Chapter 9

# Microbiological, Physical, and Chemical Procedures to Elaborate High-Quality $SO_2$-Free Wines

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**Abstract**

Sulfur dioxide ($SO_2$) is the most preservative used in the wine industry and has been widely applied, as antioxidant and antibacterial agent. However, the use of sulfur dioxide implicates a range of adverse clinical effects. Therefore, the replacement of the $SO_2$ content in wines is one of the most important challenges for scientist and winemakers. This book chapter gives an overview regarding different microbiological, physical, and chemical alternatives to elaborate high-quality $SO_2$-free wines. In the present chapter, original research articles as well as review articles and results obtained by the research group of the Wine Technology Center (VITEC) are shown. This study provides useful information related to this novel and healthy type of wines, highlighting the development of winemaking strategies and procedures.

**Keywords:** food safety, grape juice, sensory analysis, sulfur dioxide, wine

## 1. Introduction

In the last decades, the use of the sulfur dioxide ($SO_2$) has become indispensable in the food industry. This substance is widely applied as antioxidant and antibacterial in many processed foods, being the most preservative used in the wine industry. In wines, $SO_2$ prevents undesirable sensory properties and the spoilage of wines produced by chemical or microbiological agents. However, in recent times, it has been shown that the intake of $SO_2$ implicates a wide range of adverse health consequences, such as allergic reactions and cumulative harmful effects [1]. Therefore, negative perceptions toward sulfites have been induced, and a significant increase on the demand of wines with low content of $SO_2$ has been displayed by consumers in the last years [2]. For this reason, reducing the amount of $SO_2$ in wines is a decisive strategy for the wine industry and one of the current topics on the oenological science.

In wines, $SO_2$ is composed by total $SO_2$, bound $SO_2$, free $SO_2$, and molecular $SO_2$. Proper adjustment of the $SO_2$ dosage is difficult because it depends on the equilibrium between its free and bound forms. The active form is molecular $SO_2$, which depends on the concentration of free $SO_2$ and the pH [3]. This active form has the antimicrobial and antioxidant properties. In terms of antimicrobial, an insufficient addition of $SO_2$ will not ensure the wine protection, increasing the risk of yeast and bacteria proliferation. In terms of antioxidant, an inadequate dosage will allow an excessive oxidation of aromas and flavors, compromising the quality of wines [4]. Contrary, excessive dosages in wines may cause organoleptic alterations and also health reactions in consumers. Taking this into account, the International Organization of Vine and Wine (OIV) has progressively reduced the maximum limits of the total $SO_2$ in wines, which is nowadays 150 mg/L for red wines and 200 mg/L for white wines, with some exceptions depending on the sugar content (Regulation (EC) No 607/2009).

Today, there is not a commercial product or recipe able to replace the widespread $SO_2$ actions. Consequently, diverse technological strategies should be considered by winemakers in each stage of the winemaking process, according to the type of wine to be produced and the winery capabilities. From our point of view, these strategies should be addressed from three joint perspectives; microbiological strategies, physical technologies, and chemical treatments. In this sense, the Wine Technology Centre (VITEC) has been working in this research field since 2012. Our studies have been focused in red and white wines, especially regarding Tempranillo and Albariño grape varieties.

## 2. Microbiological strategies to elaborate $SO_2$-free wines

From a microbiological point of view, many factors should be taken into account to reduce the quantity of $SO_2$ in wines. First, it should be considered that an endogenous content of $SO_2$ is naturally produced by yeasts during alcoholic fermentation. Second, grape juice composition, yeast nutrition, and fermentation management may strongly influence the ability of yeasts to produce sulfites. Finally, microbiological stability of the $SO_2$-free wines remains uncertain yet.

As mentioned above, the European Union regulates the levels of total sulfites in wines following the Regulation (EC) 607/2009. Therefore, wines must be labeled with the indication "contains sulfites," when the total content of $SO_2$ is over 10 mg/L, either exogenous or endogenous. Most organisms produce sulfites as a normal intermediate during digestion or synthesis of the sulfur-containing amino acids, such as methionine and cysteine [5]. Sulfites are minor by-products of yeast fermentation, and therefore, they are natural wine constituents. The ability of yeasts to form $SO_2$ has been reported in different types of wines and geographical areas, and it was known long time ago and investigated intensively over the years [6, 7].

One of the most important factors to elaborate $SO_2$-free wines is the choice of the suitable yeast strains used for the development of the alcoholic fermentation. During winemaking process,

sulfur (naturally available as sulfate in grape juice) is used by yeasts in the synthesis of amino acids. In particular, *Saccharomyces cerevisiae* produces sulfite as an intermediate product during the assimilatory reduction of sulfate to sulfide, via adenosine-5′-phosphosulfate [6, 8]. The available sulfide ($S^{2-}$) can be used in the synthesis of amino acids, as well as being excreted as hydrogen sulfide ($H_2S$). Eventually, the sulfur amino acid biosynthesis (SAAB) pathway plays a crucial role in the active transport of sulfate ($SO_4^{2-}$) into the cell, as well as in the reduction and production of $SO_2$ and in the resistance of yeasts against this additive [9]. Yeast strains differ in their capacity to form $SO_2$, estimating a total average content ranged from 0 to 115 mg/L [10–14]. Most strains of *S. cerevisiae* produce between 10 and 30 mg/L of total $SO_2$. However, some of them may produce less than 10 mg/L, which were commonly called "low sulfite-forming strains" [6]. On the opposite side, "high sulfite-forming strains" are able to produce more than 100 mg/L. These classifications according to their ability to form $SO_2$ during the alcoholic fermentation have been reported by several authors over the time [6, 7, 12, 14].

