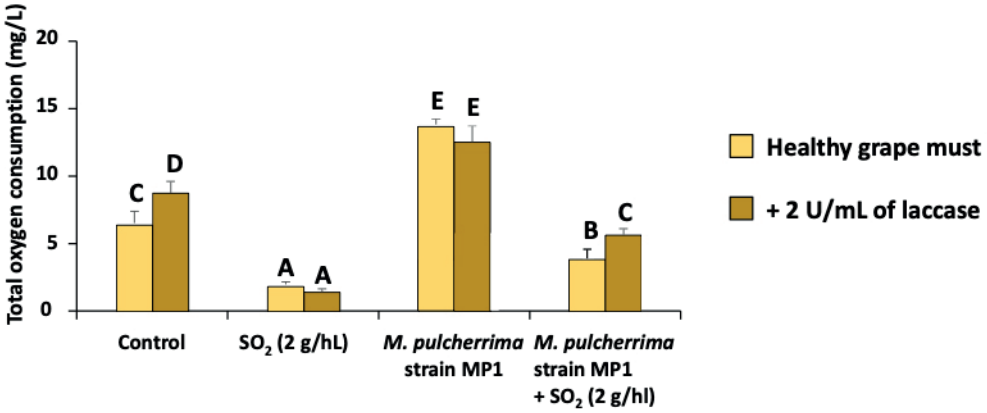

FIGURE 2. Influence of inoculation with M. pulcherrima strain MP1 on the Total Oxygen Consumption (TOC).

This graph confirms the conclusions drawn from Figure 1: briefly, that supplementation with sulphur dioxide significantly reduced TOC, whereas supplementation with *Metschnikowia pulcherrima* strain MP1 did the opposite. Supplementation with laccase significantly increased the TOC of the control and of the samples supplemented with the combination of sulphur dioxide and *Metschnikowia pulcherrima* strain MP1, but not the TOC of the other experimental groups.

2. Hydroxycinnamic acids and GRP

Figure 3 shows the hydroxycinnamic acids and GRP concentrations after oxygen consumption of the diluted grape must without supplementation (the control), and supplemented with sulphur dioxide, with the *Metschnikowia pulcherrima* strain MP1 and with the combination of the two agents in the absence or presence of 2 U/mL of laccase.

In healthy grape must, the levels of hydroxycinnamic acids and GRP were very low in the absence of sulphur dioxide. In contrast, when sulphur dioxide was added, the levels of hydroxycinnamic acids and GRP were significantly higher. This indicates that in the absence of sulphur dioxide, tyrosinase oxidised nearly all the hydroxycinnamic acids present in the grape must. By contrast, the presence of sulphur dioxide inhibited this enzyme and protected hydroxycinnamic acids from oxidation. However, the levels of GRP in the presence of sulphur dioxide were significantly higher than in its absence, which is difficult to explain. One possibility is that before sulphur dioxide completely inhibits tyrosinase, some of the caftaric acid present in the grape must is transformed into ortho-quinones, which react with the natural glutathione present in the grape must to form GRP (Singleton *et al.*,1985; Kritzinger *et al.*, 2013). This GRP would also be formed in

