

Article

Fumaric Acid as an Alternative to Sulphur Dioxide in Conventional and Skin-Macerated White Winemaking

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Abstract

The production of white-skin-macerated wines without sulphur dioxide (SO₂) has increased in recent years, although it presents challenges such as uncontrolled alcoholic fermentation (AF) or malolactic fermentation (MLF) and the production of undesirable compounds. Fumaric acid (FA) is being explored as an alternative to SO₂ due to its antimicrobial and acidifying properties. This study evaluated the effects of vinification type (white skin contact vs. conventional white wine) and different treatments: (i) control without additives, (ii) 0.6 g/L FA, and (iii) 40 mg/L SO₂. After AF, MLF was conducted under three conditions: spontaneous fermentation and two inoculated trials with 10⁶ cells/mL of *Oenococcus oeni*, one without lees and one with 1 g/L lees from AF. Yeast and lactic acid bacteria dynamics during AF were monitored, along with chemical composition, colour, and sensory characteristics of the wines. Results revealed that the development of AF and final wine compositions were influenced by the vinification type and treatment. During MLF, residual FA from the AF was able to delay its progress but was not sufficient to completely inhibit it. The sensory analysis did not reveal significant differences between treatments. Fumaric acid is a viable alternative to SO₂ for managing MLF in high-pH white wines (including those with skin maceration), allowing for lower SO₂ concentrations and enhanced freshness without impacting sensory quality.

Keywords: white skin contact; fumaric acid; SO₂; spontaneous alcoholic fermentation; malolactic fermentation; lees



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1. Introduction

In recent years, interest in ancestral winemaking techniques has resurfaced in the wine industry, driven by the search for wines with greater sensory complexity and market differentiation [1,2]. Among these techniques, white skin-contact wines, also known as *Orange wines*, have gained attention because of their unique profile, which results from the prolonged maceration of the grape must with the skins of white grapes during alcoholic fermentation (AF) [3]. This method, in which the grape skins and seeds remain in contact with the grape must, allows for greater extraction of phenolic compounds, polysaccharides, and aromatic precursors, giving the wine a more pronounced tannic structure and greater sensory complexity compared to conventional white wines, in which skin contact is minimal or non-existent [4–7].

Moreover, climate change presents new challenges for wine production. This leads to rising global temperatures, which affect grape ripening, resulting in higher sugar levels that are converted into ethanol during AF, thereby increasing the alcohol content and pH of wine [8]. Additionally, an increase in pH can cause microbiological instability in wine. Meanwhile, there is also a reduction in the natural acidity of the grape must, which is composed of various acids such as L-malic acid that can later be degraded by lactic acid bacteria (LAB) in a process known as malolactic fermentation (MLF) [9]. MLF is a process in which L-malic acid is converted into L-lactic acid and CO₂ and is generally carried out by LAB species *Oenococcus oeni*, which can originate from grape skins or the environment, but can also be deliberately inoculated. Additionally, *Lactiplantibacillus plantarum* may also be used as a starter for this fermentation [10]. This fermentation can occur spontaneously during or after AF. While MLF is desirable in red wines to soften the acidity and add complexity, it can be problematic in white and white skin-contact wines, as it significantly reduces the acidity and alters the aromatic profile [11]. To prevent its development, sulphur dioxide (SO₂) has traditionally been used throughout the winemaking process as an antimicrobial and antioxidant compound that inhibits LAB proliferation and protects wine from oxidation. However, the use of SO₂ has been debated due to its potential health effects, including allergic reactions, leading to growing consumer demand for wines with lower degrees of chemical intervention. Consequently, the search for new strategies to preserve acidity and prevent unwanted MLF has become a fundamental aspect of modern winemaking [12].

An emerging strategy, although already proven in the 1970s [13,14] in winemaking, is the addition of fumaric acid (FA), a dicarboxylic organic acid with acidifying and antimicrobial properties that is present naturally and is completely safe [15–17]. It has already been applied in multiple industries, such as food production, and its use in wine was regulated by the International Organisation of Vine and Wine (OIV) at a maximum recommended dose of 0.6 g/L (RESOLUTION OIV-OENO 581A-2021) and 0.8 g/L in grape must (RESOLUTION OIV-OENO 738-2025). Additionally, its use at low concentrations has proven effective in improving wine acidity [18–20], making it a viable alternative for regulating acidity in wines with high pH levels resulting from climate change. Therefore, FA may offer a viable alternative to SO₂ as an antimicrobial agent, reducing reliance on this preservative and enabling the production of wines with less oenological intervention [21].

In this context, the use of lees has emerged as an alternative strategy to improve the chemical and organoleptic stability of wine. Lees, which are autolysed yeasts after AF, release nitrogen compounds, polysaccharides, and mannoproteins that can enhance the body, improve protein stability, and contribute to oxidation protection of wines [22,23]. However, recent studies have shown that certain compounds, such as mannoproteins released by lees, may promote LAB growth and MLF development [24,25].

Therefore, this study aimed to evaluate the impact of the type of vinification used during AF (white skin-contact vs. white wine) and the treatment strategy (e.g., control without additives, FA, and SO₂) on the spontaneous microbiota present, chemical composition, microbiological stability, and sensory quality of wine. Additionally, the effects of yeast lees and their impact on MLF after AF were studied.

2. Materials and Methods

2.1. The Preparation of Grapes and Grape Must

Muscat of Alexandria grapes were harvested from the experimental cellar Mas dels Frares in August 2023 (Faculty of Oenology, Rovira i Virgili University, Tarragona, Spain). For the experiment, two types of vinification were applied: white skin-contact fermentation and a traditional white fermentation process (including pressing and grape must clarifi-

cation). For white wine preparation prior to AF, 10 kg of grapes were pressed, and the resulting grape must was treated with 200 mg/L Lallzyme-C-Max pectinase (Lallemand Inc., Montreal, QC, Canada) at 8 °C and clarified after 24 h. For fermentation by contact with the white skin, 10 kg of destemmed and crushed grapes were introduced into a plastic food container.

For each type of vinification, three treatment conditions were established: a control condition with no additives, a treatment with 0.6 g/L FA (Ravago Chemicals, Barcelona, Spain), and another with 40 mg/L of SO₂ in the form of BAKTOL Liquid (Martin Vialatte, Magenta, France) (Table 1). Each of these six conditions was performed in triplicate. The L-malic acid content was adjusted to approximately 2 g/L for skin contact fermentation, with the initial level being 1.14 ± 0.16 g/L ($n = 9$); for grape must after decantation, it was 1.97 ± 0.02 g/L ($n = 9$). The pH was standardised to 3.12 across all conditions with 1 M KOH (Sigma–Aldrich, Barcelona, Spain). The initial grape must density ranged from 1083 to 1085 g/L, with a citric acid content of 0.26 g/L and a yeast assimilable nitrogen (YAN) level of 90 mg/L.

Table 1. Conditions of vinification, treatments, and inoculation for alcoholic fermentation and malolactic fermentation were tested. FA: Fumaric acid; LAB: lactic acid bacteria. Each of these six conditions was performed in triplicate.

Alcoholic Fermentation		Malolactic Fermentation	
Vinification	Treatment	Inoculation	Lees
White skin contact	Control	Spontaneous	-
		Inoculation LAB (10 ⁶ cell/mL)	-
		Inoculation LAB (10 ⁶ cell/mL)	+1 g/L
	FA (0.6 g/L)	Spontaneous	-
		Inoculation LAB (10 ⁶ cell/mL)	-
		Inoculation LAB (10 ⁶ cell/mL)	+1 g/L
	SO ₂ (40 mg/L)	Spontaneous	-
		Inoculation LAB (10 ⁶ cell/mL)	-
		Inoculation LAB (10 ⁶ cell/mL)	+1 g/L
White	Control	Spontaneous	-
		Inoculation LAB (10 ⁶ cell/mL)	-
		Inoculation LAB (10 ⁶ cell/mL)	+1 g/L
	FA (0.6 g/L)	Spontaneous	-
		Inoculation LAB (10 ⁶ cell/mL)	-
		Inoculation LAB (10 ⁶ cell/mL)	+1 g/L
	SO ₂ (40 mg/L)	Spontaneous	-
		Inoculation LAB (10 ⁶ cell/mL)	-
		Inoculation LAB (10 ⁶ cell/mL)	+1 g/L

2.2. Alcoholic Fermentation

All the AFs were carried out spontaneously, without inoculation, at 20 °C under static conditions in 5 L plastic containers, with each condition performed in triplicate. After AF completion, the wines were processed. In the case of white skin-contact fermentation, the grape skins were pressed after fermentation to obtain the final wine. The resulting wines were centrifuged for 10 min at 10,000 rpm using a Sorvall centrifuge (Thermo Scientific, Waltham, MA, USA) and stored at 4 °C for one week to undergo tartaric stabilisation with 15 mg/L bentonite MIRACOL (Martin Vialatte, Magenta, France). An aliquot from each triplicate of each condition was subsequently collected and stored at −20 °C. The AF lees,

obtained before the addition of bentonite, were also preserved by freeze-drying at $-20\text{ }^{\circ}\text{C}$ using a LyoQuest freeze dryer (Telstar, Barcelona, Spain) for two days.

