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Yeast stress and death caused by the synergistic effect of ethanol and SO_2 during the second fermentation of sparkling wines

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ABSTRACT

Problems can often arise at the beginning of the second fermentation (*prise de mousse*) of sparkling wines, such as no start, a long lag period or slow fermentation. These problems are generally associated with yeast stress when inoculated in a base wine with high ethanol content and low pH. However, few studies focus on sulphites, which are often added to base wines to prevent malolactic fermentation, microbiological instability, and wine oxidation. This study aimed to evaluate the joint effect of ethanol and sulfur dioxide on yeasts during the second fermentation. For this purpose, yeasts (*Saccharomyces cerevisiae* EC1118) were subjected to ethanol, sulfur dioxide and ethanol/sulfur dioxide at the beginning of fermentation, and their vitality and viability, as well as the accumulation of intracellular reactive oxygen species and intracellular pH, were evaluated by flow cytometry. Furthermore, the expression of genes involved in sulfur transport and metabolism was determined. The results showed high mortality, ROS accumulation and intracellular pH reduction in fermentations with both ethanol and sulfur dioxide. The negative effect of ethanol, sulfur dioxide and ethanol/sulfur dioxide on yeasts was found to be dose-dependent and high in those commonly found in some base wines. Cells treated with ethanol/sulfur dioxide showed over-expression of genes involved in sulphite transport (*SUL1* and *SUL2*), efflux pump (*SSU1* and *FZF1*) and metabolism of sulfur amino acids (*MET14*). Altogether, our data indicate that ethanol and sulfur dioxide have a synergistic effect on yeasts, which may be the root cause of the problems encountered at the beginning of the second fermentation of sparkling wines, and should thus be seriously taken into consideration by winemakers.

KEYWORDS

vitality, viability, ROS, intracellular pH, gene expression

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INTRODUCTION

Sparkling wines develop over two consecutive fermentations: the first one converts grape must into base wine, and the second favours CO_2 incorporation (Di Gianvito *et al*., 2019). The base wines are characterised by an alcohol content of 9.5 % - 11.5 % (v/v) and relatively higher acidity (compared to still wines), with a pH index of 2.8 - 3.3 (Togores, 2018). The second alcoholic fermentation or *"prise de mousse"* (literally "foam creation"), is carried out in closed vessels (hermetic tanks or bottles) causing the incorporation of carbon dioxide into the liquid (Carrascosa *et al*., 2011). This second fermentation can be performed using either the traditional method (fermentation in the bottle) or the Charmat method (fermentation in a tank). For this purpose, together with the base wine, a mix (*liqueur de tirage*) is added with a specific amount of sugars per litre of wine (usually sucrose cane or beet sugar at 20 - 25 g/L) and yeast starter culture for the fermentation (Di Gianvito *et al*., 2019).

The chemical composition of the base wine and the conditions of the second fermentation usually stressing factors for the yeast inoculum: for example, high ethanol concentration, presence of glycerol and low pH (Borrull *et al*., 2015), presence of sulfur dioxide (SO_2) (Sudraud *et al*., 1985), presence of acetic acid (Giannattasio *et al*., 2013), relatively low fermentation temperatures and concentration of nutrients (Kemp *et al*., 2020; Martí-Raga *et al*., 2015), and presence of endogenous CO_2 (Porras-Agüera *et al*., 2020). Therefore, the adaptation of the yeasts to the base wine is important for the success of the second fermentation (Benucci *et al*., 2016; Borrull *et al*., 2016; Martí-Raga *et al*., 2015). This adaptation procedure is known as *pied-de-cuve* (in the French language) and consists of two important phases: adaptation to the alcoholic medium and active growing phase (Benucci *et al*., 2016). This process can last for several hours or days, and usually with a gradually increasing concentration of ethanol and sugar (Benucci *et al*., 2016; Borrull *et al*., 2016; Martí-Raga *et al*., 2016). Traditional yeast adaptation and inoculation protocols for the second fermentation recommend a final inoculum of approximately 1.5 x 10^6 cells/mL of base wine (Ribéreau-Gayon *et al*., 2006). According to these authors, it is possible for levels well below this to cause sluggish fermentations and leave unfermented sugars. Conversely, higher levels (above 2 x 10^6 cells/mL) speed up fermentations; however, depending on the strain, they can result in excessive yeast taste in the final product due to yeast autolysis.

Wine is a hydroalcoholic solution in which ethanol is the second most abundant compound (Waterhouse *et al*., 2016) and an inhibitor for several microorganisms. In the conditions of the second fermentation of sparkling wines, ethanol is described as the main environmental factor to have an influence on yeast transcriptional responses (Penacho *et al*., 2012). Among several factors tested in a study by, ethanol in the base wine was considered to be the main stress factor for yeasts in the second fermentation of sparkling wines. Tolerance to ethanol varies widely among yeast species (Lin *et al*., 2020), and within the *Saccharomyces cerevisiae* species, tolerance may vary depending on the strain (Borrull *et al*., 2015). The presence of ethanol can cause structural changes in yeasts cells, which can impact the fluidity of the plasmatic membrane (Navarro-Tapia *et al*., 2018), and consequently cell morphology (Dinh *et al*., 2008).

Another important compound pointed out as being a stress factor in base wines is SO_2, which is used as a preservative in these wines until the second alcoholic fermentation. Sulfur dioxide is added to grape musts and wines to reduce the medium (antioxidant activity) and to inhibit undesirable microorganisms (antimicrobial action) (Blouin and Peynaud, 2006; Gould and Russell, 2003). Although the use of SO_2 for the conservation of wines is an old practice (Gould and Russell, 2003), ingestion of sulphites through the consumption of food and drink can cause some related adverse clinical effects (Vally *et al*., 2009) ; for this reason, there is a worldwide movement towards decreasing the concentration of sulphites in wines. Although several studies have shown alternatives for SO_2 and sought to reduce its use (Capece *et al*., 2020; Christofi *et al*., 2021; Marchante *et al*., 2019; Shih *et al*., 2020; Simonin *et al*., 2020; Zara and Nardi, 2021), to date, no other physical technique or chemical additive can provide the efficacy and broad spectrum of action of this compound (Lisanti *et al*., 2019).

The microbial inhibition by SO_2 in yeasts has been attributed to several cellular changes, such as the modification of membrane transport activity by binding to membrane proteins (Divol *et al*., 2012); the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) - a critical enzyme in the glycolysis pathway (Hinze and Holzer, 1986) - and other enzymes like ATPase,

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alcohol dehydrogenase and NAD-glutamate dehydrogenase (Maier *et al*., 1986), causing a decrease of the ATP content in cells (Hinze and Holzer, 1986; Maier *et al*., 1986; Schimz and Holzer, 1979); the modification of the expression of many genes correlated with cell metabolism (Park and Hwang, 2008); the degradation of available thiamine (Labuschagne and Divol, 2021); the binding of metabolites (acetaldehyde, pyruvate, glucose, dihydroxyacetone-phosphate, oxaloacetic acid, and α-ketoglutaric acid), thereby preventing their further use as substrates for metabolic pathways (Rankine and Pocock, 1969). However, wine yeasts, particularly *S. cerevisiae*, have a certain tolerance to SO_2 (García-Ríos and Guillamón, 2019) and this tolerance varies between strains (Nadai *et al*., 2016). SO_2 tolerance is a desired trait in wine yeasts which has been unconsciously selected in wine-making practices over time (Zimmer *et al*., 2014), and can be considered an evolutionary advantage (García-Ríos *et al*., 2019).

To reduce the toxicity of sulfur dioxide, yeasts use several mechanisms, such as increasing the production of acetaldehyde to bond with SO_2 and thus reducing the free fraction (Cheraiti *et al*., 2010; Park and Hwang, 2008); activating the Ssu1p sulphite pump encoded with the *SSU1* gene (Marullo *et al*., 2020; Zara and Nardi, 2021); activating sulfur amino acid biosynthesis (Divol *et al*., 2012) ; and/or modifying the overall metabolic and cell cycle that lead to a "viable but non-culturable" cell behaviour (Divol and Lonvaud-Funel, 2005; Salma *et al*., 2013).

The effect of ethanol and sulfur dioxide on the individual antimicrobial action and resistance to stress of wine yeast of the *Saccharomyces cerevisiae* species has been studied in the past decades; however, relatively few studies discuss the joint effect of these compounds on yeast cells (Chandra *et al*., 2015). Long lag phases and lazy fermentations during the second fermentation of sparkling wines are often reported in the industry. The results of a study on the preparation of starter cultures (*pied de cuve*) for sparkling wine production indicate that differences in fermentative kinetics may be caused by differences in the content of ethanol and SO_2 in base wines (Benucci *et al*., 2016). As reported in spoiling wine yeasts (Chandra *et al*., 2014; 2015; Edwards and Oswald, 2018), the presence of ethanol and SO_2 can modify yeast behaviour and viability.