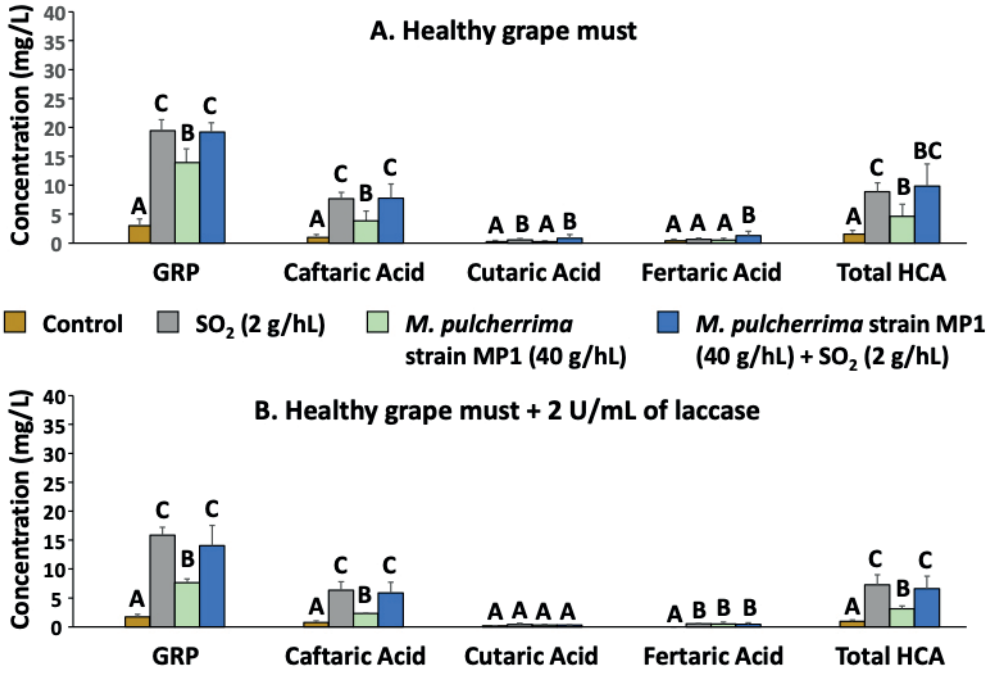

FIGURE 3. Influence of inoculation of M. pulcherrima strain MP1 on the wine GRP and hydroxycinnamic acid concentration.

the absence of sulphur dioxide and therefore would be present in the grape must, because tyrosinase, unlike laccase, cannot oxidise GRP (Kritzinger *et al*., 2013). However, the low GRP levels found in these conditions suggest that the GRP was subsequently incorporated into the brown polymers formed as a result of the polymerisation associated with enzymatic browning reactions.

When *Metschnikowia pulcherrima* strain MP1 was present, the levels of hydroxycinnamic acids and GRP were significantly higher than in control conditions, but significantly lower than in the presence of sulphur dioxide. It seems therefore that the high oxygen consumption of *Metschnikowia pulcherrima* strain MP1 reduced the availability of oxygen for polyphenol oxidase, and therefore protected the hydroxycinnamic acids against the action of this enzyme. When sulphur dioxide and *Metschnikowia pulcherrima* strain MP1 were added together, the results were very similar to those obtained with only sulphur dioxide.

Very similar results were obtained when the samples were supplemented with 2 U/mL of laccase. The only remarkable difference was that the levels of hydroxycinnamic acids and GRP of the samples were lower than without laccase, probably because laccase can oxidize hydroxycinnamic acids and GRP to a greater extent (Oliveira *et al*., 2011; Steel *et al*., 2013; Claus *et al*., 2014).

3. Colour parameters

Figure 4 shows the absorbance at 420 nm (A420nm) and the CIEL*a*b* blue–yellow component (b*) of the samples without supplementation (the control), and supplemented with sulphur dioxide, with *Metschnikowia pulcherrima* strain MP1 and with a combination of both in the absence and presence of laccase as indicators of browning intensity.

The supplementation with sulphur dioxide had a clear protective effect on the grape must against browning, since the values of A420 and b* were significantly lower than in the control sample. This again confirms the previously described protective effect of this additive against enzymatic browning (Ough and Crowell, 1987; Ribéreau-Gayon *et al*., 2006a; Oliveira *et al*., 2011). The supplementation with *Metschnikowia pulcherrima* strain MP1, both alone and combined with sulphur dioxide, also significantly decreased A420 and b* with respect to the control; this confirms that this non-*Saccharomyces* yeast protects grape must against enzymatic browning, even in the absence of sulphur dioxide.

In the presence of laccase, all the samples showed significantly higher values of A420 and b* than their corresponding controls without laccase. This confirms the high oxidative capacity of this polyphenol oxidase. It also indicates that it is more difficult to completely protect against browning in grapes affected by grey rot, even using sulphur dioxide.

Figure 5 shows the total colour difference (ΔEab*) between the different samples in relation to the grape must supplemented with sulphur dioxide.

The sample supplemented with sulphur dioxide was used as the reference for comparison with all the other samples, because sulphur dioxide addition is the most usual procedure for protecting grape must from browning. It is generally accepted that when ΔEab* is lower than 3 units the human eye cannot perceive the visual difference between two samples in terms of colour (Martínez *et al*., 2001; Pérez-Magariño and González-Sanjosé, 2003). Consequently, ΔEab* between a sample and its control reference will indicate whether the browning intensity can or cannot be perceived by the human eye, depending on whether it is greater or lower than 3 units.