2.3. Malolactic Fermentation

To perform MLF, multiple conditions were prepared, as shown in Table 1. For each type of vinification (white skin-contact and white wine) and for each treatment condition (Control, FA, or SO_2), three MLF conditions were established: a spontaneous control condition, an inoculated condition without lees, and an inoculated condition with the addition of 1 g/L of lees obtained from the AF. Inoculation was carried out with 10^6 cell/mL of the *O. oeni* VP41 strain (Lallemand, Inc.). All experimental conditions were maintained at $20\text{ }^{\circ}\text{C}$ under static conditions, and the experiments were performed in triplicate.

2.4. Monitoring of Fermentation and Populations of Microorganisms

2.4.1. Monitoring of Alcoholic Fermentation

For the monitoring of AF, daily measurements of grape must density were performed using a densimeter (Densito 30PX Portable Density, Mettler-Toledo, Columbus, OH, USA). AF was considered complete when the residual sugar concentration (glucose + fructose), which was enzymatically determined using the Y15 enzymatic multianalyser (Biosystems S.A., Barcelona, Spain), was less than 2 g/L. In addition to density, the evolution of L-malic acid was measured at the following points: initial, 1060, 1040, 1020, 1000 and end of AF. The specific types of chemical analyses performed will be described in detail later in Section 2.5.

2.4.2. Monitoring of Malolactic Fermentations

MLF was monitored by measuring the L-malic acid content every 7 days using an enzymatic kit with a Y15 enzymatic multianalyser (Biosystems S.A.). MLF was considered complete when the L-malic acid content was $<0.1\text{ g/L}$ or when it remained stable for 7 days.

2.4.3. Enumeration of Total Microorganisms During Alcoholic Fermentation

The populations of total viable and culturable yeasts and LAB were monitored at densities of 1085, 1060, 1040, and 1020 g/L and at the end of AF by performing serial dilutions and plating on solid media. To determine the total yeast population, YPD agar media (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 17 g/L agar, Panreac Química SLU, Castellar del Vallés, Spain) were used and incubated at $28\text{ }^{\circ}\text{C}$ for 48 h. The counts of non-*Saccharomyces* yeasts were carried out on lysine media plates (Oxoid, Basingstoke, UK), which were also incubated at $28\text{ }^{\circ}\text{C}$ for 48 h. On the other hand, the LAB were counted on MRS medium plates (Difco Laboratories, Detroit, MI, USA) [26], which were incubated at $27\text{ }^{\circ}\text{C}$ for 7 days in the presence of 10% CO_2 .

2.4.4. Identification of Yeast and Lactic Acid Bacteria During Alcoholic Fermentation

Identification of yeast. A total of 180 colonies were analysed, with 10 colonies randomly selected from each of the following 6 conditions in YPD media: white skin contact and white wine, as well as the control, FA, and SO_2 treatments, at three stages: AF start, middle (1040 g/L density), and end. The selected strains were individually isolated and cultured on solid YPD media. PCR analysis was then performed, targeting the ITS-5.8S rDNA region, and the restriction patterns were analysed following the method described by [27].

Identification of lactic acid bacteria. Due to the lack of LAB growth during AF, only five colonies could be isolated at the beginning of the AF from each of the 6 conditions (white skin-contact or white wine) and (control, FA, or SO_2). Additionally, 5 colonies were isolated from the middle of the AF (1040 g/L density) only in the control condition for both types of winemaking (white skin-contact and white wine). The colonies were selected and grown in modified MRS liquid media for 7 days at $28\text{ }^{\circ}\text{C}$ with 10% CO_2 . Afterward, DNA

was extracted from the obtained pellets using a High Pure PCR Template Preparation Kit (Roche, Barcelona, Spain). Two types of LAB were identified; the non-*Oenococcus* isolates were analysed by 16S-ARDRA gene and *MseI* digestion, after which sequencing [28]. For the *O. oeni* isolates, a specific PCR was used [29].

2.5. Main Compounds of Chemical Analysis

The pH was measured using a Crison Micro pH 2002 pH metre (Hach Lange Spain, l'Hospitalet, Spain). Several compounds, including acetic acid, citric acid, glucose + fructose, L-malic acid, D-lactic acid, L-lactic acid, and yeast assimilable nitrogen, were enzymatically measured with a Y15 enzymatic multianalyser (Biosystems S.A.). FA levels were analysed enzymatically [30]. Glycerol, ethanol, and succinic acid were quantified by high-performance liquid chromatography (HPLC) (Agilent 1100, Agilent Technologies, Waldbronn, Germany) equipped with a multiwavelength RID detector [31]. All analyses and measurements were performed in triplicate.

2.6. Colour Analysis and Total Polyphenolic Index (TPI)

To determine the colour and total polyphenolic index (TPI), the absorbances of the wines were measured at different wavelengths using an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, Amersham, UK). To evaluate the yellow colour, the absorbances at 420 nm were measured [32]. Additionally, TPI was calculated by measuring the absorbance at 280 nm [33]. Additionally, CIELab analysis was performed to determine colour coordinates, such as hue (H^*), lightness (L^*), and chroma (C^*) [34]. CIELab coordinates were calculated using the MSCV software (version 7, University of La Rioja, Spain) (<https://www.unirioja.es/color/descargas.shtml> (accessed on 20 January 2025)). All the samples were prepared and placed in 10 mm or 1 mm thick cuvettes made of plastic or quartz, respectively, with no dilution or after diluting the wine to 1:10, previously centrifuged, and all the measurements were performed in triplicate.

2.7. Sensory Analysis

The wines after alcoholic fermentation and cold stabilisation for one month at 5 °C were sensorially evaluated by fifteen third-year oenology students (3 women and 12 men) aged 22 to 26 from the University Rovira i Virgili (Tarragona, Spain). No additional SO₂ was added to the wines. The tasting took place in a controlled environment (20 °C) to minimise bias and ensure reproducibility of results, and 35 mL of wine was served in standard black glasses. All experimental wine conditions ($p = 6$) were evaluated using the Friedman method (ranking method) by assigning a score of 1 to the lowest perception of the assessed attribute and a score of 6 to the highest intensity, according to the criteria of aromatic intensity, oxidation, acidity, and wine quality. When the Friedman test yielded a significant result ($p < 0.05$), a multiple comparison procedure was performed to identify pairs of wines exhibiting specific differences. The absolute difference between the sums of the ranks of two specific wines was then calculated. Thus, if the difference between two wines is greater than the critical value, the wines are considered to be statistically different; if it is less, the wines are identical for the attribute considered [35].

2.8. Statistical Analysis

All the data were statistically analysed using XLSTAT 2022.5.1.1388 software (Addinsoft, Paris, France). Statistical differences in the analysis of variance (ANOVA) results with Tukey's test (HSD, honestly significant difference) were considered significant at $p < 0.05$. Additionally, principal component analysis (PCA) was performed to examine the relationships between winemaking method and the three treatments after AF. For the first PCA, which examined the presence of yeast species at the beginning and middle of

alcoholic fermentation, the data matrix included 8 variables (yeast species) and 18 observations (3 replicates $\times$ 6 conditions). According to the results of this first PCA, a dendrogram was performed to assess differences in yeast species at the onset and midpoint of AF, using agglomerative hierarchical clustering (AHC). For the second PCA, it comprised 10 variables (chemical parameters) and 18 observations (3 replicates $\times$ 6 conditions) and the data matrix consisted of 10 variables (pH, glucose + fructose, L-malic acid, citric acid, L-lactic acid, D-lactic acid, acetic acid, FA, succinic acid, glycerol, and ethanol) and 18 observations (3 replicates $\times$ 6 conditions).

3. Results

3.1. Alcoholic Fermentation

The results of spontaneous AF (Figure 1A) revealed that fermentation with skin contact had a shorter duration than white fermentation, which lasted between 11 and 13 days.