In the last years, the use of yeast strains with a low capacity to produce $SO_2$ has been one of the most used strategies to reduce the amount of $SO_2$ in wines [15]. Several studies have compared the amount of $SO_2$ produced during alcoholic fermentation by different commercial and indigenous yeast strains. In 1985, Suzzi et al. [13] investigated the biological sulfite role in the stabilization of white wines by comparing 1700 strains of *Saccharomyces* isolated from spontaneous fermentations. The majority of them produced less than 10 mg/L of total $SO_2$, around 350 produced between 10 and 20 mg/L, 52 strains produced between 20 and 40 mg/L, and just two strains produced more than 40 mg/L. More recently, an experiment carried out at industrial scale by Werner et al. [14] showed two distinguishable groups of yeasts, among 22 commercial strains. The first one produced under 10 mg/L of total $SO_2$, and the second one produced between 10 and 20 mg/L. Significant differences among yeasts strains in production of $SO_2$ (free and bound-$SO_2$) were also described by Wells and Osborne [7]. In this case, values ranged from 25 to 60 mg/L of bound-$SO_2$ were observed. In 2015, Miranda-Castilleja et al. [11] studied the production of total $SO_2$ of 52 indigenous species of *Saccharomyces* from Querétaro (Mexico), and the obtained results ranged from 37 to 115 mg/L. More recently, VITEC has investigated the natural production of $SO_2$ of 21 selected yeast strains (commercial and indigenous). Fermentations were conducted using Muscat grape juice at 18 and 25°C. These results showed a total $SO_2$ production lesser than 10 mg/L in all cases. The results in agreement with other works which also showed diverse yeast strains are able to produce small amounts of total $SO_2$ (<1.4 mg/L) [16, 17]. Thus, several commercial and indigenous yeast strains have proved to be able to produce $SO_2$-free wines. However, other considerations should be taking into account, such as the organoleptic properties and microbial stability of this type of wines.

The formation of $SO_2$ by yeasts is influenced by a complex interaction of genetic, physiochemical, and metabolic factors. $H_2S$ is one of the most undesirable metabolites derived from the alcoholic fermentations due to its unpleasant smell and taste. It should be noted that the biosynthesis and the production of $H_2S$ and $SO_2$ are linked [18, 19]. As occurs in the case of $SO_2$, the formation of $H_2S$ varies widely depend on the yeast strains [20, 21]. The release of $H_2S$

by yeast during the fermentation is a long-standing problem that has been extensively studied in comparison to the $SO_2$ production. There has been an ever-growing interest in wine yeasts with low production in $H_2S$. The selection of suitable strains has so far been the principal way of limiting excessive $H_2S$ formation. Other engineering strategies have been used for limiting its production, which generally consisted of overexpression or inactivation of some genes involved in the sulfate reduction pathway [22–24].

Both sulfites and hydrogen sulfides are produced during the biosynthesis of the sulfur containing amino acids, methionine, and cysteine, starting from sulfate assimilation. Given the metabolic link between $H_2S$ and $SO_2$, such kind of biotechnological and engineering strategies firstly applied to reduce $H_2S$ production could also be applied to decrease $SO_2$ formation by yeasts. Nonetheless, few works have been aimed to obtain both low $SO_2$ and low $H_2S$ production. Three strains with low $SO_2$ production ($SO_2$ < 10 mg/L) and with reduced $H_2S$ production were selected by De Vero et al [25]. These authors proposed a strategy that combines sexual recombination and specific selective pressure to generate nongenetically-modified *S. cerevisiae* with desired oenological characteristics. More recently, new insight into the regulation of sulfur metabolism in wine yeasts by the identification of variants of MET2 and SKP2 genes within SAAB has been reported to modulate the production of sulfites and sulfides [26]. These results provide novel targets for the improvement of wine yeast strains orientated to produce $SO_2$-free wines. This knowledge on the sulfate pathway provides a chance to successfully apply engineering strategies to select "low sulfite-forming" yeast strains. However, as we previously highlighted, the production of sulfites by yeast during fermentation not only depend on metabolic factors but also on the environment, including nutrients and fermentation management, among others. Hence, grape juices composition is an imperative factor that should be considered in order to elaborate this type of wines. The insoluble solids contained in the grape juice also appeared to have an effect on the $SO_2$ content, and wines with the higher insoluble solids obtained lower values of $SO_2$ [27]. In contrast, results obtained in our experimental cellar showed that grapes with higher content of soluble solids produced higher content of total $SO_2$ (**Figure 1**). The biplot of the principal component analysis (PCA) shows that the amount of $SO_2$ produced during the alcoholic fermentation is mainly favored by a high amount of sugars and a low quantity of nitrogen. Furthermore, musts fermented at low temperatures (18°C), and a low titratable acidity may contribute on the production of $SO_2$.

In addition, the supplementation of musts with amino acids can significantly affect $SO_2$ and $H_2S$ production depending on the amount added, the time of addition, and the nitrogen concentration [26, 28]. Individual amino acids such as methionine, cysteine, asparagine, and arginine have been shown to influence sulfite formation [18, 28]. Higher the concentration of methionine and cysteine in the grape must, lower the formation of $SO_2$ [6]. Under ammonia limitations, the addition of nonsulfur amino acids tended to increase the formation of $SO_2$ (but inhibits the formation of $H_2S$). The addition of cysteine seems to increase the $H_2S$ content but inhibits the sulfite formation, and the addition of methionine inhibits both $SO_2$ and $H_2S$ formation [28]. More recently, it was stated that methionine repressed the cysteine-induced increase in the $H_2S$ production but had no effect on the formation of $SO_2$. Both compounds were produced in greater quantities by yeast when grown in the presence of increasing concentrations of cysteine [18]. It has been reported that yeasts produce higher concentrations

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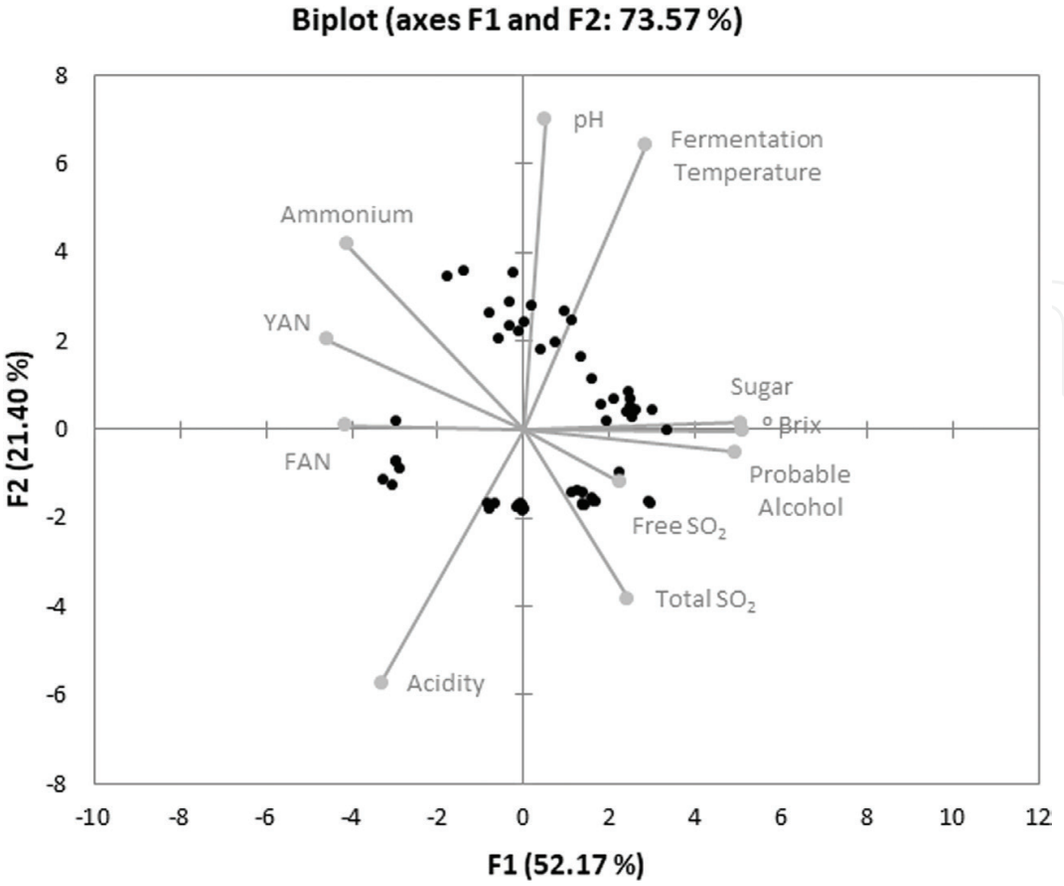