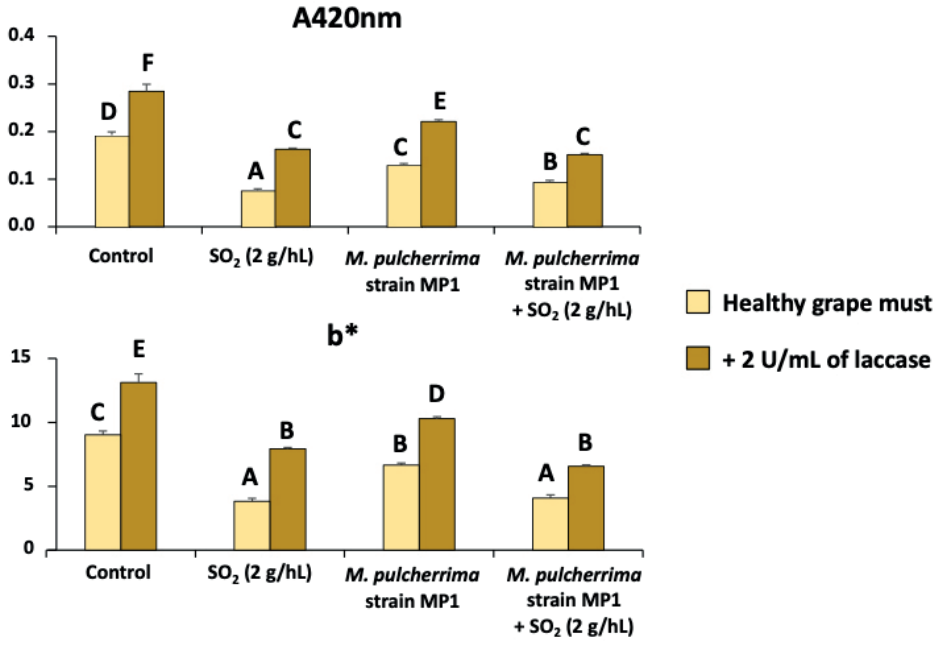

FIGURE 4. Influence of inoculation of M. pulcherrima strain MP1 on the intensity of the yellow colour (A420nm and b*) of the wine.

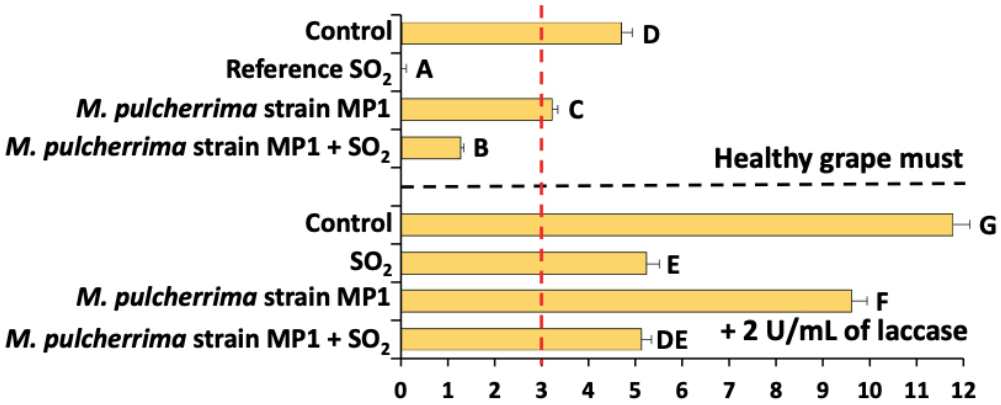

FIGURE 5. Influence of inoculation of *M. pulcherrima* strain MP1 on the total colour difference (DEab*).

