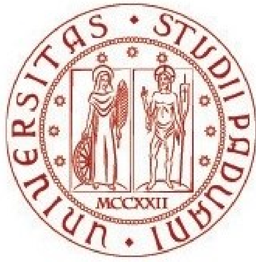

UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI AGRONOMIA, ANIMALI, ALIMENTI, RISORSE NATURALI E AMBIENTE

Corso di laurea triennale in Scienze e Cultura della Gastronomia

Use of glutathione to minimize oxidation in Verdejo musts during fermentation

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Abstract

This study examines the impact of glutathione-enriched yeast (*lev-GSH*) on the oxidation, metabolic activity, and sensory attributes of Verdejo wine obtained by fermenting the must with two yeast strains, *Saccharomyces cerevisiae* and *Lachancea thermotolerans*. Key characteristics were examined under various fermentation circumstances including varied amounts of sulfur dioxide (SO_2) and *lev-GSH*, ethanol generation, reducing sugars, pH, acid content, volatile acidity, oxygen levels, redox potential, and sensory qualities. The results indicated that glutathione supplementation influenced ethanol production, sugar metabolism, and acid regulation, particularly in *L. thermotolerans*, which exhibited higher reducing sugar levels and lower ethanol production. GSH effectively controlled oxidation in *L. thermotolerans*, maintaining lower oxygen levels and enhancing sensory attributes, such as aromatic intensity and fruity notes. Additionally, *lev-GSH* impacted wine color intensity and stability. These findings suggest that *lev-GSH* can be a valuable tool in winemaking, especially in combination with *L. thermotolerans*, to improve oxidative stability and sensory quality. Future research should explore the long-term effects of *lev-GSH* on wine stability and the molecular mechanisms underlying its interaction with different yeast strains.

1. Introduction

1.1. Climate change effects in wine production

The current generation exhibits heightened concern regarding climate change due to the disproportionate rise in temperatures, impacting various sectors on a daily basis, including oenology (Mira de Orduña, 2010). Recent years have seen a substantial impact of rising temperatures on wine production, affecting vine phenology, grape composition, vinification processes, and sensory characteristics (Mira de Orduña, 2010).
Warm weather leading to increased sugar concentration in grapes results in higher alcohol content and reduced acidity, posing challenges during fermentation due to its osmotic influence on yeasts and resulting in the development of unwanted co-products such as acetic acid (Mira de Orduña, 2010).
Budbreak and fruit ripening dates are now earlier in several regions, consequently advancing harvest dates by 2-3 weeks compared to the past (Stock et al., 2005).
Studies in various regions like Alsace, Baden, the Palatinate, and parts of California have noted similar advancements in grape harvest due to rising temperatures (Duchêne & Schneider, 2005).
While temperature rise contributes to premature ripening, the accumulation of sugars beyond a certain threshold is mainly attributed to concentration by evaporative loss (Keller, 2023).

However, warm temperatures significantly impact total acidity, with high temperatures leading to decreases in malic acid levels and alterations in grape pH due to potassium accumulation (Coombe, 1987; Tarara et al., 2008; Williams & Biscay, 1991). Lower acidity levels and higher pH values increase the susceptibility of wines to microbial contamination, necessitating higher doses of sulfur dioxide for prevention (Chidi et al., 2018;Giacosa et al., 2018).

Acidity not only influences contamination hazards but also affects wine sensory qualities, though direct correlation with pH is challenging due to various factors including human physiology and gustative equilibrium during wine tasting (Comuzzo & Battistutta, 2018;Gambuti et al., 2022).
The typical aromas of white wines, such as those given by isoprenoids and pyrazines, develop preferably in mild climates, which means they decrease with high temperatures.
Furthermore, the development of typical aromas in white wines is hindered by high temperatures (Mira de Orduña, 2010).

Climate change exacerbates risks of contamination and oxidative reactions due to elevated temperatures, posing additional challenges in wine production (Mira de Orduña, 2010). Malolactic fermentation, crucial for wine aging and stabilization, is also affected by climate change, potentially leading to excessively high pH levels in wines from warm climates if acidity is not adjusted (Lonvaud-Funel, 1999; Mira de Orduña, 2010).

This intricate interplay of climate change and its effects on wine chemistry underscores the importance of exploring strategies to mitigate oxidation risks and maintain wine quality.

1.2. Chemical oxidation in wine

Oxidation is a chemical reaction in which a substance loses electrons, resulting in an increase in its oxidation state. This process often involves the addition of oxygen or the removal of hydrogen from a compound (Waterhouse & Laurie, 2006).
The product resulting from oxidation is called ROS, i.e., Reactive Oxygen Species.
In wine, ROS are produced from reduced transition metal ions donating an electron to triplet oxygen (O_2). Since the pH of wine is acidic, the superoxide radical anion becomes protonated, forming the hydroperoxyl radical (HOO). Another electron transfer occurs, leading to the production of peroxide anion (O_2^{-2}), which will in turn be transformed to its protonated form hydrogen peroxide (H_2O_2). One more reduction step takes place, leading to the formation of the hydroxyl radical (HO), a highly reactive oxidant that can abstract a hydrogen atom from organic compounds producing water (Oliveira et al., 2011).
A series of reactions starts and generates reactive oxygen species, which can have various effects on the chemical composition of the wine and its sensory characteristics. The involvement of ferrous ions and oxygen in these reactions is common in oxidative processes and can contribute to both desirable and undesirable changes in wine.

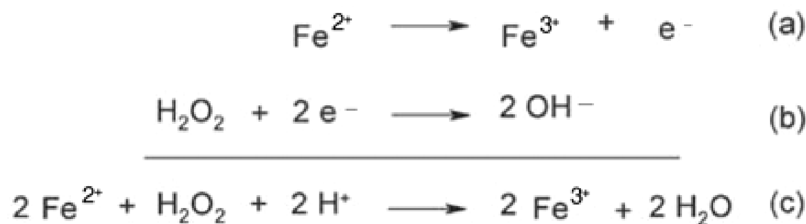

Figure 1: Redox system: oxidation of ferrous to ferric ion (**a**); reduction of hydrogen peroxide to hydroxide (**b**); and overall redox reaction (**c**) **(Waterhouse & Laurie, 2006)**.

Oxidation is an inevitable phenomenon during the production of wine, resulting from the encounter of grape juice with oxygen.
Sometimes it is chosen to make the wine adhere to a precise style, while other times it occurs involuntarily, and it can ruin an entire production negatively by affecting all sensory characteristics (smell, taste, color).
In the case of white wine, the phenomenon of 'browning' occurs: the color abandons greenish hues in favor of yellowish ones and gradually turns brown.
In the case of red wine, oxidation reduces the intensity of the typical red color, which is gradually replaced by orange hues.
The taste is also strongly influenced by oxidation, due to the polymerization of phenolic compounds, which leads to an increased sensation of dryness in the mouth.
The aroma, however, is the sensory component most affected by oxidation. Fresh aromas of fruits, flowers and grass are erased prematurely, thus eliminating everything that derives from the type of grape used and leaving only the aromas due to fermentation.
Oxidation affects the entire wine manufacturing process, from the extraction of juice from the grapes to bottle storage.
The important link between wine and oxygen was first discovered by Louis Pasteur, who, through a series of experiments, observed both the negative effects on young wines and the positive effects of limited oxygenation on ageing wines (Comuzzo & Zironi, 2013).

The ability of a must to absorb less or more oxygen depends on the variety of grapes from which it is derived and on the must composition. One study showed, for example, that a Sauvignon

must (poor in phenolic compounds) oxidize much faster than a Pinot gris (Comuzzo et al., 2015).

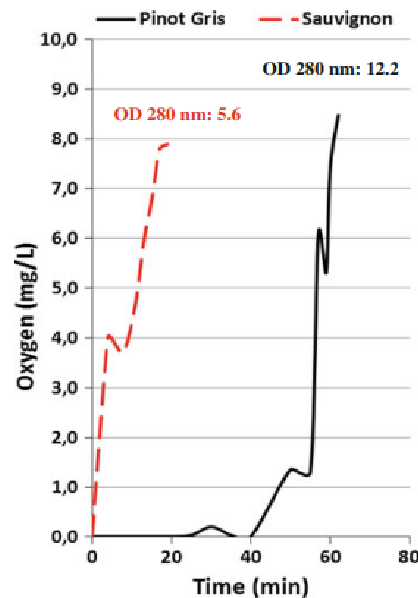

Figure 2: Oxygen levels during hyperoxygenation in Pinot gris and Sauvignon blanc **(Comuzzo & Zironi, 2013a)**

The solubility of oxygen in wine is also influenced by the temperature and the composition of the gas to which the wine is exposed: for an air-saturated wine values of oxygen solubility of 8.6 mg/L at room temperature and atmospheric pressure were given. Since we are talking about air, and not pure oxygen, the result is not high. Talking about pure oxygen it would have been 5 times higher than the one obtained, like in the case of micro-oxygenation. Regarding temperature, it was seen that at low temperatures (5°C) the solubility of oxygen increases about 10% (Waterhouse & Laurie, 2006).

Oxidation can be divided into two types: enzymatic oxidation, which occurs mainly in must, and non-enzymatic oxidation, which occurs in both must and wine**,** although it is more prominent in wine.

Enzymatic browning is due to the presence of atmospheric oxygen and polyphenol oxidase (PPO) and concerns the initial phases of the process. PPO is one of the main oxidoreductases causing browning together with peroxidase (POD). PPO is responsible for oxidizing substrates characteristic of tyrosinase and laccase. Tyrosinase is naturally present in grape berries and catalyzes the oxidation of monophenols and *o*-diphenols, while laccase is produced by molds (e.g. *Botrytis cinerea*) and oxidizes various substrates, especially 1,2- and 1,4-dihydroxyphenene.

Enzymic browning involves the oxidation of hydroxylcinnamates such as caffeoyltartaric acid (caftaric acid) and *p*-coumaroyltartaric acid (coutaric acid), which are oxidised by the PPO producing caffeoyltartaric acid *o*-quinones (CTAQ). These latter are responsible for changes in wine tone and color intensity.

In the meantime, o-quinones undergo more reactions, oxidize substrates with lower potentials, react with amino derivatives and water, forming browning pigments.