**Figure 1.** Biplot performed by 74 wines produced from Tempranillo and Albariño musts.

of $SO_2$ under higher yeast assimilable nitrogen (YAN) quantities [7, 29]. The supplementation on nitrogen using ammonium salts (sulfate or phosphate) allows higher growth rates and biomass yielding and also the stimulation of the fermentative activity [30, 31]. The addition of diammonium phosphate (DAP) significantly decreases $H_2S$ production and improves the kinetics of fermentation and aroma profile of wine [32]. In the last 5 years, VITEC has been studying the effect of ammonium sulfate and DAP addition on the amount of $SO_2$ produced by yeast along of the alcoholic fermentation. Results obtained showed that the addition of the N-sources slightly increases the total content of $SO_2$ in wines. The addition of ammonium sulfates and DAP using low sulfite-forming strains to ferment musts showed no significant differences. In the case of musts fermented by "high sulfite-forming" strains, the addition of DAP significantly increased the total content of $SO_2$ [33].

Other important consideration to elaborate $SO_2$-free wines is the management of the alcoholic fermentation. In this sense, it has been stated that temperature has several effects on biochemical and physiological properties in yeast cells. Some changes in the sulfur assimilation pathway by *S. cerevisiae* depending on temperature may occur [34]. Our results are in agreement with other authors, who reported that at low temperature, the $SO_2$ production increases [26]. $SO_2$ and $H_2S$ production is also affected by pH (acidic pH facilitate $SO_2$ uptake) and concentration of some minerals (copper and zinc) and vitamins,

such as pantothenate or thiamine [9, 26, 35]. Thiamine is a vitamin used as a co-enzyme in the alcoholic fermentation pathway. It stimulates yeast growth, speeds up fermentation, and reduces production of $SO_2$ binding compounds. Thiamine supplementation allows the transformation of pyruvic acid to acetaldehyde and limits the accumulation of ketonic compounds on wine being considered a factor to reduce the $SO_2$ amount on wines [36]. A deficiency in thiamine may reduce yeast growth, slow fermentation, and promote the accumulation of pyruvic acid and acetaldehyde, the components responsible of wine oxidation. The effect of major $SO_2$ binding compounds (acetaldehyde, pyruvic, and $\alpha$-ketoglutarate) on the production of $SO_2$ by different yeasts strains is still poorly understood, and more studies should be performed to better understand their role on the $SO_2$ production [7]. In this way, the results obtained in VITEC are in agreement with the results obtained by Comuzzo and Zironi [33, 36], who showed that the addition of DAP + thiamine reduced the production of $\alpha$-ketoglutarate.

## 3. Physical technologies to replace the use of $SO_2$ in the wine industry

From a physical point of view, different technologies have been used to ensure the wine microbiological stability and to prevent oxidations [37]. The main advantage of using physical methods is the nonaddition of chemical substances that may affect human health. By these technologies, the preservation of the organoleptic properties of wines and the antimicrobial effect should be produced at the same time. Pulsed electric fields (PEF), ultraviolet radiation (UV), high hydrostatic pressure (HHP), and flash-pasteurization lead an antimicrobial result, while the use of ultrasounds (US) or inert gases does not share this property [38–41]. The PEF consists in the application of short electric pulses of high intensity between two electrodes, producing electroporation of the cell membranes increasing their permeability. It has been shown that this technique is effective to inactivate both bacteria and yeasts [42]. Thus, PEF may be applied to eliminate undesirable microorganisms at different winemaking stages, for example, before bottling. It has been stated that the treatments with PEF also reduces the activity of enzymes, such as polyphenol oxidases and peroxidases, increases the extraction of phenolic compounds and affects the aromas of white wines [42, 43]. VITEC has evaluated the antimicrobial effect of PEF, HHP, US, and EMR (electromagnetic radiation). **Figure 2** shows the obtained results after the quantification of viable yeasts and acetic acid bacteria (AAB) in Petri dishes culture. The PEF conditions were electric field 35 kV/cm, voltage 23 kV, pulse rate 0.65 kHz, pulse duration 2.5 μS, initial conductivity 5.04 mS/cm, flow 25 l/h, and initial temperature 20.8°C. The PEF 1 and PEF 2 differed on the final temperature of the treatment which was 23 and 31°C, respectively. Worthy results of PEF as antimicrobial technique were obtained, although high colony-forming units of yeast were observed in the case of PEF 1.