The second fermentation of sparkling wines is a delicate step in the process, which can impact the final product. Predicting the progress of this step and any problems that may occur can be crucial for obtaining a quality sparkling wine. Many empirical reports express the concern of winemakers regarding this initial stage of the process, as well as their lack of full and sound understanding of what causes viability reduction and the increase in lag phase. For these reasons, we decided to explore the topic more deeply by determining whether the presence of both ethanol and SO_2 molecules causes synergism to the extent of modifying homeostasis and physiological stress responses in yeasts during inoculation and at the beginning of the second alcoholic fermentation of sparkling wines.

MATERIALS AND METHODS

The *Saccharomyces cerevisiae* yeast strain Lalvin EC-1118® (Lallemand, Canada) was used in all assays. This yeast strain is recommended and conventionally used in the second fermentation of sparkling wines. Moreover, its genome is already sequenced (Novo *et al*., 2009), a factor that facilitated molecular analyses.

1. Experimental designs

1.1. Synthetic wine and inoculum preparation

All experiments evaluating the synergism between SO_2 and ethanol were conducted in a synthetic wine (Martí-Raga *et al*., 2016) with 4 g/L tartaric acid, 0.5 g/L citric acid, 0.5 g/L malic acid, 0.134 g/L sodium acetate, 1.7 g/L YNB (with ammonium sulfate and without amino acids) and 4.0 g/L glycerol. The final pH of the solution was adjusted to 3.1 using sodium hydroxide. Different ethanol and sulfur dioxide concentrations were added to this medium following the experimental design.

For the adaptation of yeast (*pied de cuve*) a modified protocol was followed (Benucci *et al*., 2016). Briefly, a yeast colony (Lalvin EC-1118) was added to the YPD broth (2 % yeast extract, 2 % glucose, 1 % peptone, pH 6.5) and grown and shaken (150 rpm) at 28 °C for 20 hr. Next, for the adaptation of the inoculum, 10 % (v/v) ethanol, 50 g/L of sucrose and 3.5 g/L of dibasic ammonium phosphate were added to the synthetic wine. An equal volume of the base wine was added to the initial culture (1:1 v/v) and kept static at 20 °C for 24 h. This culture was then diluted with the synthetic wine to a ratio of 1:3 and maintained at a temperature of 20 °C for another 24 h. After this, in all treatments, 24 g/L of sucrose

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(for fermentation) was added to the synthetic wine and inoculated with 1.4 x 10^6 cells/mL of adapted yeasts.

1.2. The effect of ethanol and SO_2 during fermentation

The treatments were divided into four groups in triplicate: i) no EtOH or SO_2 (the control), ii) with 20 mg/L SO_2 (from potassium metabisulphite solution), iii) with 10 % v/v EtOH, and iv) with 20 mg/L SO_2 + 10 % (v/v) EtOH. The fermentations were monitored at a controlled temperature of 15 °C for 28 days (Figure 1). In this experiment, the yeast growth and viability were microscopically monitored as described in Section 2.1.

1.3. Evaluation of stress markers

The treatments were applied as previously described. In this experiment, the fermentations were also carried out under a controlled temperature of 15 °C for 6 days (Figure 2). Yeast growth and viability were determined by microscopic methods and colony-forming units (CFU) evaluated on YPD agar (methods described in Section 2.1.). Cell membrane integrity, intracellular ROS and intracellular pH were analysed by flow cytometry as described in Section 2.2.

1.4. Treatments with different concentrations of ethanol and SO_2

In this experiment, the treatments were divided into eight groups in duplicate: i) no added EtOH or SO_2 (the control), ii) 10 mg/L SO_2, iii) 20 mg/L SO_2, iv) 10 % EtOH, v) 10 % EtOH + 10 mg/L SO_2, vi) 5 % EtOH + 20 mg/L SO_2, vii) 10 % EtOH + 20 mg/L SO_2, and viii) 12 % EtOH + 20 mg/L SO_2. All the treatments were inoculated with 1.4 x 10^6 cells/mL of adapted yeasts. The fermentation temperature was held at 20 °C for 96 h (Figures 3, 4, 5 and 6; Table 2). The cell membrane integrity/cellular vitality, intracellular ROS, intracellular pH, concentrations of reducing sugars, free SO_2 and acetaldehyde were evaluated as described in Sections 2.2. and 2.3. Gene expression was also quantified (see Section 2.4. for the method) and related to the presence of sulfur dioxide in the fermentation environment at the sample collection points closest to the time of inoculation (after 12 h and 24 h).

1.5. Second fermentation on an industrial scale

In the second industrial scale fermentation (traditional method), the base wine comprised a blend (*assemblage*) of wines from the Chardonnay grape varieties (36 %), Riesling Italic (30 %), and Pinot Noir (34 %) (white vinified). The wine alcohol concentration was 11.2 % v/v, and it had a pH of 3.27, 80 mg/L of total SO_2 and 16.5 mg/L of free SO_2. Approximately 22 g/L of sucrose (*liqueur de tirage*) and an adapted inoculum were gradually added to the base wine following a specific company protocol. The percentage of ethanol in the inoculum at the time of inoculation was 13.3 % (v/v) and the total yeast population was 3.5 x 10^7 cells/mL with a viability of approximately 70 %. The base wine was inoculated with 1.4 x 10^6 cells/mL; that is 4 % (v/v) of the final fermentation volume. The bottles were kept at 12 °C for 21 days and three bottles were analysed weekly. The cell membrane integrity/cellular vitality and free SO_2 were analysed (methods described in Sections 2.2. and 2.3.).

2. Analyses performed

2.1. Yeast growth and viability assays

Growth and viability of yeast cells (exclusion tests) were performed using a light microscope (Olympus IX71) with a x 400 magnification using a Neubauer chamber, and viability was determined by staining with a 0.1 % Trypan Blue solution (Thermo Fisher Scientific, MA, EUA) (McGahon *et al*., 1995). Moreover, viable and culturable cells were determined by serial dilution, plating and colony counting on YPD agar (1 % yeast extract, 2 % peptone, 2 % glucose and 2 % agar). The plates were incubated at 28 °C for 24 h, the colony units were counted and data expressed as colony-forming units (CFU/mL) (Fugelsang and Edwards, 2007).

2.2. Flow cytometer analyses

To carry out the analyses with the flow cytometer, samples were centrifuged to separate the cells (4629 x g for 5 min). Once separated, the yeasts were washed in phosphate buffered saline with a pH of 7.2 (PBS) and stained with specific fluorescent dyes.

Flow cytometry analyses were performed in a FACSCalibur flow cytometer (Becton-Dickinson, CA, USA) equipped with an argon-ion laser emitting at 488 nm. The flow cytometer data of 20,000 cells were acquired using CellQuest Pro software (BD Bioscience) and data analysis was carried out using FlowJo v.10 software (TreeStar, Inc). All samples were incubated for 30 min in the dark before analysis.

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The cellular vitality and cell membrane integrity were determined using the LIVE/DEAD™ FungaLight™ Yeast Viability Kit (Thermo Fisher Scientific), which includes 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA) - cleaved by nonspecific esterases resulting in a fluorescent product - and propidium iodide (PI), which only penetrates membrane damaged cells. The staining and flow cytometry analyses were performed according to the manufacturer's recommendations.

The intracellular ROS (Reactive Oxygen Species) was analysed with 2′-7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma, MO, USA). A two-electron oxidation of DCFH-DA results in the formation of a fluorescent product, dichlorofluorescein (DCF) (Kalyanaraman *et al*., 2012) ; the fluorescence intensity of DCF is related to the amount of ROS within the cells. Stock solutions were prepared by dissolving DCFH at 5 mg/ml in DMSO (dimethylsulfoxide). Staining was performed in 500 µl of sample and 5 µg/ml of dye solution.

The intracellular pH was determined using the fluorescent probe pHrodo Green AM Intracellular pH Indicator (Thermo Fisher Scientific). The pHrodo Green is slightly fluorescent at neutral pH and fluorescent in acid conditions. The sample preparation procedure was performed according to the manufacturer's recommendations. The results were expressed in relative fluorescence, because, due to the low pH of the wines, part of the treatments remained outside the ideal range of pH variation detectable by pHrodo Green (pH 9-4).

The sizes of the cells were compared using forward scatter measurement (FSC); the intensity of the FSC is proportional to the diameter of the cell and is mainly due to the diffraction of light around the cell (Leif, 1986).

2.3. Physicochemical analyses

These analyses were performed together from samples of the supernatant (samples centrifuged 4629 x g for 5 min) which had been frozen at the time point of each collection. Total reducing sugars (g/L) were evaluated via the hydrolysis of sucrose in an acid medium and colorimetric method using 3,5-dinitrosalicylic acid (DNS) and microplate reader (absorbance 595 nm) (Dos Santos *et al*., 2017). The acetaldehyde (ethanal) concentration (mg/L) was quantified using the colorimetric method (acetaldehyde reacts with sodium nitroferricyanide and piperidine solution) using a spectrophotometer (570 nm absorbance), and the calibration curve was obtained directly using acetaldehyde in different concentrations, as adapted from the OIV method (OIV, 2009). Free SO_2 (mg/L) was estimated according to the Ripper titrimetric method using iodate (Zoecklein *et al*., 1999).