Oxidation in must is however affected by the presence of glutathione (GSH), which can trap the quinones produced. Glutathione (GSH) reacts with CTAQ to produce 2-S-glutathionyl caftaric acids (GRP), limiting oxidative browning up to a certain point.

Nevertheless, SO_2 can inactivate catecholase, avoiding the production of GRP and consequently maintaining more more caftaric acid and *p*-coutaric acid available with browning potential.
Furthermore, copper reacts with GSH, competing with CTAQ and enhancing the browning of grape must (Li et al., 2008).
Non-enzymatic oxidation, also knew as chemical oxidation, is so called because it does not involve enzymes. Polyphenols containing a catechol ring or a galloyl group, such as catechin, epicatechin, gallocatechin, gallic acid or caffeic acid, act as the initiators of the oxidation (Danilewicz, 2003).
The latter are oxidised to semiquinones and benzoquinones, while oxygen is reduced to hydrogen peroxide. Mediating the entire process is the redox cycle of Fe^{3+}/ Fe^{2+} and Cu^{3+}/ Cu^{2+}: oxygen does not directly react with phenolic compounds without the presence of these metal ions (Danilewicz, Seccombe, & Whelan, 2008).
The quinones resulting from the oxidation of polyphenols are unstable, which is why they may be subject to further reactions. For instance, they can react with nucleophilic compounds forming dimers or polymers that are more easily oxidized because of their lower redox potentials (Li et al., 2008).
This may result in an acceleration of the polymerization process (Boulton, Singleton, Bisson, & Kunkee, 2001; Zhai, Du, Guan, Qiao, & Pan, 2001).
A further scenario is the Fenton reaction, i.e. the reaction in which hydrogen peroxide binds to ferrous ions generating hydroxyl radicals (Waterhouse Andrew L. & Laurie Felipe, 2006).
These radicals can oxidize almost any organic molecule found in wine, such as ethanol, tartaric acid, glycerol, sugars, and organic acids (Danilewicz J. C., 2003; Li et al., 2008;
Oliveira et al., 2011).

Oxidation being an irreversible reaction, it is essential to look for preventing methods.
Prevention of oxidation can be applied in different stages:

- In the treatment of the grapes, for example by opting for a soft pressing that limits the extraction of phenolics compounds from the skins.
- By limiting the wine's contact with oxygen, using inert gases or yeast lees as protectors.
- By blocking oxidation reactions.

Understanding the significant implications of climate change-induced oxidation on wine chemistry highlights the critical need for effective preventive measures. In the following section, we delve into various methods, including the use of sulfites and yeast derivatives, aimed at mitigating oxidation risks, and preserving wine quality.

1.3. Sulfur dioxide (SO_2) and its roles in winemaking

Sulfites marked the beginning of modern oenology in the second half of the 19th century, and they are still the most used antioxidant in wines. Used throughout the whole wine-making process, it protects the wine from browning, but not only that: it also has an antimicrobial effect that prevents the growth of dangerous yeasts or bacteria in the wine (Ribéreau-Gayon P. et al., 2006).
Although *S. cerevisiae* naturally produces SO_2, in an amount of approximately 0-100mg/L (but typically between 10 and 20 mg/L), sulfites remain a mainly exogenous compound (Eschenbruch R., 1974; Comuzzo & Zironi, 2013).
The amount of added SO_2 varies between 50mg/L and 200mg/L (Oliveira et al., 2011b) and it is present in wine under an acid-base equilibrium, constituted of the undissociated molecular form (SO_2) and the dissociated bisulfite ion (HSO_3^-), which is the most common at pH wine. Among the two, the molecular form is the most active at the microbiological and oxidation control level, but it is also the least present (Ribéreau-Gayon P. et al., 2006).

In wine, sulfur dioxide is divided into free SO_2 and bounded SO_2, the latter being the one that reacts with unsaturated compounds.

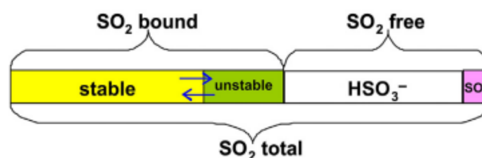

Figure 3: Sulfur dioxide fractions in wine(Giacosa et al., 2018b)

The action of sulfites is directed at quinones, which are returned to their phenolic form (Danilewicz J. C., 2003; Danilewicz J. C. et al., 2008).
Furthermore, sulfites can inhibit aldehyde formation by binding to hydrogen peroxide (Elias R. J. et al., 2010).

Sulfur dioxide can be added to wine in various forms: in gaseous form, in aqueous solution or by salts that release SO_2, by combustion of sulfur. This latter being a traditional method, it is commonly used in winemaking, particularly for sanitizing barrels. After thorough cleaning and drying, barrels are sulfurized to eliminate unwanted microorganisms present on the inner surface or in wood cavities affected by wine penetration (Giacosa et al., 2018b).
The gaseous sulfur dioxide released during combustion can effectively penetrate the oak's porosity, making it suitable for use in empty containers, even at high doses. However, the actual yield of sulfur dioxide is typically lower than the weight of burned sulfur due to impurities or incomplete combustion (Giacosa et al., 2018b). Perforated disks, suspended in barrels with a metal wire and ignited, are commonly used for this purpose, as they effectively prevent and combat the presence of undesirable microorganisms such as Brettanomyces, particularly in red wine production where the presence of 4-ethylphenols is a concern (Chatonnet et al., 1993).
Despite its effectiveness, the combustion of sulfur poses risks for operators and requires proper training and personal protective equipment due to potential corrosion. It's important to monitor sulfite levels in stored wines as some sulfur dioxide may dissolve into the wine during barrel filling (Giacosa et al., 2018b).

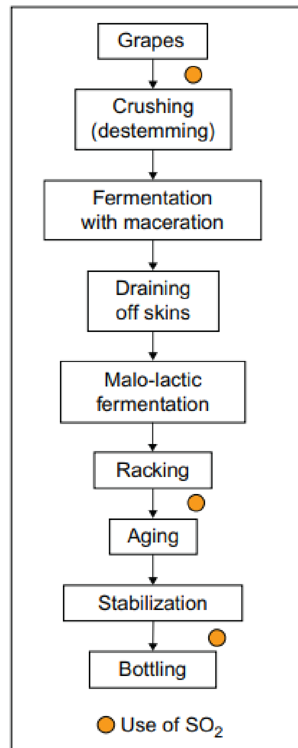

Figure 4: Flow-chart of red winemaking, indicating the main phases where SO_2, is generally used (Giacosa et al., 2018b)

Sulfur dioxide maintains an indispensable role in contemporary winemaking processes owing to its multifaceted functionalities. Its roles can be succinctly categorized as follows:

1. Antiseptic: due to its dual antiseptic activities, including selective inhibition of must microflora and antimicrobial action during wine aging, thus ensuring preservation.
2. Antioxidant: acting as an inhibitor of dissolved oxygen, sulfur dioxide effectively shields wines from chemical oxidation, safeguarding polyphenols, and aromatic compounds from deterioration.
3. Antioxidasic: by impeding the activity and potential denaturation of oxidasic enzymes like polyphenoloxidase (PPO), sulfur dioxide safeguards musts from pre-fermentation oxidations, ensuring the integrity of the winemaking process.
4. Solubilizing: in higher concentrations, sulfur dioxide facilitates the diffusion of color compounds from grape skins by creating microscopic openings in cell walls, thereby enhancing the release of anthocyanins.
5. Binding agent: sulfur dioxide enhances the sensory profile of wines by binding with compounds contributing to pungent odors or tastes, such as acetaldehyde and pyruvic acid, rendering them imperceptible to the palate.
6. Fining agent: with its mild clarifying action, sulfur dioxide aids in the coagulation of colloidal substances, thereby augmenting the spontaneous precipitation of lees and contributing to wine clarity.

These functions underscore the indispensable nature of sulfur dioxide in modern winemaking practices (Giacosa et al., 2018b).

The widespread disappearance of defective or altered wines from the market can be attributed to advancements in winemaking techniques, improved hygiene standards, and the ubiquitous use of an antiseptic, sulfur dioxide, which has remained an undisputed additive for over a century (Giacosa et al., 2018b). However, concerns over the use of sulfites have prompted producers to seek ways to reduce or eliminate their presence, aiming to enhance the perceived

naturalness of wines and mitigate health risks for sulfite-sensitive consumers (Giacosa et al., 2018b). European Community legislation mandates the labeling of wines containing sulfites above 10 mg/L, primarily due to concerns about allergic reactions in sensitive individuals (Giacosa et al., 2018). While allergic reactions are the primary concern, the World Health Organization has established a safety limit for daily sulfite intake at 0.7 mg/kg of body weight, underscoring potential health risks associated with excessive consumption (Giacosa et al., 2018b). Studies have revealed that the LD_{50} of sulfur dioxide varies between 0.7 to 2.5 g/kg body weight depending on the species, highlighting the toxicity of sulfites (Ribéreau-Gayon P. et al., 2006).
According to the Food and Drug Administration (FDA) 1% of population is sensitive to sulfites: for individuals with asthma, sulfites can irritate the respiratory tract, and they may also induce headaches in sensitive individuals, although these symptoms can be exacerbated by other compounds present in wine, such as histamine, tyramine, flavonoids, and alcohol (Giacosa et al., 2018b). Yet, it is important to remember that these symptoms are often aggravated by compounds produced in the wine itself such as histamine, tyramine, flavonoids, and alcohol. Consequently, regulations such as Regulation (EC) 607/2009 mandate the labeling of wines containing sulfites above 10 mg/L (Comuzzo & Zironi, 2013b).

These potential side effects have spurred research into alternative additives. In this study, we focus on glutathione as a potential substitute for sulfites.

1.4. Sulfur dioxide substitutes

In recent years, there has been a growing interest in reducing sulfur dioxide levels in wine using alternative additives. Various molecules permitted by wine legislation have been explored as potential substitutes or supplements to SO_2. Additionally, novel products are continuously being assessed to compensate for the functions of SO_2.
The antimicrobial and antioxidant activities of these substitutes are being studied and examined in order to understand their action. While some enological products aid in extracting secondary metabolites from grape pomace, they do not directly replace SO_2 in its antimicrobial and antioxidant roles (Giacosa et al., 2018b).