The use of high hydrostatic pressures (HHP) was evaluated in our studies at different pressures (from 400 to 600 MPa) and times (1, 3, 5, and 10 min). HHP results showed that the inhibition of microorganism by this methodology depends not only on the time and pressure

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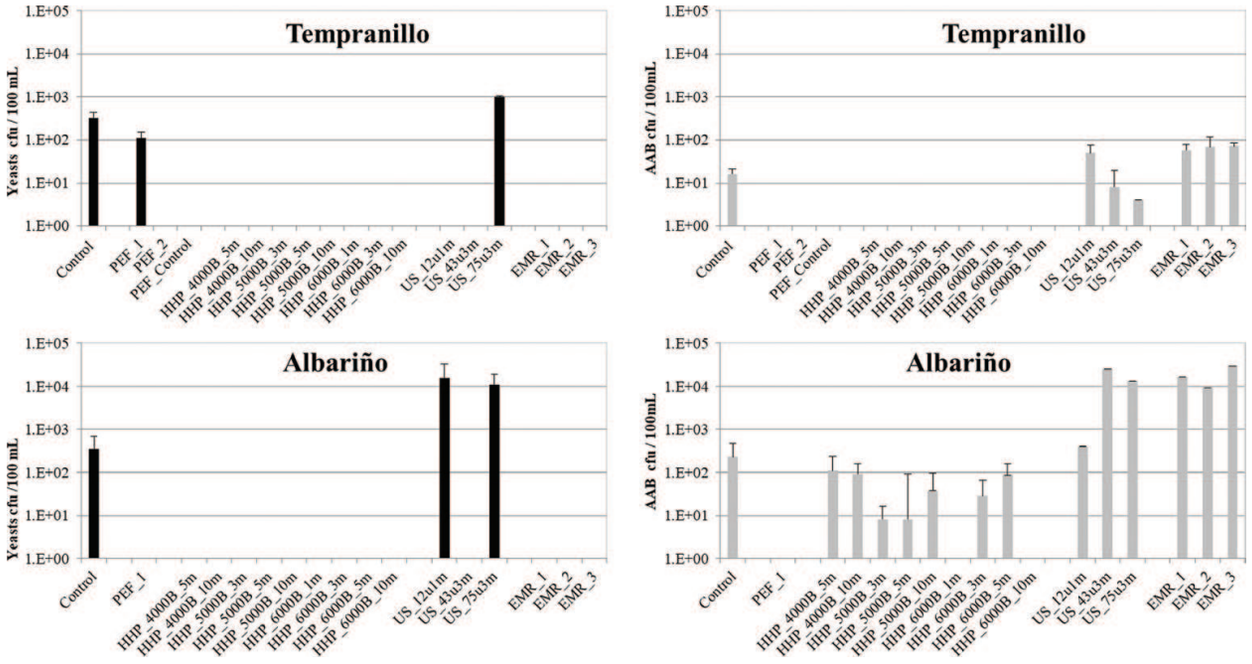


**Figure 2.** Evaluation of different physical treatments in Tempranillo and Albariño wines (at the end of the alcoholic fermentation) by the quantification of viable yeasts and acetic acid bacteria in Petri dishes culture (cfu, colony forming units).

applied but also on the variety and the type of microorganisms (**Figure 2**). Tempranillo and Albariño yeast growth were inhibited by all pressures and times applied. However, in the case of acetic acid bacteria, the HHP treatment was very efficient for Tempranillo but not for Albariño wines. Even so, low levels of viable AAB ($10^2$ cfu/100 mL) were found. According to Bartowsky et al. [44], AAB populations from either spoiled or unspoiled wines ranged between $10^2$ and $10^3$ cfu/mL. According to the literature, pressures above 700 MPa may inhibit the polyphenol oxidase, although lower values of pressure are enough to inactivate yeasts and bacteria [45]. In our experiments, HHP results as a very effective technique against yeast and lactic acid bacteria and a lesser extent against AAB. At the studied conditions, HPP and PEF showed a noteworthy preservation of the organoleptic properties of wines (data not shown), according to other authors [45–47].

Other techniques, such as ultrasounds (US) and EMR, were also evaluated. The EMR is one of the most recent physical technologies evaluated in wines, which has shown a good potential in food processing, such as fruits, vegetables, and juices. This technique allows increasing the wine temperature for a short time period without any external heating source. EMR allows achieving the reduction of microorganisms with low effect on the organoleptic properties of wines, when compared with other heating techniques, such as flash pasteurization. However, recently studies have shown that the application of lower power microwave exposures may increase the growth of *Brettanomyces* cells [48]. In agreement, **Figure 2** shows an increase on AAB after the treatment with EMR in both cases. The application of US at different conditions considering time of application (from 1 to 3 min)

and wavelengths (12, 43 and 75 μm) inhibited the yeasts growth but not the bacteria population (**Figure 2**). The effectiveness of US resulted lower than HHP, at least at the experimental conditions studied. As occurred with EMR treatment, an increase on the colony-forming units was observed after the treatment with US. Ultraviolet radiation reduces the population of wine microorganisms, but different resistances to the radiation have been stated depending on species. It appears to be an effective method against *Brettanomyces*, *Saccharomyces*, *Acetobacter*, *Lactobacillus*, and *Pediococcus* [46]. Furthermore, it has been described that phenolic compounds can absorb UV radiation and is therefore less effective in red wines. This technique seems to be more effective in white wines at the end of fermentation, when wines present low turbidity. In order to increase the total polyphenol, it could be also applied at maceration stage [38, 49].

In general, all the physical treatments assessed clearly affect the viability of lactic acid bacteria in Tempranillo and Albariño varieties. In both cases, only viable lactic acid bacteria were detected in the control (data not shown). The employed treatments reduced the viability of yeasts and lactic and acetic acid bacteria. However, in this study, both US and EMR were not effective enough to reduce the population of viable acetic acid bacteria. According to the results, AAB were more resistant to the treatments than lactic acid bacteria (LAB). Regarding techniques, a higher antimicrobial effect of HHP and EMR was observed in comparison to the other methodologies employed. Besides, some wines produced by US and EMR showed oxidation characteristics. As occurred in the antimicrobial assays, the optimization of methods and experimental conditions is an imperative action to avoid adverse effects on the sensory quality of wines. It should be noted that some of these physical techniques are commonly used in food industry, but their implementation on the wine sector is so far to be available for a daily work routine, mainly due to economic and technique questions.

The oxidation is one of the main processes that affect $SO_2$-free wines. Apart from the mentioned technologies and despite of its antimicrobial effect is limited, the use of inert gases is more and more applied throughout the winemaking process. The oxygen control by the management of the inert gases during the winemaking process must be considered because they have an important impact on the organoleptic properties. Caps are the ultimate physical barrier to preserve wines during storage, and so their oxygen permeability should be considered. The long-term protection is one of the most concerns for wineries in bottled wines with reduced $SO_2$ content [50]. The assays carried out in VITEC using argon and carbon dioxide showed valuable sensory results (**Figure 3**). The $SO_2$-free red wines produced by the use of Ar and $CO_2$ showed higher significant color intensity, tannic intensity, and dryness. Greater aroma intensity and mouthfeel were also found, although values did not show significant differences. In general, Tempranillo-bottled $SO_2$-free wines obtained higher global punctuations than wines with $SO_2$ addition.