2.4. Gene expression

RNA extraction was performed according to a protocol specific for *Saccharomyces cerevisiae* (Shedlovskiy *et al*., 2017). The extracted RNA was treated with DNase I (Thermo Fisher Scientific) following the manufacturer's protocol. The absence of contaminant genomic DNA in the RNA was checked before cDNA synthesis using RNA as a template for a PCR assay. The RNA was reversed-transcribed into cDNA with the M-MLV Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer's protocol. The primers for qRT-PCR are shown in Table 1. Their sequences were obtained from published studies and by using the Primer designing tool system on the NCBI website (Primer-BLAST). Primers were purchased from Thermo Fisher Scientific.

Real-time PCR was performed in Applied Biosystems StepOne qRT-PCR (Thermo Fisher Scientific) using SYBR Green as a fluorophore. Reactions were carried out in 20 µL of mix containing 10 µL of Platinum ™ SYBR ™ Green qPCR SuperMix-UDG dye (Thermo Fisher Scientific), 2.0 µL of primer mix (200 nM final concentration), and 8 µL of cDNA. The thermocycling programme consisted of one hold at 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. After the cycles, melting-curves data were collected to verify PCR specificity and contamination. Moreover, in an initial experiment, three potential housekeeping genes were evaluated: *ACT1*, *TEF1,* and *IPP1*. As all of them gave similar Ct values for the different samples, *ACT1* was selected for further analysis.

With the values obtained from the expression of each gene in the treatments, ΔCт was calculated by subtracting C_T (threshold cycle) of the reference gene (*ACT1*) from C_T of the target gene. Afterwards, ΔΔCт was calculated [ΔC_T (target sample) - ΔC_T (sample reference)], subtracting the ΔCт of the genes from the treatment samples (20 mg/L SO_2 without EtOH, – 10 % EtOH without SO_2, 10 % EtOH + 20 mg/L SO_2) by the control sample (without EtOH and without SO_2). Then the formula for the comparative method was applied

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TABLE 1. Genes and primers used in qRT-PCR

Gene	Primer F (5' - 3')	Primer R (5' - 3')	Size (bp)	Function	Reference
ACT1	TCGCCTTGGACTTCGAACAA	CAAAGCTTCTGGGGCTCTGA	128	Housekeeping gene	This paper
IPP1	AGCCAGTTTCTGCCTTCCACGA	TGGTGATTTCTAACTTGGCGTTGGT	111	Housekeeping gene	This paper
TEF1	GGTTACTCTCCAGTTTTGGATTGTC	ACGAACTTGACCAAAGCAGC	149	Housekeeping gene	(Nardi *et al*., 2010b)
COM2	AGCCTTGGTTGTGAACCCAT	GCGTGGTCACTCTCATCACT	185	SO_2 tolerance regulator	This paper
FZF1	CCAGAGAGTTACTGGTCGGATA	CGTGTGGTCATAGTGGTCAAT	102	SO_2 chemical induction	(García-Ríos *et al*., 2019)
MET14	AGAAGTCGCTGAGCAAAGGG	TTCAACCGTCTTCTGGTCGG	145	Sulfur assimilation pathway	This paper
SUL1	CACTGGGTTGGGTATACTGC	ATGAGAGCCGGAATTTGACC	120	Sulphate permease	(Chen *et al*., 2018)
SUL2	AAGGGAGAACGACCCTGAAT	TGGCCTTTCTCAAATCAACC	161	Sulphate permease	(Jennings and Cui, 2012)
SSU1	TTTGCGTTTGTTGGTCAATTCTATGCCTTTTA	TCCACGCTTTCAATGCTGTTATACGGAGAA	151	SO_2 influx of compound	(Nardi *et al*., 2010a)

$(2^{-\Delta\Delta C_T})$ (Livak and Schmittgen, 2001), giving a value for the relative expression of the genes or RQ.

3. Statistical analysis

Statistical analyses were performed by two-way ANOVA and Tukey's multiple comparisons test, using a level of significance of 95 %. Graphs and statistical analyses were performed using the Graphpad Prism® software (GraphPad, CA, USA).

RESULTS

1. The effect of ethanol and SO_2 during fermentation and evaluation of stress markers

In the first experiment, it was possible to verify the impact caused by SO_2 and ethanol on yeast viability and cell growth. As can be observed in Figure 1, yeast cells grown on the control medium showed high viability throughout the experiment and exhibited a typical growth behaviour, attaining the stationary phase with approximately 5 x 10^7 cells/mL after 8 days. On the medium supplemented with SO_2 (20 mg/L), the yeast population underwent a small (approximately 20 %) reduction in viability in the first few days (Figure 1A), which was maintained until the end of fermentation. This initial reduction in yeast viability caused a delay in population growth, but it reached almost the same final cell density as the control (Figure 1B). However, in the medium with EtOH (10 %), cell viability remained high throughout the fermentation, but yeast growth was drastically reduced (Figure 1A and 1B).

Conversely, in the medium containing both EtOH and SO_2 (10 % EtOH + 20 mg/L SO_2), a drastic reduction in yeast viability during the first few days was observed, reaching 2 % on the sixth day (Figure 1A). After this point, the cell population started to grow slowly (Figure 1B), remaining relatively low compared to the control. At the end of the experiment (28 days) the yeast population in EtOH + SO_2 medium was just 1/6 of the control and SO_2 treatments, and 1/2 of the EtOH treatment (Figure 1B). These data indicate that EtOH and SO_2 have a synergistic effect on yeast viability and growth.

To better understand the effect of ethanol and SO_2, and their synergistic effect on yeasts, we conducted a new acute experiment (144 h), monitoring cell viability, vitality, intracellular ROS and intracellular pH. As shown in Figure 2 (A, B, C and D), this experiment confirmed the synergistic effect of ethanol and sulfur dioxide, which together cause a drastic reduction in cell growth and viability. Yeast growth, determined by microscopic cell counting (Figure 2A) and colony-forming units (Figure 2B) showed a considerable reduction in yeast growth in the presence of sulfur dioxide and, in particular ethanol. However, yeast growth was completely inhibited by the presence of both ethanol and sulfur dioxide in the synthetic wines.

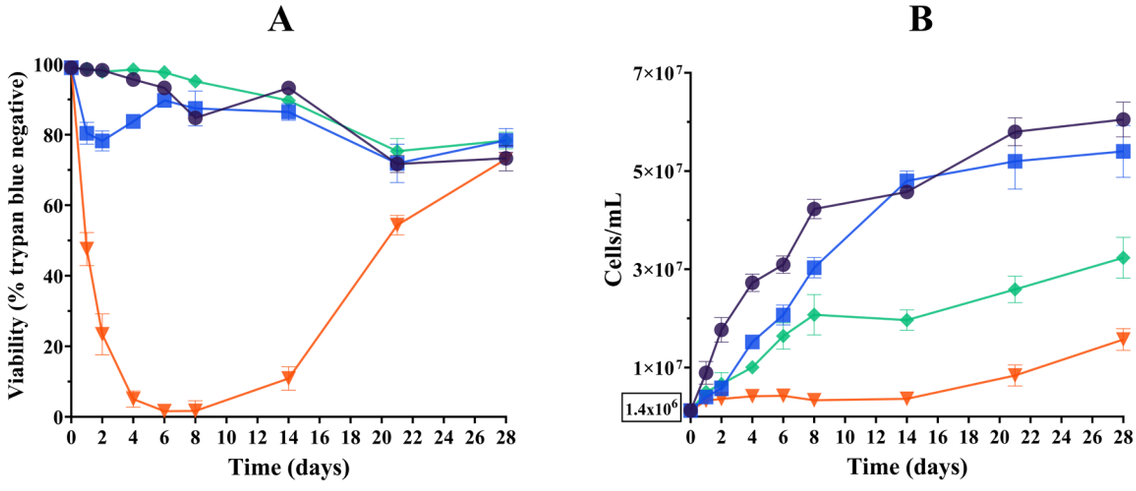


FIGURE 1. Synergism, viability, and growth in synthetic wine. Cell membrane integrity (viability) was assessed with a light microscope and Neubauer chamber, and using trypan blue exclusion dye (A); population growth was estimated using light microscope and Neubauer chamber (B). (●) Control; (■) 20 mg/L SO_2; (■) 10 % (v/v) EtOH; (▼) 10 % EtOH + 20 mg/L SO_2. The error bars in the line graphs represent the standard deviation obtained from triplicate samples within the same experiment.

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The cell viability results obtained using trypan blue (Figure 2C) and PI (Figure 2D) showed high viability (> 95 %) in the control- and ethanol-containing medium.

Yeasts cultivated in the medium supplemented with SO_2 exhibited an initial reduction in viability (after 24 and 48 h), followed by an increase in viability associated with population growth. Conversely, yeasts cultivated in EtOH + SO_2 medium showed a drastic and rapid reduction in viability.

The cytometric analysis of intracellular ROS using the DCFH-DA dye showed a basal low ROS concentration for yeast cells grown in the control medium and in the media that contained just ethanol or SO_2.