Dimethyl dicarbonate

Dimethyl dicarbonate (DMDC), proposed as a substitute for sulfur dioxide in winemaking (Divol et al., 2005; Ough, 1975), acts as a chemical inhibitor of microbes by inhibiting various enzymes and methoxycarbonylating nucleophilic residues (Renouf et al., 2008). Despite its effectiveness in halting cell development, DMDC quickly converts to methanol upon addition to wine, with minor byproducts like methyl carbonate and alkyl carbonate (Divol et al., 2005). Studies have shown that the methanol levels generated by DMDC are not toxicologically significant (Divol et al., 2005). However, DMDC's efficacy is influenced by factors such as temperature, ethanol content, pH, and microbial species and strains (Costa et al., 2008).

Unlike SO_2, which suppresses yeast proliferation, DMDC effectively kills yeast cells, making it a promising yeast growth inhibitor (Divol et al., 2005). However, its transient effects, with complete conversion to methanol within hours, make it unsuitable for wine storage (Divol et al., 2005).
Moreover, relying solely on DMDC in winemaking may not be sufficient to replace SO2 entirely, as it may fail to inhibit the growth of certain bacteria and oxidize wine (Divol et al., 2005). Therefore, while DMDC shows promise as a supplement to SO_2 in inhibiting yeast growth, its limitations necessitate further research into alternative additives for sulfite-free winemaking.

Bacteriocins

Bacteriocins, such as nisin, pediocin, and plantaricin, are small polypeptides produced by specific lactic acid bacteria (LAB) that inhibit the growth of other bacterial species (Yurdugül & Bozoglu, 2002).
Nisin, the only commercially available bacteriocin, is effective against wine LAB, particularly *Oenococcus oeni*, but has limited impact on yeasts (Rojo-Bezares et al., 2007).
Pediocin and plantaricin also target various LAB strains, with pediocin showing promise in removing biofilms from stainless steel surfaces in wine (Nel et al., 2002).

Combining bacteriocins with metabisulfite has been suggested to control spoilage bacteria growth in wine, potentially reducing sulfur dioxide levels (Yurdugül & Bozoglu, 2002). Additionally, a combination of bacteriocins and DMDC may offer alternatives to SO_2 addition, targeting bacteria and yeasts, respectively (Yurdugül & Bozoglu, 2002). However, the use of bacteriocins in wine is not yet authorized, and their impact on wine properties requires further research (Yurdugül & Bozoglu, 2002).

Phenolic compounds

Phenolic compounds are crucial in wine production, influencing its color, astringency, and health benefits due to their antioxidant properties (García-Ruiz et al., 2008). These compounds, including phenolic acids, flavonoids, stilbenes, tannins, and anthocyanins, scavenge free radicals and contribute to wine's antioxidative capacity (García-Ruiz et al., 2008).

Recent studies have explored the potential of phenolic compounds as replacements for SO_2 in winemaking, given their antioxidant and antimicrobial properties (Bautista-Ortín et al., 2005). Enological tannins, for instance, have been investigated for their ability to inhibit enzymes and scavenge radicals, potentially replacing SO_2 without affecting the fermentation process (Bautista-Ortín et al., 2005; García-Ruiz et al., 2008).
However, the effectiveness of different tannins on wine characteristics varies, with some causing undesirable changes in color and aroma (Bautista-Ortín et al., 2005). Phenolic extracts from winemaking byproducts have also shown antimicrobial activity against various pathogens, indicating their potential as natural preservatives (Bautista-Ortín et al., 2005).
Nevertheless, their antimicrobial effect often requires higher doses than those naturally present in wines, raising concerns about their impact on wine properties (García-Ruiz et al., 2008).
Despite these challenges, combining phenolic compounds with other antimicrobial agents like bacteriocins and DMDC could offer a promising approach to preserving wine without the use of SO_2 (Bautista-Ortín et al., 2005; García-Ruiz et al., 2008).

Further research is needed to optimize the use of phenolic compounds in winemaking and ensure their compatibility with wine characteristics (García-Ruiz et al., 2008).

Lysozyme

Lysozyme, derived from egg albumen, has gained attention in winemaking for its antimicrobial properties (Chung & Hancock, 2000). Operating optimally in the pH range of 2.8–4.2, lysozyme effectively inhibits bacterial growth in wines, particularly against gram-positive bacteria (Delfini et al., 2004). However, its efficacy varies among LAB species and is less effective against gram-negative bacteria (Delfini et al., 2004).
Lysozyme's activity is more pronounced in white wines compared to red wines, due to polyphenolic compounds in red wine that can bind lysozyme (Bartowsky et al., 2004; Delfini et al., 2004).

While lysozyme treatments show promise in reducing volatile acidity and biogenic amine content in wines, they may also lead to wine haze formation and a decrease in color density and phenolic content in red wines (Bartowsky et al., 2004; Isabel et al., 2009). Combining lysozyme with other compounds like nisin and tannins has been explored to reduce or replace SO_2 usage in winemaking, resulting in wines with improved sensory impact (Chung & Hancock, 2000; Isabel et al., 2009). However, lysozyme's use adds significant costs for winemakers, and its allergenic nature requires labeling even when used as a processing aid (Regulation (EC) No 607/2009) (Chung & Hancock, 2000).

Recent studies have investigated alternative antimicrobial enzymes, such as lytic cocktails of *Streptomyces* spp. and β-glucanases, as potential substitutes for lysozyme in controlling LAB and yeast in wine production (Chung & Hancock, 2000; Isabel et al., 2009). These enzymes offer efficient microbial control with minimal impact on enological parameters (Chung & Hancock, 2000; Isabel et al., 2009).

Yeast derivatives

Before wine was marketed globally and with so much production, fermentation took place naturally thanks to the microflora present in grapes (Rainieri, 2000). It was only in the second half of the 19th century that yeasts were identified as being responsible for ethanol production (Demain & Solomon, 1981). From this time onwards, several studies demonstrated the crucial role these microorganisms play in determining the body, viscosity, color, flavor, and aroma of wine (Rainieri, 2000).

It is the thermal inactivation of one yeast, *Saccharomyces cerevisiae*, that leads to yeasts derivatives (YDs). Yeast extracts are a soluble extract of inactivated yeast or yeast autolysate; purified mannoproteins are obtained from yeast cell walls, with varying degrees of purification; inactivated dry yeasts are obtained after thermal inactivation followed by drying; yeast autolysates are obtained after thermal inactivation and enzyme processing; centrifugation of yeast autolysate yields yeast cell walls; and yeast extracts are a mixture of inactivated yeast and yeast autolysate (Shurson, 2018).

YDs are frequently employed in the winemaking process to eliminate unwanted wine constituents or to enhance technological and sensory aspects (Pozo-Bayón et al., 2009), as they alter the color, texture, and chemical-physical stability of wine (Comuzzo et al., 2006; Escot et al., 2001). In red wines, yeast mannoproteins stabilize the color by increasing the stability of phenolic components (Escot et al., 2001; François et al., 2007; Riou et al., 2002). Furthermore, research has demonstrated that these products can adsorb browning agents and phenolic compounds found in white wines (Razmkhab et al., 2002).

YDs influence on wine aroma is due to the impact on wine volatile compounds, that arises from multiple phenomena:

a. wine volatiles are adsorbed by yeast walls (Lubbers et al., 1994);
b. YDs volatile compounds are released (Comuzzo et al., 2006);
c. soluble colloids that influence wine aroma compound’s volatility are released (Comuzzo et al., 2006; Pozo-Bayón et al., 2009).

Numerous variables, including the chemical composition of the wine, the properties of YDs, and the dosage of YDs, influence the existence of these concurrent events (Comuzzo et al., 2006).

Glutathione

Recently, yeasts with specific compositions were created to fight oxidation in wine (Bahut et al., 2019). The primary cause of their protective effect was attributed to glutathione (γ-L-Glutamyl-L-cysteinyl-glycine) that the yeasts contained.

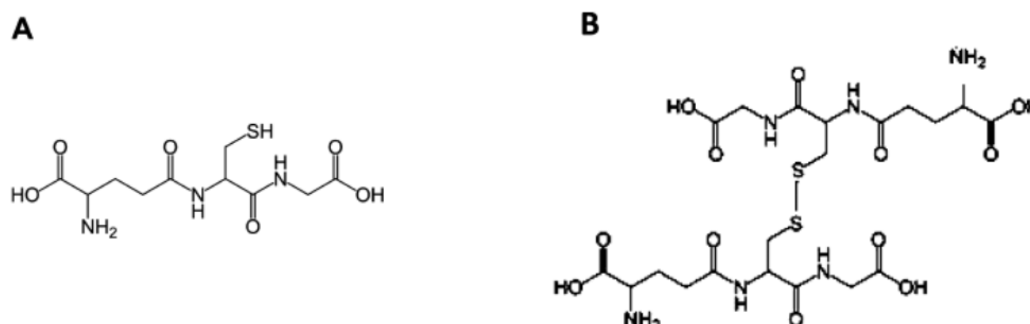

Figure 5: Molecular structures of (A) glutathione (GSH) and (B) glutathione disulfide (GSSG).

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is a tripeptide which contains three constitutive amino acids: glutamate, cysteine, and glycine. Its structure confers it two features: protection against hydrolysis, thanks to the γ peptide bond between glutamate and cysteine, and the antioxidant effect, due to the presence of a sulphydryl group (Chaya et al., 2014).

Glutathione (GSH) showed an ability to prevent the enzymatic browning and the development of off-flavor compounds, drawing the OIV's attention (International Organisation of Vine and Wine). Nevertheless, its pure use is still strictly forbidden. At present, it is only allowed the use of yeast derivatives enriched in GSH (IDY) (Chaya et al., 2014). The antioxidant role of GSH in wine is since it captures o-quinones (CTAQ), correlated to the increase of browning pigments, and that it can protect other aromatic molecules such as terpenes, volatile esters and thiols, responsible for the typical ageing off-flavours (Oliveira et al., 2011b).