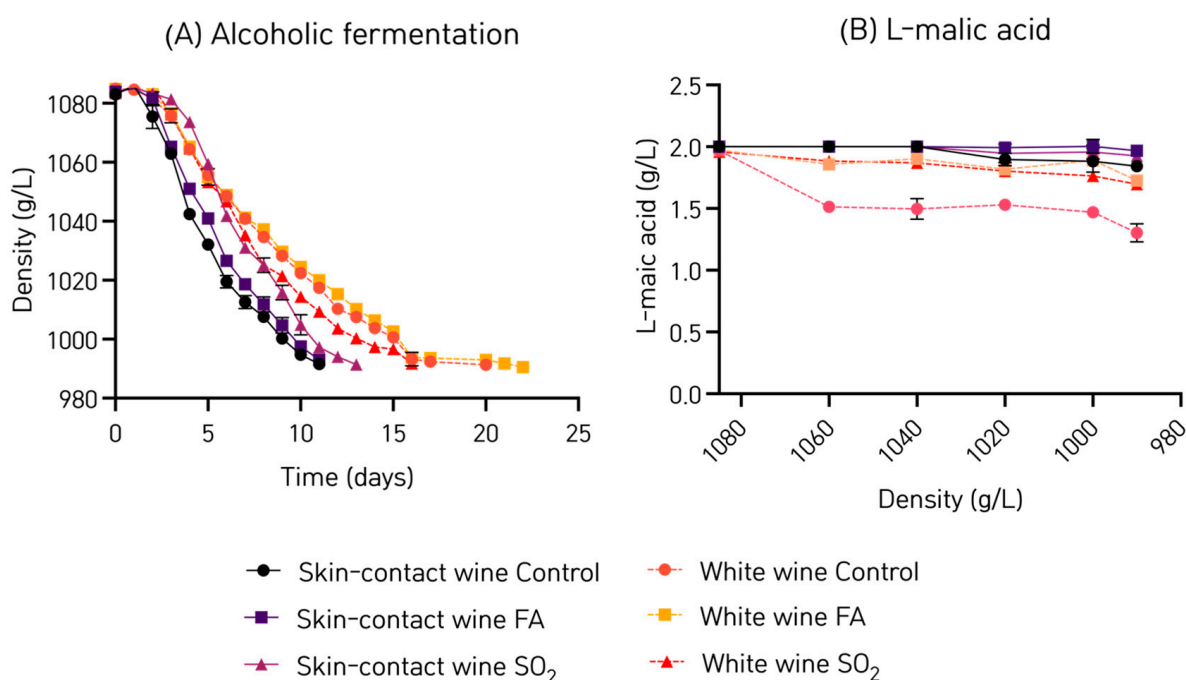


Figure 1. Evolution of density (g/L) (A) and L-malic acid (g/L) (B) during alcoholic fermentation under two vinification conditions: white skin-contact fermentation and traditional white fermentation. The three treatments: control (no additives), FA (0.6 g/L fumaric acid), and SO₂ (40 mg/L sulphur dioxide).

On the other hand, white fermentation without skin contact took longer: the control lasted 20 days, the FA treatment extended to 22 days, and the SO₂ treatment reduced the duration to 16 days. In this case, we observed that the presence of SO₂ improved AF development, which may be due to competition between species. SO₂ acts as an antimicrobial agent, reducing the presence of non-*Saccharomyces* yeasts, whereas *S. cerevisiae*, which is generally more resistant, dominates [36]. As a result, there is no interspecies competition in the early stages of AF, when most nutrients are consumed [37]. Overall, white skin contact enhances AF development, likely through the release of yeast-assimilable compounds that promote AF growth during maceration. These compounds, such as lipids from grape skins, fatty acids such as linoleic acid, and sterols such as β -sitosterol, increase yeast growth by increasing resistance and biomass [38,39]. Fermentations in which part of the grape remains intact also involve anaerobic processes, during which partial intracellular fermentation takes place [40].

With respect to the evolution of L-malic acid (Figure 1B) during AF, significant consumption was observed only in the white wine control condition, where its concentration decreased to 1.30 g/L. This suggests a partial occurrence of MLF and LAB metabolism [8]. Under the other conditions, a slight decrease in L-malic acid was observed, which was more pronounced in white wine but with a reduction of less than 0.3 g/L. This could be attributed to yeast metabolism, as certain yeasts are capable of degrading L-malic acid in the medium during AF [41].

3.2. Evolution of Yeast and Lactic Acid Bacteria During Alcoholic Fermentation

To understand the evolution of AF, the evolution of yeasts and LAB was studied at different stages of the process (start, 1080, 1060, 1040, 1020, and 1000 g/L density, and end of AF). Figure 2 illustrates the evolution of total yeasts (2A), non-*Saccharomyces* yeasts (2B), and LAB (2C).

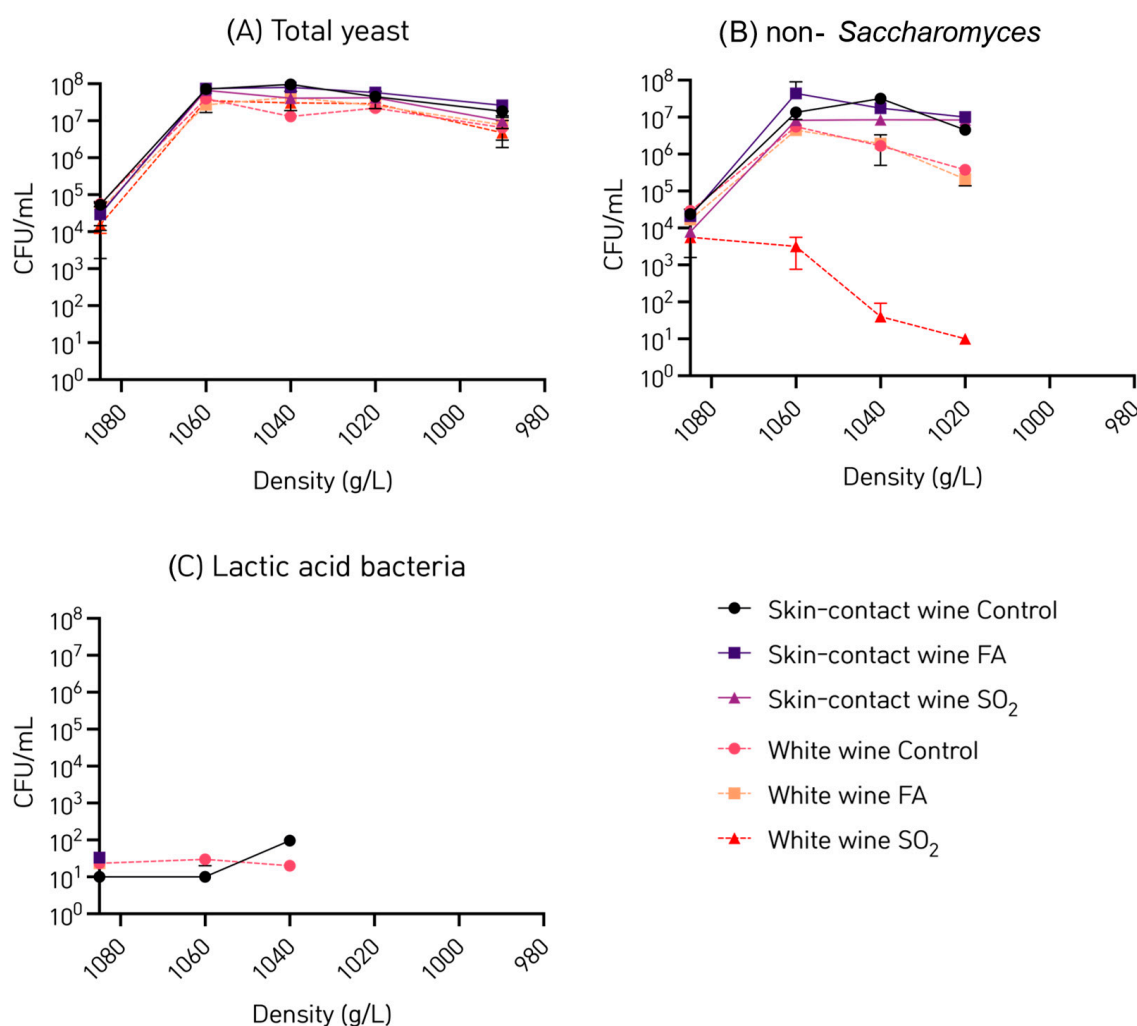


Figure 2. Evolution of total yeasts (A), non-*Saccharomyces* yeasts (B), and LAB (C) during the alcoholic fermentation populations (CFU/mL) under two vinification conditions: white skin-contact fermentation and traditional white fermentation. The three additional treatments include a control (no additives), FA (0.6 g/L fumaric acid), and SO_2 (40 mg/L sulphur dioxide).