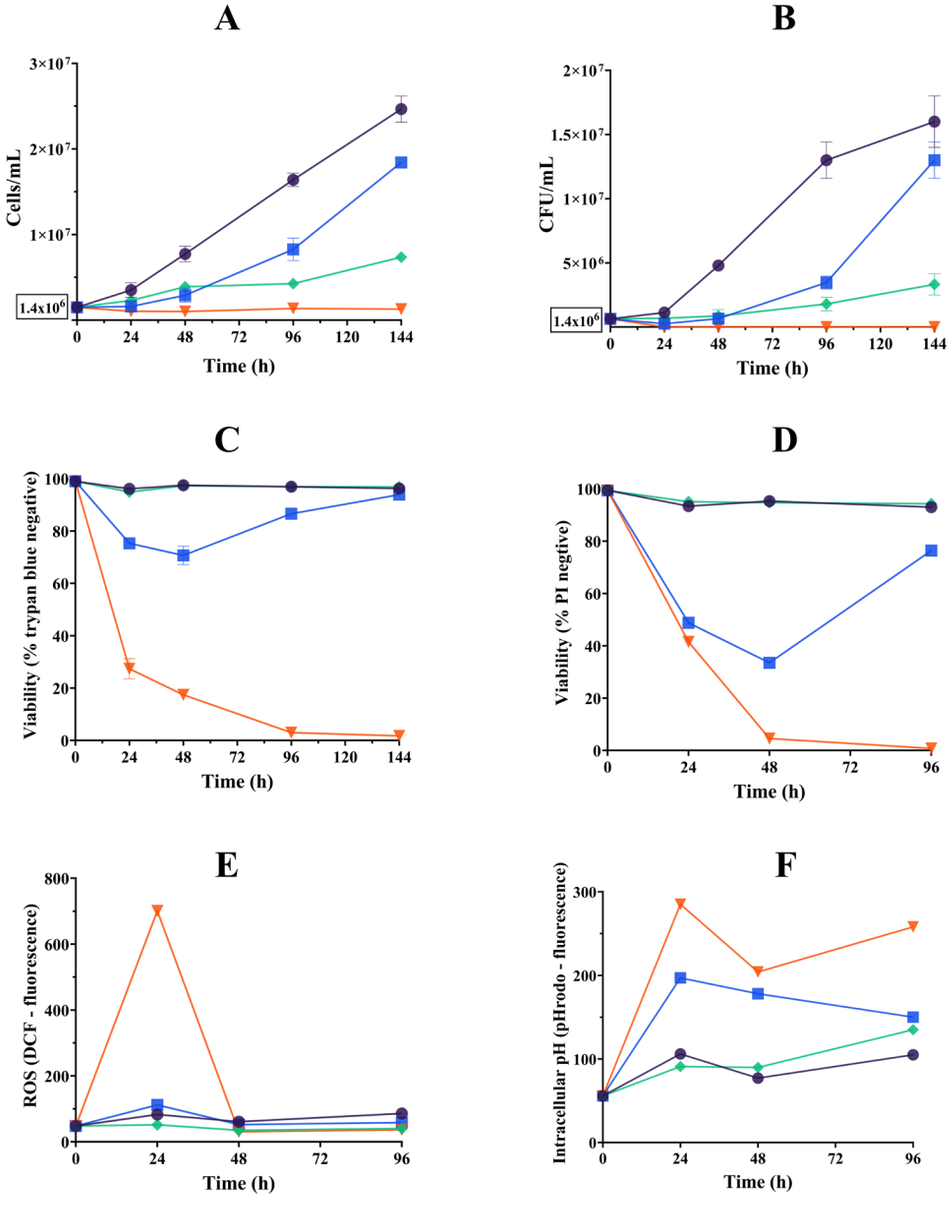


FIGURE 2. Stress caused by synergism between EtOH and SO_2. Estimation of population growth using the light microscope and Neubauer chamber (A); viability test and growth with colony-forming units (CFU) (B); cell membrane integrity (viability) assessed with the light microscope, Neubauer chamber and using trypan blue exclusion dye (C); cell membrane integrity (viability) assessed with flow cytometer + PI (D); intracellular ROS analysed with flow cytometer + DCFH-DA (E); intracellular pH analysed with flow cytometer + pHrodo Green AM (F). (●) Control; (■) 20 mg/L SO_2; (■) 10 % EtOH; (▼) 10 % EtOH + 20 mg/L SO_2. The error bars in the line graphs (A, B and C) represent the standard deviation obtained from triplicate samples within the same experiment.

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However, a peak in intracellular ROS occurred on the first day of the treatment supplemented with both ethanol and SO_2 (Figure 2E), suggesting that together these compounds cause greater oxidative stress and may have also influenced the drastic reduction in cell viability.

The analysis of intracellular pH and treatments with SO_2 and with EtOH + SO_2 revealed considerable changes in the fluorescence of the dye (the higher the fluorescence, the lower the pH). However, the EtOH + SO_2 treatment exhibited the highest fluorescence (lower pH) throughout the experiment. This corroborates the results of the other analyses (Figure 2) which showed that the treatment with both molecules intensified the stress in yeast cells, indicating a synergistic effect had taken place. At the end of the experiment, the SO_2 treatment contained 5 mg/L of free SO_2, compared to 10 mg/L in the EtOH + SO_2 treatment.

2. The interaction of different concentrations of ethanol and sulfur dioxide concentrations in yeasts

The control, 10 mg/L SO_2, 10 % EtOH and 10 % EtOH +10 mg/L SO_2 treatments showed almost the same behaviour for all variables, except for sugar consumption (Figure 3B), with the presence of ethanol decreasing fermentation rate.

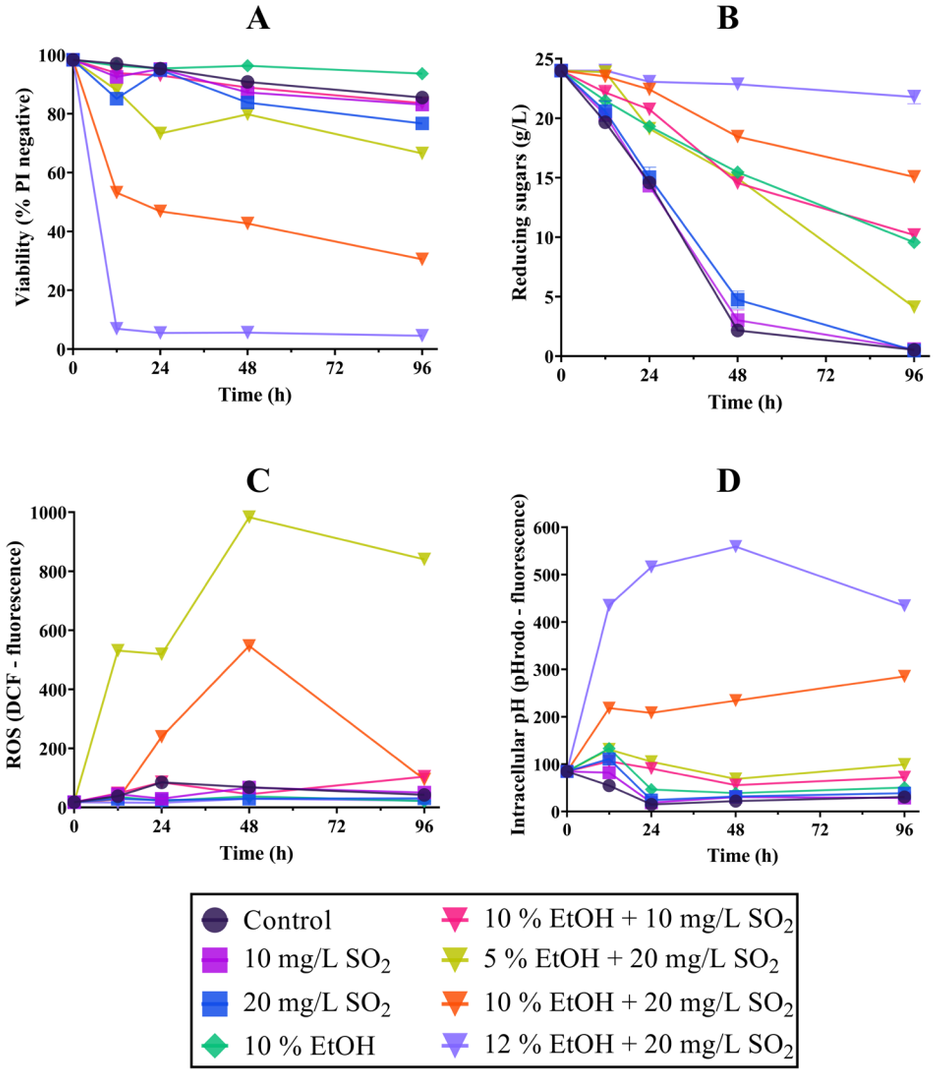


FIGURE 3. Different concentrations of EtOH and SO_2. Cell membrane integrity (viability) assessed with flow cytometer + PI (A); monitoring of consumption of total reducing sugars (B); intracellular ROS analysed with flow cytometer + DCFH-DA (C); intracellular pH analysed with flow cytometer + pHrodo Green AM (D). The error bars in the line graphs (B) represent the standard deviation obtained from duplicate samples within the same experiment.