Glutathione (GSH) occurs spontaneously also in grapes and musts. Depending on the grape variety, maturity level, vineyard techniques, and fermentation circumstances, GSH concentration in wines can range from 0.1 to 70 mg/L (Dubourdieu & Lavigne-Cruege, n.d.; Kritzinger et al., 2013).

These factors led to the next study's analysis of a Verdejo must's oxidation, which included varying amounts of GSH-enhanced yeast.

2. Objectives

Main objective/general objective

This project aims at examining the kinetic of oxidation of a Verdejo must during fermentation, and to compare the impact on wine composition of both the addition of two glutathione-enriched yeast derivatives and sulfites.

Specific objectives

- To compare the oxidation phenomena in fermentations performed with *S. cerevisiae* vs. *L. thermotolerans*
- To benchmark the antioxidant activity of glutathione-enriched yeast derivatives vs. the use of SO_2
- To characterize the physico-chemical composition of the wines treated

3. Materials and methods

The analyses were conducted in the food engineering laboratory of the Universidad Politécnica de Madrid, in the period between October 2023 and January 2024.

3.1. Materials

3.1.1. Frozen must

The study was conducted using defrosted Verdejo must which was kept frozen for better preservation.

In Spain, the wine grape known as Verdejo has gained a lot of popularity, frequently taking the place of the well-liked Sauvignon Blanc. Castilla and Léon are the primary growing regions, with the Rueda Denomination of Origin situated south of Valladolid (700-900 meters above sea level) being the most productive. The latter has lengthy, chilly winters and cloggy, dry summers with little to no precipitation. The grape's sugar content and acidity can be balanced by the altitude because the heat helps the former develop and the nighttime cold helps the latter. Because this variety of grape is highly sensitive to oxidation, the winemaking process needs to be done carefully and under strict control. Looking at the aromatic side, a wine made from the Verdejo grape will be characterized by typical green fruit flavors such as apple, lime, and a hint of melon. Typical aromas are fresh grass, pine, and a slight touch of fennel (Sánchez-Palomo et al., 2010).

After defrosting the must, the following analysis have been conducted:

- ✓ Baumé
- ✓ pH
- ✓ Amino nitrogen
- ✓ Gluconic acid
- ✓ Tartaric acid
- ✓ Total acidity
- ✓ Ammonia nitrogen
- ✓ Malic acid
- ✓ Total reducing sugars
- ✓ Reduction and oxidation potential
- ✓ Oxygen
- ✓ Refractometry
- ✓ Total Polyphenol Index (TPI)

3.1.2. Yeasts strains and antioxidants agents

Two types of yeasts were used in the study: *S. cerevisiae* and *L. thermotolerans.*
Two tests were conducted, the first one using commercial activated dry yeast and the second one using cryogenic yeast from the Food Technology Laboratory in the Escuela Técnica Superior de Ingenieria Agronomica (ETSIAAB).

For the first test, a Lalvin QA23 *S. cerevisiae* yeast, sourced from the Vinhos Verdes region of Portugal, was employed. Known for its suitability in low assimilable nitrogen environments and fermentative safety, Lalvin QA23 exhibits a medium lag phase, rapid fermentation, and fructophilic characteristics, thriving at temperatures ranging from 15 to 32 °C. This yeast produces minimal sulfites and requires minimal oxygen. On the other hand, *L. thermotolerans*

L3.1 demonstrates a moderate lag phase and a low alcohol tolerance (<10%v/v). Its optimal fermentation temperature falls within the range of 14°C to 18°C, yielding high glycerol, low volatile acidity, and high NFA requirements.

For the second test, 7VA *Saccharomyces cerevisiae* and L3.1 *Lachancea thermotolerans* both grown in YPD agar medium were used for the fermentation trial. Both strains are part of the cryogenic yeast collection of the Food Technology Laboratory in the Escuela Técnica Superior de Ingenieria Agronomica (ETSIAAB).

Additionally, the effects of antioxidation capacity were examined using glutathione $GLUTASTAR^{TM}$, an additive aimed at preventing wine oxidation. $GLUTASTAR^{TM}$ is intended to be added to grapes or must prior to fermentation, enhancing aromatic expression, freshness, and preserving thiols and esters. Developed through collaborative research with the Institut Universitaire de la Vigne et du Vin de Dijon (France), $GLUTASTAR^{TM}$ undergoes an optimized production process to maximize the release of reduced glutathione and nucleotides with potent free radical scavenging properties.

Finally, the study also involved the use of metabisulfites as an additive. Metabisulfites are commonly employed in winemaking for their antimicrobial and antioxidant properties. They serve to inhibit unwanted microbial growth and prevent oxidation during the winemaking process. Potassium metabisulfite ($K_2S_2O_5$) as a source of SO_2 in these experiments (Sigma-Aldrich, Barcelona).

3.2. Methods

Saccharomyces cerevisiae was used for few of the fermentations, and *Lachancea thermotolerans* was also used, fermented in succession with *Saccharomyces cerevisiae*. O-lev-GSH was employed at two different doses (200 and 400 mg/L), in comparison to an untreated control and another that received a 50 mg/L dose of SO_2.

Every trial was run in triplicate, and various analyses were done on a regular basis or at the conclusion of the fermentation process (Table 1).

Table 1. Experimental setup for the oxidation test

Yeast (x3)	Added antioxidants	Analysis					
		enzymatic	FOSS	pH	color	O_2	sensorial
Sc			R	X	R	R	X
Sc	SO_2 50mg/L	X	R	X	R	R	X
Sc	O-lev-GSH 200mg/L	X	R	X	R	R	X
Sc	O-lev-GSH 400mg/L	X	R	X	R	R	X
Lt -> Sc		X	R	X	R	R	X
Lt -> Sc	SO_2 50mg/L	X	R	X	R	R	X
Lt -> Sc	O-lev-GSH 200mg/L	X	R	X	R	R	X
Lt -> Sc	O-lev-GSH 400mg/L	X	R	X	R	R	X

X: analysis at the beginning/end of the fermentation; R: regular analysis

3.2.1. Sample preparation

Test 1

As illustrated in Table 1 the aim was to achieve a yeast quantity of 50mg per bottle (200mL), resulting in a total of 600mg for 12 bottles.

Two little beakers were used: one for *S. cerevisiae* and another for *L. thermotolerans*, both being treated with the same process.
Considering that weighing 600mg is a minute amount, a practical approach is adopted. Specifically, 1g of each yeast is weighed and reconstituted by combining it with 9mL of water and 1mL of must, to prevent osmotic shock. This process results in the creation of a solution comprising 10mL, containing 1g of yeast.
To ensure that each bottle receives the targeted 50mg of yeast, the following formula is employed to calculate the requisite quantity (in mL) for each bottle:

$$x = \frac{10mL \ x \ 0{,}05g}{1g} = 0{,}5\text{mL} = 500\mu\text{L}$$

Therefore, the inoculation method was the following, repeated twelve times for each type of yeast (Figure 6).

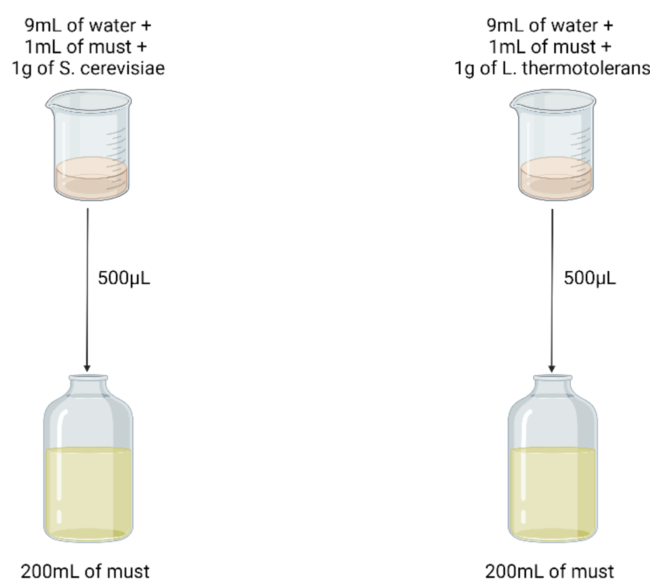

Figure 6. Inoculation procedure in test 1

Following the preparation of the must with the respective yeasts, the subsequent step involved the addition of sulfites and glutathione. This phase necessitated the fulfillment of three distinct conditions:

1. Six bottles required the incorporation of 10mg of sulfites each (5mg/L).
2. Another six bottles mandated the inclusion of 40 mg of O-lev-GSH for bottle (200mg/L).
3. Additionally, six bottles were designated to receive a dosage of 80mg of glutathione each (400mg/L).

As for sulfites, 100mg in the form of potassium metabisulphite were dissolved in 10mL of water. In this way, 1mL of dissolution was the amount that had to be included to ensure that there were 10mg of sulfites in each 200ml must bottle.

While for glutathione there was a need to create two dissolutions of 100mL of must each: the first one containing 2g of O-lev-GSH and the second one containing 4g.
To create the 6 bottles containing a dosage of 200mg/L of O-lev-GSH, 2mL of the first dissolution have been added, plus 2mL of must.

Regarding the 6 bottles containing a dosage of 400mg/L of O-lev-GSH, 4mL of the second dissolution has been added.

Figure 7: Inoculation procedure 1

Test 2

For the second trial, plates with cryopreserved yeasts were prepared. These yeasts were preserved with cryoprotectants so that they do not develop genetic changes, as it cannot reproduce.

The first step consisted in the preparation of six YPD medium of 100mL (three bottles for *S. cerevisiae* and three bottles for *L. thermotolerans*). The following substances were needed:

- 2% or 2g glucose (20g/L) (Sigma-Aldrich, Barcelona)
- 2% or 2g peptone (20g/L) (Condalab, Madrid)
- 1% or 1g of yeast extract
- Distilled water

The YPD preparations were then autoclaved for 15 minutes at 120°C.

Vials of YPD medium were then inoculated with one colony each under sterile conditions.
After 48h at 20°C, the culture pass was performed: 20mL were taken from each inoculated vial and inserted into new YPD vials of 200mL.
It was observed under an optical microscope that the population from each of the two yeasts strains was above 4log CFU/ml (Table 2).