With respect to total yeasts, the initial population of 10^4 CFU/mL was not affected by the treatments. Throughout the AF, no significant differences were detected in the total yeast populations across the different vinification methods or treatments, reaching approximately 10^7 CFU/mL. These findings suggest that the conditions were suitable

for yeast multiplication. However, differences were observed in the evolution of non-*Saccharomyces* yeasts, particularly in white skin-contact wines, which presented greater proportions. This may be because grape skins serve as a significant reservoir for various non-*Saccharomyces* species. During maceration, these yeasts can be transferred to the grape must, increasing their presence in the early stages of fermentation [42].

Due to the additional source of nutrients and microorganisms from the grape skins, white skin-contact vinification appears to mitigate the effects of these treatments. Additionally, wines produced under these vinification conditions typically present greater microbial diversities and higher nutrient loads, likely due to the increased extraction of compounds from the skins. In white wines, the treatment differences were more pronounced. Specifically, in white wines treated with SO₂, the non-*Saccharomyces* populations were drastically reduced. For example, research on spontaneous wine fermentation has demonstrated that SO₂ addition significantly affects the dynamics of wild yeast populations, leading to a prevalence of *S. cerevisiae* [43,44].

LAB were detected only at the beginning of AF, with populations of approximately 10¹ CFU/mL. Their growth was limited, showing only a slight increase in the control condition without FA or SO₂ for both vinification types. The lack of LAB growth or their low populations could be attributed to the low pH of the grape must, the sanitary state of the grapes, the competition between yeast and LAB, and the fermentation temperature, which are factors that do not favour LAB development during AF [45–47]. Therefore, FA has been shown to be as effective as SO₂ in inhibiting LAB growth during fermentation [48].

The analysis of the evolution of yeast species during AF in two types of winemaking (white and white skin-contact) and three types of treatment (control, FA, and SO₂) revealed differences in species diversity and dominance throughout the fermentation process. These differences are strongly influenced by the type of winemaking, the treatments applied, and the fermentation stage (Figure 3).

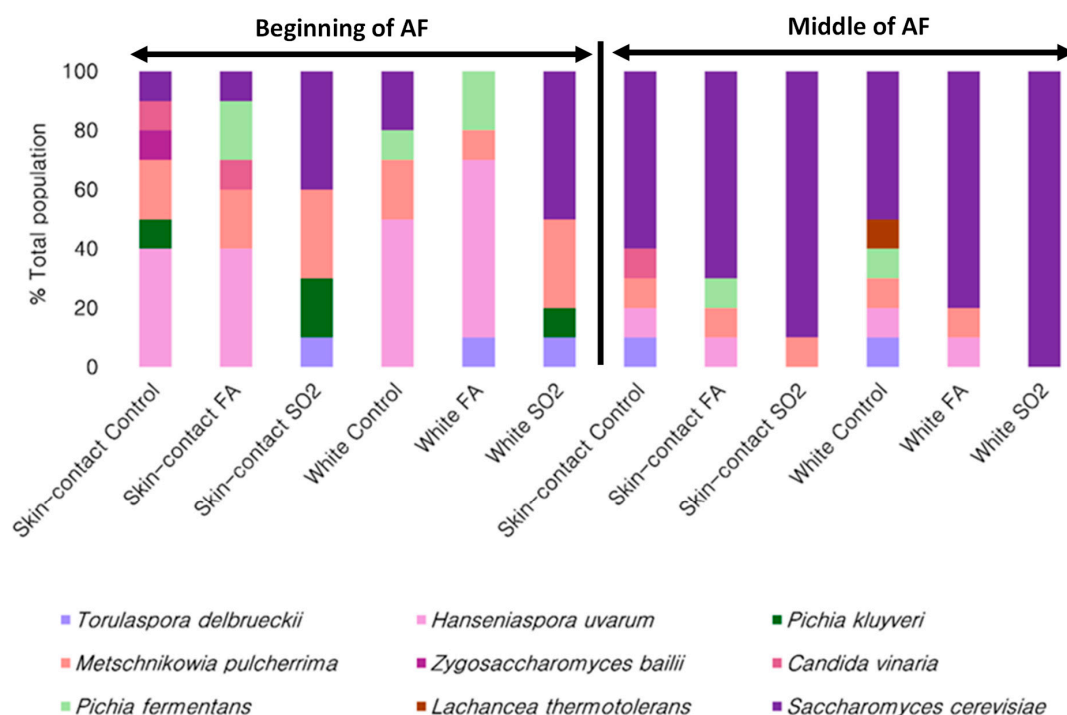


Figure 3. Evolution of the species of yeasts at the start (1085 g/L of density) and the middle (1040 g/L of density) of the alcoholic fermentation expressed in percentage under two vinification conditions: white skin-contact fermentation and traditional white fermentation. The three treatments include a control (no additives), FA (0.6 g/L fumaric acid), and SO₂ (40 mg/L sulphur dioxide).

In the initial stage of fermentation (AF Start), a high species diversity is observed, with greater diversity under skin-contact conditions. These species include *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *S. cerevisiae*, *Hanseniaspora uvarum*, *Zygosaccharomyces bailii*, and *Pichia fermentans*. This may be due to the transfer of yeasts from the skins, as explained previously [35,49]. However, in the treatment with SO₂, the levels of non-*Saccharomyces* yeasts were lower in both types of vinification, leading to a strong selective effect in favour of *S. cerevisiae* implantation throughout the entire AF process. As reported in previous studies, SO₂ use influences the grape must microbiota and reduces the presence of non-*Saccharomyces* yeasts [45,50].

As AF progresses, in the middle stage of fermentation (AF Middle) at a density of 1040 g/L, the species diversity progressively decreases, and *S. cerevisiae* begins to dominate under most experimental conditions, particularly in the FA and SO₂ treatments. Residual populations of *Candida vinaria*, *Lachancea thermotolerans*, and *H. uvarum* still persist. Finally, in the last phase of fermentation (AF End), *S. cerevisiae* becomes the dominant species under all conditions, confirming its key role in AF and its tendency to prevail in the final stages of AF [35,48,49].

With respect to the evolution of LAB, only *Lactiplantibacillus mali*, *Lactiplantibacillus plantarum*, *Lactiplantibacillus hilgardii*, and *O. oeni* were identified for both control vinification conditions at the beginning of AF (Figure S1). In the early stages of AF, the addition of SO₂ and FA reduced the presence of non-*Oenococcus* LAB in both types of vinification. By the middle of the AF (1040 g/L density), *O. oeni* became the only detectable species in the control condition without additives for both types of vinification, reaching 100% of the LAB population. This confirms the inhibitory effects of FA and SO₂ on LAB, as well as the dominance of *O. oeni* due to its ability to adapt to the stressful conditions of wine [50–52]. The mechanism of action of FA on LAB is due primarily to its intracellular entry, which increases the acidity of the medium, altering metabolism and reducing viability [53]. Additionally, intracellular FA has been shown to decrease the efficiency of the glutamate decarboxylase (GAD) system, which is responsible for regulating the intracellular pH and counteracting acidification by increasing the pH [54]. On the other hand, SO₂ acts as an inhibitor of ATPase activity, which is essential for energy production, thereby disrupting metabolism and LAB viability [55].

To understand the statistical trends and effects of winemaking and treatments, a principal component analysis (PCA) was performed for the early and middle stages of AF (Figure 4), along with cluster analysis using a dendrogram (Figure S2). The PCA biplots display the sample distributions on the basis of yeast composition, showing clear differences between the FA and SO₂ treatments compared with the control.

In the initial AF phase, a clear separation between treatments was observed: the control and FA conditions were grouped on the positive F1 axis, whereas the SO₂ conditions were grouped on the negative F1 axis. Additionally, white skin-contact conditions are positioned on the positive F2 axis, whereas white winemaking conditions are positioned on the negative F2 axis. In general, *S. cerevisiae* is predominantly associated with SO₂ treatments in both vinification types. However, *T. delbrueckii* and *M. pulcherrima* are linked to SO₂ treatments, suggesting that these non-*Saccharomyces* yeasts were the most resistant to SO₂ of all the yeasts tested. For example, a study showed that among different non-*Saccharomyces* strains, *T. delbrueckii* and *M. pulcherrima* were two of the species that adapted best to SO₂ [56]. *H. uvarum* and *P. fermentans* were more strongly related to the white control and FA treatments. *Candida vinaria* and *Z. bailii* were closely associated with the skin-contact control group. The dendrogram (Figure S2) results clearly indicate the presence of two distinct clusters, where the SO₂ treatment acts as the primary differentiator at this stage of AF.