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However, treatments that include both ethanol (5 to 12 %) and sulfur dioxide (20 mg/L) exhibited a reduction in fermentation rate and cell viability proportional to the ethanol concentration. As can be observed in Figures 3A and 3B, the treatment with 5 % EtOH + 20 mg/L SO_2 showed a 27 % reduction in yeast viability in the first 24 h, which was maintained until the 96 h time point, resulting in sluggish fermentations. Treatments with 10 % and 12 % ethanol + 20 mg/L of SO_2 showed a remarkable decrease in cell viability in the first 12 h (by 45 and 95 % respectively) and a proportional inhibition of sugar consumption.

Yeast cells grown in 5 % EtOH + 20 mg/L SO_2 and 10 % EtOH + 20 mg/L SO_2 exhibited a considerable increase in intracellular ROS (Figure 3C): In the former treatment, the ROS which had accumulated during the first 48 h remained high until the end of the experiment, while in the latter, ROS showed a constant increase during the first 48 h, followed by a sharp decrease thereafter. Interestingly, the yeasts in the more severe treatment (12 % EtOH + 20 mg/L SO_2) did not accumulate intracellular ROS - a fact that may be related to the high mortality (> 95 %) observed in this treatment - indicating that ROS accumulation depends on metabolic activity.

Regarding the intracellular pH, data in Figure 3D show that yeast cells cultivated in the highest concentrations of EtOH and SO_2 (10 % EtOH + 20 mg/L and 12 % EtOH + 20 mg/L SO_2 showed greater fluorescence (lower pH intracellular) than the control and the other treatments. This decrease seems to be correlated with the percentage of cells with a damaged membrane, which may indicate the entry of a greater amount of SO_2 into the intracellular environment and/or the failure of proton efflux.

The results of the analysis of yeast using two fluorescent markers (CFDA and PI; Figure 4) showed that EtOH (10 %) did not interfere with yeast cell enzyme activity (vitality) immediately after inoculation (12 h), corroborating the data presented in Figures 1A, 2C, 2D and 3A. Conversely, treatments with 20 mg/L of SO_2, and 10 % EtOH + 20 mg/L of SO_2, had completely different behaviour. In the treatment of 20 mg/L of SO_2, initial stress was observed with a drop in vitality (lower CFDA fluorescence) and a small increase in cells with a damaged membrane (PI positive). In the second treatment (10 % EtOH + 20 mg/L SO_2), yeast cells showed four populations distributed in different quadrants, indicating a higher level of stress, with a considerable increase in the number of cells with the damaged membranes (quadrant Q3). Moreover, an increase of just 2 % EtOH (12 % EtOH + 20 mg/L SO_2) resulted in a significantly higher number of cells with membrane disfunction even after a short 12 h exposure (more than 90 %).

The comparison of cell size using direct dispersion measurement (FSC) by flow cytometry analysis (Figure 5) showed that yeast cells in the control and the 10 and 20 mg/L SO_2 treatments exhibited a "normal" distribution, while yeast cells subjected to 10 % EtOH showed a small percentage of larger cells (Figure 5A). The treatments that cause a more marked reduction in yeast viability (10 % EtOH + 20 mg/L SO_2, 12 % EtOH + 20 mg/L SO_2), showed a high percentage of cells with reduced size (Figure 5D), thus indicating modifications having been made to cell permeability and water content or a possible modification to the metabolism of the membrane lipids (phospholipids).

One of the most important compounds involved in sulfur dioxide neutralisation is acetaldehyde, which is produced during alcoholic fermentation and combines with sulfur dioxide, thus reducing the amount of free SO_2. The results of the analyses of free SO_2 and acetaldehyde (Table 2) show that only the treatments with considerable fermentative activity (Figure 3B) showed a large decrease in free SO_2; meanwhile, the treatment with 12 % EtOH + 20 mg/L SO_2, which exhibited a dramatic inactivation of yeast, showed the lowest acetaldehyde production and maintained high levels of free SO_2 throughout the fermentation process. The treatments with SO_2 showed an increase in acetaldehyde concentration (mainly within 12 h) in comparison to the control, as did those with ethanol alone. The treatments with both molecules (10 % EtOH + 10 mg/L SO_2 and 5-10 % EtOH + 20 mg/L SO_2) showed higher concentrations of acetaldehyde than the other treatments.

Yeast responds to a stress factor (in this case SO_2 and ethanol) with a differential expression of genes that contribute to cell viability and homeostasis. As shown in previous figures, in mid- or non-lethal concentrations of ethanol and sulfur dioxide, a yeast population can adapt and grow, albeit slowly. This "adaptation" involves the differential expression of genes, the products of which contribute to yeast cell homeostasis, metabolism and eventual division. In this context, we evaluated the expression of several genes associated with sulfur transport and metabolism. As can be observed in Figure 6A, the *SSU1*,

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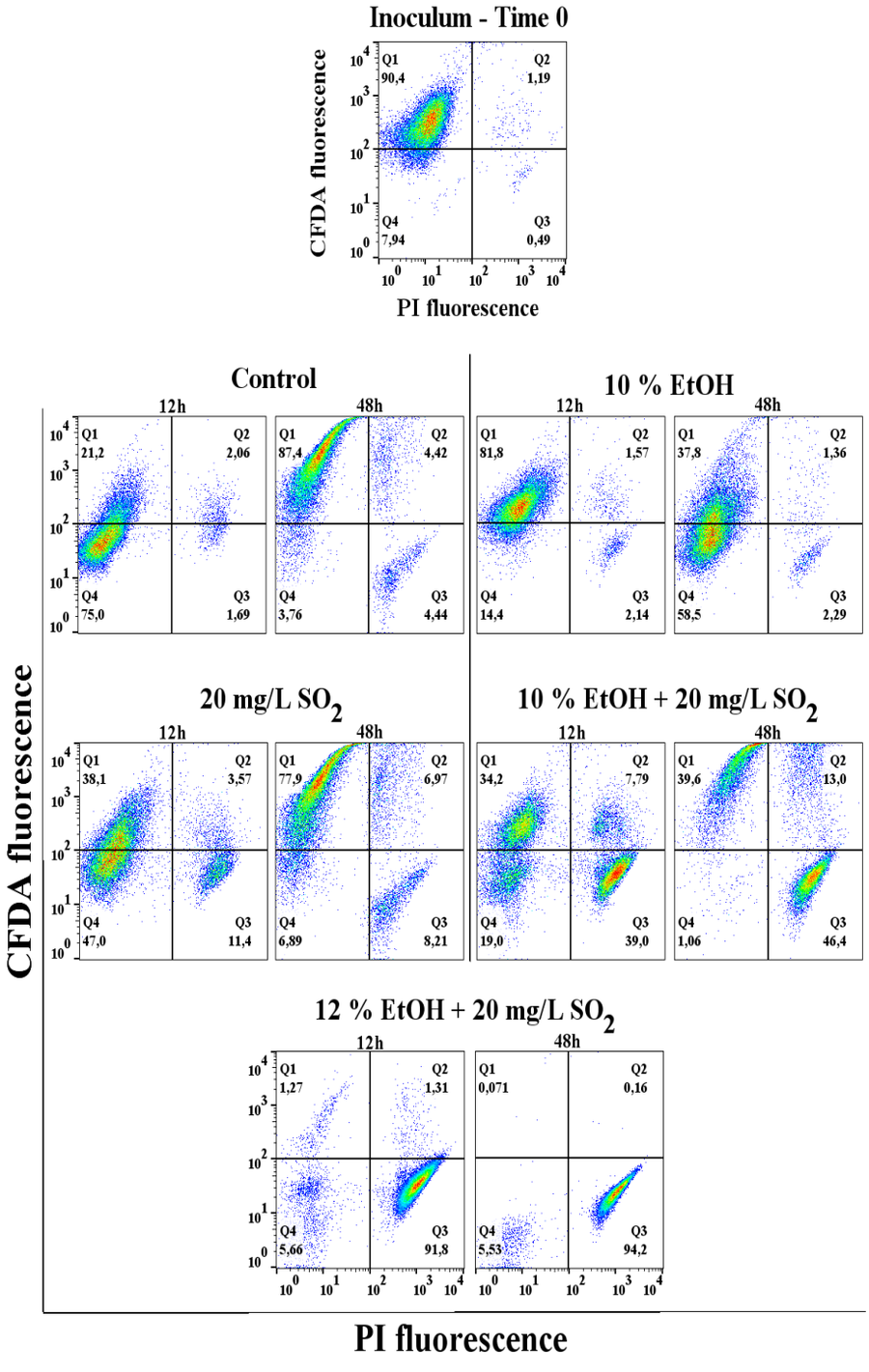


FIGURE 4. Vitality versus viability. Flow cytometer analysis of the third test using the LIVE/DEAD™ FungaLight™ Yeast Viability Kit (CFDA/PI).

MET4, and *FZF1* genes were hyper-expressed in yeasts grown in 10 % EtOH + 20 mg/L SO_2 compared with the treatments that contained just ethanol or SO_2. However, the *SUL1* gene showed higher expression in both 10 % EtOH and 10 % EtOH + 20 mg/L SO_2 treatments, while *SUL2* exhibited higher expression in the treatments that contained SO_2. The *COM2* gene expression did not vary among treatments.