Table 2. Population observed for strains in test 2

Yeast	Population
S. cerevisiae – 7VA	$3{,}5x10^6$ UFC/mL
L. thermotolerans – L3.1	$1{,}5x10^4$ UFC/mL

After 72h at 20°C, it was possible to move on to must inoculation. The following method was repeated twelve times for each type of yeast (Figure 8).

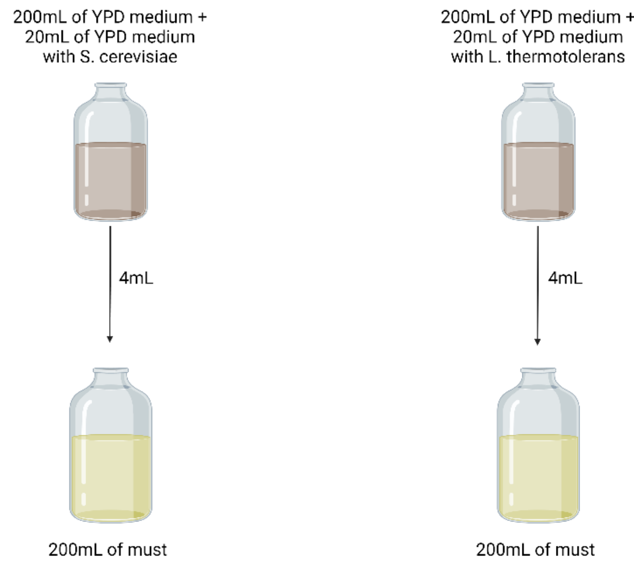

Figure 8. Inoculation procedure in test 2

As in Test 1, the next step is the addition of sulphites or glutathione.
As for sulphites, a dissolution of 100mL of must containing 200mg of metabisulphites was prepared, of which 10mL was inoculated for each bottle (three for *S. cerevisiae* and three for *L. thermotolerans*) to obtain a final concentration of 50mg/L of metabisulphites.
Meanwhile, we prepare a dissolution of 200mL of must with 1,6g of glutathione; 5mL were inoculated in each vial with a desired final concentration of 200 mg/L and 10 mL for the ones with a desired final concentration of 400 mg/L.

3.2.2 General enological parameters – FTIR (foss)

The FTIR technique (Fourier Transform Infrared Spectroscopy) was used to assess oenological parameters thanks to an $OenoFoss^{TM}$ device. Measurements of the wines' ethanol, glucose, fructose, pH volatile acidity, and Total Polyphenol Index (TPI) were made periodically.
For this study, one milliliter of the material was needed, and once the analysis was finished, it was used to measure the color.
While sugars and acids were represented as g/L, the ethanol content was expressed as % v/v. To eliminate measurement errors and degas CO_2, all samples were combined using a vortex mixer prior to measurement.

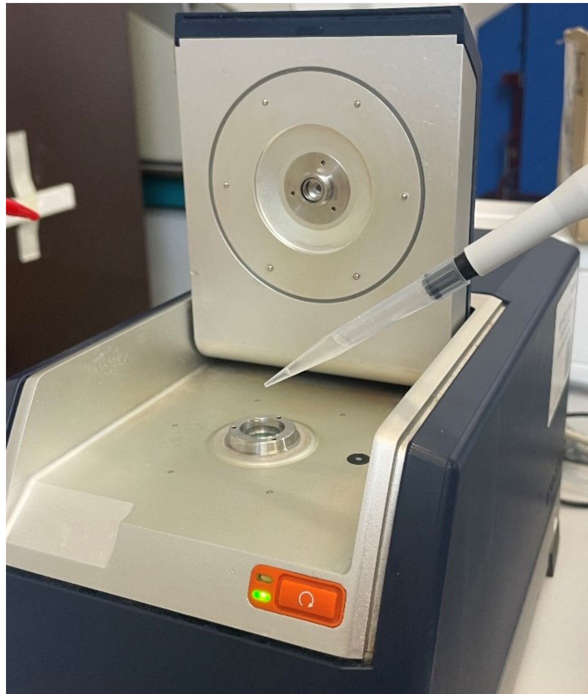

Figure 9: $OenoFoss^{TM}$ device

3.2.3 Enzymatic analyzer for PAN determination (Amino Nitrogen)

An automatic enzymatic analyzer Y25 from the Biosystem company was used to determine lactic acid through the determination of NADH produced after the reaction catalyzed with L-lactate dehydrogenase, from which L-lactic acid produces pyruvate. Lactic acid was expressed as g/L.
This analyzer was made especially for the laboratory of oenology. It's an open analyzer that adjusts to the many sample kinds and output levels needed by the industry.

Pre-programmed methodologies and specialized reagents reduce user intervention, making the work of the oenologist easier from the start.

3.2.4 pH measurements

The pH of each sample was measured with a Professional Bench Meter XS Instruments.

3.2.5 Reduction and oxidation potential analysis

To measure the redox potential we have used the Hanna HI98120 ORP/REDOX meter which is supplied with the HI73120 ORP electrode, HI73128 electrode removal tool, batteries and instructions, and waterproof temperature.

3.2.6 Dissolved O_2 measurement

Dissolved oxygen has been measured with an oximeter HANNA HI98198 (Hanna instruments, Rhode Island, United States). Once the sensor was inserted, one waited about one minute for a constant value to stabilize.

3.2.7 Color determination – spectrophotometry

A DNA Phone Smart Analysis device was used to analyze the color of the wine. A cuvette with a 1 mm route length and a photodiode array detector are used in the spectrophotometer. Three absorbance measurements were made in the discrete wavelength mode: 420, 520, and 620 nm. By adding up the absorbance values at these three wavelengths and multiplying the result by ten, the color intensity was determined.

By dividing the absorbance at 420 nm by the absorbance at 520 nm, the hue of the color was found. Using CIELab coordinates, the DNA Phone produced values for the brightness (L), green-red (a), and blue-yellow (b) components, providing a color representation. Additionally, hue (h) and chroma (C) were compared using CIELChuv cylindrical coordinates.

3.2.8 Total polyphenolic index -TPI -Uv – vis

The total polyphenol index (TPI) was determined using an Expectrophotometer Agilent 8453 UV-visible System (Agilent Technologies, California, United States). A spectrophotometer was used to obtain the IPT at a wavelength of 280nm. Quartz cuvettes of 1 cm were used.

3.2.9 Sensorial

A sensory analysis was carried out with 8 untrained tasters, ages 20 to 50, four men and four women. The following parameters were evaluated: intensity, hue, limpidity, aromatic quality, herbal, floral, fruity, reduced, and oxidized scents, body, astringency, bitterness, acidity, and overall perception and scored from 1 to 5.

3.2.10 Data processing and statistical analysis

The study’s results were processed utilizing Statgraphics 19 (Statgraphics Technologies Inc., Virginia, USA).

Utilizing the multiple range test, an analysis of variance (ANOVA) was used to compare each parameter and their differences. The significance criterion of $p < 0.05$ was applied.

4. Results and discussion

4.1 Evaluation of the antioxidant effect of glutathione

4.2.2 Physico-chemical characterization of Verdejo must

The following analyses were carried out before any treatment with the aim of studying the characteristics of the must.

Table 3: Analysis of Verdejo must before any treatment

<table>
<tr><td>Parameter</td><td colspan="6">Result</td></tr>
<tr><td>Baumé (°Bé)</td><td colspan="6">34,98</td></tr>
<tr><td>pH</td><td colspan="6">3,6</td></tr>
<tr><td>N. Amino (mg/L)</td><td colspan="6">250,2</td></tr>
<tr><td>A. Glucon. (g/L)</td><td colspan="6">0,7</td></tr>
<tr><td>A. tart. (g/L)</td><td colspan="6">3,9</td></tr>
<tr><td>FOSS Aci. Total TH2 (g/L)</td><td colspan="6">5,13</td></tr>
<tr><td>FOSS Aci. Volatil (g/L)</td><td colspan="6">0,1</td></tr>
<tr><td>FOSS N. Amoinia (mg/L)</td><td colspan="6">0,0</td></tr>
<tr><td>FOSS A. malic (g/L)</td><td colspan="6">3,4</td></tr>
<tr><td>FOSS Az. Reduct. Total (g/L)</td><td colspan="6">202,8</td></tr>
<tr><td>PAN (Amino Nitrogen)</td><td colspan="6">OUT</td></tr>
<tr><td>pH</td><td colspan="6">3,48</td></tr>
<tr><td rowspan="2">Reduction and oxidation potential</td><td colspan="3">T °C</td><td colspan="3">mV</td></tr>
<tr><td colspan="3">18,9</td><td colspan="3">256</td></tr>
<tr><td rowspan="2">O_2</td><td colspan="2">T °C</td><td colspan="2">%sat</td><td colspan="2">Mg/L</td></tr>
<tr><td colspan="2">19,3</td><td colspan="2">69</td><td colspan="2">5,8</td></tr>
<tr><td>Refractometry (°Brix)</td><td colspan="6">20</td></tr>
<tr><td>Total Polyphenol Index (TPI) (Abs 280nm)</td><td colspan="6">1,282</td></tr>
</table>

4.2.3 General enological parameters

FTIR analysis provided insights throughout the fermentation process into various enological parameters including ethanol content, glucose, fructose, pH, volatile acidity, and Total Polyphenol Index (TPI), using an OenoFoss instrument (FOSS Iberia, Barcelona, Spain), a Fourier transform infrared spectrophotometer (FTIR).
Monitoring enological parameters using FTIR enabled real-time assessment of fermentation progress and metabolic changes. Changes in ethanol concentration, sugar consumption, and acidity levels reflect the metabolic activity of yeast strains and the influence of antioxidant treatments on wine composition.

Table 4: General oenological parameters. Values in the same column with the same letter do not show significant differences (p>0.05).

