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Effect of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* Co-Inoculation on Alcoholic Fermentation Behavior and Aromatic Profile of Sauvignon Blanc Wine

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Abstract: Enhancing the sensory profile of wines by exposing the aromas of the grape variety through the involvement of microorganisms has always been a challenge in winemaking. The aim of our work was to evaluate the impact of different fermentation schemes by using mixed and pure cultures of different *Saccharomyces* species to Sauvignon blanc wine chemical composition and sensory profile. The Sauvignon blanc must has been inoculated with mixed and pure cultures of *S. pastorianus* and *S. cerevisiae* strains. For the mixed fermentation schemes, one strain of *S. pastorianus* has been inoculated with different proportions of *S. cerevisiae* (*S. pastorianus* to *S. cerevisiae*: 99%–1%, 95%–5%, 90%–10%, 80%–20% and 70%–30% *w*/*w*) in co-inoculation with two commercial strains of *S. cerevisiae*. A total of 13 fermentations trials, three monocultures and 10 mixed cultures were performed in biological triplicate. The fermentation kinetics have been controlled by density measurement and classical oenological analyses were performed based on the International Organisation of Vine and Wine (OIV) analytical methods. The population dynamics were evaluated by the specific interdelta PCR reaction of the *Saccharomyces* species at the beginning and at the end of the fermentation process. The volatile compounds of the wine aroma, such as the esters, higher alcohols and thiols were analyzed by GC/MS. Sensory assessment by trained panel was carried out for all produced wines. Complete depletion of the sugars was achieved between 10 and 13 days for all the fermentation trials. The population dynamics analysis revealed that the *S. cerevisiae* strain was the most predominant at the end of the fermentation process in all inoculation ratios that were tested. The wines that were fermented with *S. pastorianus*, either in pure or mixed cultures, were characterized by significantly lower acetic acid production and higher malic acid degradation when compared to the wines that were fermented only with *S. cerevisiae* strains. The aroma profile of the produced wines was highly affected by both inoculation ratio and the *S. cerevisiae* strain that was used. The presence of *S. pastorianus* strain enhanced the production of the varietal thiols when compared to the samples that were fermented with the *S. cerevisiae* pure cultures. The mixed inoculation cultures of *Saccharomyces* species could lead to wines with unique character which can nicely express the varietal character of the grape variety.

Keywords: *S. pastorianus*; yeast interaction; Sauvignon blanc; varietal aromas; wine typicity

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1. Introduction

During winemaking, the consortium of microorganisms rapidly evolves and adapts to the environmental changes. A plethora of genera and species have been identified, and many biotic and abiotic factors shape their population structure and dynamics. The unique microbiota composition of each region is affected mainly by the microclimate, the viticulture and the winemaking techniques that are used. In this regard, the microorganisms play an essential role, which can affect the final product in a positive or negative way [1]. Without a doubt, *Saccharomyces cerevisiae* is the yeast species that is considered to be adapted to the winemaking conditions, which is reflected in its ability to carry out the alcoholic fermentation and also create the ideal organoleptic profile that is demanded by the market and the consumers [2,3]. In terms of the increasing insights into the grape and the wine microecosystem, the non-*Saccharomyces* species dominate the population in the beginning of the alcoholic fermentation in spontaneously fermented wines, while their interactions at molecular and chemical levels with the *S. cerevisiae* strains can lead to wines with enhanced sensorial properties [4].

The compatibility between the yeast species as well as their effect on the wine quality is far from being controlled in winemaking, even if several studies have been carried out. The interactions between the yeast species are mainly antagonistic, thus leading to off-flavor by-products or sluggish fermentations. The use of pure cultures results in the development of different metabolomic fingerprint compared to the respective resulting from the use of mixed cultures, while the metabolites that are produced may also be affected by cell-to-cell yeast contact in multi-starter wine fermentation [4,5]. Additionally, other parameters, such as the temperature, the inoculation ratio, the available nitrogen sources and the amount of produced acetic acid may configure the microbial dynamics during winemaking [1,4–7].

Sauvignon blanc is, worldwide, one of the most used varieties for the production of aromatic white wines, with a typical varietal profile due to the production of thiols. Three major aromatic volatile thiols, namely, 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), were identified as being responsible for the box tree, grapefruit and passion fruit aromas, respectively, of Sauvignon blanc [8,9]. The yeast strains that produced the highest levels of the volatile-free thiols fermented the wines with the highest perceived intensity of fruitiness, and these wines were the ones preferred by the tasting panels [10]. While the green character in the Sauvignon Blanc wines can be manipulated through vineyard management, the tropical fruity characters appear to be largely dependent on the wine yeast strain that is used during fermentation. Therefore, the choice of yeast strain for alcoholic fermentation offers great potential to modulate the wine aroma profiles as well as to direct the definable styles that are adapted to the consumer market specifications [11,12].

In our previous study [7] we have shown for the first time the oenological potential of the *S. pastorianus* species for Sauvignon blanc wine production as well as the effect of the inoculation scheme for the expression of varietal aromas. In our present study, we exhaustively studied the interaction between *S. pastorianus* and *S. cerevisiae* by comparing the pure and mixed cultures of the *Saccharomyces* species under different inoculation ratios. One *S. pastorianus* strain was tested under five co-inoculation combinations schemes with two commercial *S. cerevisiae* strains in Sauvignon blanc must. For all fermentation trials, the population dynamics of the inoculated and indigenous yeasts, the aromatic compounds with high oenological interest and the sensory profile of the produced wines were analyzed.

2. Materials and Methods

2.1. Yeast Strains and Culture Conditions

Saccharomyces pastorianus strain Sp2, which is currently under industrial development, and two commercial strains of *S. cerevisiae* SafŒno™ CK S102 (Fermentis, France) and SafŒno™ SH 12 (Fermentis, France) which are preconized for Sauvignon blanc wines and characterized for intensifying their aromatic profiles were chosen for this study (Table 1). The Sp2 and the CK S102 are registered in the CNCM collection (Collection Nationale de

Cultures de Microorganismes) under the reference numbers I-5474 and I-5473, respectively. The SH12 is registered in the collection of the Institut Francais de la Vigne et du Vin (CRB-Centre de Ressources Biologiques) under the reference number NL17912. All strains were inoculated as active dry yeasts according to their population (CFU/g) in order to obtain the correct inoculum ratio. The cell activation took place at 30 °C for 20 min; the cell viability and population were verified for each inoculum by plate count in YPDA medium (20 g/L glucose, 10 g/L yeast extract, 10 g/L peptone and 25 g/L agar). The inoculum level of the pure and mixed cultures was approximately 10^6 CFU/mL.

Table 1. *Saccharomyces* yeast species in pure and mixed cultures in different inoculation ratios.

Yeast Strains in Pure Cultures	
Code	Yeast strains
Sp2	*S. pastorianus* Sp2
Sc1	*S. cerevisiae* CK S102
Sc2	*S. cerevisiae* SH 12
Yeast strains in mixed cultures	
Code	Yeast strains
Sp2 70%–Sc1 30%	*S. pastorianus* Sp2 70%–*S. cerevisiae* CK S102 30% (*w*/*w*)
Sp2 80%–Sc1 20%	*S. pastorianus* Sp2 80%–*S. cerevisiae* CK S102 20% (*w*/*w*)
Sp2 90%–Sc1 10%	*S. pastorianus* Sp2 90%–*S. cerevisiae* CK S102 10% (*w*/*w*)
Sp2 95%–Sc1 5%	*S. pastorianus* Sp2 95%–*S. cerevisiae* CK S102 5% (*w*/*w*)
Sp2 99%–Sc1 1%	*S. pastorianus* Sp2 99%–*S. cerevisiae* CK S102 1% (*w*/*w*)
Sp2 70%–Sc2 30%	*S. pastorianus* Sp2 70%–*S. cerevisiae* SH 12 30% (*w*/*w*)
Sp2 80%–Sc2 20%	*S. pastorianus* Sp2 80%–*S. cerevisiae* SH 12 20% (*w*/*w*)
Sp2 90%–Sc2 10%	*S. pastorianus* Sp2 90%–*S. cerevisiae* SH 12 10% (*w*/*w*)
Sp2 95%–Sc2 5%	*S. pastorianus* Sp2 95%–*S. cerevisiae* SH 12 5% (*w*/*w*)
Sp2 99%–Sc2 1%	*S. pastorianus* Sp2 99%–*S. cerevisiae* SH 12 1% (*w*/*w*)

2.2. Must Preparation and Fermentation Kinetics

The grape must of the Sauvignon blanc grapes (Asprokampos Nemea, Greece), with 12 °Be initial density and total acidity of 6 g tartaric acid/L was supplemented with sulfites (50 mg/L), clarified by gravity using pectolytic enzymes at 3 g/hL (Safizym Pres, Fermentis, France), followed by cold treatment for 12 h, and decanted to the fermenters made of glass (30 L). The yeasts were inoculated as pure and mixed cultures at approximately 10^6 CFU/mL final concentration. The different inoculation ratios in the mixed cultures were achieved by weighting different proportions of the dry yeast cells. Twenty-four hours after yeast addition, 200 mg/L of organic nutrient (SpringFerm™, Fermentis, France) was added. The alcoholic fermentations were performed at 18 °C. During the fermentation, the samples were taken aseptically at 24 h intervals for further analyses, while sugar depletion (Glucose < 2 g/L) signified the end of the process. The fermentations were carried out in triplicate.