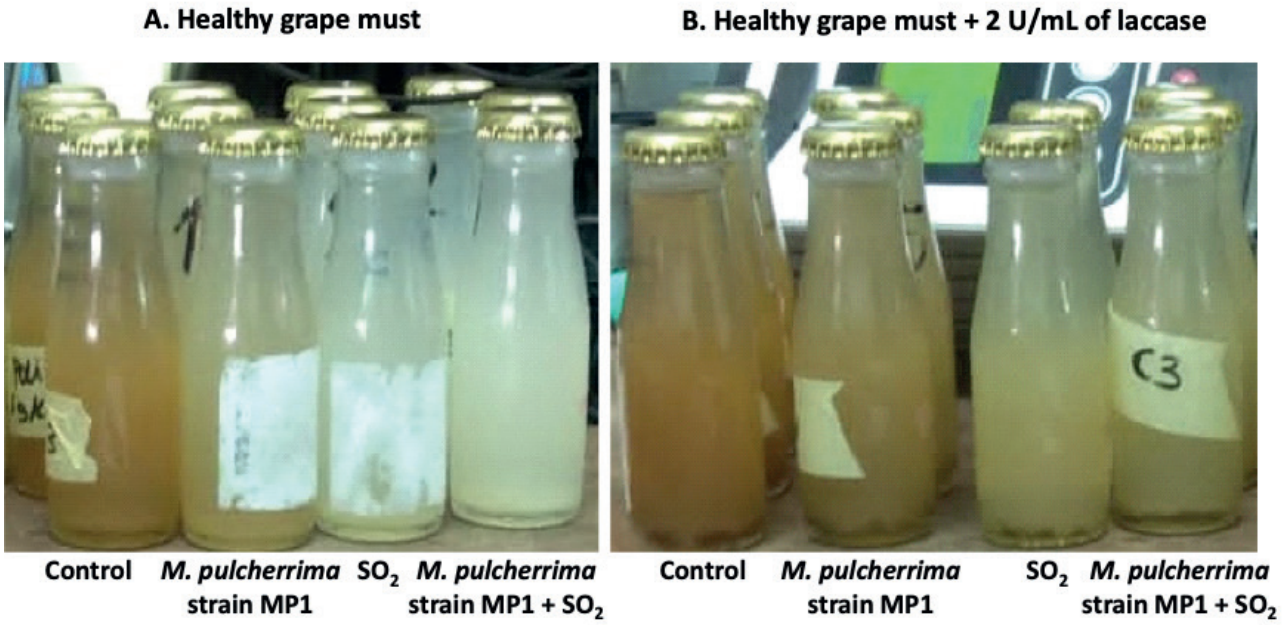

FIGURE 6. Influence of inoculation using *M. pulcherrima* strain MP1 on the intensity of the yellow colour of the wine.

In the absence of laccase, ΔEab* between the control sample and the reference was clearly higher than 3 units, which indicates that without sulphur dioxide the intensity of the browning was high enough to be clearly perceived by the human eye. By contrast, total colour difference of the sample inoculated with *Metschnikowia pulcherrima* strain MP1 was slightly above 3 units, which indicates that the intensity of browning was very low and hardly perceptible. This confirms that *Metschnikowia pulcherrima* strain MP1 exerts a real protective effect against enzymatic browning, at least in healthy grape must conditions. When sulphur dioxide and *Metschnikowia pulcherrima* strain MP1 were added in combination, the ΔEab* was much lower than 3 units and therefore the difference in colour with respect to the reference was imperceptible.

In the presence of laccase, the ΔEab* values were much higher than in the healthy grape must. This means that browning was evident, even in the presence of sulphur dioxide.

Figure 6 shows photographs of the different samples after the experimental process.

In healthy grape must, the control sample without anything added to it clearly underwent a browning process. By contrast, the samples supplemented with sulphur dioxide, whether alone or combined with *Metschnikowia pulcherrima* strain MP1, were much lighter in appearance, which confirms that this additive completely prevents the grape must from browning. The sample supplemented with only *Metschnikowia pulcherrima* strain MP1 was slightly browner than the samples supplemented with sulphur dioxide, but it was much lighter in colour than the control without any additive. However, in the presence of laccase the protective effectiveness of *Metschnikowia pulcherrima* strain MP1, and even that of sulphur dioxide, were not enough to prevent browning. This confirms that supplementation with *Metschnikowia pulcherrima* strain MP1 exerts a protective effect on grape must against browning in healthy grape conditions.

CONCLUSIONS

In this work, we analysed how supplementation with sulphur dioxide and/or *Metschnikowia pulcherrima* strain MP1 influence the oxygen consumption kinetics, hydroxycinnamic acids, GRP concentrations, and browning intensity in two situations: i) grape must from healthy grapes, and ii) grape must supplemented with the enzyme laccase to reproduce what occurs when the grapes are infected with *Botrytis cinerea*. The results show that sulphur dioxide drastically reduces oxygen consumption rate, protects hydroxycinnamic acids against oxidation and prevents grape must from browning even in the presence of laccase, although less effectively than in the absence of laccase. By contrast, the supplementation with *Metschnikowia pulcherrima* strain MP1 dramatically increases the oxygen consumption rate, which reduces the oxygen available for polyphenol oxidases. In the absence of laccase, this results in a decrease in browning and in a certain degree of protection of the hydroxycinnamic acids.