Strategy	Ethanol (%vol)	pH	Reducing Sugars Tot (g/L)	Malic Acid (g/L)	Volatile Acidity (g/L)
S. cerevisiae	$12{,}21 \pm 0{,}04^{cd}$	$3{,}49 \pm 0{,}00^{b}$	$1{,}43 \pm 0{,}09^{a}$	$2{,}03 \pm 0{,}03^{b}$	$0{,}36 \pm 0{,}03^{a}$
S. cerevisiae + SO_2	$12{,}08 \pm 0{,}11^{bcd}$	$3{,}55 \pm 0{,}01^{b}$	$1{,}93 \pm 0{,}32^{a}$	$2{,}30 \pm 0{,}06^{bc}$	$0{,}27 \pm 0{,}05^{a}$
S. cerevisiae + 200 mg/L lev-GSH	$12{,}10 \pm 0{,}14^{bcd}$	$3{,}50 \pm 0{,}02^{b}$	$1{,}50 \pm 0{,}10^{a}$	$2{,}10 \pm 0{,}06^{b}$	$0{,}28 \pm 0{,}05^{a}$
S. cerevisiae + 400 mg/L lev-GSH	$12{,}32 \pm 0{,}03^{d}$	$3{,}49 \pm 0{,}01^{b}$	$1{,}27 \pm 0{,}12^{a}$	$2{,}10 \pm 0{,}06^{b}$	$0{,}38 \pm 0{,}02^{a}$
L. thermotolerans	$11{,}6 \pm 0{,}17^{abc}$	$3{,}45 \pm 0{,}02^{b}$	$3{,}77 \pm 1{,}92^{a}$	$2{,}17 \pm 0{,}12^{bc}$	$0{,}44 \pm 0{,}03^{ab}$
L thermotolerans + SO_2	$11{,}56 \pm 0{,}14^{ab}$	$3{,}49 \pm 0{,}01^{b}$	$9{,}27 \pm 0{,}57^{b}$	$2{,}63 \pm 0{,}03^{c}$	$0{,}60 \pm 0{,}04^{b}$
L. thermotolerans + 200 mg/L lev-GSH	$11{,}58 \pm 0{,}16^{ab}$	$3{,}28 \pm 0{,}05^{a}$	$3{,}83 \pm 1{,}00^{a}$	$1{,}30 \pm 0{,}25^{a}$	$0{,}40 \pm 0{,}02^{a}$
L. thermotolerans + 400 mg/L lev-GSH	$11{,}28 \pm 0{,}08^{a}$	$3{,}24 \pm 0{,}02^{a}$	$2{,}33 \pm 0{,}79^{a}$	$1{,}13 \pm 0{,}07^{a}$	$0{,}33 \pm 0{,}04^{a}$

The investigation sheds light on the effects of glutathione (GSH) supplementation and the metabolic behavior of *Saccharomyces cerevisiae* and *Lachancea thermotolerans*.

First off, under various settings, *S. cerevisiae* maintained a pretty consistent level of ethanol production—a critical component of fermentation—with values ranging from 12.08% to 12.32% alcohol by volume. On the other hand, *L. thermotolerans* showed somewhat lower ethanol levels (11.28% to 11.6%) in comparison to *S. cerevisiae*.
This phenomenon may be explained by multiple elements that are intrinsic to the metabolic processes and physiological traits of individual yeast species. During alcoholic fermentation, *L. thermotolerans* can transform part of the fermentable sugars (glucose + fructose) into L-lactic

acid at the expense of ethanol, whereas *S. cerevisiae* has highly active and efficient glycolytic and fermentation pathways that enable it to convert sugars rapidly into ethanol and carbon dioxide.

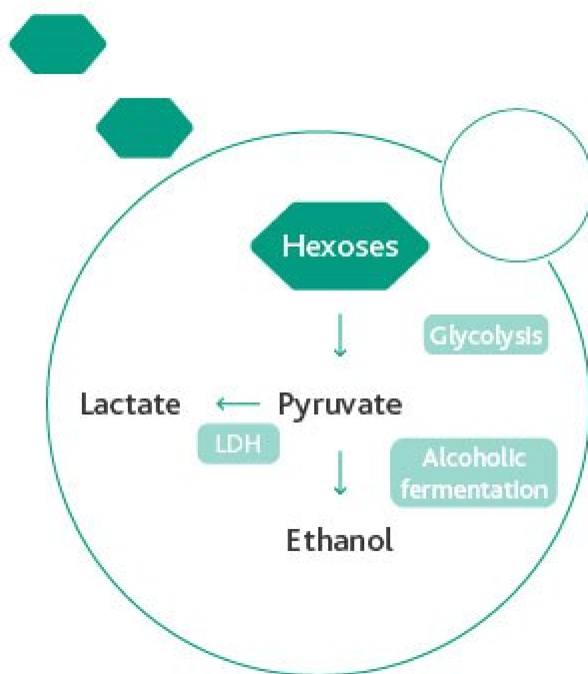

Figure 10: Production of L-lactic acid by *Lachancea thermotolerans* through the enzymatic activity of lactate dehydrogenase (LDH; Hranilovic et al.,2018).

It is interesting to note that when large concentrations of *lev-GSH* (400 mg/L) were added to *S. cerevisiae*, ethanol production significantly increased as compared to the control, but it significantly decreased in *L. thermotolerans*. This implies that, depending on the yeast strain employed, the impact of *lev-GSH* supplementation on ethanol production may change.

The reducing sugar content for *L. thermotolerans* alone (3.77 ± 1.92) was relatively low compared to when supplemented with SO_2 (9.27 ± 0.57). This significant increase in reducing sugar content with SO_2 supplementation suggests a potential impact on sugar metabolism. However, supplementation with *lev-GSH* did not lead to significant changes in reducing sugar content compared to the control for both 200 mg/L (3.83 ± 1.00) and 400 mg/L (2.33 ± 0.79) concentrations.

The analysis of reducing sugars revealed instead a notable distinction between the two yeast strains, confirming the different sugar utilization pattern. *S. cerevisiae* exhibited relatively low levels of reducing sugars, with minor decreases observed with *lev-GSH* supplementation. Conversely, *L. thermotolerans* consistently showed significantly higher levels of reducing sugars across all conditions, indicating that *L. thermotolerans* might have a higher capacity for sugar metabolism or a less efficient fermentation process, resulting in the accumulation of reducing sugars even with *lev-GSH* supplementation.

The pH values were relatively consistent within each yeast strain across different treatments, except for the significant decrease observed with *lev-GSH* supplementation in *L. thermotolerans*.

Additionally, while SO_2 addition showed a slight increase in pH in some cases, it did not lead to significant variations compared to the control.

Furthermore, malic acid content varied significantly between the two strains and in response to *lev-GSH* supplementation. *S. cerevisiae* typically metabolizes malic acid through the tricarboxylic acid (TCA) cycle, converting it into ethanol and carbon dioxide during fermentation. This metabolic pathway may contribute to its moderate production of malic acid compared to other yeast strains.
In contrast, *L. thermotolerans*, known for its ability to ferment at higher temperatures, may possess alternative metabolic pathways or regulatory mechanisms for malic acid metabolism. These pathways could lead to variations in malic acid production levels between yeast strains. Additionally, physiological differences between *S. cerevisiae* and *L. thermotolerans*, such as pH tolerance, may further influence malic acid metabolism. For instance, *S. cerevisiae*'s tolerance to low pH conditions may affect malic acid stability and metabolism during fermentation.

In addition, the wine obtained from *L. thermotolerans* exhibited higher malic acid levels, particularly pronounced in the presence of SO_2. However, the addition of *lev-GSH* led to a reduction in malic acid content, suggesting a regulatory effect on acid metabolism. *Lev-GSH*, acting as an antioxidant and regulator of cellular redox balance, may modulate enzymes involved in malic acid metabolism. This regulatory effect could lead to a decrease in malic acid production observed with *lev-GSH* supplementation. Additionally, *lev-GSH* supplementation may reduce oxidative stress in yeast cells, allowing them to allocate resources towards other metabolic pathways rather than malic acid production.

Lastly, volatile acidity, an important indicator of fermentation quality, showed slight variations between the strains. *S. cerevisiae* displayed relatively stable levels of volatile acidity across different conditions, while *L. thermotolerans* exhibited slightly higher levels.
SO_2 supplementation resulted in a significant increase in volatile acidity in both yeast strains, suggesting a potential impact on the production of volatile acids (e.g. acetic acid).
Lev-GSH supplementation did not lead to significant changes in volatile acidity in *S. cerevisiae*, but it resulted in slight decreases in volatile acidity in *L. thermotolerans*, although these changes were not statistically significant.

In summary, while both yeast strains showcased distinct metabolic profiles, the addition of *lev-GSH* had limited effects on most parameters. Exceptions were observed in reducing sugar levels in *S. cerevisiae* and malic acid content in *L. thermotolerans*, suggesting potential regulatory roles of *GSH* in specific metabolic pathways. These findings provide valuable insights into the dynamics of fermentation and the potential applications of *GSH* in improving product quality and process efficiency.

4.2.4 Dissolved oxygen and redox potential

Periodic measurements of dissolved oxygen content in fermenting juices and in finished wines

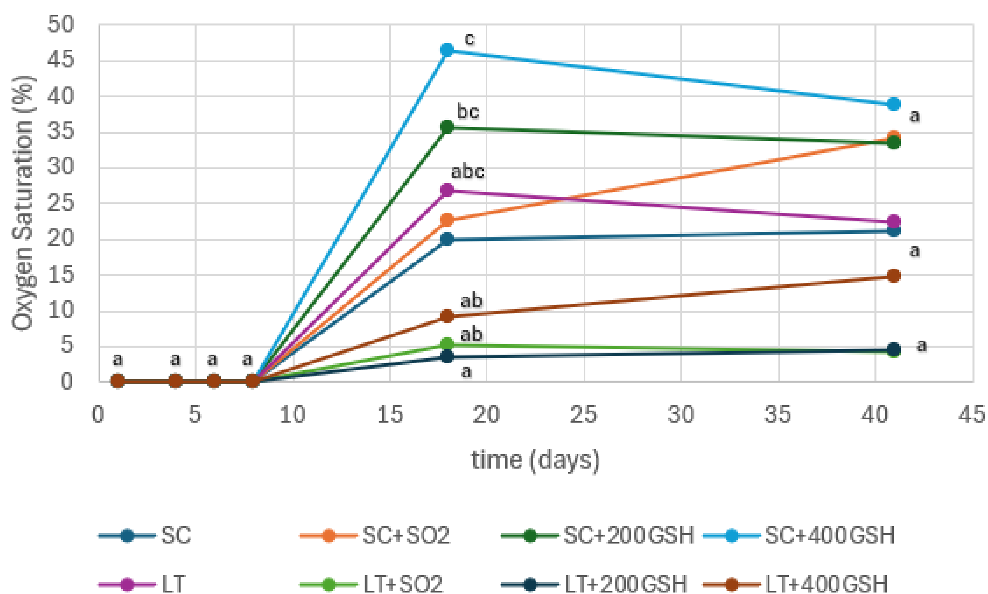

Figure 11: Evolution of oxygen saturation (%) during the fermentation process.

were performed with the aims of tracking the alcoholic fermentation in relation to the dissolved oxygen drop associated with it. When the final measurements were made 45 days following the yeast inoculation, they revealed that the wines produced with *Lachancea thermotolerans* contained less oxygen than wines produced with *S. cerevisiae,* indicating that *Lachancea thermotolerans* needed more oxygen to complete the fermentation than *S. cerevisiae*.