2.3. Microbiological Analyses and Yeast Molecular Identification

Samples from all fermenters were taken every 48 h for enumeration of the yeast population, while after 48 h of yeast inoculation, at the middle and at the end of fermentation for molecular typing. From each fermenter, 1 mL sample per time point was taken to study the microbial growth. A sample taken from a fermenter without yeast inoculation was used as negative control during the whole fermentation procedure. Each sample was serially diluted in sterile saline. The measurements of the *Saccharomyces* yeast and indigenous non-*Saccharomyces* yeast population levels were accomplished by plating serial dilutions on Wallerstein Laboratory Nutrient agar (WLN) or Lysine medium agar, respectively. All samples that were grown in WLN medium were plated in duplicate, with one plate being incubated at 37 °C for 24 h, and the other being incubated at 28 °C for 48 h, as *S. pastorianus* cells are thermosensitive and cannot grow significantly at temperatures that are above 30 °C. The incubation of Lysine agar plates was performed at 28 °C for 48–72 h. The cell

growth of total and wild yeast populations was measured in terms of colony-forming units (CFU/mL). After the colony enumeration of each plate, the WLN plates that were incubated at 28 °C were stored at 4 °C in order for them to be used for a yeast genetic fingerprint analysis.

The DNA fingerprinting took place after 48 h, at the middle and at the end of the fermentation process in order to confirm that the dominated strains of the *Saccharomyces* species were the inoculated ones and not the indigenous ones. *Saccharomyces* strains that were responsible for the fermentation in each fermenter were identified by inter-delta sequence profile analysis using δ12 (5′-TCAACAATGGAATCCCAAC-3′) and δ2 (5′-GTGGATTTTTATTCCAACA-3′) primers [7,13]. A single colony was picked from the WLN plate of interest (the one that was incubated at 28 °C and stored at 4 °C), and the colony cells were resuspended in 20 μL of 0.02 N NaOH. Then, the cells were lysed through heat, after 10 min of incubation at 98 °C in their resuspension solution, and 3 μL of lysed cells were used as template for each PCR reaction. A total of 16 colonies per fermenter and per time point, corresponding to at least half of the colonies that were enumerated, were tested through PCR reaction with δ12/δ2 primer set, as well as 16 colonies from the negative control fermenter. Each PCR reaction was performed in 25 μL volume, containing 1 U of KapaTaq polymerase, 1× Buffer B, 1 mM $MgCl_2$ (final concentration of $MgCl_2$ at 2.5 mM), 0.2 mM dNTPs and 800 nM of each primer. The PCR program was carried out as follows: 4 min at 94 °C, 35 cycles of 30 sec at 94 °C, 30 sec at 49 °C and 60 sec at 72 °C, and this was followed by a final 10 min extension step at 72 °C. The PCR results were obtained by separation in 2% agarose gel containing 0.01% Midori Green in 1 × SB buffer (5 mM sodium borate decahydrate) and visualization under UV light. The agarose gels were run at 120 V for 35 min in 1 × SB buffer. The resulting DNA profiles were compared to the respective ones of each of the strains that were employed. For the preparation of the latter, the cells of the starter cultures (Sp2, CK S102 or SH 12) were resuspended in water at 100 mg/mL concentration, and serial dilutions were plated on WLN plates and incubated at 28 °C for 24–48 h. Single colonies from the control cultures were used as a template for the positive control reactions in PCR analysis.

2.4. Chemical Analyses

2.4.1. Analysis of Classical Oenological Parameters

The must was analyzed immediately after the crushing of the grapes for the following parameters: glucose/fructose, total acidity, pH, malic acid and Yeast-Assimilable Nitrogen (YAN) using enzymatic kits that were adapted for a Y15 Biosystems auto-analyser (Barcelona, Spain), while the free and total SO_2 were determined by titrimetric methods. The fermentations were monitored by daily enzymatic measurements of glucose and fructose [14]. The alcohol content of the wines was analyzed by NIR spectrometry [15].

2.4.2. Higher Alcohols, Acetates and Esters Quantification

Major and minor fermentation volatile compounds of wine aroma were analyzed by GC–MS using the Head-Space Solid-Phase Micro-Extraction (HS-SPME) procedure which was recently adapted and described in [7]. Twenty-five micrograms of the internal standard, namely 3-octanol (1 g/L), were added to 25 mL of wine which were placed in a 40 mL vial, and then supplemented with 3 g of NaCl, a magnetic stir bar, and then, sealed using a screw-top cap with a silicon septum. The vial was placed on a heating stir plate, and the samples were equilibrated by stirring them at 750 rpm for 10 min at 40 °C. The SPME needle was inserted automatically (HTA3000A S.R.L., Brescia, Italy) through the septum and the DVB/CAR/PDMS 75 μm fiber was used to absorb the volatile compounds of the head-space for 30 min at 40 °C. The SPME needle was removed from the vial and inserted into the injector of GC for thermal desorption for 10 min. The analysis was performed using an Agilent 7890A GC that was equipped with an Agilent 5873C MS detector. The column that was used was an DBWAX capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and the gas carrier was helium with a flow rate of 1.2 mL/min. The injector and

MS-transfer line were maintained at 250 °C and 260 °C, respectively. The oven temperature was held at 30 °C for 5 min and then, it was raised to 220 °C at 4 °C/min and held at this temperature for 20 min. The selective ion monitoring (SIM) mode was applied, and the quantifier ions that were used were: ethyl isobutyrate (m/z 88), ethyl butyrate (m/z 71), ethyl 2 methyl butyrate(m/z 102), isoamyl acetate (m/z 87), isobutyl acetate (m/z 116), 3-octanol (m/z 59), ethyl hexanoate (m/z 88), ethyl octanoate (m/z 88), ethyl decanoate (m/z 157), 2 phenyl ethyl acetate (m/z 104), hexyl acetate, (m/z 69), isoamyl alcohol (m/z 87), 2-methyl-1-propanol (m/z 87), hexanol-1 (m/z 84), 2-phenylethanol (m/z 122) and 3-(methylthio)-1-propanol (m/z 96).

2.4.3. Varietal Thiols Quantification

The varietal thiols, 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA) and 4-methyl-4-methylpentan-2-one (4MMP) were quantified using the method that was described by Tominaga et al. [9].

2.5. Sensory Analyses

The wines were evaluated by Descriptive Analysis [16]. The sensory assessment was performed by a group of 8 trained judges with previous experience. The panelists were instructed to avoid eating, drinking and smoking for 1 h prior to the sessions. The panelists attended these sessions over a period of 1 month, twice per week. The training consisted of smelling of standard odors and description of wines by using references standards from an aroma box [17]. After 2 weeks, the panelists chose some attributes characterizing the wines. During the following sessions, the panelists were trained using appropriate solutions. The panelists were provided with 30 mL of samples in ISO wine glasses that were coded with random three-digit numbers at room temperature (18–20 °C). The intensity of the sensory attributes that were examined was evaluated using a 10-point scale (1: null; 10: very strong), and scored manually. The sensory test was repeated twice on two different days. Additionally, the Odor Active Value (OAV) was calculated as a ratio between the compound concentration and the odor threshold of each volatile compound based on bibliographic references [18–20].

2.6. Statistical Analyses

The fermentations were carried out in triplicate, and the results are presented as mean values ± standard deviation. Significant differences between the chemical profiles of the wines that were produced under different inoculation schemes were evaluated by One- and Two-way Analysis of Variance (ANOVA), which was followed by Tukey's post hoc test ($p < 0.05$). A principal component analysis (PCA) was applied to the chemical parameters to unravel the relationships between the samples and variables. All statistical analyses were performed using JMP version 3.1.5 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Yeast Growth and Ooenological Parameters during Fermentation

The alcoholic fermentations of the Sauvignon blanc clarified must were conducted at a laboratory scale under different inoculation schemes by using *Saccharomyces* strains in pure and mixed cultures. The monocultures consisted of a *S. pastorianus* strain (Sp2) and two *S. cerevisiae* strains, Sc1 and Sc2. The co-inoculation of the *Saccharomyces* strains took place in different inoculation ratios (70%–30%, 80%–20%, 90%–10%, 95%–5% and 99%–1% w/w) of Sp2:Sc1 and Sp2:Sc2 (Table 1). The fermentation kinetics were followed by sugar consumption, while the yeast population dynamics were assessed by plate counting combined with molecular methods, which were performed in order to discriminate the inoculated from the indigenous yeast strains.

All fermentation trials lead to complete sugar consumption. The inoculation mode affected the growth kinetics of the inoculated strains as well as of the indigenous yeast microbiota (Figure 1). *Saccharomyces pastorianus* reached 6.3×10^7 CFU/mL after seven

days of fermentation, when it was used as a monoculture; while the *S. cerevisiae* strains reached the same population levels after four days.