However, in the presence of laccase, supplementation with *Metschnikowia pulcherrima* strain MP1 was not effective enough to prevent browning. It can therefore be concluded that *Metschnikowia pulcherrima* strain MP1 can be considered an adequate alternative tool to reduce, and maybe eliminate, the addition of sulphur dioxide at the beginning of winemaking for preventing browning in white grape must made from healthy grapes.

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REFERENCES

Agarbati, A., Canonico, L., Ciani, M., & Comitini, F. (2023) *Metschnikowia pulcherrima* in cold clarification: biocontrol activity and aroma enhancement in Verdicchio wine. *Fermentation*, 9, 302. https://doi.org/10.3390/fermentation9030302

Ayala, F., Echavarri, J.F., & Negueruela A.I. (1997) A new simplified method for measuring the color of wines. ii. white wines and brandies. *American Journal of Enology and Viticulture, 48,* 364–369. https://doi.org/ 10.5344/ajev.1997.48.3.364

Bahut, F., Romanet, R., Sieczkowski, N., Schmitt-Kopplin, P., Nikolantonaki, M., & Gougeon, R. D. (2020). Antioxidant activity from inactivated yeast: Expanding knowledge beyond the glutathione-related oxidative stability of wine. *Food Chemistry*, 325 (2020) 126941 https://doi.org/10.1016/j.foodchem.2020.126941

Canonico, L., Agarbati, A., Galli, E., Comitini, F., & Ciani, M. (2023) *Metschnikowia pulcherrima* as biocontrol agent and wine aroma enhancer in combination with a native *Saccharomyces cerevisiae*. *LWT - Food Science and Technology*, 181 (2023)114758. https://doi.org/10.1016/j.lwt.2023.114758

Canonico, L., Comitini, F., & Ciani, M. (2019) *Metschnikowia pulcherrima* selected strain for ethanol reduction in wine: influence of cell immobilization and aeration condition. Foods, 8, 378. https://doi.org/10.3390/foods8090378

Chacón-Rodríguez, L., Joseph, L., Nazaris, B., Coulon, J., Richardson, S., & Dycus, D.A. (2020) Innovative use of non-*Saccharomyces* in bio-protection: *T. delbrueckii* and *M. pulcherrima* applied to a machine harvester. *Catalyst: Discovery into Practice*, 4, 82-90. https://doi.org/10.5344/catalyst.2020.20003

Claus, H., Sabel, A., & König, H. (2014). Wine phenols and laccase: an ambivalent relationship, p. 155-185. In Rayess YE (ed.), Wine - Phenolic Composition, Classification and Health Benefits. Nova Science Publishers: New York, NY, USA.

Contreras, A., Curtin, C., & Varela, C. (2015). Yeast population dynamics reveal a potential 'collaboration' between *Metschnikowia pulcherrima* and *Saccharomyces uvarum* for the production of reduced alcohol wines during Shiraz fermentation. *Applied and Environmental Microbiology*, 99, 1985-1895. https://doi.org/10.1007/s00253-014-6193-6

D'Amico, M., Di Vita, G., & Monaco, L. (2016). Exploring environmental consciousness and consumer preferences for organic wines without sulfites. *Journal of Cleaner Production*, *120*, 64–71. https://doi.org/10.1016/j.jclepro.2016.02.014

Diéval, J. B., Vidal, S., & Aagaard, O. (2011). Measurement of the oxygen transmission rate of co-extruded wine bottle closures using a luminescence-based technique. *Packaging Technology and Science*, *24*(7), 375–385. https://doi.org/10.1002/pts.945

Di Gianvito, P., Englezos, V., Rantsiou, K., & Cocolin, L. (2022) Bioprotection strategies in winemaking. *International Journal of Food Microbiology*, 364, 109532. https://doi.org/10.1016/j.ijfoodmicro.2022.109532

Du Toit, W. J., Marais, J., Pretorius, I. S., & du Toit, M. (2006). Oxygen in must and wine: A review. *South African Journal for Enology and Viticulture, 27,* 76–94. https://doi.org/10.21548/27-1-1610