The oxygen control during all the production process of this type of wines is an imperative engagement. It is important to take into account that wines without sulfite addition are exposed to physicochemical and microbiological alterations. Considering the techniques available in any winery, to avoid microbiological alterations, sterilizing filtration may be an alternative. However, this technique could reduce the sensorial quality of the wine

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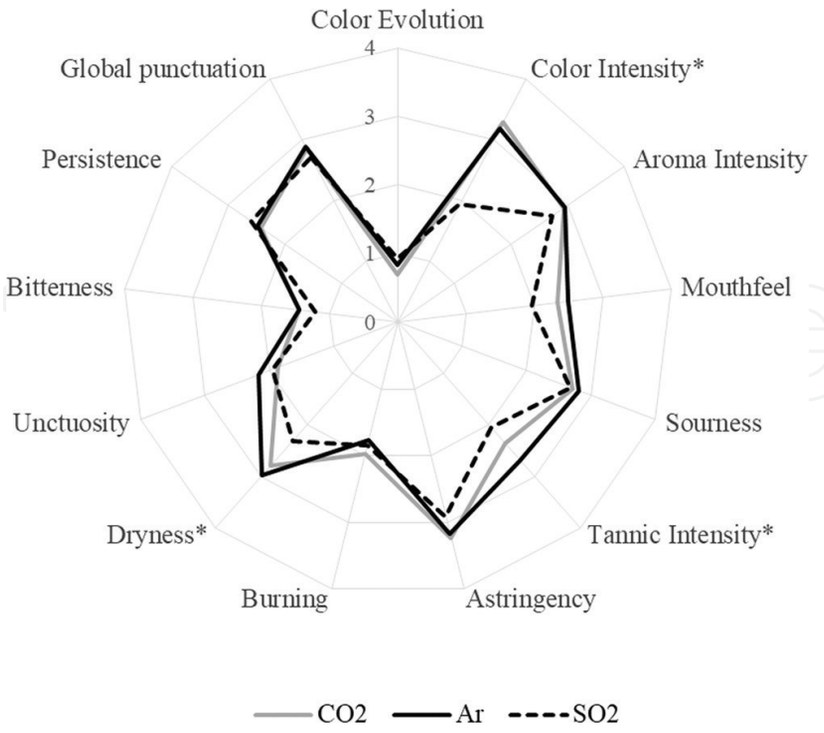


**Figure 3.** Comparison of the sensory evaluation of Tempranillo wines elaborated using argon (Ar), carbon dioxide ($CO_2$) and sulfur dioxide ($SO_2$). * Significant differences by HSD Tukey test ($p < 0.05$).

because it is a very oxidative process. To ensure a correct conservation of the $SO_2$-free wines, the amount of oxygen incorporated into wine should be controlled, especially at bottling, where concentrations from 0.2 to 4 mg/L may be incorporated, depending on conditions [51]. The amount of oxygen incorporated at bottling is the sum of the dissolved oxygen and the headspace oxygen, which is called TPO (total packaged oxygen). By our experience, between 0.5 and 1.5 mg/L of dissolved $O_2$ is usually incorporated at this process. Moreover, the oxygen in the headspace changes depending on the type of closure. In submerged caps, the headspace height is commonly 1–2 cm, and the normal values of dissolved oxygen ranged from 0.5 mg/L (with the use of inert gases) to 2 mg/L (without inertization). In the case of screw caps, the headspace height is higher, about 4 to 6 cm, and the oxygen values ranged from 2 to 6 mg/L. In summary, in submerged caps, values of TPO around 1 or 2 mg/L could be optimum, but values over 3 mg/L are not suitable. In screw caps, TPO values around 2.5 mg/L are optimum, but values over 7 mg/L are not suitable. The type of caps employed not only changes the amount of oxygen incorporated at bottling but also is the ultimate barrier physic to protect wines during the storage period. Thus, a correct cap should be selected depending on the type of wine, and also its permeability to oxygen should be measured to estimate the optimum storage period. The measure of the oxygen transmission rate (OTR) helps to carried out these purposes. **Figure 4** shows "high" and "low" oxygen permeability of different types of caps measured in VITEC by the MOCON® equipment. The OTR measurement corresponds to two natural corks stoppers. As can be seen in the figure, the cork stopper represented in green reached the stability of the oxygen permeability at 24 h, while the stopper represented in red did not reach this stability until the third day. Moreover, once reached the stability, the values of OTR

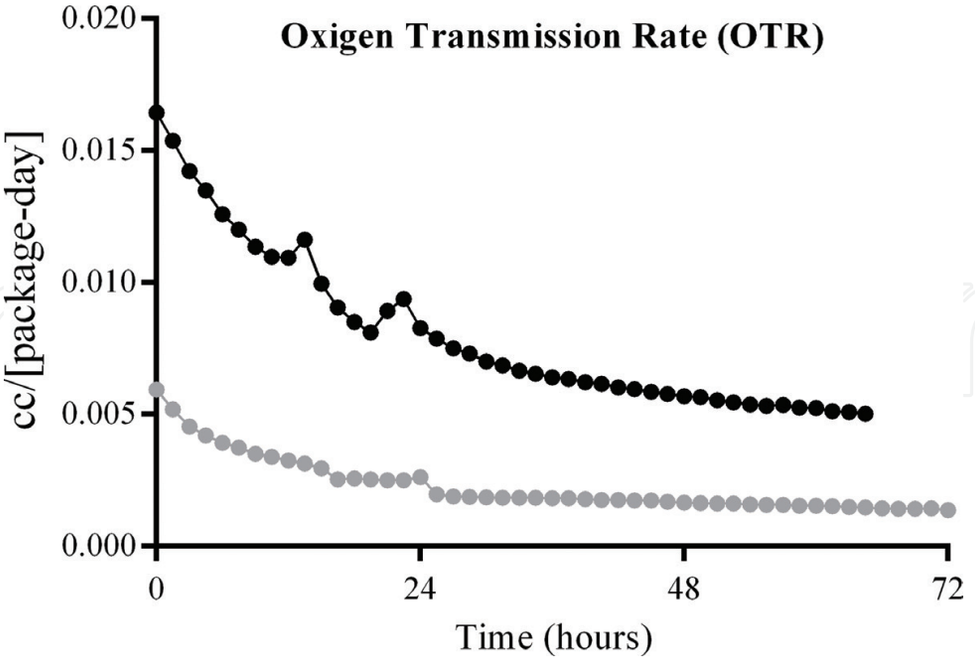


**Figure 4.** Representative oxygen transmission rate (OTR) of caps with different oxygen permeability.

were 4 times higher for "red" stopper than for "green". It can be also observed a great decrease in the case of the "red" stopper, likely due to higher content of oxygen inside of the cork and therefore higher porosity.