After 24 h (Figure 6B), the yeast cells subjected to 10 % EtOH and 10 % EtOH + 20 mg/L SO_2 showed higher expression of *SSU1* and, in particular, *MET14* genes than the control and the SO_2 treatments. However, the high expression of *SUL1* and *SUL2* observed in the first few hours was not evident after cell adaptation to ethanol and sulfur dioxide stress.

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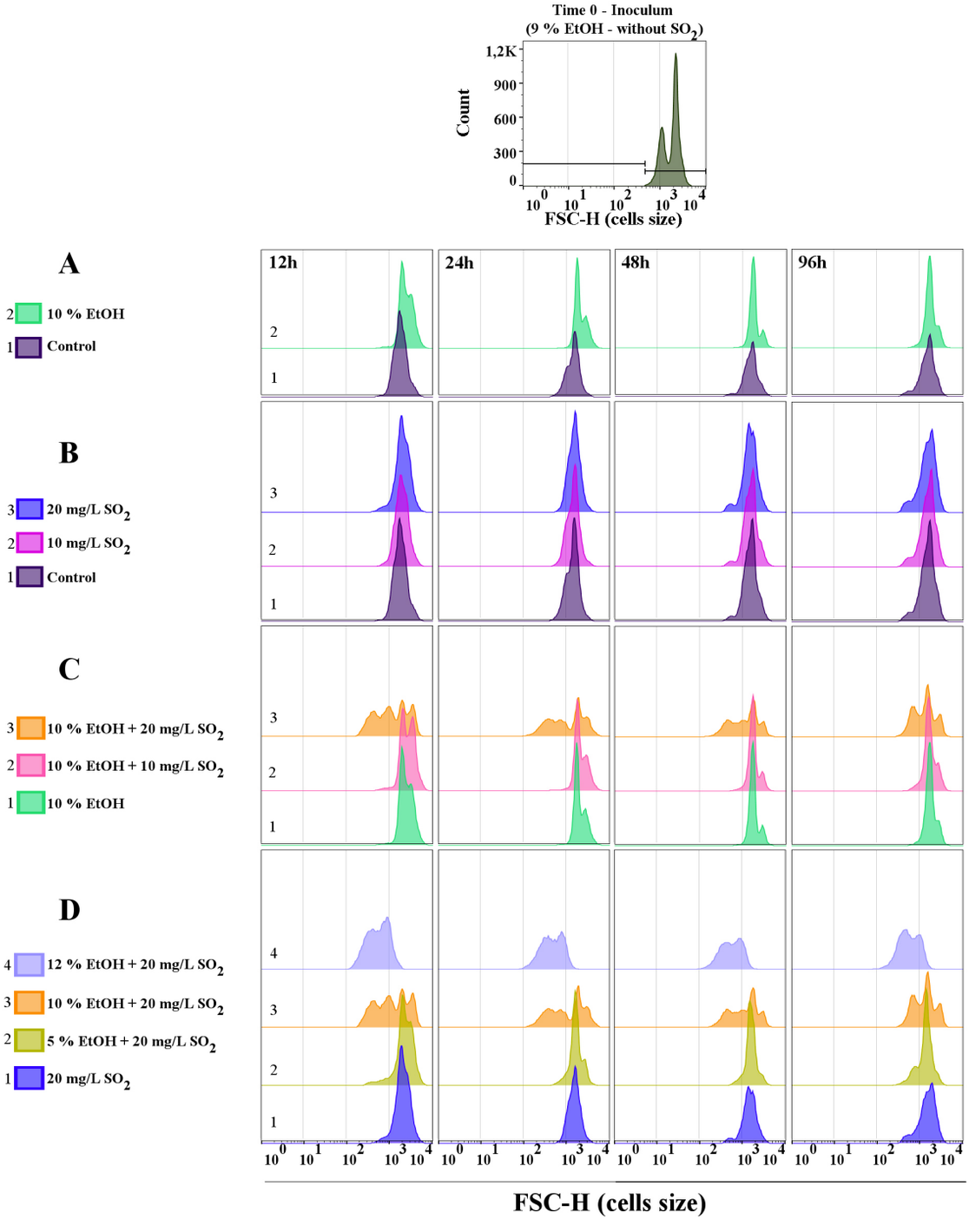


FIGURE 5. Differences in cell size. Analysis was performed with flow cytometry using direct dispersion measurement (FSC). The intensity of the FSC is proportional to the diameter of the cell.

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TABLE 2. Analysis of free SO_2 and acetaldehyde over time.

SAMPLE	FREE SO_2 (mg/L)					ACETALDEHYDE (mg/L)			
	Time					Time			
	T0	12 h	24 h	48 h	96 h	12 h	24 h	48 h	96 h
Control	-	-	-	-	-	27.4 ± 2^{ef}	23.3 ± 8^{ce}	45.5 ± 5^{d}	53.8 ± 7^{d}
10 mg/L SO_2	10 ± 0^{A}	3.2 ± 1^{B}	3.2 ± 1^{B}	3.2 ± 1^{B}	3.2 ± 0^{B}	40.8 ± 2^{de}	39.3 ± 2^{c}	52.8 ± 12^{cd}	70.3 ± 1^{cd}
20 mg/L SO_2	20 ± 0^{A}	3.8 ± 0^{B}	3.8 ± 0^{B}	3.2 ± 0^{B}	3.2 ± 0^{B}	62.1 ± 1^{cd}	42.4 ± 3^{c}	64.1 ± 22^{bcd}	78.1 ± 1^{cd}
10 % EtOH	-	-	-	-	-	99.3 ± 5^{b}	68.3 ± 5^{b}	62.1 ± 1^{bc}	78.6 ± 7^{c}
10 % EtOH + 10 mg/L SO_2	10 ± 0^{A}	3.2 ± 1^{B}	3.2 ± 1^{B}	3.2 ± 0^{B}	3.2 ± 0^{B}	125.2 ± 2^{a}	82.8 ± 1^{ab}	77.6 ± 2^{b}	105.5 ± 7^{b}
5 % EtOH + 20 mg/L SO_2	20 ± 0^{A}	3.84 ± 0^{B}	3.2 ± 1^{B}	3.2 ± 0^{B}	3.2 ± 0^{B}	125.2 ± 1^{a}	68.8 ± 1^{ab}	84.8 ± 5^{b}	130.4 ± 5^{a}
10 % EtOH + 20 mg/L SO_2	20 ± 0^{A}	4.8 ± 0^{BC}	3.8 ± 0^{C}	3.2 ± 0^{D}	3.2 ± 0^{D}	68.8 ± 2^{c}	93.1 ± 1^{a}	111.2 ± 1^{a}	119.0 ± 6^{ab}
12 % EtOH + 20 mg/L SO_2	20 ± 0^{A}	15.4 ± 0^{B}	14.8 ± 1^{B}	14.8 ± 1^{B}	12.5 ± 0^{C}	8.1 ± 0^{f}	7.2 ± 1^{e}	4.5 ± 1^{e}	0.1 ± 0^{e}

*The values are shown with mean ± standard deviation (SD) obtained from replicated samples (duplicate) within the same experiment. In the free SO_2 column, statistical calculations were performed on each row (differences in the sample itself over time), which are represented by capital letters. In the columns with acetaldehyde analyses, treatments at each time point are compared (differences per column at each time point) and are represented with lower-case letters. Distinct letters are significantly different according to the Tukey test ($P \leq 0.05$).