Englezos, V., Di Gianvito, P., Peyer, L., Giacosa, S. Río Segade, S., Edwards, N., Rolle, L., Rantsiou, K., & Cocolin, L. (2022) Bioprotective effect of *Pichia kluyveri* and *Lactiplantibacillus plantarum* in winemaking conditions. *American Journal of Enology and Viticulture*, 73, 294-307. https://doi.org/10.5344/ajev.2022.22008

Escribano-Viana, R., González-Arenzana, L., Garijo, P., Fernández, L., López, R., Santamaría P., & Gutiérrez, A.R. (2022) Bioprotective effect of a *Torulaspora delbrueckii*/*Lachancea thermotolerans*-mixed inoculum in red winemaking. *Fermentation, 2022, 8*, 337. https://doi.org/10.3390/fermentation8070337

Friedman, M. (1996). Food browning and its prevention: an overview. *Journal of Agricultural and Food Chemistry, 44,* 631–653. https://doi.org/10.1021/jf950394r

Fronk, P., Hartmann, H., Bauer, M., Solem, E., Jaenicke, E., Tenzer, S., & Decker, H. (2015). Polyphenoloxidase from Riesling and Dornfelder wine grapes (*Vitis vinifera*) is a tyrosinase. *Food Chemistry*, *183*, 49–57. https://doi.org/10.1016/j.foodchem.2015.03.016

Gabrielli, M., Aleixandre-Tudo, J. L., Kilmartin, P. A., Sieczkowski, N., & du Toit, W. J. (2017). Additions of glutathione or specific glutathione-rich dry inactivated yeast preparation (DYP) to sauvignon blanc must: Effect on wine chemical and sensory composition. *South African Journal for Enology and Viticulture, 38,* 18–28. https://doi.org/10.21548/38-1-794

Giménez, P., Anguela, S., Just-Borras, A., Pons-Mercadé, P., Vignault, A., Canals, J. M., Teissedre, P. L., & Zamora, F. (2022). Development of a synthetic model to study browning caused by laccase activity from Botrytis cinerea. *LWT - Food Science and Technology*, 154 (2022) 112871. https://doi.org/10.1016/j.lwt.2021.112871

Giménez, P., Just-Borras, A., Pons, P., Gombau, J., Heras, J. M., Sieczkowski, N., Canals, J. M., & Zamora, F. (2023). Biotechnological tools for reducing the use of sulphur dioxide in white grape must and preventing enzymatic browning: glutathione; inactivated dry yeasts rich in glutathione; and bioprotection with *Metschnikowia pulcherrima*. *European Food Research and Technology*, *249*(6), 1491–1501. https://doi.org/10.1007/s00217-023-04229-6

Grassin, C., & Dubourdieu, D. (1986). Optimisation de la méthode de dosage de l'activité laccase de *Botrytis cinerea* par la syringaldazine. *Journal International des Sciences de la Vigne et du Vin, 20,* 125–130. https://doi.org/10.20870/oeno-one.1986.20.2.1298

Kritzinger, E. C., Bauer, F. F., & Du Toit, W. J. (2013). Role of glutathione in winemaking: A review. *Journal of Agricultural and Food Chemistry, 61*(2), 269–277. https://doi.org/10.1021/jf303665z

Ky, I., Lorrain, B., Jourdes, M., Pasquier, G., Fermaud, M., Gény, L., Rey, P., Doneche, B., & Teissedre, P. L. (2012). Assessment of grey mould (Botrytis cinerea) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. *Australian Journal of Grape and Wine Research*, *18*(2), 215–226. https://doi.org/10.1111/j.1755-0238.2012.00191.x

Lago-Vanzela, E. S., Da-Silva, R., Gomes, E., García-Romero, E., & Hermosín-Gutiérrez, I. (2011). Phenolic composition of the Brazilian seedless table grape varieties BRS clara and BRS morena. *Journal of Agricultural and Food Chemistry*, *59*(15), 8314–8323. https://doi.org/10.1021/jf201753k

Li, H., Guo, A., & Wang, H. (2008). Mechanisms of oxidative browning of wine. *Food Chemistry, 108*(1), 1-13. https://doi.org/10.1016/j.foodchem.2007.10.065