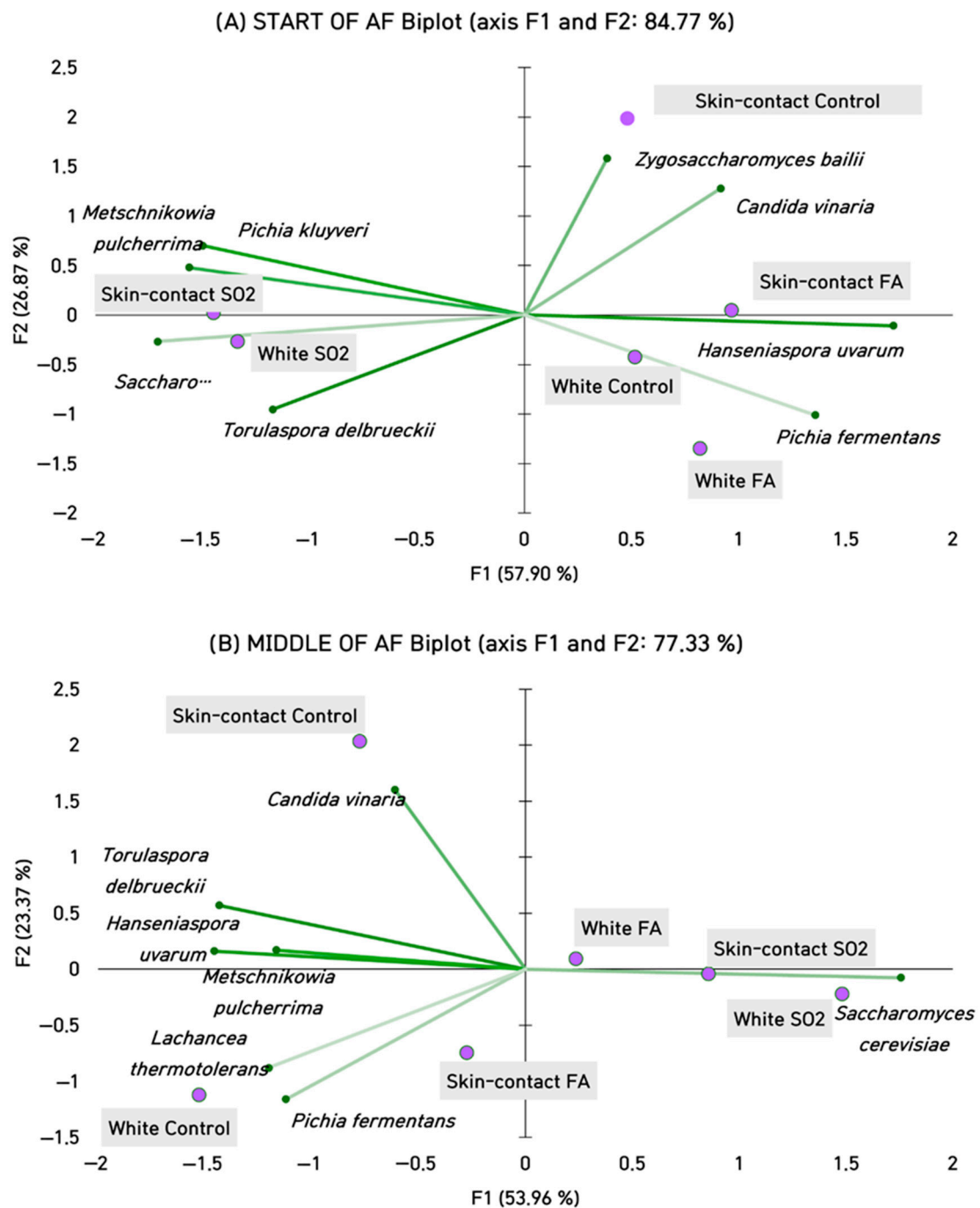


Figure 4. Principal component analysis biplot of alcoholic fermentation during white skin-contact vinification at two stages: (A) early fermentation (density 1085 g/L) and (B) mid-fermentation (density 1040 g/L). The analysis displays the distribution of samples according to yeast species, treatment (control (no additives)), FA (0.6 g/L fumaric acid), SO₂ (40 mg/L sulphur dioxide), and type of vinification (white skin-contact fermentation and traditional white fermentation). Points represent the wine samples (observations), and vectors indicate the contribution and direction of the original variables to the principal components.

In the middle phase of fermentation, *S. cerevisiae* became dominant in most treatments, particularly the SO₂ treatment. *Candida vinaria* remains associated with the skin-contact control, suggesting that in the absence of SO₂ or FA, some non-*Saccharomyces* yeasts can persist until this stage. At this point in the AF, there was no clear distribution based on winemaking type, indicating that the presence of nutrients or interspecies competition did

not follow distinct patterns. The dendrogram (Figure S2) at this fermentation stage shows that the conditions are distributed into four clusters, where the control conditions for both winemaking types are separated. However, the FA and SO₂ treatments did not result in differences between winemaking types.

3.3. Composition of Wines After Alcoholic Fermentation

After the AFs were conducted, the wines were analysed, and it was observed that there were significant differences between the conditions in terms of their final compositions (Tables 2 and S1). The pH values ranged from 3.05 to 3.18, with significant differences between the vinification methods. Compared with white wines, skin-contact wines presented a significantly greater pH (average of 3.14–3.07). This difference can be attributed to a greater extraction of potassium from contact with the grape skins during alcoholic fermentation, which can lead to the precipitation of tartaric acid, insoluble as the alcohol content increases. This generally results in an increase in pH [57]. Significant differences were also observed between the treatments. The control wine had the highest pH, whereas the wines treated with FA and SO₂ presented slightly lower values, with no significant differences between them. The decreased pH in FA-treated wines may be due to the direct addition of this organic acid, which contributes to the total acidity of the wine [1,57]. On the other hand, SO₂ might indirectly influence pH reduction by inhibiting microorganisms that could increase pH during fermentation or by reacting with specific compounds in the medium [58].

Table 2. Significant differences in chemical parameters after alcoholic fermentation in wines obtained through two types of vinification (white skin-contact and white) and three different treatments: control (without additives), addition of 0.6 g/L of fumaric acid (FA), and addition of 40 mg/L of sulphur dioxide (SO₂). All the values were expressed in g/L, except pH and ethanol % *v/v*. The *p*-value column indicates the significance of the effect of vinification or treatment for each parameter. The different letters indicated a significant difference.

	Vinification			Treatments			
	White Skin-Contact	White	<i>p</i> -Value	Control	FA	SO ₂	<i>p</i> -Value
pH	3.14	3.07	<0.0001	3.14 ^a	3.09	3.09 ^b	<0.0001
Glucose + Fructose	1.66	1.70	0.7560	1.58 ^a	1.79 ^a	1.67 ^a	0.7560
L-malic acid	1.85 ^a	1.58 ^b	<0.0001	1.54 ^b	1.79 ^a	1.81 ^a	<0.0001
Citric acid	0.23 ^a	0.20 ^b	0.0060	0.22 ^a	0.21 ^a	0.22 ^a	0.0060
L-lactic	0.02 ^b	0.09 ^a	0.0090	0.10 ^a	0.01 ^b	0.05 ^{ab}	0.0090
D-lactic	0.13 ^a	0.07 ^b	<0.0001	0.11 ^a	0.10 ^b	0.09 ^b	<0.0001
Acetic acid	0.39 ^a	0.34 ^b	0.0800	0.38 ^a	0.35 ^a	0.37 ^a	0.0800
Fumaric acid	0.07 ^a	0.02 ^a	0.1970	0.00 ^a	0.13 ^a	0.01 ^a	0.1970
Succinic acid	1.70 ^a	1.43 ^b	0.0000	1.39 ^b	1.81 ^a	1.48 ^b	0.0000
Glycerol	7.06 ^a	5.38 ^b	<0.0001	6.37 ^a	6.24 ^a	6.04 ^b	<0.0001
Ethanol	13.44 ^b	14.08 ^a	<0.0001	13.72 ^a	13.80 ^a	13.75 ^a	0.0001

The glucose and fructose levels ranged from 1.51 to 1.88 g/L, with no significant differences between the vinification methods or treatments applied, indicating that the addition of FA and SO₂ did not substantially impact the yeast fermentation efficiencies under these conditions. Significant differences in the L-malic acid levels were detected between the different types of vinification and the different treatments. A general consumption of L-malic acid was observed during AF, which is a normal process since yeasts can metabolise this acid during AF [59].

However, the L-malic acid levels were greater in white skin-contact wines, possibly because the extraction of compounds from grape skins could influence bacterial activity and contribute to acid retention. In particular, wines treated with SO₂ presented the highest L-malic acid levels, whereas white wines presented lower concentrations, with significant differences between vinification types. In both cases, *S. cerevisiae* was the predominant yeast. Additionally, some studies have reported that non-*Saccharomyces* yeasts can consume greater amounts of L-malic acid than can *S. cerevisiae* [60].