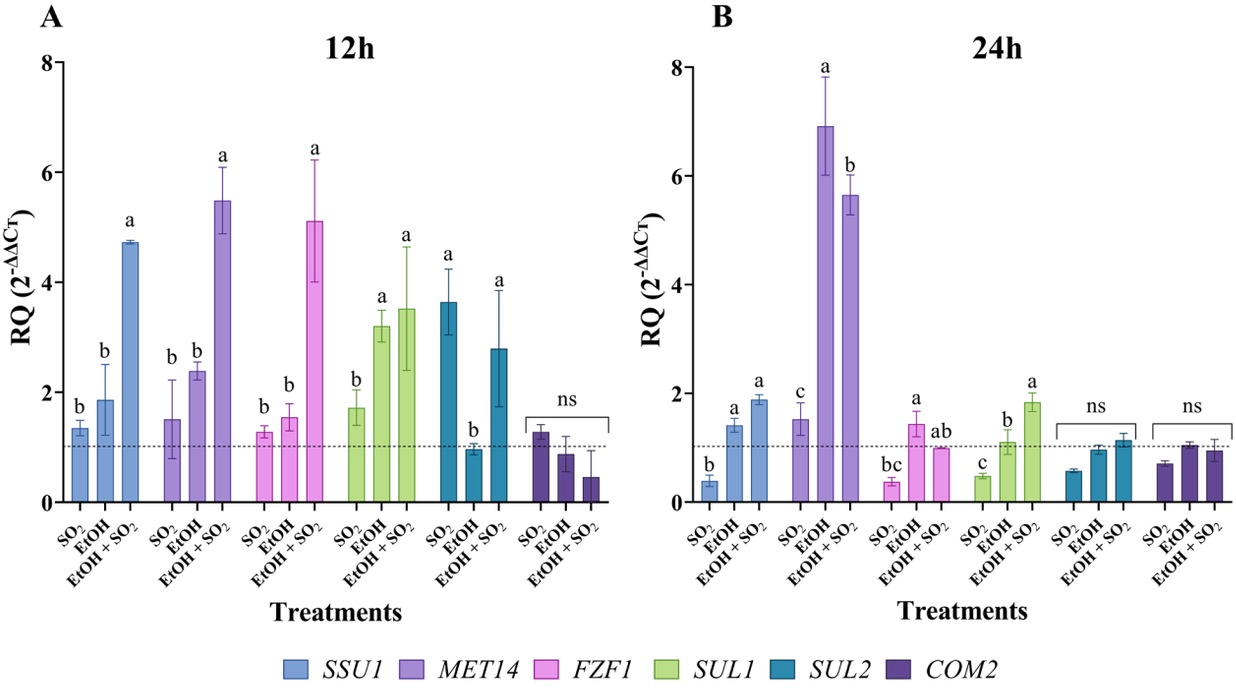


FIGURE 6. Analysis of relative expression (RQ). The dotted line represents Value 1 of the control treatment. Bars represent RQ values of each gene relative to the control treatment (dotted line). SO_2 = 20 mg/L SO_2; EtOH = 10 % EtOH; EtOH + SO_2 = 10 % EtOH + 20 mg/L SO_2. The error bars are the standard deviation of three replications of the same treatment. Different letters denote significantly different mean values by the Tukey test ($P \leq 0.05$); ns = not significant.

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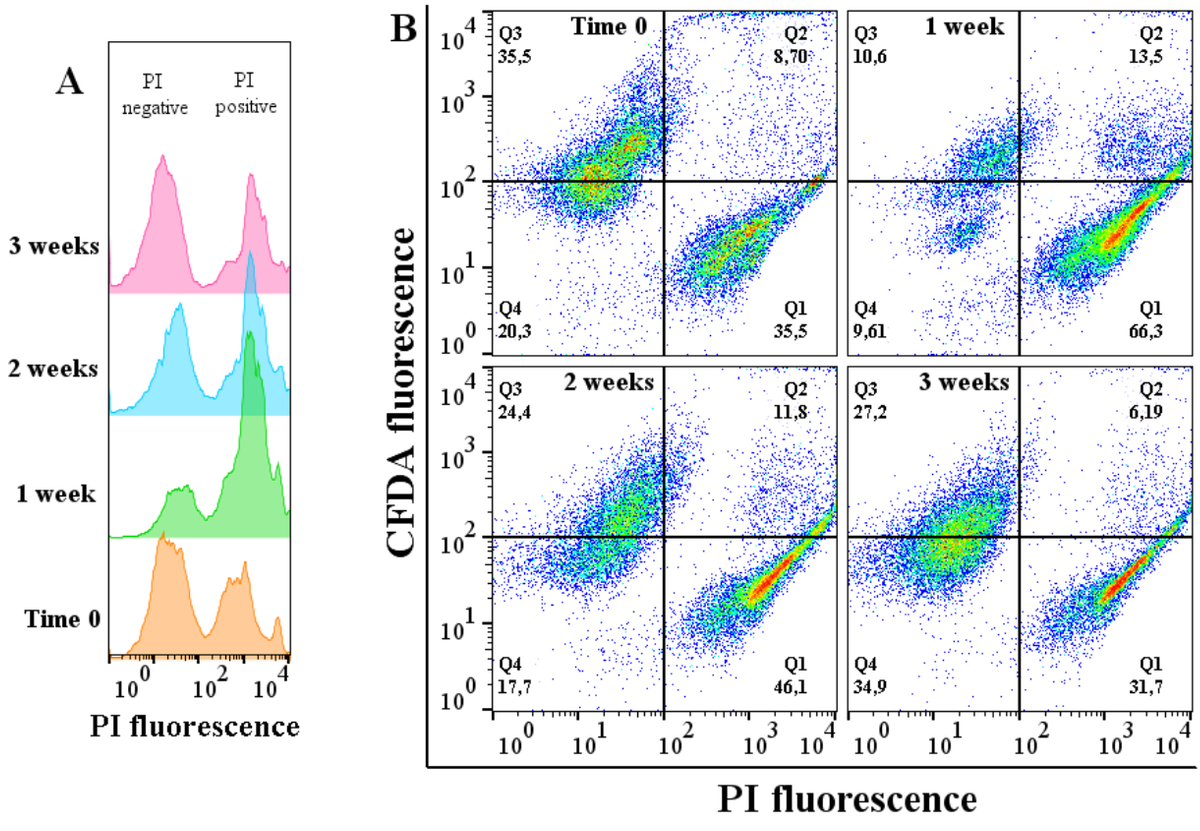


FIGURE 7. Flow cytometer analysis of the first four weeks of fermentation using the LIVE/DEAD™ FungaLight™ Yeast Viability Kit. The graph as a histogram (PI) (A). Cell concentration map (CFDA/PI) (B).

3. Vitality and viability of yeasts on an industrial scale

Figure 7 shows yeast vitality and viability during the first three weeks of the second fermentation of a sparkling wine made by the traditional method. As can be observed, just before inoculation, a lower number of cells were stained with PI, indicating a higher number of cells with an intact cell membrane (inoculum at time 0). However, one week after inoculation, there was a considerable increase in cells with damaged cell membranes (PI positive cells). Moreover, at this point, the living cells (PI negative) exhibited a lower esterase activity than the control, which reflects a reduction in cell metabolism. In the second and third weeks, the number of viable (PI negative) and overall metabolism (CFDA fluorescence) increased. In the 2nd and 3rd week, there was an increase in cells with damaged plasma membrane that prevented the entry of the dye, as well as an increase in cells containing the active esterase enzyme. During this period, free SO_2 decreased from 16.5 mg/L (time 0) to 5.5 mg/L (after 2 weeks). The behaviour of the yeasts during the industrial scale second fermentation of a real wine showed a similar effect (decrease in vitality and viability) to that in synthetic wines with similar concentrations of ethanol and free SO_2 (Figure 4).

DISCUSSION

Empirical observations in wineries report frequent problems at the start of and during the second fermentation of sparkling wines in both traditional and Charmat processes. In general, the practical solutions adopted in these cases are pre-adaptation and reinoculation with yeasts, which is costly and laborious, especially for traditional sparkling wines. Furthermore, industrial data show that these problems are associated with a considerable reduction in yeast viability. In an attempt to explain the occurrence of these problems, we carried out a series of experiments in synthetic wine to determine the effects of high concentrations of ethanol and the presence of free sulfur dioxide, and the synergism between the two, on the vitality, viability and other parameters of yeasts.

The present study shows that the synergism between ethanol and SO_2 can be considered

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to be the main stress factor for yeasts at the beginning of the second fermentation (lag phase) in the production of sparkling wines. Depending on the concentrations of ethanol and SO_2, this synergism modifies cellular homeostasis, deregulates intracellular pH (loss of internal buffering capacity), increases oxidative stress, and interferes with the regulation of gene expression, which can negatively impact the vitality and viability of yeasts, slowing or even interrupting the fermentation process.

The data obtained for the synthetic wine that were supplemented with ethanol, sulfur dioxide and ethanol/sulfur dioxide (Figures 1, 2, 3 and 4), and real base wines (Figure 7) showed the synergistic effect of ethanol and sulfur dioxide on the vitality and viability of yeasts. Despite the yeasts having been adapted, the results show that whether inoculated in an environment with an ethanol concentration close to that of base wines or in an industrial base wine, yeast grows slowly but maintains high viability. This behaviour may be due to fluidisation (Huffer *et al*., 2011; Jones and Greenfield, 1987), the depolarisation of membrane potential by an increased passive proton flux and inhibition of nutrient uptake (Casey and Ingledew, 1986) and a delay in the cell cycle (Kubota *et al*., 2004). Moreover, cells grown in synthetic wine without ethanol and supplemented with 20 mg/L of SO_2 maintained a normal growth rate and high viability, as has been observed in grape must fermentations (Ferreira *et al*., 2017). Conversely, yeast inoculated in wine containing both ethanol and sulfur dioxide exhibited a rapid and strong decrease in cell viability, indicating their synergistic effect on the yeast. The low number of cells that remained viable after the initial shock slowly resumed growth, resulting in a long lag phase and slow fermentation.

The results of the experiment on synthetic musts supplemented with different concentrations of sulfur dioxide show that there was a dose-dependent longer lag phase; the yeasts started to multiply when free SO_2 concentration decayed to approximately 5 mg/L on combination with yeasts and fermentation-derived products, such as acetaldehyde (Ochando *et al*., 2020). However, in the presence of high ethanol concentrations (> 10 % v/v), yeast growth is limited (Jing *et al*., 2018), and consequently free SO_2 concentrations remain high for longer periods of time. Moreover, the presence of ethanol in real wine leads to an increase in SO_2 pKa, which, according to the expression of Henderson–Hasselbalch [free SO_2/1 + 10(pH-pKa1)] increases the molecular SO_2 fraction and consequently its antimicrobial activity.