Lester, M. R. (1995). Sulfite sensitivity: Significance in human health. *Journal of the American College of Nutrition*, *14*(3), 229–232. https://doi.org/10.1080/07315724.1995.10718500

Martínez, J.A., Melgosa, M., Pérez, M.M., Hita, E., & Negueruela, A.I. (2001) Visual and Instrumental Color Evaluation in Red Wines. *Food Science and Technology International*, 7, 439–444. https://doi.org/10.1106/VFAT-5REN-1WK2-5JGQ

Martinez, M. V., & Whitaker, J. R. (1995). The biochemistry and control of enzymatic browning. *Trends in Food Science & Technology, 6,* 195–200. https://doi.org/10.1016/S0924-2244(00)89054-8

Morata, A., Loira, I., Escott, C., del Fresno, J.M., Bañuelos, M.A., & Suárez-Lepe, J.A. (2019) Applications of Metschnikowia pulcherrima in wine biotechnology. *Fermentation*, 5, 63. https://doi.org/10.3390/fermentation5030063

Morata, A., Loira, L., González, C., & Escott, C. (2021) Non-*Saccharomyces* as biotools to control the production of off-flavors in wines. *Molecules*, 2021. 26, 4571. https://doi.org/10.3390/molecules26154571

MSCVes.zip. (2013). Retrieved from http:// www. unirioja. es/ color/ descargas.shtml. Accessed July 9, 2023.

Oliveira, C. M., Ferreira, A. C., De Freitas, V., & Silva, A. M. (2011). Oxidation mechanisms occurring in wines. *Food Research International, 44*(5), 1115–1126. https://doi.org/10.1016/j.foodres.2011.03.050

Ough, CS., & Crowell, E. A. (1987). Use of sulphur dioxide in winemaking. *Journal of Food Science ,52,* 386–389. https://doi.org/10.1111/j.1365-2621.1987.tb06620.x

Pérez-Magariño, S., & González-Sanjosé, M.L. (2003). Application of absorbance values used in wineries for estimating CIELAB parameters in red wines. *Food Chemistry, 81*, 301-306. https://doi.org/10.1016/S0308-8146(02)00509-5

Pons-Mercadé, P., Anguela, S., Giménez, P., Heras, J. M., Sieczkowski, N., Rozès, N., Canals, J. M., & Zamora, F. (2021). Measuring the oxygen consumption rate of some inactivated dry yeasts: Comparison with other common wine antioxidants. *Oeno One*, *55*(2), 147–158. https://doi.org/10.20870/oeno-one.2021.55.2.4618

Puyo, M., Simonin, S., Klein, G., David-Vaizant, V., Quijada-Morín, N., Alexandre, H., & Tourdot-Maréchal, R. (2023). Use of oenological tannins to protect the colour of rosé wine in a bioprotection strategy with *Metschnikowia pulcherrima*. *Foods*, 12, 735. https://doi.org/10.3390/foods12040735

Ribéreau-Gayon, P., Glories, Y., Maujean, A., & Dubordieu, D. (2006a). The use of sulphur dioxide in must and wine treatment (chapter 8). In John Wiley & Sons (Eds.), *Handbook of Enology: The Microbiology of Wine and Vinifications, Volume 1, 2nd Edition* (pp. 281-304). https://doi.org/10.1002/0470010363.ch8

Ribéreau-Gayon, P., Glories, Y., Maujean, A., & Dubordieu, D. (2006b). Products and methods complementing the effect of sulphur dioxide. In John Wiley & Sons (Eds.), *Handbook of Enology: The Microbiology of Wine and Vinifications, Volume 1, 2nd Edition* (pp. 223-240). https://doi.org/10.1002/0470010363.ch9

Rubio-Bretón, P., Gonzalo-Diago, A., Iribarren, M., Garde-Cerdán, T., & Pérez-Álvarez, E.P. (2018) Bioprotection as a tool to free additives winemaking: Effect on sensorial, anthocyanic and aromatic profile of young red wines. *LWT - Food Science and Technology*, 98, 458-464. https://doi.org/10.1016/j.lwt.2018.08.050

Ruiz, J., Belda, I., Beisert, B., Navascues, E., Marquina, D., Calderon, F., Rauhut, D., Santos, A., & Benito, B. (2018). Analytical impact of Metschnikowia pulcherrima in the volatile profile of Verdejo white wines. *Applied and Environmental Microbiology*, 102, 8501-8509. https://doi.org/10.1007/s00253-018-9255-3