## 4. Chemical treatments to elaborate $SO_2$-free wines

The addition of chemical substances to wines is the most used alternative to reduce the $SO_2$ addition in wines. Over the years, the addition of several chemical substances has been allowed by the OIV with different purposes. Accordingly, new antioxidant and antimicrobial additives have been evaluated as possible alternatives to the use of the $SO_2$ [37, 52]. Particularly, the addition of dry yeasts enriched in glutathione, chitosan, and dimethyl dicarbonate, and different hydrolyzed and condensed tannins were evaluated by our research group. The most relevant results and some considerations related to these practices are summarized below.

In the last years, the potential application of glutathione (GSH) has increased the attention of many winemakers and researchers. The addition of reduced glutathione to grape juices or wines is allowed by OIV up to 20 mg/L (OIV OENO 445/2015). The use of GSH in the wine production was reviewed in 2013 by several authors [36, 53]. Following studies also demonstrated that the combination of $SO_2$ and GSH involves a notable protective effect in wines [54]. Recent studies have shown that the addition of glutathione-rich dry inactivated yeast to grape juices modifies the white wine aroma influencing the concentrations of some volatile compounds and precursors with some benefits on its preservation [55–57]. The GSH amount of wine changes depending on the winemaking period. Hence, this compound decreases after wine aging and storage; at pressing could increase its content up to 20 times [58].

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Chitosan is a natural polymer formed by deacetylation of chitin, which has a wide range of applications in different field research, such as agriculture, food, and pharmaceutical industry, among others [59]. The use of this polysaccharide in oenology was approved in 2009 by the OIV to fining musts (OIV-OENO 336A-2009). Moreover, it also used as antimicrobial and antioxidant. Chitosan allows the growth of *Saccharomyces* strains but is an antimicrobial against *Brettanomyces*, acetic, and lactic acid bacteria [60–63]. Commonly, it is used to preserve wine from oxidation and also as fining agent for white wine protein stabilization [64, 65]. **Figure 5** shows the potential of chitosan as antimicrobial. In this case, a significant decrease on yeasts, LAB, and AAB after the addition of 10 g/hL of chitosan to Tempranillo wines (after alcoholic fermentation) was observed. This effectiveness was greater for yeasts, decreasing up to $1 \times 10^4$ cfu/100 mL.

Dimethyl dicarbonate (DMDC) was also accepted by European Union to be used in wine with a maximum limit amount of 200 mg/L (Regulation (EC) No 643/2006). DMDC is an organic chemical compound, which acts inhibiting the growth of microorganisms [9, 66]. When it is added to wines, it is quickly transformed to methanol and produces certain content on methyl and alkyl carbonates as products reaction by polyphenols or organic acids. These products are usually found at a low concentration, and so the quality of wine, flavors and aromas, should not be affected [67]. DMDC seems to be more effective against yeasts than against

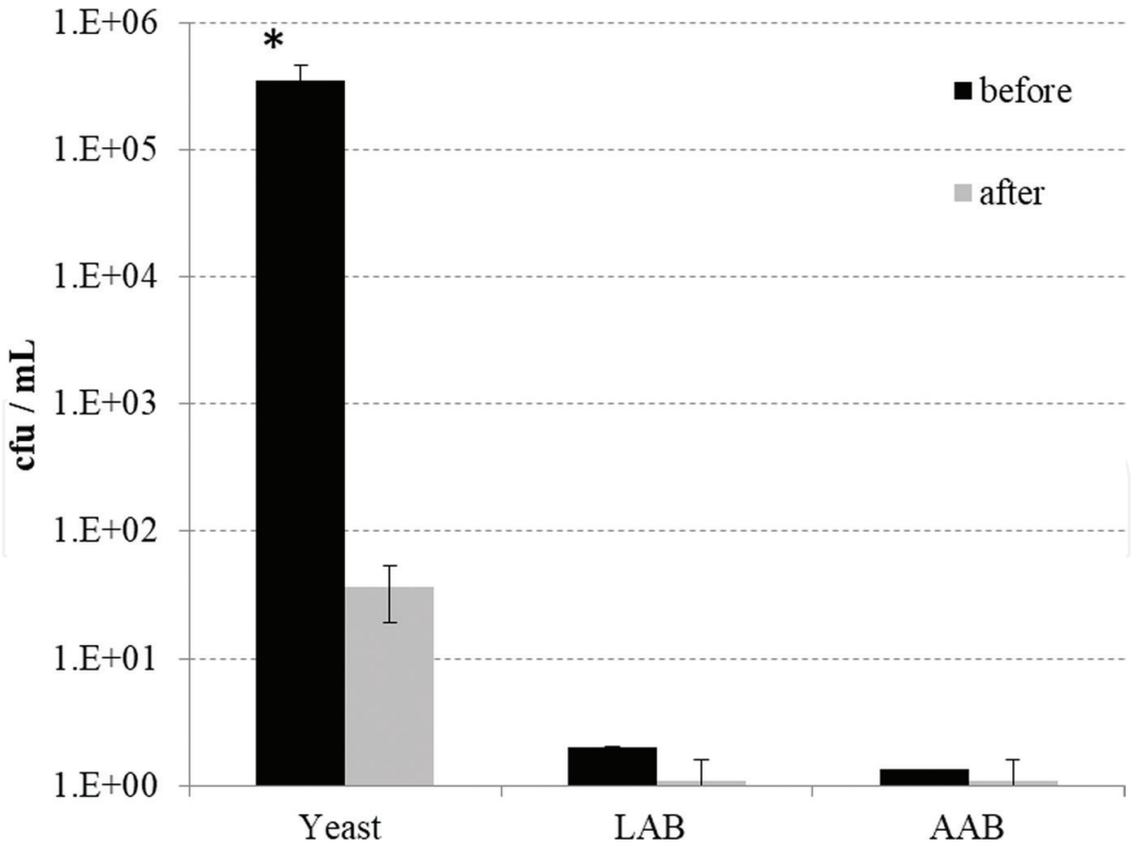


**Figure 5.** Viable yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) quantified in Petri dishes culture (cfu; colony-forming units) from Tempranillo wines before and after a treatment with chitosan (10 g/hL). *Significant differences by HSD Tukey test ($p < 0.05$).