Slight variations in citric acid levels were observed, with significant differences between vinification types, indicating that skin-contact wines had higher average citric acid contents than white wines did. Fermentation with skin contact may favour the retention of this acid by inhibiting bacterial consumption due to the presence of phenolic compounds extracted from the skins [61]. However, no significant differences were found between the treatments, although the wines treated with SO₂ presented the highest citric acid concentrations. Compared with those in the FA and SO₂ treatments, apparent levels of L-lactic acid were detected only in white wines, with the highest values observed in the control, in which a slight increase in the LAB population was observed at the start of AF. This increase occurred because L-lactic acid is the product of MLF, although some yeasts can produce it [41,62], and both FA and SO₂ have strong antimicrobial effects [18,33,47]. Significant differences were also observed between vinifications, with white wines having a relatively high concentration. This may be because white wines contain relatively low concentrations of phenolic compounds that can inhibit the growth of *O. oeni* [63]. D-lactic acid was detected at higher concentrations in white skin-contact wines than in white wines, with significant differences. Additionally, the ANOVA results for the different treatments also revealed significant differences, with the white wine in the control treatment having the highest concentration compared with those in the FA and SO₂ treatments. The greater presence of D-lactic acid in the control treatment could be related to a different microbiota, favouring the action of some non-*Saccharomyces* yeasts, such as *L. thermotolerans*, which produce more of this isomer during fermentation [64–66]. The acetic acid concentrations did not significantly differ across the values. Additionally, no differences were detected between vinification types or treatments. FA was detected only in the FA treatment, as expected, with levels reaching 0.20 g/L in the white skin-contact wines and 0.04 g/L in the white wines, with no differences between the contact with the skin and the treatments, with high standard deviations in the case of the FA content of the white skin-contact wine. The almost complete disappearance of FA under the conditions in which it was added may be due to the degree of dissociation in which it is found at low pH values, such as 3.12, where FA has high bioavailability and can enter yeast cells [54,67,68]. Higher levels of succinic acid were detected in white skin-contact wines, especially those subjected to FA and SO₂ treatments. The results revealed significant differences between vinification types. Succinic acid is produced during AF, and the greater nutrient availability in the skins of white skin-contact wines likely favours succinate production by yeasts [69]. There were also significant differences between the treatments, with the FA treatment resulting in the highest concentration of succinic acid. The higher concentrations of succinic acid in wines treated with FA may also be linked to yeast metabolism because yeasts can transform FA into succinic acid through the tricarboxylic acid cycle [15]. Glycerol levels significantly differed between vinification types, with white skin-contact wines exhibiting relatively high levels. Additionally, there were significant differences between the treatments, with the control and FA treatments resulting in relatively high glycerol values. In white skin-contact wines, yeast fermentation may have been more stressful, promoting higher glycerol production [70]. In contrast, white wines had higher alcohol contents, as demonstrated in a study by [71], where traditional white wines presented higher alcohol contents than

did skin-contact wines. However, no significant differences in ethanol production were observed between the treatments.

Additionally, Figure 5 presents PCA plots, each displaying different combinations of principal axes, F1 and F2.

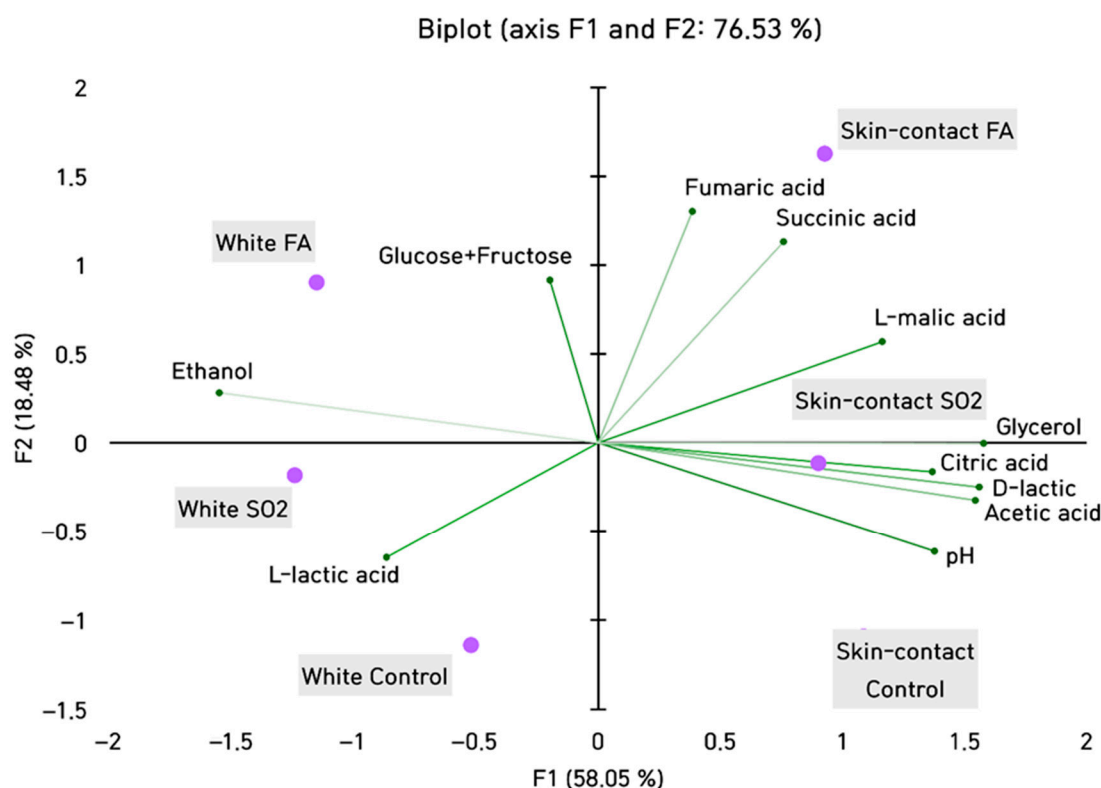


Figure 5. Biplot of the principal component analysis after alcoholic fermentation under two vinification conditions: white skin-contact fermentation and traditional white fermentation, and three addition treatments include a control (no additives), FA (0.6 g/L fumaric acid), and SO₂ (40 mg/L sulphur dioxide). Points represent the wine samples (observations), and vectors indicate the contribution and direction of the original variables to the principal components.

The purple points represent the active observations, which are different wine conditions. The green arrows in the biplots represent the active variables, which are chemical measures such as glucose + fructose, various organic acids (e.g., succinic acid, FA, L-malic acid, and acetic acid), and glycerol, all expressed in g/L, ethanol, and pH. The PCA revealed that the analysed chemical variables were strongly correlated with the differentiation of the wines, suggesting that the vinification method plays a principal significant role in shaping their chemical compositions. These PCA results confirmed that different vinification and addition conditions modulated the final wine compositions.

3.4. Colour of Wine After Alcoholic Fermentation

The analysis revealed significant differences in yellow colour ($A_{420\text{nm}}$), total phenolic index (TPI), and CIELAB parameters, depending mainly on the vinification method rather than on the treatment applied (Table 3). The white skin-contact vinification significantly enhanced the extraction of phenolic compounds and orange-yellow pigments. As a result, the wines obtained presented high TPI values and increased $A_{420\text{nm}}$. These findings are consistent with previous studies demonstrating that white skin-contact fermentation promotes the solubilization of tannins (flavanol family) and flavonols, thus changing the colour and phenolic complexity of wine [72–74]. In contrast, wines produced through white fermentation presented considerably lower TPIs and significantly lower colour intensities.

The lower extraction of phenolic compounds can be attributed to the pressing and clarification steps prior to AF, which decrease the number of suspended solids that contribute to wine colouration and phenolic complexity. Other studies have indicated that traditional white fermentation yields lighter-coloured wines with lower colour saturations [75]. With respect to the effects of the different treatments, the colour intensity and oxidation levels of the control wines were greater than those of the control wines. Wines treated with FA displayed oxidation levels similar to those of those treated with SO₂, as well as comparable colour intensities.

Table 3. Colour parameters after alcoholic fermentation in wines obtained through two vinification conditions (white skin-contact and white) and three different treatments: control (without additives), addition of 0.6 g/L of fumaric acid (FA), and addition of 40 mg/L sulphur dioxide (SO₂). Mean values $\pm$ standard deviation are presented for each condition. Capital letters for each parameter indicate a significant effect of the two effects (winemaking and conditions) using two-way ANOVA at $p < 0.05$. AU, Arbitrary Unit.