Simonin, S., Alexandre, H., Nikolantonaki, M., Coelho, C., & Tourdot-Maréchal, R. (2018) Inoculation of *Torulaspora delbrueckii* as a bio-protection agent in winemaking. *Food Research International*, 107, 451-461. https://doi.org/10.1016/j.foodres.2018.02.034

Simonin, S., Honoré-Chedozeau, C., Monnin, L., David-Vaizant, V., Bach, B., Alexandre, H., Chatelet, B., & Tourdot-Marechal, D. (2022). Bioprotection on Chardonnay grape: Limits and impacts of settling parameters. *Australian Journal of Grape and Wine Research,* 2022, Article ID 1489094. https://doi.org/10.1155/2022/1489094

Simonin, S., Roullier-Gall, C., Ballester, J., Schmitt-Kopplin, P., Quintanilla-Casas, B., Vichi, S., Peyron, D., Alexandre, H., & Tourdot-Maréchal, R. (2020) Bio-protection as an alternative to sulphites: Impact on chemical and microbial characteristics of red wines. *Frontiers in Microbiology*, 2020. 11, 1308. https://doi.org/10.3389/fmicb.2020.01308

Singleton, V.L., Salgues, M., Zaya, J., & Trousdale, E. (1985). Caftaric acid disappearance and conversion to products of enzymic oxidation in grape must and wine. *American Journal of Enology and Viticulture, 36*, 50-56. https://doi.org/10.5344/ajev.1985.36.1.50

Sipiczki, M., (2006) Metschnikowia strains isolated from botrytized grapes antagonize fungal and bacterial growth by iron depletion. *Applied and Environmental Microbiology*, 72, 6716–6724. https://doi.org/10.1128/AEM.01275-06

Steel, C. C., Blackman, J. W., & Schmidtke, L. M. (2013). Grapevine bunch rots: Impacts on wine composition, quality, and potential procedures for the removal of wine faults. *Journal of Agricultural and Food Chemistry, 61,* 5189–5206. https://doi.org/10.1021/jf400641r

Vignault, A., Pascual, O., Jourdes, M., Moine, V., Fermaud, M., Roudet, J., Canals, J. M., Teissedre, P. L., & Zamora, F. (2019). Impact of enological tannins on laccase activity. *OENO One*, *53*(1), 27–38. https://doi.org/10.20870/oeno-one.2019.53.1.2361

Vignault, A., Gombau, J., Jourdes, M., Moine, V., Canals, J. M., Fermaud, M., Roudet, J., Zamora, F., & Teissedre, P. L. (2020). Oenological tannins to prevent Botrytis cinerea damage in grapes and musts: Kinetics and electrophoresis characterization of laccase. *Food Chemistry*, *316*. https://doi.org/10.1016/j.foodchem.2020.126334

Windholtz, S., Nioi, C., Coulon, J., & Masneuf-Pomarède, I. (2022) Assessment of O_2 consumption, a new tool to select bioprotection yeast strains. *IVES Conference Series-WAC. 2022*. https://ives-openscience.eu/14802/

Windholtz, S., Nioi, C., Coulon, J., & Masneuf-Pomarede, I. (2023). Bioprotection by non-Saccharomyces yeasts in oenology: Evaluation of O_2 consumption and impact on acetic acid bacteria. *International Journal of Food Microbiology*, 405 (2023) 110338. https://doi.org/10.1016/j.ijfoodmicro.2023.110338

Windholtz, S., Redon, P., Lacampagne, S., Farris, L., Lytra, G., Cameleyre, M., Barbe, Coulon, J., Thibon, C., & Masneuf-Pomarède, I. (2021). Non-*Saccharomyces* yeasts as bioprotection in the composition of red wine and in the reduction of sulfur dioxide. *LWT - Food Science and Technology*, 149 (2021) 111781. https://doi.org/10.1016/j.lwt.2021.111781

Yao, M., Wang, F., & Arpentin, G. (2023). Bioprotection as a tool to produce natural wine: Impact on physicochemical and sensory analysis. *BIO Web of Conferences*, 56, 02019 (2023). https://doi.org/10.1051/bioconf/20235602019

Zimdars, S. (2020). *Role of glutathione and Botrytis cinerea laccase activities in wine quality*. Gotinga, Germany: Cuvillier Verlag, ISBN 978-3736972544.