The reduction in yeast viability during the beginning of fermentation in the presence of sulfur dioxide, and particularly sulfur dioxide and ethanol, is associated with the loss of cell membrane integrity (Figure 2), which is considered a marker of necrotic death (Wloch-Salamon and Bem, 2013). Furthermore, the cells which survived the exposure to ethanol/SO_2 showed a large increase in the intracellular concentration of ROS (Figures 2E and 3C). Experimental data (Figure 3C) indicates that ROS accumulation in ethanol/sulfur dioxide interaction depends on cell metabolism. The accumulation of ROS is one of the main determinants of apoptotic cell death (Farrugia and Balzan, 2012). Apoptosis can function as a defence and preservation mechanism of cell populations in the face of stress (viral pathogens, homeostasis change, nutrient insufficiency and other adverse conditions), ensuring that part of the cells survive to propagate their genome (Fröhlich and Madeo, 2000). When comparing both results obtained regarding ROS accumulation (Figures 2E and 3C), it is possible to observe a difference in fluorescence formation kinetics and intensity. This difference may be related to the temperature at which both experiments were conducted (15 °C Figure 2 and 20 °C figure 3) at the time of the analysis; the populations were at different theoretical stages of fermentation, since the fermentation temperature modifies metabolism and fermentation kinetics (Alexandre, 2019).

It is known that the intracellular pH of yeasts decreases in the presence of sulfur dioxide (Pilkington and Rose, 1988). In an aqueous solution, SO_2 (acid oxide-sulfur dioxide) in its molecular form (SO_2) enters cells more easily, because it has no charge, and the molecule rapidly dissociates to form bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) anions (Divol *et al*., 2012). SO_2 behaves like a weak acid in aqueous environments (Waterhouse *et al*., 2016). Moreover, studies of changes to cell membrane caused by ethanol have shown that it increases passive water transport (Madeira *et al*., 2010), modifies cell membrane fluidity and decreases H^+-ATPase activity, which is responsible for maintaining intracellular pH (Aguilera *et al*., 2006). This modification to membrane structure may facilitate the diffusion of molecular SO_2 into the cytoplasm, which has no charge. The molecular SO_2 fractions found in a solution depends on the pH for a

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modification of the dissociation constant to occur ($SO_2 + H_2O \leftrightarrow SO_2 \cdot H_2O$; $SO_2 \cdot H_2O \leftrightarrow HSO_3^- + H^+$; $HSO_3^- \leftrightarrow SO_3^{2-} + H^+$) (Divol *et al*., 2012; Gould and Russell, 2003; Ribéreau-Gayon *et al*., 2006; Waterhouse *et al*., 2016). The reduction of intracellular pH (loss of internal buffering capacity) may be the result of a "snowball effect", because the lower the cell's intracellular pH, the higher the percentage of the molecular form of SO_2 that would theoretically remain active within the cell, leading to the disruption of cell homeostasis and thus cell death. This hypothesis is reinforced by ethanol having a dose-dependent effect when in association with sulfur dioxide (Figure 3).

Another interesting fact is the size of yeast cells depends on their environment. In the presence of ethanol, there is a tendency for cell structure to change and for part of the cell population to become larger (it becomes swollen) (Figure 5); such an effect on the diameter of *Saccharomyces cerevisiae* cells has been described in other studies (Dinh *et al*., 2008; Kubota *et al*., 2004). However, death caused by the presence of SO_2 and ethanol decreases cell size (Figure 5). Our results indicate that there may be a relationship between cell size and loss of plasma membrane integrity (PI + cells); the percentage of inactivated cells is almost the same as that of cells that become smaller in treatments with 10 % EtOH + SO_2 20 mg/L and 12 % EtOH + SO_2 20 mg/L.

In alcoholic fermentation, the production of acetaldehyde by yeasts plays an important role when in combination with free SO_2 (Rankine and Pocock, 1969), because it decreases the antimicrobial action of SO_2 (Liu and Pilone, 2000). The excess of acetaldehyde produced by the decarboxylation of pyruvate during fermentation is secreted, instead of being used in the production of ethanol or acetic acid (Liu and Pilone, 2000). Increased acetaldehyde has been reported in the second fermentation of sparkling wines (Pozo-Bayón *et al*., 2003), and more acetaldehyde has been found to form in some yeasts (including the EC1118 strain) in the presence of exogenous SO_2 in must fermentations (Li and Mira de Orduña Heidinger, 2020; Li and Mira de Orduña, 2017). However, in the literature, no correlation between the increase in extracellular acetaldehyde and greater stress during the second fermentation of wines in the presence of EtOH and EtOH + SO_2 has been made, in contrast to the present study (Table 2). Our results point to a greater formation of acetaldehyde when ethanol and SO_2 are present together, which corroborates with the results that show an increase in stress when both molecules are present. Another important factor is the relationship between the formation of acetaldehyde by yeasts and their overcoming stress and consequently leaving the lag phase. This was clearly seen in the treatment with 10 % EtOH + 20 mg/L of SO_2 in which there was a delay in acetaldehyde production, probably due to reduced viability (Figures 3A and 4). In the treatment with 12 % EtOH + 20 mg/L of SO_2 practically no acetaldehyde was formed to combine SO_2 and overcome stress, and almost the entire yeast population was inactivated (Figures 3 and 4).

The regulation of gene expression plays an important role in an organism's development and its response to physiological and environmental changes; it can be responsible for the survival of a group of cells and its growth after a period of latency. The results obtained in terms of relative gene expression showed that, compared to the other treatments, there was an increase in the expression of several of the genes involved in sulfur sensing and metabolism in the 10 % EtOH + 20 mg/L SO_2 treatment after 12 and 24 hours (Figure 6). This agrees with the results of the other analyses that show a synergistic increase in stress in this treatment.

The *SUL1* and *SUL2* genes encode proteins located in the plasma membrane with sensory and sulphate (SO_4^{2-}) transport functions from the external environment into the cell (Kankipati *et al*., 2015). While the *SUL2* was the most expressed in treatments with SO_2 only and *SUL1* the most expressed in ethanol only, both these genes were hyper expressed in the EtOH + SO_2 treatment (Figure 6). Moreover, in the EtOH + SO_2 treatment, *FZF1* and *SSU1* are more expressed than in the other treatments. The *FZF1* gene encodes a plasma membrane protein (Fzf1p) involved in the expression of the *SSU1* sulphite efflux pump (Avram *et al*., 1999; Park and Bakalinsky, 2000). In turn, the *SSU1* gene, a marker of adaptive evolutionary advantage that is found in oenological yeast as a result of the use of sulphites in winemaking (García-Ríos *et al*., 2019; Zimmer *et al*., 2014), is positively correlated with the tolerance and detoxification mechanism of sulphite (Marullo *et al*., 2020; Zara and Nardi, 2021).

The *MET14* gene, which encodes the APS kinase enzyme involved in the sulfur assimilation pathway that reduces sulphate to sulphide (Donalies and Stahl, 2002; Noble *et al*., 2015), was hyper-expressed in the first 12 h of the EtOH + SO_2

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treatment. However, after 24 h, gene expression seems to have been more influenced by the presence of ethanol only. As it is a metabolic route gene linked to the biosynthesis of sulfur amino acids, other factors - in addition to the presence of exogenous SO_2 - may influence its greater expression, such as the formation of sulphites by the yeast (Donalies and Stahl, 2002). Surprisingly, no change in expression was found for *COM2*, a gene whose product controls, directly or indirectly, the expression of more than 80 % of the genes activated by SO_2 (Lage *et al*., 2019).

In general, the increase in the expression of these genes may indicate a response to a greater amount of intracellular SO_2 caused by the synergistic effect of ethanol/sulfur dioxide, as, theoretically, more proteins related to this stress would need to be synthesised to create an efficient sulphite efflux in order to survive.

In practical terms, the data obtained in this study show that winemakers should be particularly careful when adding sulphites to base wines for the production of sparkling wines; depending on their concentrations, free sulfur dioxide in the presence of ethanol can lead to the death of a large part of yeast population, and consequently to either the non-development of the second fermentation or a slow fermentation with a long latency phase. It is worth remembering that, when added to base wines to avoid microbial instability and oxidation, sulfur dioxide will remain as free SO_2, since most of the compounds which commonly bind to sulfur dioxide are removed from the lees of the first fermentation and during the stabilisation and filtering processes. Moreover, to attain 11 to 12 % ethanol in sparkling wines, the ethanol concentration of base wines must contain more than 10 % ethanol, which - as seen in this study - interacts with sulfur dioxide, thus increasing the risk of problems in the second fermentation. Therefore, sulphites may literally be responsible for headaches in consumers (Silva *et al*., 2019), but their high concentrations in the production of sparkling wines can also be the cause of "headaches" for winemakers.

CONCLUSIONS

Depending on their concentrations, the presence of both ethanol and SO_2 in the fermentation environment causes synergism and increases stress on yeasts by modifying intracellular homeostasis, deregulating intracellular pH and increasing oxidative stress, thus leading to cell death. Moreover, ethanol/sulfur dioxide causes yeasts to hyper-express genes related to sulphite tolerance in an attempt to overcome their negative effects. A reduction in the vitality and viability of a yeast population can either prevent the second fermentation of sparkling wines from occurring at all or increase its lag phase, thus causing serious technological problems that are difficult to overcome.

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