The observed differences in sugar utilization patterns between the two yeasts strains can be partly explained by the longer aerobic or "breathing" phase exhibited by *L. thermotolerans*. Several studies have highlighted that *L. thermotolerans* typically has an extended initial growth phase where it utilizes oxygen more efficiently before transitioning to anaerobic fermentation. This phase allows it to build up biomass and prepare for fermentation, which can lead to a slower but more sustained reduction of sugars. Research shows that *non-Saccharomyces* yeasts, including *L. thermotolerans*, often exhibit different metabolic behaviors compared to *S. cerevisiae*. For instance, *L. thermotolerans* can produce significant amounts of lactic acid, which affects the overall fermentation dynamics and can influence the duration and effectiveness of sugar consumption. This prolonged aerobic phase allows *L. thermotolerans* to metabolize sugars more steadily and potentially manage oxidative stress better, leading to less rapid but more efficient sugar utilization throughout the fermentation process (Shekhawat et al., 2018). Additionally, *L. thermotolerans'* ability to thrive in environments with lower oxygen levels and its different metabolic pathways contribute to its unique fermentation profile. The yeast's capacity to produce secondary metabolites and its distinct transcriptional responses during mixed-culture fermentations with *S. cerevisiae* further underline its different fermentation kinetics (Shekhawat et al., 2018).

These factors collectively suggest that *L. thermotolerans'* fermentation process is more gradual and prolonged, leading to the observed higher levels of reducing sugars in comparison to S. cerevisiae, which typically exhibits a quicker shift to anaerobic fermentation and rapid sugar consumption.

The sample inoculated with *L. thermotolerans* and added with 200 mg/L of glutathione-enriched yeast had the lowest oxygen concentration. Overall, nonetheless, no discernible antioxidant impact of glutathione-enriched yeasts was seen.
The data collected on oxygen levels during fermentation offer valuable insights into the effectiveness of the oxidation control strategies.

On day 18 of fermentation, *L. thermotolerans* + 200 *lev-GSH* and *L. thermotolerans* + SO_2 were the treatments showing the lowest oxygen levels, indicating they are highly effective at controlling oxidation. Their oxygen levels are 3.4 and 5.3, respectively, grouping them into the most effective category. *L. thermotolerans* + 400 *lev-GSH*, with an oxygen level of 9.2, was slightly less effective but still significantly better than the *S. cerevisiae* treatments. *S. cerevisiae* and *S. cerevisiae* + SO_2 show some control over oxidation but are not as effective as the *L. thermotolerans* treatments with antioxidants. *S. cerevisiae* + 200 *lev-GSH* and *S. cerevisiae* + 400 *lev-GSH* have the highest oxygen levels (35.6 and 46.4), indicating that higher doses of GSH with SC yeast are not as effective in controlling oxidation.

On day 41, *L. thermotolerans* + 200 *lev-GSH*, *L. thermotolerans* + 400 *lev-GSH*, and *L. thermotolerans* + SO_2 continue to show low oxygen levels over time (11.3, 15.9, and 21.6, respectively), proving their long-term effectiveness in oxidation control.
S. cerevisiae and *S. cerevisiae* + SO_2 show higher oxygen levels (29.8 and 56.3), showing moderate effectiveness. Their ability to control oxidation diminishes over time compared to *L. thermotolerans* treatments. Despite being in the same statistical group as other treatments, *S. cerevisiae* + 200 *lev-GSH* and *S. cerevisiae* + 400 *lev-GSH* still show high oxygen levels (59.0 and 71.5), indicating they are less effective in controlling oxidation.
The use of *L. thermotolerans* yeast strain with antioxidants (*lev-GSH* and SO_2) consistently results in the lowest oxygen levels, demonstrating strong antioxidant activity and effective oxidation control both in the short term and long term. Numerous characteristics of the *L. thermotolerans* yeast strain and the function of antioxidants such SO_2 and glutathione (GSH) are linked to the observed efficacy of *L. thermotolerans*. The oxygen uptake rate of *L. thermotolerans* is lower than that of the *S. cerevisiae* yeast strain. As a result, *L. thermotolerans* consumes oxygen more gradually during fermentation, lowering the total amount of oxidative stress. In contrast, *S. cerevisiae* yeast has a higher oxygen uptake rate, which can lead to increased oxidation of fermenting must and, consequently, higher oxygen levels.
The *S. cerevisiae* yeast strain with SO_2 shows some oxidation control but is less effective than *L. thermotolerans* treatments. Its effectiveness decreases over time, as evidenced by higher oxygen levels by day 41.

The data clearly indicate that the *L. thermotolerans* yeast strain combined with antioxidants (SO_2, 200 *lev-GSH*, and 400 *lev-GSH*) is the most effective strategy for managing oxidation during fermentation, thus confirming the object of this study. These treatments maintain low oxygen levels consistently, ensuring better preservation of wine quality.
On the contrary, the *S. cerevisiae* yeast strain, particularly with higher doses of glutathione (GSH), shows less effective oxidation control, making it a less favorable option for winemaking.

4.2.5 Sensory analysis

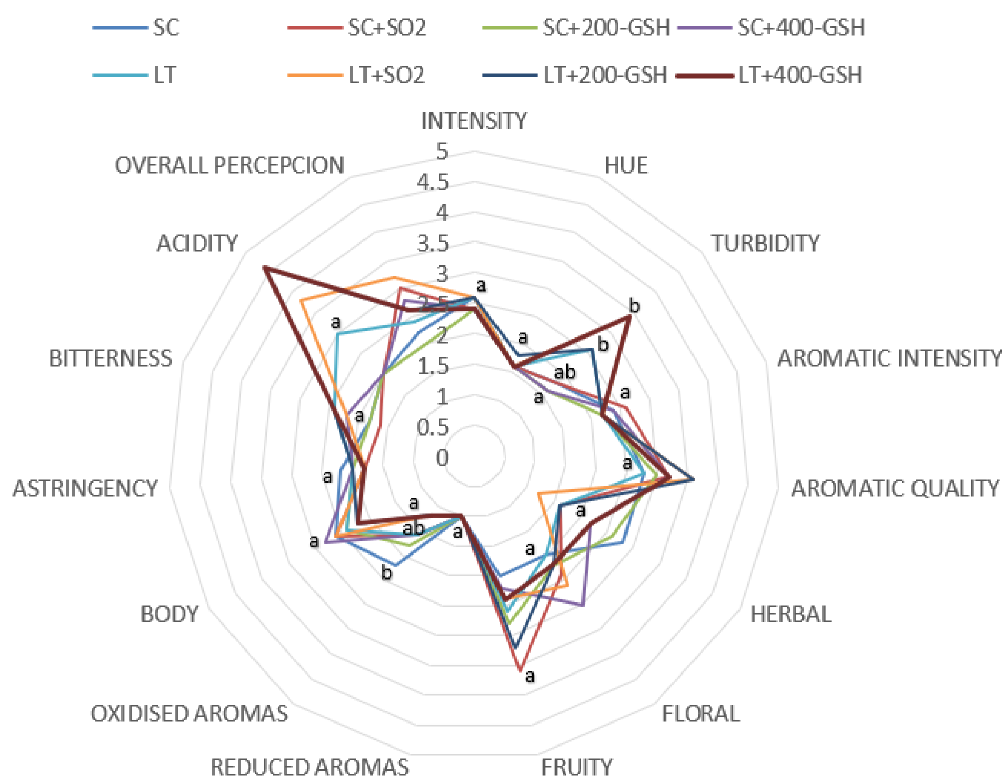

Figure 12: Radar chart of the sensory analysis' results

A sensory study was conducted, assessing factors related to taste, smell, and vision. The intensity, hue, limpidity, aromatic quality, herbal, floral, fruity, reduced, and oxidized scents, body, astringency, bitterness, acidity, and overall perception were the factors that were analyzed. They belonged to the employees of the Department of Food Chemistry and Technology (ETSIAAB).

Each sample's data was averaged across multiple judges to provide a comprehensive understanding of their sensory characteristics. Below is an interpretation of the findings and their potential implications.

Overall, *Saccharomyces cerevisiae* demonstrated a balanced profile with moderate intensity and aromatic qualities with minimal oxidized aromas, indicating effective control of oxidation. The balance in body, astringency, and acidity contributed to a pleasant overall perception, suggesting that the winemaking process successfully preserved freshness. Like *S. cerevisiae*, *S. cerevisiae* + SO_2 exhibited minimal oxidized aromas, suggesting good freshness. Its slightly higher aromatic intensity and fruity notes indicate a vibrant wine, potentially benefiting from protective measures against oxidation, such as the use of SO_2 or other antioxidants.

S. cerevisiae + 200 *lev-GSH* had a more complex aromatic profile with pronounced herbal notes but maintained minimal oxidized aromas. The presence of herbal and floral notes, along with good freshness indicators, suggests effective oxidation management.

S. cerevisiae + 400 *lev-GSH* showed consistent intensity and slightly higher turbidity, but with low oxidized aromas. This indicates that despite a fuller body, the wine maintained its freshness, likely due to effective antioxidant use during production.