	White Skin-Contact			White		
	Control	FA	SO ₂	White Control	White FA	White SO ₂
Total Phenolic Index (AU)	3.52 $\pm$ 0.01 ^A	3.56 $\pm$ 0.14 ^A	3.15 $\pm$ 0.03 ^B	1.87 $\pm$ 0.02 ^C	1.80 $\pm$ 0.08 ^C	1.69 $\pm$ 0.18 ^C
Yellow colour (420 nm)	0.96 $\pm$ 0.01 ^A	0.60 $\pm$ 0.15 ^B	0.67 $\pm$ 0.02 ^B	0.15 $\pm$ 0.00 ^C	0.16 $\pm$ 0.00 ^C	0.18 $\pm$ 0.00 ^C
L*	71.9 $\pm$ 1.18 ^B	64.47 $\pm$ 0.49 ^C	73.2 $\pm$ 6.55 ^B	96.6 $\pm$ 0.17 ^A	96.60 $\pm$ 0.17 ^A	95.23 $\pm$ 0.23 ^A
C*	19.37 $\pm$ 5.64 ^B	31.34 $\pm$ 0.54 ^A	20.02 $\pm$ 7.18 ^B	9.08 $\pm$ 0.32 ^C	9.08 $\pm$ 0.32 ^C	9.48 $\pm$ 0.38 ^C
h*	59.59 $\pm$ 33.92 ^B	83.29 $\pm$ 0.49 ^{AB}	78.59 $\pm$ 18.13 ^{AB}	96.81 $\pm$ 4.37 ^A	96.81 $\pm$ 4.37 ^A	94.77 $\pm$ 0.59 ^A

Additionally, in skin-contact wines, the addition of SO₂ reduced phenolic extraction and decreased the colour intensities. In white wines, SO₂ further lowered TPI, suggesting its role as an antioxidant, protecting phenolic compounds from oxidation and preventing colour darkening.

These results are consistent with other studies demonstrating that SO₂ preserves chromatic stability in white wines and prevents oxidative reactions [72,73]. CIELab values revealed that white wines presented higher (L*) values, indicating greater clarity, whereas skin-contact wines presented lower luminosities, reflecting darker and more saturated colours (Table 3). The colour saturation (C*) was significantly higher in the skin-contact wines than in the white wines, where the lower phenolic concentrations resulted in reduced chromatic intensities. The hue angle (h*) varied between yellow and orange tones in skin-contact wines, whereas white wines presented cooler and more homogeneous hues. These results are consistent with studies indicating that white skin-contact fermentation enhances the extraction of phenolic compounds, leading to wines with more intense and saturated colours [76].

3.5. Sensory Analysis

Using the Friedman test, a non-parametric statistical method used to detect differences in treatments across multiple test attempts, wines obtained by two types of winemaking and three treatments (control, addition of fumaric acid, or sulphite dioxide (SO₂)), were differentiated according to four attributes: aromatic intensity, oxidation, acidity, and overall quality. As shown in Table 4, wines were significantly different for three attributes except the aromatic intensity. From these results, a multiple pairwise comparison was performed to identify which specific pairs of wines differ. Regarding the strange absence of significant difference for the aromatic intensity attribute, maceration by contact with the skins (orange wines) generally increases the extraction of aromatic precursors, in particular terpene precursors of the Moscatel of Alexandria grape variety [5]. On the other hand, the oxidation

attribute allows a significant grouping of pellicular maceration wines with respect to other classic white wines, especially with respect to control wines and the treatment with the addition of fumaric acid (Table 4). Regarding acidity, it would seem that the panellists perceived the orange wines as more acidic than the other wines, regardless of the acid effect that the addition of fumaric acid or sulphite dioxide could cause. However, they are both significantly more acidic. Finally, in terms of overall quality, white wines and skin-macerated white wines with added fumaric acid formed a significant and preferred compact group among the panellists.

Table 4. Comparative sensory profile: Skin-contact vs. conventional white wines (assessors = 15) using the Friedman test and pairwise comparisons (post hoc analysis). Sum of ranks and *p*-value determined by the Friedman test; BrC: skin-contact (Control); BrFA: skin-contact fumaric acid; BrSO₂: skin-contact (SO₂); BIC: white wine (Control); BIFA: white wine (fumaric acid); BISO₂: white wine (SO₂). n.s.: no significant. Different lowercase letters (a, b, c) indicate significant groups within the same attribute.

Attribute	Wines						<i>p</i> -Value
	BrC	BrFA	BrSO ₂	BIC	BIFA	BISO ₂	
Aromatic Intensity	61.5	53.5	50.5	49	57	43.5	n.s.
Oxidation	72.0 ^a	67.0 ^a	57.0 ^{ab}	51.5 ^{bc}	37.5 ^c	32.0 ^c	<0.001
Acidity	55.5 ^{ab}	68.5 ^a	64.5 ^a	48.0 ^{bc}	43.0 ^{bc}	35.5 ^c	<0.01
Overall Quality	31.5 ^b	52.5 ^a	35.5 ^b	66.5 ^a	65.0 ^a	64.0 ^a	<0.001

In conclusion, sensory analysis indicated that oxidation, acidity, and overall quality perception were the only criteria showing significant differences between the wines. According to Figure 6, regardless of the treatment applied, the “classic” white wines appeared to be preferred to the skin-contact wines, the latter being judged to be more oxidised and acidic.

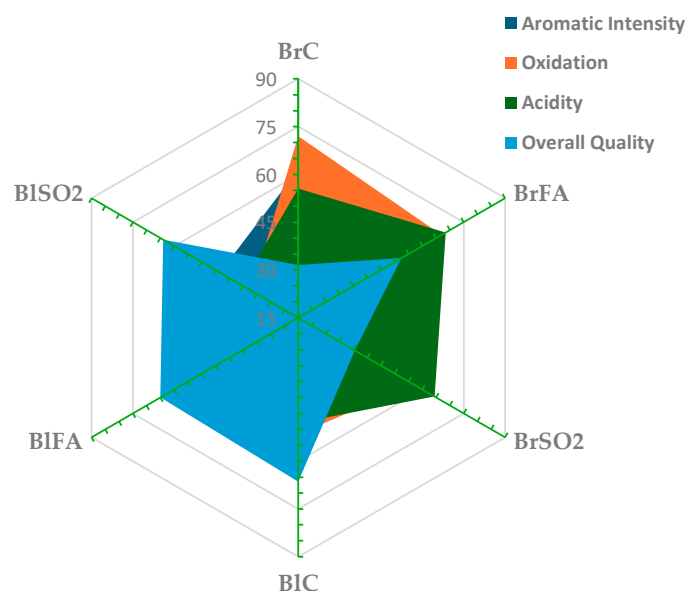


Figure 6. Radar graph of the results of the classification test performed by 15 panellists on 4 attributes. BrC: skin-contact (Control); BrFA: skin-contact fumaric acid; BrSO₂: skin-contact (SO₂); BIC: white wine (Control); BIFA: white wine (Fumaric acid); BISO₂: white wine (SO₂). The scores represent the total sum on a scale of 15 to 90 for six wines.

3.6. Malolactic Fermentation

To observe whether FA has an inhibitory or delaying effect on the development of MLF compared with SO₂, MLF was carried out under multiple conditions. For each type of wine, either skin-contact or white, and for each additive condition, three MLF conditions were established: a spontaneous control condition, an inoculated condition without lees, and an inoculated condition with the addition of 1 g/L of yeast lees. The results revealed differences in MLF development depending on the vinification type (Figure 7).

