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Sequential Non-*Saccharomyces* and *Saccharomyces cerevisiae* Fermentations to Reduce the Alcohol Content in Wine

Margarita García [1], Braulio Esteve-Zarzoso [2], Juan Mariano Cabellos [1] and Teresa Arroyo [1,*]

[1] Department of Food and Agricultural Science, IMIDRA, 28800 Alcalá de Henares, Spain; margarita_garcia_garcia@madrid.org (M.G.); juan.cabellos@madrid.org (J.M.C.)

[2] Department of Chemistry and Biotechnology, Rovira i Virgili University, 43007 Tarragona, Spain; braulio.esteve@urv.cat

* Correspondence: teresa.arroyo@madrid.org; Tel.: +34-918-879-486

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Abstract: Over the last decades, the average alcohol content of wine has increased due to climate change and consumer preferences for particular wine styles that resulted in increased grape sugar levels at harvest. Therefore, alcohol reduction is a current challenge in the winemaking industry. Among several strategies under study, the use of non-conventional yeasts in combination with *Saccharomyces cerevisiae* plays an important role for lowering ethanol production in wines nowadays. In the present work, 33 native non-*Saccharomyces* strains were assayed in sequential culture with a *S. cerevisiae* wine strain to determine their potential for reducing the alcohol content in Malvar white wines. Four of the non-*Saccharomyces* strains (*Wickerhamomyces anomalus* 21A-5C, *Meyerozyma guilliermondii* CLI 1217, and two *Metschnikowia pulcherrima* (CLI 68 and CLI 460)) studied in sequential combination with *S. cerevisiae* CLI 889 were best able to produce dry wines with decreased alcohol proportion in comparison with one that was inoculated only with *S. cerevisiae*. These sequential fermentations produced wines with between 0.8% (*v/v*) and 1.3% (*v/v*) lower ethanol concentrations in Malvar wines, showing significant differences compared with the control. In addition, these combinations provided favorable oenological characteristics to wines such as high glycerol proportion, volatile higher alcohols, and esters with fruity and sweet character.

Keywords: alcohol reduction; native yeast; non-*Saccharomyces*; sequential fermentation; wine

1. Introduction

At present, the increasing alcohol content in wines is closely related to climate change and consumer choice for full-bodied, rich, and ripe fruit flavor profiles, which often involve increased grape maturity [1–3]. In recent years, the worldwide trend towards more frequent warm periods during the grapevine growing season has increased sugar content in grapes and therefore the alcohol concentration in wines [4]. Thus, the average alcohol level has risen about 2% (*v/v*) over the past few decades in warm areas, and it is not uncommon to find wines with an alcohol content higher than 16% (*v/v*) [5]. Excessive alcohol concentration in wines can alter the sensory profile of wines, increasing bitterness, astringency, and hotness perception and masking some volatile compounds [6,7]. Additionally, wines with elevated alcohol content can lead to harmful health effects [8] and also increase costs in markets where taxes are linked to the ethanol level in many countries [9].

Among the various methodologies aimed at the reduction of alcohol content in wines, microbiological approaches may be promising to preserve organoleptic characteristics and quality in wines. In addition, they are profitable and easy to implement strategies that do not require the need

for specialized equipment [10,11]. *Saccharomyces cerevisiae* is the principal microorganism selected for winemaking. This species completes fermentation of sugars due to its ability to produce and tolerate high concentrations of alcohol [12,13]. Unlike *S. cerevisiae*, non-*Saccharomyces* yeasts are not generally able to complete the fermentation process; thus, mixed or sequential inoculations with *S. cerevisiae* are required for this purpose [14–17]. Research efforts have therefore focused on developing new *S. cerevisiae* strains that produce less ethanol in wine [18] and on using non-*Saccharomyces* yeasts that metabolize sugar without producing ethanol or that do so with less efficiency [19].

Several investigations have employed non-*Saccharomyces* co-cultures as a tool for reducing the ethanol concentration in wine [19–29]. Here, the early inoculation of non-*Saccharomyces* strain transforms sugar to produce biomass and by-products, decreasing ethanol formation before addition of *S. cerevisiae* [2,30]. This action plan is particularly adequate to winemaking in warm regions, as in the case of the Madrid winegrowing region (Spain) under study in the present work. The climate in the Denomination of Origin (D.O.) "Vinos de Madrid" presents temperatures ranging from −8 °C in winter to 41 °C in summer, and rainfall ranges between 461 and 658 mm [31]. Winemakers in this region are working hard in order to elaborate new styles of wine that are more competitive in the market [32]. The knowledge and selection of native yeasts is a very important achievement to confer typicity and originality to the wine [33,34], and its use is also considered a reactive adaptation practice to climate change [35].

In this work, 33 native non-*Saccharomyces* strains from 13 different wine yeast species were tested with the aim of identifying yeasts that, in sequential fermentation with *S. cerevisiae*, could be used for reducing alcohol content in Malvar white wines, and additionally evaluating their positive impact on the quality of these wines. Moreover, no previous investigations have been carried out to select non-*Saccharomyces*/*S. cerevisiae* combinations with native yeasts from D.O. "Vinos de Madrid" (Madrid, Spain) directed towards ethanol reduction in wines.