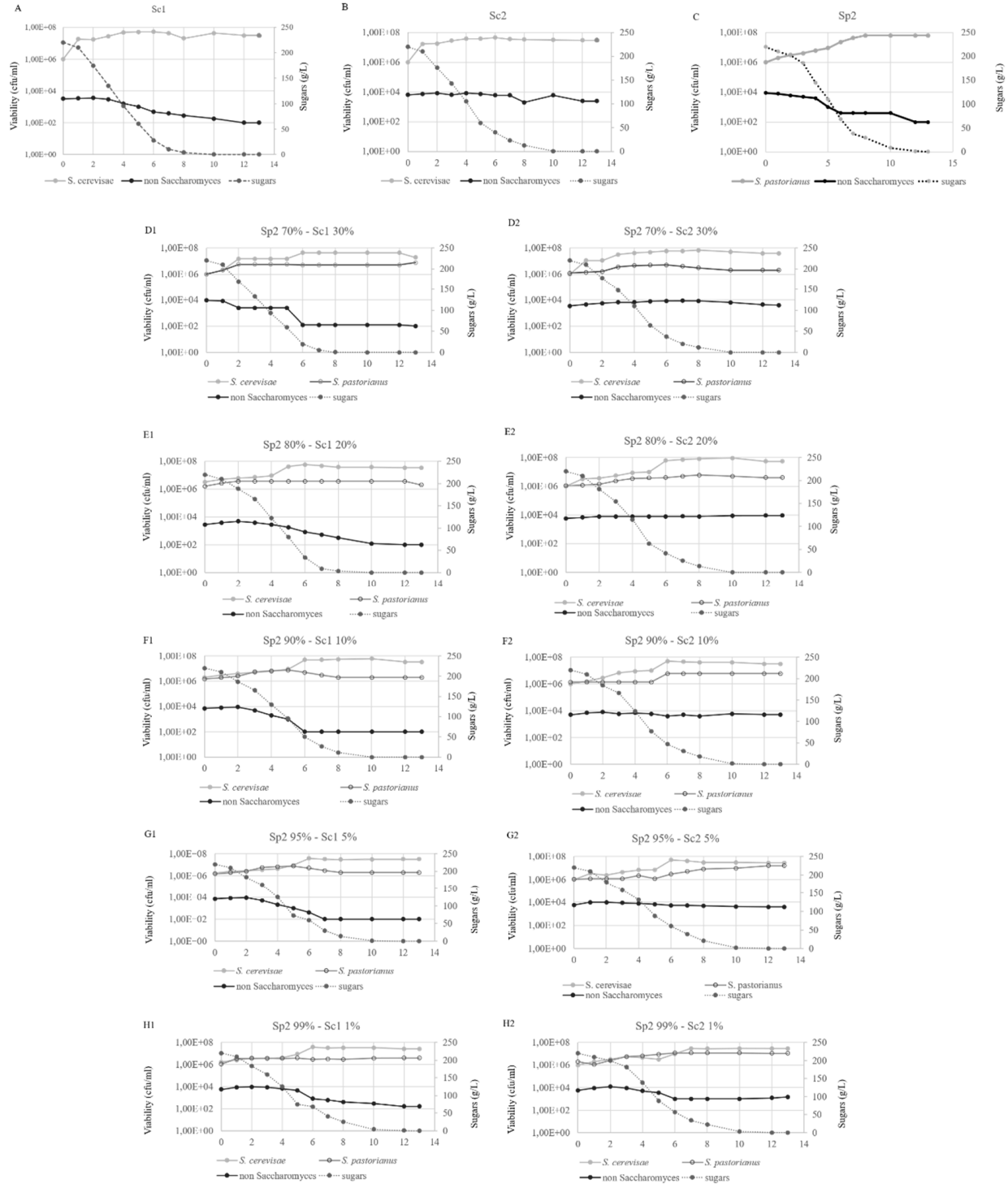

Figure 1. Fermentation kinetics and yeast population dynamics of Sauvignon blanc must which was inoculated with pure (**A**–**C**) and mixed (**D**–**H**) cultures of *Saccharomyces* species. The *S. cerevisiae*-inoculated strains (Sc1 and Sc2) are indicated with grey lines, and the *S. pastorianus*-inoculated strains (Sp2) are indicated with white circles, while the indigenous wild yeast is indicated with black lines. The fermentation follow-up through sugar consumption is indicated with a dashed line. Values are means of two independent experiments.

Additionally, the indigenous yeast population was close to 10^4 CFU/mL when the strain Sc2 was inoculated, and it was close to 10^3 CFU/mL in the case of Sp2 and Sc1 pure cultures (Figure 1A–C). In the mixed cultures, in terms of the population level, the *S. pastorianus* strain was always below (approximately 1 log CFU/mL) that of the *S. cerevisiae* co-inoculated strain, except in the case of Sp2 99%–Sc2 1% (Figure 1 H2), in which both Sp2 and the Sc2 followed comparable growth pattern during the entire fermentation period. The indigenous non-*Saccharomyces* yeasts exhibited a different growth profile according to the inoculated *Saccharomyces* strains. When the *S. cerevisiae* strain 1 was present, the population of the non-*Saccharomyces* yeast was close to 10^3 CFU/mL, while the presence of the Sc2 strain increased (CFU/mL) the indigenous yeast population by 1 log.

The oenological parameters were evaluated according to the OIV protocols at the end of the fermentation trials for all produced wines (Supplementary Table S1). The fermentation duration lasted between 10 and 13 days according to the inoculation mode. Statistically significant, but technologically slight differences were observed for the level of the pH and acidity values among the trials. The pure culture of *S. pastorianus* presented a significantly lower volatile acidity production (0.06 ± 0.03 g/L), which was increased in all cases with the presence of the *S. cerevisiae* strains. As far as the glycerol production was concerned, the presence of the *S. pastorianus* strain in the mixed inoculation cultures increased ($p < 0.05$) the produced levels in comparison to pure cultures of *S. cerevisiae* for both tested strains. On the contrary, the presence of Sp2 in the mixed cultures significantly decreased ($p < 0.05$) the degradation of the L-malic acid compared to the pure cultures of Sc1 and Sc2.

3.2. Volatile Compounds Analyses

The quantification of the thiols, higher alcohols and esters was performed for all fermentation trials (Table 2). 3-mercaptohexan-1-ol, 3-mercaptohexyl acetate, and 4-methyl-4-mercaptopentan-2-one, which are intrinsic compounds of the Sauvignon blanc variety, were quantified. The total thiols production values ranged from 211 ± 4.6 ng/L to 561.3 ± 1.7 ng/L under the fermentation scheme of Sp2 99%–Sc1 1% and Sp2 70%–Sc2 30%, respectively. The presence of the Sc2 strain significantly increased the production of the total thiols both in the monoculture and co-inoculation conditions. The only condition in which the presence of Sc1 strain exhibited a high thiols production level (328 ± 6 ng/L) was when it was inoculated with Sp2 at a ratio of 70%–30% (Sp2/Sc1). When the three tested strains were inoculated in pure cultures, they presented significant differences in the thiols production values; the *S. pastorianus* strain Sp2 showed an intermediate level by producing 293.5 ± 16.6 ng/L. In particular, the highest value of 3MH production (424 ± 5.6 ng/L) among the tested conditions was observed under the fermentation scheme Sp2 70%–Sc2 30%. The same fermentation condition also showed the highest level of 3MH production when the strain Sc1 was inoculated. The volatile compound 3MHA was detected in all cases with the exception of the 99%–1% scheme regarding both *S. cerevisiae* strains, and the 90%–10% and 95%–5% regarding the *S. cerevisiae* strain Sc1. The presence of the *S. cerevisiae* strain Sc2 significantly increased the production of 4MMP in all fermentation trials. The maximum level that was observed was 126 ± 3.6 ng/L under the scheme of Sp2 70%–Sc2 30%, and the lower that the percentage of Sc2, the lower the production of 4MMP in the mixed cultures.

Table 2. Volatile compounds of the wines produced by pure and mixed cultures in different inoculation ratios of *S. cerevisiae* and *S. pastorianus* strains. Values with different roman letters (a–h) in the same row are significantly different according to Tukey's post hoc test ($p < 0.05$).