Lachancea thermotolerans exhibited higher turbidity and fruit-forward notes with minimal oxidized aromas. The complexity and freshness of this wine suggest that oxidation was well-controlled, enhancing the fruit characteristics without compromising the wine's integrity. This sample may attract those who enjoy complexity in their wines. With the highest turbidity and

aromatic quality, *L. thermotolerans* + SO_2 also had minimal oxidized aromas, indicating excellent freshness. The pronounced floral and fruity notes suggest effective antioxidant measures, preserving the wine's vibrant profile. *L. thermotolerans* + 200 *lev-GSH* and *L. thermotolerans* + 200 *lev-GSH* both displayed consistent profiles with balanced herbal, floral, and fruity notes, minimal reduced and oxidized aromas, and refreshing finishes. These samples are likely to appeal to consumers who enjoy well-balanced, aromatic wines with a refreshing finish.

The sensory analysis focused on oxidation revealed that all samples maintained minimal oxidized aromas, indicating successful management of oxidation across different winemaking strategies. The use of SO_2 and glutathione (GSH) likely played a crucial role in preserving the wines' freshness and aromatic integrity. Samples with higher aromatic intensity and fruity notes, such as *S. cerevisiae* + SO_2 and *L. thermotolerans* + SO_2, particularly benefited from effective oxidation control, highlighting the importance of antioxidants in maintaining wine quality.
In general, *L. thermotolerans* was perceived to be more acidic although it was not statistically significant.
Overall, the balanced profiles and minimal oxidized aromas across all samples underscore the effectiveness of the strategies used to mitigate oxidation, ensuring a high-quality sensory experience.

4.2.6 Color determination

Table 5: Colors parameters obtained with a DNA Phone Smart Analysis device

Strategy	Color intensity (absorbance units)	Hue (dimensionles s)	Chroma	Hue (*)	L	a	b
S. cerevisiae	$0{,}14 \pm 0{,}03^a$	$4{,}10 \pm 0{,}91^a$	$5{,}60 \pm 0{,}42^a$	$105{,}00 \pm 1{,}30^a$	$98{,}00 \pm 1{,}00^a$	$-1{,}44 \pm 0{,}04^a$	$5{,}40 \pm 0{,}40^a$
S. cerevisiae + SO_2	$0{,}14 \pm 0{,}00^a$	$2{,}80 \pm 0{,}40^a$	$5{,}10 \pm 0{,}60^a$	$103{,}00 \pm 5{,}00^a$	$98{,}60 \pm 0{,}30^a$	$-1{,}00 \pm 0{,}30^a$	$5{,}00 \pm 0{,}70^a$
S. cerevisiae + 200 mg/L lev-GSH	$0{,}18 \pm 0{,}01^a$	$2{,}21 \pm 0{,}18^a$	$5{,}00 \pm 0{,}20^a$	$107{,}00 \pm 1{,}00^a$	$96{,}30 \pm 0{,}40^a$	$-1{,}42 \pm 0{,}07^a$	$4{,}70 \pm 0{,}20^{ab}$
S. cerevisiae + 400 mg/L lev-GSH	$0{,}17 \pm 0{,}00^a$	$2{,}29 \pm 0{,}22^a$	$5{,}00 \pm 0{,}50^a$	$102{,}00 \pm 4{,}00^a$	$96{,}70 \pm 0{,}30^a$	$-1{,}00 \pm 0{,}20^a$	$4{,}90 \pm 0{,}50^a$
L. thermotolerans	$0{,}16 \pm 0{,}05^a$	$2{,}41 \pm 0{,}61^{ab}$	$4{,}20 \pm 0{,}60^a$	$99{,}00 \pm 4{,}00^a$	$96{,}50 \pm 1{,}40^a$	$-0{,}06 \pm 0{,}30^a$	$4{,}10 \pm 0{,}40^a$
L thermotolerans + SO_2	$0{,}19 \pm 0{,}03^b$	$2{,}00 \pm 0{,}33^b$	$4{,}20 \pm 0{,}60^a$	$100{,}00 \pm 4{,}00^a$	$96{,}0 \pm 1{,}00^a$	$-0{,}80 \pm 0{,}30^a$	$4{,}10 \pm 0{,}60^a$
L. thermotolerans + 200 mg/L lev-GSH	$0{,}22 \pm 0{,}02^a$	$1{,}56 \pm 0{,}07^a$	$3{,}80 \pm 0{,}50^a$	$98{,}00 \pm 4{,}00^a$	$94{,}80 \pm 0{,}50^a$	$-0{,}60 \pm 0{,}40^a$	$3{,}70 \pm 4{,}00^a$
L. thermotolerans + 400 mg/L lev-GSH	$0{,}17 \pm 0{,}01^a$	$2{,}54 \pm 0{,}18^a$	$5{,}40 \pm 0{,}60^a$	$99{,}00 \pm 4{,}00^a$	$97{,}00 \pm 0{,}40^a$	$-0{,}80 \pm 0{,}30^a$	$5{,}30 \pm 0{,}50^a$

Multiple parameters were measured to analyze the changes in wine color during fermentation: hue, chroma, CIELAB color values (L*, a*, b*), color intensity, and hue. To comprehend central tendency and variability across several treatments, the mean and standard deviation for each parameter were computed.

For color intensity, the mean values ranged from 0.139 to 0.217 absorbance units, with *L. thermotolerans* + 200 mg/L *lev-GSH* showing the highest mean color intensity (0.217 ± 0.023). This suggests that the addition of *lev-GSH* at 200 mg/L enhances the color intensity of the wine. ANOVA was performed to determine if there were significant differences in color intensity between treatments. The results of the ANOVA indicated significant differences ($p < 0.05$), suggesting that the treatments indeed affected the color intensity of the wine.

Similarly, for hue, chroma, and CIELAB color values, descriptive statistics were calculated, and bar charts were created to visualize the data. ANOVA was also conducted for these parameters to identify any significant differences between treatments.
The hue analysis showed that the values varied from 1.56 to 4.1, with S. cerevisiae exhibiting the highest hue (4.1 ± 0.9). The chroma values varied between 3.8 and 5.6, with S. cerevisiae exhibiting the greatest chroma of 5.6 ± 0.4.

With L* values ranging from 94.8 to 98.6, a* values from -1.44 to -0.06, and b* values from 3.7 to 5.4, the CIELAB color values revealed variances between treatments.
These values indicate differences in lightness, green-red, and blue-yellow components of the wine color.

In summary, the addition of *lev-GSH* and SO_2 had notable effects on the color parameters of the wine. *Lev-GSH* at 200 mg/L significantly enhanced color intensity, while other treatments showed variations in hue, chroma, and CIELAB values. These findings provide valuable insights into the impact of different treatments on wine color during fermentation, contributing to the optimization of winemaking processes for desired color characteristics.

5. Conclusion

Throughout this study, we explored the role of glutathione-enriched yeast as an antioxidant in winemaking, particularly its effects on the fermentation process, metabolic behavior, and wine quality when used with different yeast strains. The findings reveal nuanced interactions between GSH supplementation, yeast metabolism, and the overall characteristics of the resulting wines.

Firstly, we observed that *lev-GSH* supplementation influenced ethanol production differently depending on the yeast strain. In *Saccharomyces cerevisiae*, higher concentrations of *lev-GSH* (400 mg/L) resulted in a significant increase in ethanol production, whereas in *Lachancea thermotolerans*, the same concentration led to a decrease. This highlights the strain-specific responses to *lev-GSH*, suggesting that glutathione may interact with the metabolic pathways of different yeast strains in unique ways.

The impact of glutathione on reducing sugar levels was minimal for *S. cerevisiae*, whereas *L. thermotolerans* consistently exhibited higher levels of reducing sugars across all conditions. This indicates that *L. thermotolerans* has a less efficient fermentation process, which could be due to its prolonged aerobic phase and different metabolic pathways compared to *S. cerevisiae*. Additionally, *lev-GSH* supplementation in *L. thermotolerans* led to a reduction in malic acid content, suggesting a regulatory effect on acid metabolism, possibly by modulating enzymes involved in this pathway.

The dissolved oxygen and redox potential measurements provided further insights into the effectiveness of GSH as an antioxidant. *L. thermotolerans*, particularly when supplemented with *lev-GSH* or SO_2, consistently exhibited lower oxygen levels, indicating strong antioxidant activity and effective oxidation control. In contrast, *S. cerevisiae* treatments, especially those with higher doses of *lev-GSH*, were less effective in controlling oxidation. This suggests that *L. thermotolerans*, combined with antioxidants like glutathione, could be a more effective strategy for managing oxidation during fermentation.

The sensory analysis reinforced these findings, as wines made with *L. thermotolerans* and supplemented with *lev-GSH* or SO_2 demonstrated higher aromatic quality, complexity, and minimal oxidized aromas. This indicates that the antioxidant properties of glutathione, along with the characteristics of *L. thermotolerans*, contribute to preserving the freshness and enhancing the sensory profile of the wine.

The color determination research revealed that lev-GSH supplementation increased wine color intensity, especially at 200 mg/L in *L. thermotolerans*. This is undesirable for white wines like Verdejo since it may be a sign of oxidation. The intricate interactions between glutathione and other wine constituents during fermentation may be the cause of this unanticipated rise in color intensity. Glutathione interacts with phenolic chemicals and other products of oxidation, which may result in increased color intensity. Furthermore, compared to other yeast strains, *L. thermotolerans* may affect these interactions in a different way, which could explain the observed outcomes. There were also variations in hue, chroma, and CIELAB values; the maximum hue and chroma were found in S. cerevisiae. These results emphasize how critical it is to optimize winemaking procedures by strategically using antioxidants such as lev-GSH and SO_2 to avert unwanted color changes and possible oxidation, thereby maintaining the intended color characteristics of white wines such as Verdejo.

In conclusion, the study demonstrates that glutathione enriched yeasts can serve as an effective antioxidant in winemaking, with its impact varying significantly depending on the yeast strain used. While glutathione supplementation in *S. cerevisiae* showed limited benefits, its use in *L. thermotolerans* proved to be more advantageous, leading to better oxidation control, improved sensory attributes, and enhanced color intensity. Future research could further investigate the molecular mechanisms underlying these strain-specific responses to glutathione, optimize glutathione dosages, and explore its use in combination with other antioxidants to enhance wine quality. Additionally, exploring the potential of other *non-Saccharomyces* yeasts in conjunction with GSH could provide broader insights into innovative winemaking practices.

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