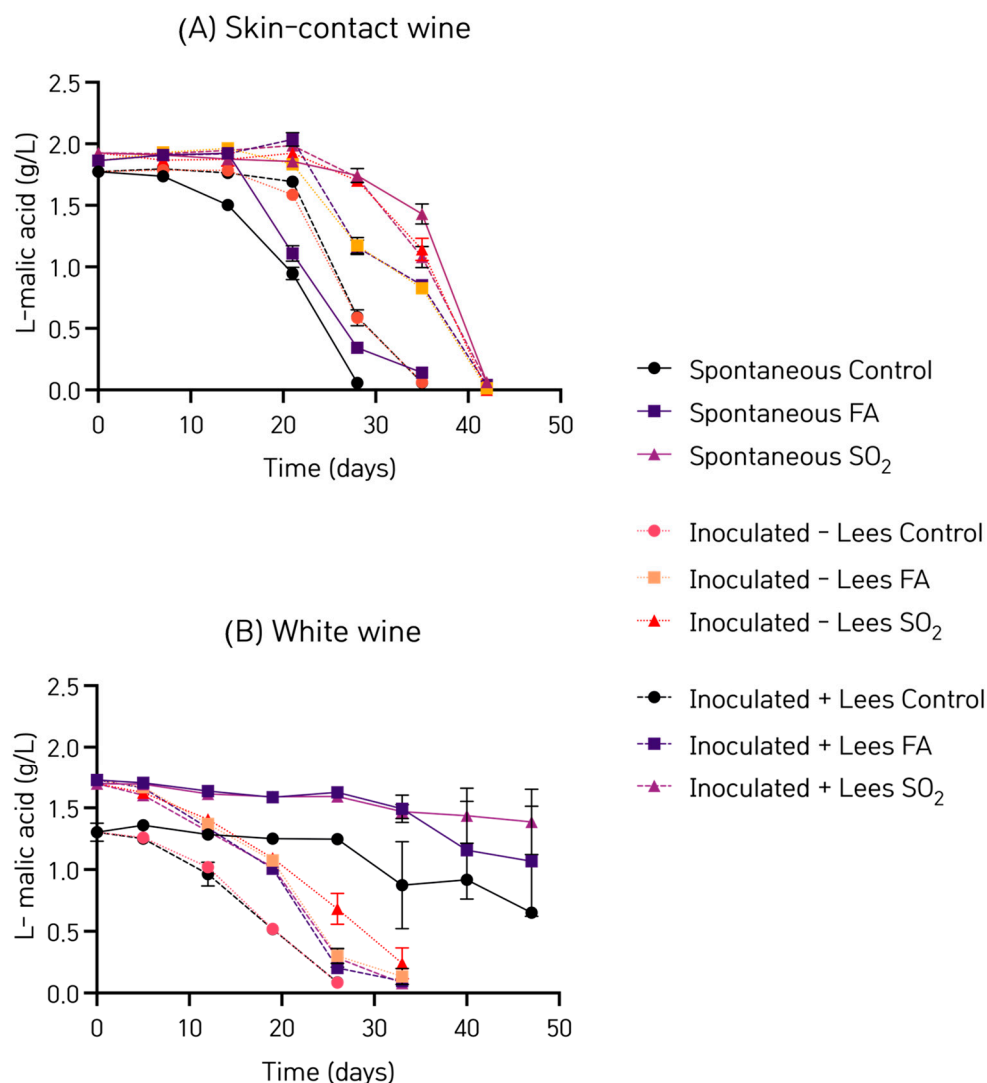


Figure 7. Evolution of L-malic acid (g/L) during malolactic fermentation under two vinification conditions: white skin-contact fermentation (A) and traditional white fermentation (B), and three addition treatments include a control (no additives), FA (0.6 g/L fumaric acid), and SO₂ (40 mg/L sulphur dioxide).

In general, skin-contact wines (Figure 7A) completed MLF in all the cases, with spontaneous conditions promoting their progression. This could be due to the adaptation of the existing LAB in wine to the environment [26]. Additionally, the presence of lees improved MLF development regardless of the treatment, likely because they provided essential nutrients such as peptides, amino acids, and growth factors, favouring LAB metabolism and thus enhancing MLF [10]. The SO₂ conditions, however, required the longest time for MLF completion, as this compound has well-documented antimicrobial effects [12,33,60,77].

In the case of the white wine conditions (Figure 7B), spontaneous fermentation did not complete MLF. In general, inoculation with *O. oeni* VP41 significantly accelerated

MLF, which may be due to an increase of up to 10^6 cells/mL, in contrast to spontaneous conditions, in which the initial concentration of LAB was not detectable. Additionally, we observed that the control conditions resulted in the highest consumption of L-malic acid, suggesting an inhibitory effect from FA and SO_2 . Furthermore, in this case, the addition of lees also improved MLF kinetics.

These findings highlight the impact of vinification methods and additive treatments on MLF efficiency. The presence of lees consistently supports LAB metabolism, enhancing MLF completion [24,25]. Moreover, the SO_2 -treated wines presented the slowest MLF progression due to their antimicrobial activities. Skin-contact wines showed greater adaptability to spontaneous MLF, whereas in white wines, inoculation was essential to achieve complete L-malic acid conversion.

4. Limitations

This study presents several limitations that should be considered when interpreting the results. First, the experiments were conducted at a pilot scale (5 L), which may not fully replicate the dynamics of industrial-scale vinification. Microbial behaviour, oxygen exposure, and extraction kinetics can differ under larger-volume conditions.

Second, only one grape variety (Muscat of Alexandria), a single maturity, and a single harvest (2023) from one vineyard were evaluated. Therefore, the findings may not be directly extrapolated to other grape varieties, vintages, or terroirs with different physicochemical characteristics and indigenous microbiota.

Third, a single concentration of FA (0.6 g/L) and SO_2 (40 mg/L) was tested. It is necessary to use 0.8 g/L of fumaric acid (maximum recommended dose to must by OIV). Dose–response relationships were not assessed, and different concentrations could potentially lead to different microbial or sensory outcomes.

Additionally, MLF was evaluated under controlled inoculation conditions and spontaneous fermentation, but broader strain variability of lactic acid bacteria was not explored.

Finally, the sensory analysis was conducted with a limited panel ($n = 15$) with wines after alcoholic fermentation, which, although trained, may not fully represent broader consumer perception. Further studies, including larger-scale trials, multiple vintages, and expanded sensory panels, are necessary to confirm the robustness and general applicability of these findings.

5. Conclusions

This study highlights the significant impact of vinification type on AF and MLF, particularly under spontaneous conditions. Compared with traditional white winemaking, white skin-contact winemaking improved the progression of fermentation, facilitating fermentation development. With respect to yeast dynamics, white skin-contact wines presented greater variability in non-*Saccharomyces* yeast populations during the early stages of AF. However, by mid-AF, the species diversity decreased in both vinification types, and *S. cerevisiae* became dominant. The final wine compositions also significantly differed, where white wines were associated with higher ethanol contents, whereas white skin-contact wines presented higher pH values, glycerol contents, and D-lactic acid concentrations, possibly because of the presence of higher concentrations of non-*Saccharomyces* yeast. Sensory analysis revealed that oxidation was the only significant difference, with greater oxidation perception in white skin-contact wines. MLF was also influenced by the type of vinification, with a decrease in time in the case of white skin-contact wines. With respect to the effects of the various treatments, no significant effects on the AF kinetics were observed. However, yeast populations were affected, with both FA and SO_2 significantly reducing yeast diversity, leading to increased dominance of *S. cerevisiae*.

Additionally, these treatments influenced the final wine composition, with FA increasing the succinic acid concentration. Therefore, skin-contact wines present a greater colour intensity, TPI, and oxidation, with a clear tendency towards intense orange and yellow tones. Furthermore, FA treatment increased the chroma and colour intensity in skin-contact wines, and the addition of SO₂ reduced the colour intensity. In terms of MLF development, residual FA from the AF delayed its progression but was not sufficient to completely inhibit it. Therefore, reintroducing FA after AF is recommended if MLF inhibition is desired. However, FA could serve as a useful tool to control spontaneous MLF during AF while still allowing its controlled development post-AF using selected LAB strains. Finally, yeast promoted MLF in all the cases.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/beverages12050056/s1>. Figure S1: Evolution of the species of lactic acid bacteria; Figure S2: Dendrogram of the ACH analysis; Table S1: Chemical parameters after alcoholic fermentation in wines.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AF	Alcoholic Fermentation
AHC	Agglomerative Hierarchical Clustering
ANOVA	Analysis of Variance
ARDRA	Amplified Ribosomal DNA Restriction Analysis
CFU	Colony Forming Units
CI	Colour Intensity
CIELab	Commission Internationale de l'Éclairage Lab colour system
CO ₂	Carbon Dioxide
DNA	Deoxyribonucleic Acid
FA	Fumaric Acid
g/L	grams per litre
HPLC	High Performance Liquid Chromatography
HSD	Honestly Significant Difference
ITS	Internal Transcribed Spacer
KOH	Potassium Hydroxide

L	Litres
LAB	Lactic Acid Bacteria
mg/L	milligrams per litre
MLF	Malolactic Fermentation
mm	millimetres
MRS	de Man, Rogosa and Sharpe medium
nm	nanometres
OIV	International Organisation of Vine and Wine
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
rDNA	Ribosomal DNA
RID	Refractive Index Detector
rpm	revolutions per minute
SO ₂	Sulphur Dioxide
TPITLA	Total Polyphenolic IndexThree Letter Acronym
YANTPI	Yeast Assimilable NitrogenTotal Polyphenolic Index
YPDYAN	Yeast extract Peptone Dextrose mediumYeast Assimilable Nitrogen
% v/vYPD	percent volume/volumeYeast extract Peptone Dextrose medium
°C% v/v	degrees Celsiuspercent volume/volume
°C	degrees Celsius

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