Aromatic Compounds	Pure Cultures			Sp2/Sc1 Mixed Cultures					Sp2/Sc2 Mixed Cultures				
	Sp2	**Sc1**	**Sc2**	**Sp2 70% Sc1 30%**	**Sp2 80% Sc1 20%**	**Sp2 90% Sc1 10%**	**Sp2 95% Sc1 5%**	**Sp2 99% Sc1 1%**	**Sp2 70% Sc2 30%**	**Sp2 80% Sc2 20%**	**Sp2 90% Sc2 10%**	**Sp2 95% Sc2 5%**	**Sp2 99% Sc2 1%**
Thiols (ng/L)													
3-mercaptohexan-1-ol	282.5 ± 15 ^{f}	249.7 ± 3 ^{g}	385.7 ± 15 ^{b}	315.3 ± 7 ^{c,d}	290.7 ± 9 ^{d,e,f}	283.3 ± 7 ^{e,f}	251.0 ± 6 ^{g}	205.7 ± 3 ^{h}	424.0 ± 5 ^{a}	373.7 ± 7 ^{b}	317.7 ± 5 ^{c}	308.7 ± 2 ^{c,d,e}	317.3 ± 4 ^{c,d}
3-mercaptohexan-1-ol Acetate	5.8 ± 0.6 ^{f}	10.0 ± 1.0 ^{b,c}	19.3 ± 1.4 ^{a}	8.0 ± 1.0 ^{c,d,e}	6.0 ± 1.0 ^{e,f}	ND ^{g}	ND ^{g}	ND ^{g}	11.0 ± 1.0 ^{b}	8.7 ± 1.5 ^{b,c,d}	6.7 ± 0.6 ^{d,e,f}	5.0 ± 0.0 ^{f}	ND ^{g}
4-methyl-4-mercaptopentan-2-one	5.2 ± 1.2 ^{f}	4.3 ± 0.6 ^{f}	73.3 ± 3.5 ^{c}	4.6 ± 0.6 ^{f}	5.0 ± 1.0 ^{f}	5.3 ± 0.6 ^{f}	5.3 ± 1.5 ^{f}	5.3 ± 1.5 ^{f}	126.0 ± 3 ^{a}	84.0 ± 3.0 ^{b}	81.3 ± 3.5 ^{b}	21.0 ± 2.0 ^{d}	12.3 ± 1.5 ^{e}
Total thiols	293.5 ± 16 ^{f}	264.0 ± 4 ^{g,h}	478.3 ± 13 ^{b}	328.0 ± 6 ^{d,e}	301.6 ± 11 ^{e,f}	288.6 ± 8 ^{f,g}	256.3 ± 8 ^{h}	211.0 ± 4 ^{i}	561.0 ± 2 ^{a}	466.3 ± 10 ^{b}	405.7 ± 1 ^{c}	334.7 ± 4 ^{d}	329.7 ± 5 ^{d,e}
Higher alcohols (mg/L)													
2- phenyl-ethanol	18.8 ± 0.7 ^{d,e,f}	28.4 ± 0.9 ^{a,b}	13.9 ± 1.1 ^{g}	28.6 ± 0.6 ^{a,b}	30.1 ± 0.9 ^{a}	24.9 ± 4.0 ^{b,c}	20.9 ± 0.9 ^{c,d}	19.8 ± 0.8 ^{d,e}	14.5 ± 0.9 ^{f,g}	13.9 ± 1.1 ^{g}	15.5 ± 1.1 ^{e,f,g}	17.0 ± 1 ^{d,e,f,g}	16.5 ± 1.0 ^{d,e,f,g}
propanol	28.3 ± 1.8 ^{a,b,c}	20.3 ± 0.7 ^{h}	23.9 ± 1.8 ^{e,f,g}	20.6 ± 0.6 ^{g,h}	25.0 ± 1.0 ^{d,e,f}	26.7 ± 1.3 ^{c,d,e}	22.2 ± 0.7 ^{f,g,h}	28.0 ± 0.8 ^{a,b,c,d}	25.5 ± 1.1 ^{d,e}	30.4 ± 0.6 ^{a}	30.4 ± 1.2 ^{a}	27.0 ± 1.0 ^{b,c,d,e}	30.1 ± 0.9 ^{a,b}
isobutanol	31.3 ± 1.2 ^{a}	19.0 ± 0.0 ^{g,h}	12.3 ± 0.8 ^{i}	22.7 ± 0.5 ^{e,f}	26.1 ± 0.8 ^{b,c,d}	27.7 ± 1.5 ^{b,c}	22.7 ± 0.6 ^{e,f}	28.2 ± 0.3 ^{b}	17.2 ± 0.9 ^{h}	18.1 ± 0.4 ^{h}	21.3 ± 1.4 ^{f,g}	24.1 ± 0.9 ^{d,e}	25.1 ± 0.9 ^{c,d,e}
butanol	1.1 ± 0.1 ^{a}	0.7 ± 0.1 ^{d}	0.9 ± 0.0 ^{b,c,d}	0.8 ± 0.1 ^{c,d}	0.9 ± 0.1 ^{a,b,c}	1.0 ± 0.1 ^{a,b,c}	1.0 ± 0.0 ^{a,b,c}	1.1 ± 0.1 ^{a,b}	0.9 ± 0.2 ^{a,b,c}	1.0 ± 0.1 ^{a,b,c}	1.0 ± 0.0 ^{a,b,c}	1.0 ± 0.1 ^{a,b,c}	1.1 ± 0.1 ^{a,b}
isoamyl alcohol	174.4 ± 5 ^{b}	173.8 ± 3 ^{b}	122.7 ± 2 ^{e}	195.2 ± 2 ^{a}	190.0 ± 3 ^{a}	193.1 ± 3 ^{a}	190.3 ± 2 ^{a}	189.7 ± 1.5 ^{a}	135.3 ± 3 ^{d}	140.0 ± 1 ^{d}	157.2 ± 4 ^{c}	159.3 ± 1 ^{c}	153.0 ± 2 ^{c}
Total alcohols	254.0 ± 7 ^{c}	242.3 ± 1 ^{d}	173.7 ± 2 ^{f}	239.4 ± 3 ^{a,b,c}	242.0 ± 2 ^{a,b,c}	247.1 ± 2 ^{a}	248.6 ± 3 ^{b,c}	236.2 ± 2 ^{a,b}	247.0 ± 1 ^{e}	178.9 ± 4 ^{e}	189.6 ± 1 ^{d}	207.9 ± 5 ^{d}	211.7 ± 4 ^{d}
Esters (mg/L)													
Isoamyl acetate	3.1 ± 0.1 ^{g}	3.5 ± 0.2 ^{e}	3.7 ± 0.0 ^{d}	3.9 ± 0.0 ^{c}	4.0 ± 0.0 ^{c}	4.2 ± 0.0 ^{b}	4.4 ± 0.0 ^{a}	3.6 ± 0.0 ^{d,e}	2.9 ± 0.0 ^{h}	3.0 ± 0.0 ^{g,h}	3.1 ± 0.0 ^{f,g}	3.2 ± 0.0 ^{f}	3.2 ± 0.0 ^{f}
Hexyl acetate	0.2 ± 0.1 ^{c}	0.2 ± 0.0 ^{b}	0.3 ± 0.0 ^{a}	0.2 ± 0.0 ^{c,d}	0.2 ± 0.0 ^{d,e}	0.2 ± 0.0 ^{c,d}	0.2 ± 0.0 ^{d,e}	0.2 ± 0.0 ^{c,d}	0.2 ± 0.0 ^{b,c}	0.2 ± 0.0 ^{f}	0.2 ± 0.0 ^{c}	0.2 ± 0.0 ^{d,e}	0.2 ± 0.0 ^{e,f}
2-phenylethyl acetate	0.3 ± 0.2 ^{e}	0.4 ± 0.0 ^{a}	0.3 ± 0.0 ^{f}	0.3 ± 0.0 ^{b,c}	0.3 ± 0.0 ^{c,d}	0.3 ± 0.0 ^{b}	0.3 ± 0.0 ^{d,e}	0.3 ± 0.0 ^{d,e}	0.2 ± 0.0 ^{g}	0.2 ± 0.0 ^{g}	0.2 ± 0.0 ^{g}	0.2 ± 0.0 ^{f}	0.2 ± 0.0 ^{f}
Ethyl isobutyrate	0.1 ± 0.0 ^{b}	0.1 ± 0.0 ^{a}	0.1 ± 0.0 ^{d}	0.1 ± 0.0 ^{b}	0.1 ± 0.0 ^{b,c}	0.1 ± 0.0 ^{b,c}	0.1 ± 0.0 ^{c}	0.1 ± 0.0 ^{b}	0.1 ± 0.0 ^{b}	0.1 ± 0.0 ^{b,c}	0.1 ± 0.0 ^{b,c}	0.1 ± 0.0 ^{b,c}	0.1 ± 0.0 ^{b}
Ethyl butyrate	0.6 ± 0.0 ^{a}	0.4 ± 0.0 ^{f}	0.5 ± 0.0 ^{c,d}	0.4 ± 0.1 ^{e}	0.4 ± 0.0 ^{e}	0.4 ± 0.0 ^{e}	0.5 ± 0.0 ^{c,d}	0.5 ± 0.0 ^{c}	0.5 ± 0.1 ^{b,c}	0.6 ± 0.0 ^{a,b}	0.5 ± 0.0 ^{c,d}	0.5 ± 0.0 ^{d}	0.5 ± 0.0 ^{c,d}
Ethyl octanoate	1.6 ± 0.1 ^{c}	1.2 ± 0.1 ^{h}	1.7 ± 0.0 ^{a}	1.3 ± 0.1 ^{g,h}	1.3 ± 0.0 ^{g}	1.2 ± 0.0 ^{h}	1.3 ± 0.0 ^{e,f}	1.4 ± 0.0 ^{d,e}	1.6 ± 0.0 ^{b,c}	1.6 ± 0.0 ^{a,b}	1.5 ± 0.0 ^{d}	1,4 ± 0.0 ^{f,g}	1.5 ± 0.0 ^{d,e}
Ethyl hexanoate	2.2 ± 0.2 ^{a}	1.8 ± 0.0 ^{e}	2.1 ± 0.0 ^{a,b,c}	1.7 ± 0.1 ^{e,f}	1.6 ± 0.0 ^{f}	1.6 ± 0.0 ^{e,f}	1.9 ± 0.0 ^{d}	2.1 ± 0.0 ^{a,b}	2.2 ± 0.0 ^{a}	2.1 ± 0.0 ^{a,b}	2.0 ± 0.0 ^{c,d}	2.0 ± 0.0 ^{b,c,d}	2.2 ± 0.0 ^{a}
Ethyl 3-hydroxy butanoate	0.4 ± 0.0 ^{b}	0.2 ± 0.0 ^{g}	0.2 ± 0.0 ^{e}	0.2 ± 0.0 ^{g}	0.3 ± 0.0 ^{e,f}	0.3 ± 0.0 ^{e}	0.3 ± 0.0 ^{d}	0.3 ± 0.0 ^{c,d}	0.3 ± 0.0 ^{c}	0.4 ± 0.0 ^{c}	0.4 ± 0.0 ^{b}	0.4 ± 0.0 ^{a}	0.4 ± 0.0 ^{a}
Total esters	8.4 ± 0.2 ^{d}	7.7 ± 0.0 ^{h}	8.8 ± 0.0 ^{b}	8.2 ± 0.0 ^{e,f}	8.1 ± 0.1 ^{e,f,g}	8.4 ± 0.0 ^{c,d}	9.0 ± 0.0 ^{a}	8.6 ± 0.0 ^{c}	8.0 ± 0.0 ^{f,g}	8.1 ± 0.0 ^{f,g}	8.0 ± 0.0 ^{g}	8.1 ± 0.0 ^{f,g}	8.3 ± 0.0 ^{d,e}