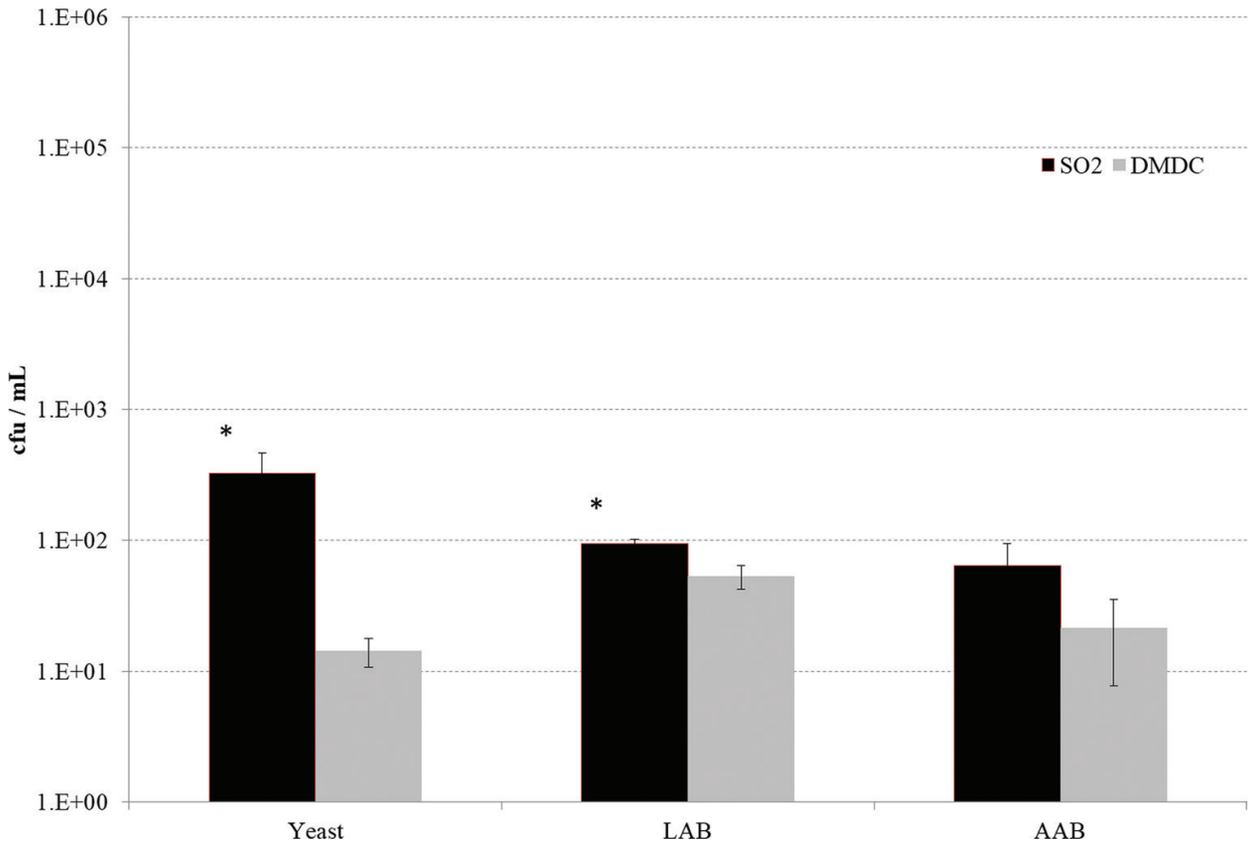


**Figure 6.** Viable yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) quantified in Petri dishes culture (cfu, colony-forming units) from Albariño musts treated with dimethyl dicarbonate (DMDC = 20 g/hL). *Significant differences by HSD Tukey test ($p < 0.05$).

bacteria, although its activity depends on several factors, such as the pH [66–68]. In this sense, **Figure 6** shows the results obtained by the addition of DMDC to Albariño musts. The above-mentioned antimicrobial effect can be observed in yeast, LAB, and AAB. However and as occurred with chitosan, DMDC treatment was clearly more effective in yeasts than in bacteria.

The addition of oenological tannins to wine is an accepted practice by the OIV (OENO 12/2002 and revisions OENO 5/2008, OENO 6/2008, OENO 352/2009, and OENO 554/2015), which mainly aims the color stabilization and the improvement of the wine mouthfeel and flavor. Quite a few studies have evaluated the influence of the tannin addition on the chemical and sensory properties of wines. However, the results obtained are not as promising as expected. In 2005, Bautista-Ortiz et al. [69] did not observe any improvement on the chromatic and sensory properties of wines treated with different oenological tannins. Harbertson and co-workers [70] observed that some additions may be unjustified and have limited or negative impacts on the wine quality. A wide range of commercial tannins exists on the market; nonetheless, a lack of information about the composition and origin of the product is a common pattern. This fact could lead to technological problems according to the expected final wine [71]. The antioxidant properties of tannins, with related health beneficial effects, and their benefits when added to wines are also well known [72]. Both characteristics make tannins a very attractive alternative to the use of $SO_2$ in wine. Some studies showed hopeful results when mixed with antimicrobials, such as lysozyme [17, 73]. The

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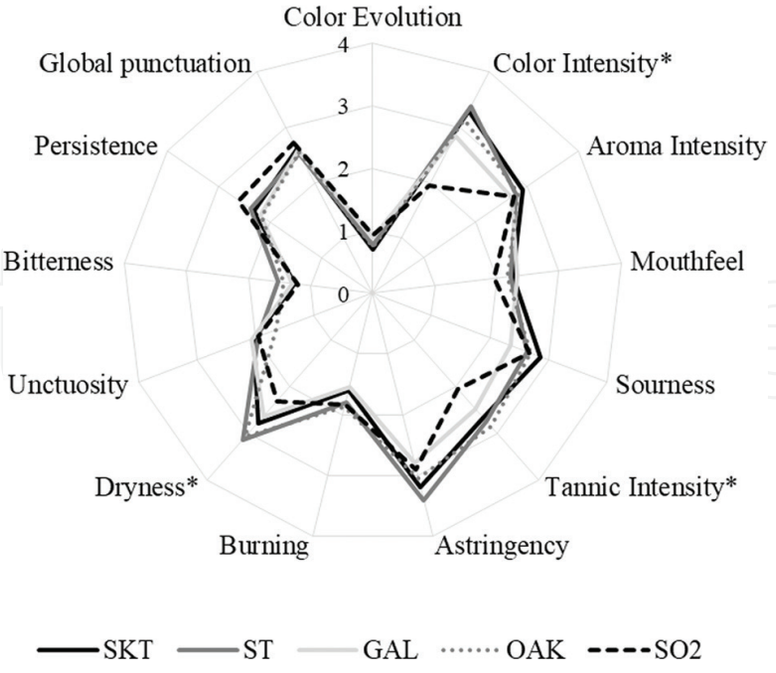