Five compounds belonging to the group of higher alcohols were quantified. When the three strains were inoculated in monocultures, the production values of the total higher alcohols were 254 ± 7.1 mg/L for the Sp2 inoculation mode, 242.3 ± 1.0 mg/L for Sc1, and 173.7 ± 2.5 mg/L for Sc2. The case of the Sp2 inoculation mode had the highest level of higher alcohols when it was compared to all the trials, while the Sc2 the lowest one. The presence of *S. pastorianus* significantly increased the production level in the mixed cultures with Sc2, compared to the monoculture of the latter. However, the production level was reduced, compared to the monoculture of the Sp2 strain. More precisely, the wines that were fermented with Sc2 produced the lowest level of 2-phenyl-ethanol (13.9 ± 1.1 mg/L), isobutanol (12.3 ± 0.8 mg/L), and isoamyl alcohol (122.7 ± 2.5 mg/L) when they were compared to all fermentation trials. All wines that were produced under different fermentation schemes were analyzed for eight volatile esters that are responsible for the floral and fruity characters of the wines. The amount of isoamyl acetate, which is known for contributing to wines the characteristic banana aroma, was increased in all co-inoculation schemes of Sc1 and Sp2 compared to the other fermentation conditions. The production level of the total esters ranged from 7.7 mg/L for Sc1 in the pure culture inoculation condition to 9 mg/L for the mixed culture of Sp2 95%–Sc1 5%. The presence of the *S. pastorianus* strain significantly increased the level of esters that were produced when it was combined with Sc1. On the contrary, the presence of Sp2 significantly decreased the ester production in the mixed cultures with Sc2 when it was compared to that of the respective of *S. cerevisiae* Sc2 pure culture.

The possible effects of both inoculation modes and the yeast strain on the volatile compounds of the Sauvignon blanc wines aroma are illustrated in Figure 2. According to the Two-way ANOVA test, significant differences ($p < 0.05$) among the samples were detected for the three aromatic groups, the higher alcohols, the esters and the thiols, which were fermented under six inoculation schemes. In the case of the higher alcohols (Figure 2A), the co-inoculation of the *S. pastorianus* strain Sp2 with Sc1 or Sc2 in any ratio resulted in the statistically significant increase in the higher alcohol level when it was compared to those of the inoculation mode of the *S. cerevisiae* pure cultures. The interaction was not statistically significant for the two *S. cerevisiae* strains that were used under the fermentation scheme 95%–5%. The Sauvignon blanc wines fermented with the mixed cultures of Sp2 and Sc1 exhibited a high production of esters, especially when the *S. pastorianus* strain was inoculated at a percentage of 95%. Inversely, the wines that were fermented with the Sc1 strain were characterized by higher esters levels than the wines that were co-fermented with Sp2. The interaction of the two tested factors for the two used strains was lower under the inoculation ratios of 80%–20% and 70%–30% (Figure 2B). Finally, for thiols generation, the production was always higher when *S. pastorianus* was inoculated with the *S. cerevisiae* strains at a ratio of 70%–30%, whereas the presence of *S. cerevisiae* Sc2 always lead to higher levels compared to *S. cerevisiae* Sc1. The interaction was less intense under the fermentation ratio 95%–5%.

3.3. Sensory Impact

We performed a PCA analysis based on the Odor Active Value (OAV) of each volatile compound to better visualize the diversity in the volatile compounds of the wine aroma that was produced using different inoculation schemes (Figure 3). The PCA analysis distinguished clearly four modalities; the wines that were fermented with the pure cultures of the two *S. cerevisae* strains, the wines that were fermented with the mixed cultures of Sc1 with the *S. pastorianus* strain, and the wines that were fermented with mixed cultures of Sc2 with the *S. pastorianus* strain. The wines that were inoculated with the *S. cerevisae* strain Sc2, in the pure or mixed cultures, were clearly loaded positively on the PC1 and connected mainly to the higher thiols levels, while the wines that were fermented by the presence of the Sc1 strain were located to the negative side of PC1, indicating lower thiols production by this strain in comparison with Sc2.

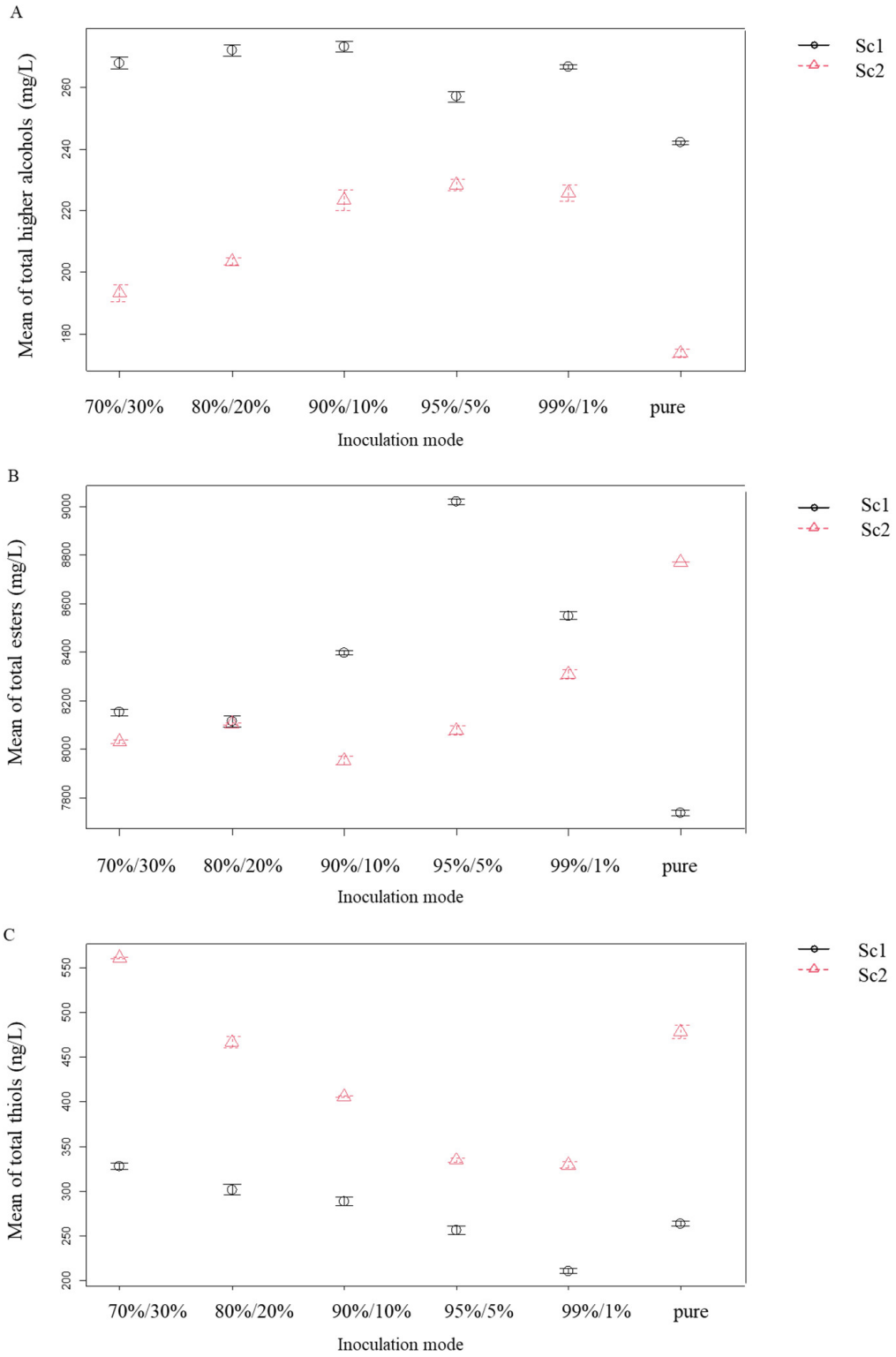

Figure 2. Interaction plot of means of aromatic compounds versus inoculation mode for *S. cerevisiae* strains Sc1 and Sc2. The compounds belonged to the group of: (**A**) Higher alcohols, (**B**) Esters, and (**C**) Thiols. The inoculation mode concerned the pure cultures of Sc1 and Sc2 and the mixed cultures of the two *S. cerevisiae* strains Sc1 and Sc2 with the *S. pastorianus* strain Sp2 under different inoculation ratio Sp2/Sc strains (70%–30%, 80%–20%, 90%–10%, 95%–5% and 99%–1%). Error bars indicate the standard error of the mean values.

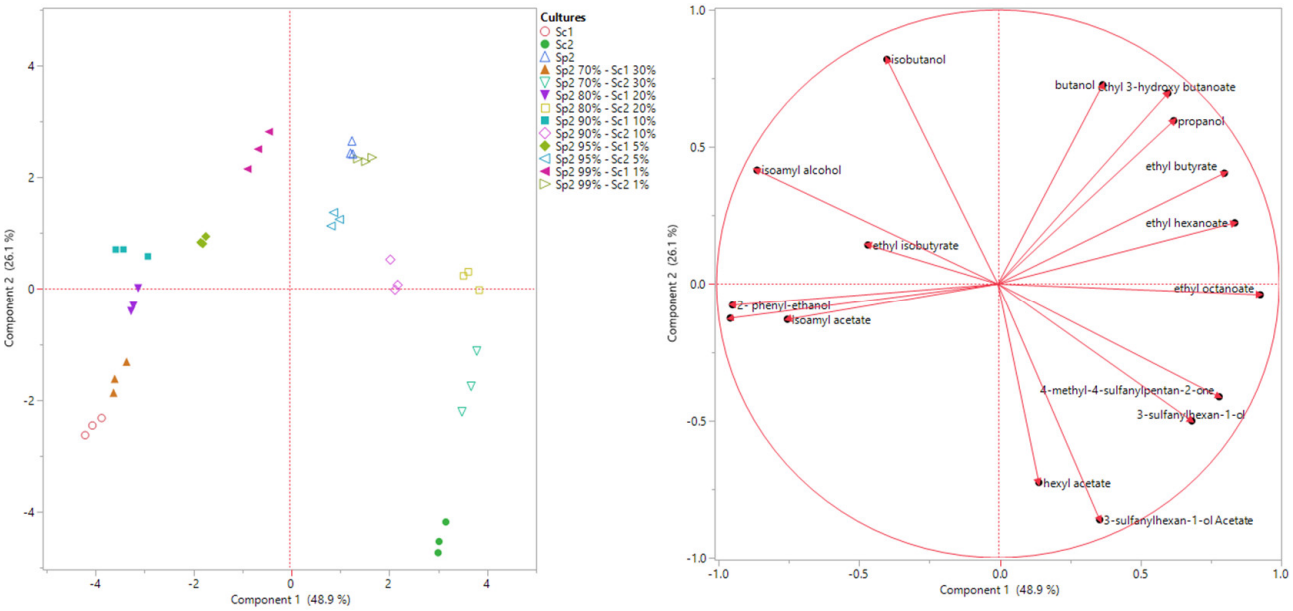

Figure 3. Principal component analysis of 39 volatile compounds analyses expressed as Odor Active Value (OAV) of Sauvignon blanc wines fermented with monocultures of *S. cerevisiae* and *S. pastorianus* (Sc1, Sc2 and Sp2) as well as with mixed cultures of both species in different inoculation ratios.

As the effect of the *S. cerevisiae* strain that was used for each fermentation was clear and evident, we further performed the sensory evaluation to find any possible differences between the wines that were fermented with the same *S. cerevisiae* strain and Sp2 but under different inoculation schemes (Supplementary Figure S1). For the strain combination Sc1 and Sp2, the produced Sauvignon blanc wines that presented an overall better sensory profile, characterized by higher complexity, balance, tropical fruit aromas and aromatic intensity, were the ones that were fermented with the ratio Sp2 95%–Sc1 5% in comparison to the other inoculation ratios that were tested (Figure S1A). When the Sauvignon blanc must was inoculated with the mixed cultures of Sp2 and Sc2, the fermentation scheme that achieved the preferred sensory profile was when Sp2 was co-inoculated at 70% with Sc2 30%. Under this inoculation scheme, the produced wines were more floral, fruity and complex (Figure S1B).

The PCA which was derived from the sensory descriptive analysis of the wines was used to reveal the diversity among the *Saccharomyces*-inoculated strains (Sc1, Sc2 and Sp2) and to individuate the specific attributed sensory wine characteristics within the inoculation scheme (Figure 4). The biplot of the sensory descriptors that were evaluated (Aroma intensity, Citrus, Floral, Tropical fruits, Vegetal/Herbaceous, Reduction, Balance and Complexity) revealed the expected diversity of the produced wines fermented under various fermentation schemes and the impact of each yeast strain. The sensory descriptors that correlate to the wine's overall quality such as Global Mark, Balance and Complexity are closely presented and distinguishable by two fermentation schemes; Sp2 95%–Sc2 5% and Sp2 70%–Sc2 30%, where the second one was evaluated with higher rate by the sensory panel. The wine that was fermented by the monoculture of the Sc2 strain was also positioned on the upper right quadrant by virtue of wines characterized by Tropical fruits aromas and Aromatic intensity. The majority of the fermentation schemes with the Sc1 strain, either in monoculture or in mixed cultures, were present on the left side of the biplot. The upper-left quadrant includes the fermentation schemes that resulted in wines with mainly negative attributes such as the Reduction and Vegetal notes. Finally, the lower-right biplot gathered the fermentation schemes that grouped the more floral wines, where both Sc1 and Sc2 strains were present in the mixed inoculation cultures.

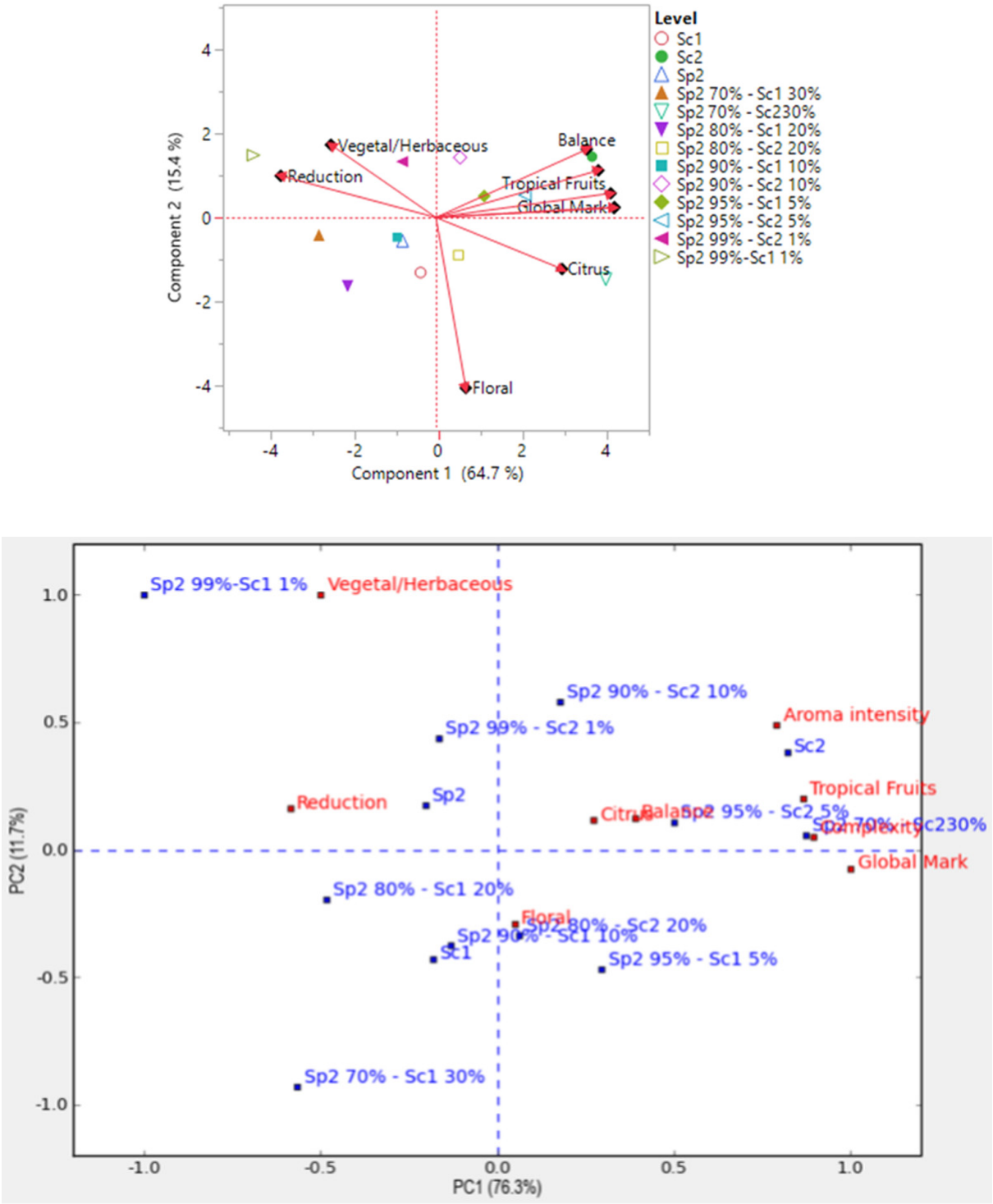

Figure 4. Biplot of the principal component analysis (PC1 vs. PC2) for descriptive sensory analysis of Sauvignon blanc wines fermented with monocultures of *S. cerevisiae* and *S. pastorianus* (Sc1, Sc2 and Sp2) as well as with mixed cultures of both species in different inoculation ratio.

4. Discussion

The grapes, the must and the wine environment are complex microecosystems that are characterized by multiple interactions, which can be detrimental for the quality of the final product. Inevitably, the environmental changes that occur throughout the winemaking process shapes the microbial community not only in terms of species/strains that are

present, but also on the metabolites' production [21,22]. The feasibility of inoculating *S. cerevisiae* with a non-*Saccharomyces* yeast (e.g., *Torulaspora delbrueckii*) in order to partially mimic the indigenous microbiota composition and promote an aromatic typicity and complexity has been widely explored [23–25]. Nevertheless, the species compatibility is not always taken for granted as antagonistic mechanisms may be developed with a controversial impact on the wine's fermentability and the quality characteristics. The objective of our study was to evaluate the compatibility of the strains belonging to the *Saccharomyces* genus as fermentation starters to promote the wine aromatic typicity. Thus, two strains of *S. cerevisiae* and one strain of *S. pastorianus* were chosen to be inoculated in different inoculation ratios in the Sauvignon blanc must.