**Figure 7.** Sensory profile of Tempranillo wines elaborated by different enological tannin additions to grape juices. $SO_2$: Wine control. ST: Grape seed tannins (40 g/hL), SKT: Grape skin tannins (30 g/hL), GAL: Tara tannin (20 g/hL), OAK: oak tannins (30 g/hL). *Significant differences by HSD Tukey test ($p < 0.05$).

studies carried out in VITEC have recently shown that the addition of tannins mixed with glutathione may be an effective alternative to the use of $SO_2$ [74]. **Figure 7** shows the sensory analysis of Tempranillo wines with addition of grape seed tannins (ST), grape skin tannins (SKT), oak tannins (OAK), and tara tannins (GAL). In general, the sensory profiles of wines produced with the addition of different tannins were similar (and even better) than wines elaborated by addition of $SO_2$. Significant higher color intensity was observed between control and treated wines. Treated wines also obtained significant dryness and tannic intensity. Astringency and mouthfeel reached higher values but not significant. Lower persistence and higher aroma intensity can also be observed. Low differences between treatments were found, which may be due not only to the different quantity of tannins added but also to their qualitative profile. Recent studies performed by other authors have confirmed the importance of the anthocyanin/tannin ratio on the wine oxidation process and especially on the acetaldehyde formation. Wines with higher tannin addition showed lower production of acetaldehyde [75].

Other chemical substances, such as ascorbic acid and lysozyme, may also be able alternatives to $SO_2$. Ascorbic acid has the ability to scavenge molecular oxygen before the oxidation of phenolic compounds occurs. It is a highly efficient antioxidant in combination with sulfur dioxide; nonetheless, a pro-oxidation effect may occur when the content of $SO_2$ and ascorbic acid is low [76]. The reaction between ascorbic acid and oxygen results in dehydroascorbic acid and hydrogen peroxide, which would be removed by sulfites. Under certain conditions, ascorbic acid both accelerates oxygen removal and reduces the $O_2$:$SO_2$ molar reaction ratio [4]. In wines, it is generally employed in winemaking stages with high oxygen dissolution, such as grape crushing, after racking or just before bottling. The addition of ascorbic

acid in white wines improves color and flavor retention during bottling aging [77]. Certain carbonyl compounds, such as furfural, acetaldehyde, glyoxal, and diacetyl, formed from the oxidation of ascorbic acid may involve the formation of brown pigments by reacting with phenolic compounds. Higher browning was observed in catechin model solutions containing ascorbic acid than in model solutions containing sulfite [78]. These oxidation products of ascorbic acid bind to $SO_2$ reducing in some extent the ratio between free and total $SO_2$ content [76]. The mixture of ascorbic acid together with $SO_2$ seems to be a better antioxidant combination than the use of $SO_2$ alone, avoiding the oxidation of wine and preserving the aroma profile. In white wines, ascorbic acid provides considerable protection against oxidation under conditions of low oxygen [79]. However, it should be highlighted that the impact of the addition of ascorbic acid to wine composition and sensory characters is far to be clarified [36, 77].

Lysozyme belongs to glycoside hydrolases, which is a type of enzyme that catalyzes the hydrolysis of bonds between N-acetyl muramic acid and N-acetyl-D-glucosamine residues in peptidoglycans, and it is found in the cell walls of bacteria, especially in Gram-positive bacteria. These enzymes are therefore destructive to many bacteria like lactic acid bacteria (LAB). In winemaking, indigenous LAB, such as *Lactobacillus brevis*, *Oenococcus oeni, Lactobacillus kunkeei*, *Pediococcus parvulus* and *Pediococcus damnosus,* can be completely inhibited by lysozyme, being this efficacy strongly affected by winemaking and dosage [80, 81]. The addition of lysozyme did not have any negative effect on yeast growth and sugar reduction and may prevent the increase of volatile acidity during the stuck/sluggish of the alcoholic fermentation [17, 81]. This substance had little or no effect on the content of alcohol, titratable acidity, and pH value and did not cause important changes on the sensory characteristics of wines. Nonetheless, it may produce esters in certain wines, contributing to their complexity [73, 82]. Lysozyme may involve changes on yeast nitrogen consumption and the amino nitrogen metabolism, although it does not appear to have an effect on the formation of biogenic amines [16]. The addition of lysozyme may produce a color loss associate with the formation of precipitates in red wines and may induce protein haze in white wines [82]. Lysozyme does not possess an antioxidant activity and therefore does not prevent the wine oxidation. Hence, it becomes necessary the addition of antioxidants, such as proanthocyanidins, in combination with lysozyme to replace the $SO_2$ actions [16, 73]. A critical point of lysozyme is the safety of wines treated with this additive, since it is an egg allergen (allergen Gal d 4 according to the International Allergen Code) that remains in bottled wine. The OIV issued limitation of 500 mg/L [83], and this quantity is removed by an efficient fining treatment using, for example, bentonite or metatartaric acid [84].

## 5. Conclusions

The use of yeast strains with a low capacity to produce $SO_2$, during the alcoholic fermentation is essential to reduce the final amount of $SO_2$ in wines. Both commercial and

indigenous yeasts strains can be used with this purpose. However, factors as grape juice composition, the management of the fermentation, and musts supplementation will be decisive. Different physical technologies and methodologies can be used to elaborate this type of wines. The replacement of the antioxidant and antimicrobial action of the $SO_2$ is a complex mission. However, the combination of different physical techniques together with a good management of inert gases to control oxygen appears to be a suitable practice to achieve this purpose. In addition, some chemical treatments will help to complete the effects caused by these practices. In general, chemical treatments should be combined at different wine production stages to complete their respective actions. The combination of chemical additions even with $SO_2$ may help to reduce its use during the winemaking. It should be noted that still today, there is a lack on the knowledge of the microbiological stability of $SO_2$-free wines during the aging period. Therefore, more research is needed to better understand the effect of the low concentration of $SO_2$ in wines as well as the use of new additives, especially regarding the wine stability after storage and the effects on the human health.

In summary, multidisciplinary approaches should be considered to elaborate high-quality $SO_2$-free wines. The combination of microbiological strategies, physical methods, and chemical treatments becomes indispensable to achieve this ambitious purpose. Several yeast strains are able to generate low quantities of $SO_2$ during alcoholic fermentations (<10 mg/L), and several physical and chemical treatments have shown their antioxidant and antimicrobial effect. Therefore, reducing the $SO_2$ amount in wine production may be achieved. Nonetheless, more research should be done to adapt winemaking procedures according to the particular working conditions and the desired product of each winery.

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