According to our results, the indigenous population of non-*Saccharomyces* yeasts showed a different growth behavior according to the inoculation scheme, and especially, the added strain of *S. cerevisiae*. The presence of Sc1 in any inoculum proportion decreased their population by approximately 2 log CFU/mL, while the presence of Sc2 kept a constant cell concentration. Additionally, the presence of *S. pastorianus* affected neither the indigenous population nor the added *S. cerevisiae* strain. During the vinification process, the niche construction is evolving and inevitably, a selection pressure is created for the microorganisms [2,4,26]. The way in which these mechanisms may impact the inter and intra species physiology depends on many factors that are more or less known [4,25,27]. In our case, Sp2 and Sc1 produced significant high amounts of SO_2, which is a strong antimicrobial agent, especially against the non-*Saccharomyces* yeast; therefore, the control of the wild yeast population is justified.

Saccharomyces pastorianus is a species that is characterized by low acetic acid production under winemaking conditions, but at the same time its fermentation capacity cannot compete with the respective one of *S. cerevisiae* [7]. Nevertheless, in our present work we proposed that the co-inoculation of *S. pastorianus* with *S. cerevisiae* in any ratio can lead to lower acetic acid levels without negatively affecting the fermentation kinetics or the other oenological characteristics. As acetic acid is correlated with volatile acidity, its concentration is critical for wine quality. The yeast cells produce acetic acid during the alcoholic fermentation and the formation level of it depends on many factors such as the yeast strain, the oxygen level, the osmotic stress or the nitrogen content [5,28,29]. Under oenological conditions, through the aspect of species competition, the production of acetic acid may act as an antimicrobial factor with a negative effect on the yeast fermentative performance [28,30]. This phenomenon is more frequent when non-*Saccharomyces* yeasts are used in mixed cultures with *S. cerevisiae* or when spontaneous fermentations take place [31–33]. Interestingly, some strains of *S. cerevisiae* are capable of metabolize acetic acid under limited aerobic conditions, thereby giving an interesting technological trait [29]. In our case, the two tested species, *S. pastorianus* and *S. cerevisiae*, showed high compatibility as only the positive oenological attributes were observed when they were used in a co-inoculation mode. Similar results were observed when *S. cerevisiae* was inoculated with *T. delbrueckii*, but only under sequential fermentation [31].

The aromatic profile of the produced Sauvignon blanc wines of the present study exhibited significant differences concerning their chemical composition and their sensory traits, highlighting the metabolic interactions not only at species and strains level, but also in different yeast inoculation ratios. The majority of the volatile metabolites that were produced by the yeasts during alcoholic fermentation process belong to the group of higher/fusel alcohols and esters [30,34–36]. The concentration of these aroma compounds is highly dependent on the factors such as the yeast species or strains that are used, but also on the grape variety and the oenological techniques that are used [34,37]. However, how these aromatic compounds contribute to the wine organoleptic profile is highly dependent on their perception threshold as well as on the forming perception interactions [22]. The wine ranked as the more complex with the greater global mark was the one fermented under the Sc2 30%–Sp2 70% inoculation scheme. Interestingly, the produced wine was characterized by intermediate higher alcohols and esters concentration when it was compared to the other

tested fermentation schemes. For instance, higher alcohols can even provoke a masking effect on the wine's fruity notes through perception interactions or giving 'heavier' aromas. The aroma perception depends on the volatiles distribution between the matrix and the gas phase highly affecting the final organoleptic characteristics [38]. Additionally, the non-volatile compounds of wine such as polysaccharides and proteins may also have an impact on the volatility and aroma perception [39].

On the other hand, the produced volatile thiols had a clear effect on the wine's sensory traits. Volatile thiols mostly develop during alcoholic fermentation through the action of yeast cells from odorless grape precursors [9,18]. As these compounds are linked to the varietal aromas of Sauvignon blanc, extended research has been focused on that grape variety and the factors affecting their generation. Inevitably, their final concentration in wine is highly dependent on the yeast species and the strain that are used as well as on environmental parameters such as the fermentation temperature, copper sulphate supplementation, the YAN levels and its infection by *Botrytis cinerea* [12,35]. Our results clearly showed that the inoculation scheme, in pure or mixed cultures of *S. cerevisiae* and *S. pastorianus* affected the production of varietal thiols and directly influenced the wine's sensory profile. Interestingly, the presence of *S. pastorianus* in the mixed cultures either with Sc1 or Sc2 in a ratio of 70%–30% significantly increased the total thiols production compared to monocultures. This result highlighted not only the species effect, but also the importance of the inoculation ratio [11]. On the contrary, the 3MHA production level was always decreased in the mixed inoculation cultures. In the case of 3MHA, similar results were obtained when *S. cerevisiae* was inoculated with *T. delbrueckii* [36]. In the latter study, the concentration level of 3MHA in the wines that were fermented with mixed cultures was significantly decreased compared to the wines that were fermented with pure cultures of the *S. cerevisiae* strain [36]. 3MHA is produced from 3MH during grape must fermentation by the action of a yeast ester-forming alcohol acetyltransferase through an acetylation reaction, which is encoded by the *atf1p* gene [35,40,41]. Besides the formation of 3MHA, the fermentation with mixed cultures did not affect the production of the other acetate esters such as isoamyl acetate, hexyl acetate and 2-phenyl ethyl acetate, thereby indicating that besides the alcohol acetyltransferase Atf1p, other enzymes are implicated in the production of 3MHA [10,41]. Regarding 4MMP production, it is noteworthy that the samples of Sc2 as well as the mixed cultures between Sp2 and Sc2 (from 70%–30% to 90%–10%) produced higher levels of it. This is extremely important as 4MMP is the most stable thiol during ageing as it is chemically a ketone, while 3MH is an alcohol, and 3MHA is an ester and it is quickly hydrolyzed to 3MH. The oxidation of 3MH leads to 3,3-dithiobis(hexan-1-ol) and related chiral oxidation products that have been identified in botrytized Sauternes wines [42]. This has an important impact on the wine aroma intensity as the wines with higher 4MMP levels could be more varietal expressive for longer periods. In addition, the levels of 4MMP were significantly higher in the co-inoculated scheme Sp2 70%–Sc2 30%, and this means that the co-inoculation enhanced the 4MMP production.

5. Conclusions

The present study focused on the effect of mixed fermentations of *S. pastorianus* with two different *S. cerevisiae* strains on the production of Sauvignon blanc wine in different inoculation ratios. Both *S. cerevisiae* strains used, and the inoculation ratios significantly influenced the enological properties and the aroma profile of the produced wines. From a global point of view, the co-inoculated scheme Sp2 70%–Sc2 30% was the one that resulted in a Sauvignon blanc wine that was mostly preferred by the sensory panel and the one that was characterized by the highest thiols concentration. It would be interesting in the future to test this inoculation scheme in order to ferment more Sauvignon blanc must from regions of different geographical origin.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8100539/s1, Figure S1: Means of the sensory characteristics of the Sauvignon blanc wines produced with different fermentation schemes inoculated with mixed cultures of *S. cerevisiae* Sc1 and *S. pastorianus* Sp2 (A) as well as with *S. cerevisiae* Sc2 and *S. pastorianus* Sp2 (B). Table S1: Mean concentration with standard deviation of oenological parameters of Sauvignon blanc wines fermented under different fermentation schemes of pure and mixed cultures of *S. pastorianus* strain Sp2 and *S. cerevisiae* strains Sc1 and Sc2. Values with different roman letters (a–h) in the same row are significantly different according to Tukey's post hoc test ($p < 0.05$).

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References

1. Belda, I.; Ruiz, J.; Alastruey-Izquierdo, A.; Navascués, E.; Marquina, D.; Santos, A. Unraveling the enzymatic basis of wine "flavorome": A phylo-functional study of wine related yeast species. *Front. Microbiol.* **2016**, *7*, 12. [CrossRef] [PubMed]
2. Ciani, M.; Mannazzu, I.; Marinangeli, P.; Clementi, F.; Martini, A. Contribution of winery-resident *Saccharomyces cerevisiae* strains to spontaneous grape must fermentation. *Antonie Van Leeuwenhoek* **2004**, *85*, 159–164. [CrossRef] [PubMed]
3. Jolly, N.P.; Varela, C.; Pretorius, I.S. Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* **2014**, *14*, 215–237. [CrossRef] [PubMed]
4. Comitini, F.; Agarbati, A.; Canonico, L.; Ciani, M. Yeast interactions and molecular mechanisms in wine fermentation: A comprehensive review. *Int. J. Mol. Sci.* **2021**, *22*, 7754. [CrossRef] [PubMed]
5. Brice, C.; Cubillos, F.A.; Dequin, S.; Camarasa, C.; Martínez, C. Adaptability of the *Saccharomyces cerevisiae* yeasts to wine fermentation conditions relies on their strong ability to consume nitrogen. *PLoS ONE* **2018**, *13*, e0192383. [CrossRef] [PubMed]
6. Albertin, W.; Masneuf-Pomarede, I.; Galeote, V.; Legras, J.-L. New insights into wine yeast diversities. In *Yeasts in the Production of Wine*; Romano, P., Ciani, M., Fleet, G.H., Eds.; Springer: Berlin/Heidelberg, Germany, 2019; pp. 117–163.
7. Dimopoulou, M.; Troianou, V.; Toumpeki, C.; Gosselin, Y.; Dorignac, É.; Kotseridis, Y. Effect of strains from different *Saccharomyces* species used in different inoculation schemes on chemical composition and sensory characteristics of Sauvignon blanc wine. *OENO One* **2020**, *54*, 745–759. [CrossRef]
8. Darriet, P.; Tominaga, T.; Lavigne, V.; Boidron, J.-N.; Dubourdieu, D. Identification of a powerful aromatic component of *Vitis vinifera* L. var. Sauvignon wines: 4-mercapto-4-methylpentan-2-one. *Flavour Fragr. J.* **1995**, *10*, 385–392. [CrossRef]
9. Tominaga, T.; Furrer, A.; Henry, R.; Dubourdieu, D. Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. Sauvignon blanc wines. *Flavour Fragr. J.* **1998**, *13*, 159–162. [CrossRef]
10. Swiegers, J.H.; Capone, D.L.; Pardon, K.H.; Elsey, G.M.; Sefton, M.A.; Francis, I.L.; Pretorius, I.S. Engineering volatile thiol release in *Saccharomyces cerevisiae* for improved wine aroma. *Yeast* **2007**, *24*, 561–574. [CrossRef]
11. Englezos, V.; Rantsiou, K.; Cravero, F.; Torchio, F.; Pollon, M.; Fracassetti, D.; Ortiz-Julien, A.; Gerbi, V.; Rolle, L.; Cocolin, L. Volatile profile of white wines fermented with sequential inoculation of *Starmerella bacillaris* and *Saccharomyces cerevisiae*. *Food Chem.* **2018**, *257*, 350–360. [CrossRef]
12. Masneuf-Pomarède, I.; Mansour, C.; Murat, M.-L.; Tominaga, T.; Dubourdieu, D. Influence of fermentation temperature on volatile thiols concentrations in Sauvignon blanc wines. *Int. J. Food Microbiol.* **2006**, *108*, 385–390. [PubMed]
13. Legras, J.-L.; Karst, F. Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. *FEMS Microbiol. Lett.* **2003**, *221*, 249–255. [CrossRef]
14. International Organisation of Vine and Wine. *Compendium of International Methods of Wine and Must Analysis*; OIV: Paris, France, 2020.
15. Cozzolino, D.; Parker, M.; Dambergs, R.G.; Herderich, M.; Gishen, M. Chemometrics and visible-near infrared spectroscopic monitoring of red wine fermentation in a pilot scale. *Biotechnol. Bioeng.* **2006**, *95*, 1101–1107. [CrossRef] [PubMed]
16. Heymann, H.; Ebeler, S.E. *Sensory and Instrumental Evaluation of Alcoholic Beverages*; Elsevier Inc.: London, UK, 2017.

17. Nanou, E.; Mavridou, E.; Milienos, F.S.; Papadopoulos, G.; Tempère, S.; Kotseridis, Y. Odor characterization of white wines produced from indigenous Greek grape varieties using the frequency of attribute citation method with trained assessors. *Foods* **2020**, *9*, 1396. [CrossRef] [PubMed]
18. Dubourdieu, D.; Tominaga, T. Polyfunctional thiol compounds. In *Wine Chemistry and Biochemistry*; Moreno-Arribas, M.V., Polo, C., Eds.; Springer: Berlin/Heidelberg, Germany, 2009; pp. 275–293.
19. Goméz, M.J.; Cacho, J.F.; Ferreira, V.; Vicario, I.M.; Heredia, F.J. Volatile components of Zalema white wines. *Food Chem.* **2007**, *100*, 1464–1473. [CrossRef]
20. Li, H. *Wine Tasting*; Science Press: Beijing, China, 2006; pp. 29–106.
21. Goddard, M.R. Quantifying the complexities of *Saccharomyces cerevisiae*'s ecosystem engineering via fermentation. *Ecology* **2008**, *89*, 2077–2082. [CrossRef]
22. Tempère, S.; Marchal, A.; Barbe, J.-C.; Bely, M.; Masneuf-Pomarede, I.; Marullo, P.; Albertin, W. The complexity of wine: Clarifying the role of microorganisms. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 3995–4007. [CrossRef]
23. Lambrechts, M.G.; Pretorius, I.S. Yeast and its importance to wine aroma—A review. *South Afr. J. Enol. Vitic.* **2000**, *21*, 97–129. [CrossRef]
24. Roudil, L.; Russo, P.; Berbegal, C.; Albertin, W.; Spano, G.; Capozzi, V. Non-*Saccharomyces* commercial starter cultures: Scientific trends, recent patents and innovation in the wine sector. *Recent Pat. Food Nutr. Agric.* **2020**, *11*, 27–39. [CrossRef]
25. Wang, C.; Mas, A.; Esteve-Zarzoso, B. The interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific. *Front. Microbiol.* **2016**, *7*, 502. [CrossRef]
26. Franco, W.; Benavides, S.; Valencia, P.; Ramírez, C.; Urtubia, A. Native yeasts and lactic acid bacteria isolated from spontaneous fermentation of seven grape cultivars from the Maule region (Chile). *Foods* **2021**, *10*, 1737. [CrossRef] [PubMed]
27. Bordet, F.; Joran, A.; Klein, G.; Roullier-Gall, C.; Alexandre, H. Yeast–yeast interactions: Mechanisms, methodologies and impact on composition. *Microorganisms* **2020**, *8*, 600. [CrossRef] [PubMed]
28. Giannattasio, S.; Guaragnella, N.; Ždralević, M.; Marra, E. Molecular mechanisms of *Saccharomyces cerevisiae* stress adaptation and programmed cell death in response to acetic acid. *Front. Microbiol.* **2013**, *4*, 33. [CrossRef] [PubMed]
29. Vilela-Moura, A.; Schuller, D.; Mendes-Faia, A.; Côrte-Real, M. Reduction of volatile acidity of wines by selected yeast strains. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 881–890. [CrossRef] [PubMed]
30. Fleet, G.H. Yeast interactions and wine flavour. *Int. J. Food Microbiol.* **2003**, *86*, 11–22. [CrossRef]
31. Benito, Á.; Calderón, F.; Benito, S. The influence of non-*Saccharomyces* species on wine fermentation quality parameters. *Fermentation* **2019**, *5*, 54. [CrossRef]
32. Renault, P.E.; Albertin, W.; Bely, M. An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 4105–4119. [CrossRef] [PubMed]
33. Tufariello, M.; Fragasso, M.; Pico, J.; Panighel, A.; Castellarin, S.D.; Flamini, R.; Grieco, F. Influence of non-*Saccharomyces* on wine chemistry: A focus on aroma-related compounds. *Molecules* **2021**, *26*, 644. [CrossRef]
34. Bordet, F.; Roullier-Gall, C.; Ballester, J.; Vichi, S.; Quintanilla Casas, B.; Gougeon, R.; Julien-Ortiz, A.; Schmitt-Kopplin, P.; Alexandre, H. Different wines from different yeasts? Saccharomyces cerevisiae intraspecies differentiation by metabolomic signature and sensory patterns in wine. *Microorganisms* **2021**, *9*, 2327. [CrossRef]
35. Dzialo, M.C.; Park, R.; Steensels, J.; Lievens, B.; Verstrepen, K.J. Physiology, ecology and industrial applications of aroma formation in yeast. *FEMS Microbiol. Rev.* **2017**, *41* (Suppl. S1), S95–S128. [CrossRef]
36. Sadoudi, M.; Tourdot-Maréchal, R.; Rousseaux, S.; Steyer, D.; Gallardo-Chacón, J.-J.; Ballester, J.; Vichi, S.; Guérin-Schneider, R.; Caixach, J.; Alexandre, H. Yeast–yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol.* **2012**, *32*, 243–253. [CrossRef] [PubMed]
37. Roullier-Gall, C.; Bordet, F.; David, V.; Schmitt-Kopplin, P.; Alexandre, H. Yeast interaction on Chardonnay wine composition: Impact of strain and inoculation time. *Food Chem.* **2022**, *374*, 131732. [CrossRef] [PubMed]
38. Cameleyre, M.; Lytra, G.; Barbe, J.-C. Static headspace analysis using low-pressure gas chromatography and mass spectrometry, application to determining multiple partition coefficients: A practical tool for understanding red wine fruity volatile perception and the sensory impact of higher alcohols. *Anal. Chem.* **2018**, *90*, 10812–10818.
39. Dimopoulou, M.; Raffenne, J.; Claisse, O.; Miot-Sertier, C.; Iturmendi, N.; Moine, V.; Coulon, J.; Dols-Lafargue, M. *Oenococcus oeni* exopolysaccharide biosynthesis, a tool to improve malolactic starter performance. *Front. Microbiol.* **2018**, *9*, 1276. [CrossRef] [PubMed]
40. Santiago, M.; Gardner, R.C. Yeast genes required for conversion of grape precursors to varietal thiols in wine. *FEMS Yeast Res.* **2015**, *15*, fov034. [CrossRef] [PubMed]
41. Swiegers, J.H.; Willmott, R.; Hill-Ling, A.; Capone, D.L.; Pardon, K.H.; Elsey, G.M.; Howell, K.S.; de Barros Lopes, M.A.; Sefton, M.A.; Lilly, M.; et al. Modulation of volatile thiol and ester aromas by modified wine yeast. *Dev. Food Sci.* **2006**, *43*, 113–116.
42. Sarrazin, E.; Shinkaruk, S.; Pons, M.; Thibon, C.; Bennetau, B.; Darriet, P. Elucidation of the 1,3-sulfanylalcohol oxidation mechanism: An unusual identification of the disulfide of 3-sulfanylhexanol in sauternes botrytized wines. *J. Agric. Food Chem.* **2010**, *58*, 10606–10613. [CrossRef]