2. Materials and Methods

2.1. Yeast Strains: Purity and Identity Control

A total of 33 non-*Saccharomyces* strains from the IMIDRA collection belonging to 10 different genera were used in this study (Table 1). All non-*Saccharomyces* strains were native from D.O. "Vinos de Madrid" vineyards and cellars [31,36]. The well-studied native strain, *S. cerevisiae* CLI 889, was employed as a control [31,34,37]. Cryogenically preserved (−80 °C) strains in 30% glycerol were subsequently seeded on YPD liquid medium (1% yeast extract, 1% meat peptone, and 2% glucose (Conda Laboratories, Madrid, Spain), w/v) and incubated for 24–48 h at 28 °C. Later, all strains were maintained at 4 °C on YPD plates.

To confirm yeast strain identifications, DNA extraction and rDNA 5.8S–ITS region PCR-RFLP analysis [38] were employed as described previously by Cordero-Bueso et al. [39]. Some of these strains were also sequenced [31,40], and the D1/D2 domain of the 26S rDNA gene was amplified using primers NL-1 and NL-4 [41].

Table 1. Yeast strains used in this study.

Species Name	Strain Code	Year of Isolation	Origin [1]	References [2]
Wickerhamomyces anomalus	CLI 1218	2007	Malvar [a]	[31,36]
	31-1C	2006	Garnacha [c]	This study
	21A-5C	2007	Garnacha [c]	[36]
	23A-6C	2007	Garnacha [c]	[36]
	5B-1C	2008	Garnacha [c]	This study
Candida stellata	6-5A	2006	Shiraz [c]	[36]
	2A-1B	2007	Shiraz [c]	This study

Table 1. *Cont.*

Species Name	Strain Code	Year of Isolation	Origin [1]	References [2]
Hanseniaspora valbyensis	CLI 194	1993	Garnacha [a]	[36]
	CLI 190	1993	Garnacha [a]	[36]
Hanseniaspora guilliermondii	CLI 417	1995	Malvar [a]	This study
	7A-3A	2007	Garnacha [c]	This study
	8A-8B	2007	Garnacha [c]	This study
	CLI 225	1994	Tempranillo [a]	[36,42]
	CLI 72	1993	Garnacha [a]	[36]
Hanseniaspora uvarum	CLI 903	1993	Airén [b]	[36,42]
Hanseniaspora vineae	CLI 3	1993	Tempranillo [a]	[36]
Torulaspora delbrueckii	LS1 FF2 3A	2009	Garnacha [a]	[33]
	LS2 FF2 1A	2009	Garnacha [a]	[33]
	CLI 918	2006	Malvar [a]	[16,40,42,43]
Metschnikowia pulcherrima	CLI 68	1993	Garnacha [a]	[36]
	CLI 457	1995	Malvar [a]	[16,36,40,42]
	CLI 463	1995	Malvar [a]	This study
	CLI 219	1994	Malvar [a]	[36,42]
	CLI 460	1995	Malvar [a]	[36,42]
	CLI 461	1995	Malvar [a]	This study
Lachancea thermotolerans	AMB FF4 10A	2009	Malvar [a]	[33]
	3-4A	2006	Shiraz [c]	[36]
	9-6C	2006	Malvar [a]	[16,40,42]
	CLI 1219	2007	Malvar [a]	[31,42]
Pichia membranifaciens	CLI 679	2006	Malvar [a]	[31,42]
Meyerozyma guilliermondii	CLI 1217	2006	Malvar [a]	[31,42]
Priceomyces carsonii	CLI 1221	2006	Malvar [a]	[31,42]
Zygosaccharomyces bailii	CLI 622	2009	Malvar [a]	[31,42]
Saccharomyces cerevisiae	CLI 889	2000	Airén [a]	[16,34,37,40,42,43]

[1] a, spontaneous fermentation; b, must; c, grape; [2] publications in which strains have been investigated.

2.2. Laboratory-Scale Fermentations

Bunches from healthy grapes of white Malvar (*Vitis vinifera* L. cv.) variety were collected from a vineyard in the Madrid winegrowing region of Spain (40°31′ N, 3°17′ W and 610 m altitude). The must was clarified by pectolytic enzymes (Enozym Altair, Agrovin, Spain) (0.01 g/L) at 4 °C and stored frozen until use. The main characteristics of Malvar must were pH 3.3; 23.3 °Brix, equivalent to about 230 g/L of reducing sugars; probable alcohol content, 13.5% (*v/v*); and yeast assimilable nitrogen (YAN), 170 mg/L.

The grape must was inoculated with a final concentration of 10^6 cells/mL from 48 h pre-cultures of each yeast strain (33 non-*Saccharomyces* and 1 *S. cerevisiae* as control strain). The fermentations were carried out in quadruplicate in 50 mL Falcon tubes containing 30 mL of sterile Malvar must. The trials were divided into two sections: Section I (pure culture), where strain growth was performed in aerobic conditions at 20 °C with continuous orbital shaking (130 rpm). The fermentation kinetic was controlled daily by weight loss. At 96 h, one duplicate of each trial was used to the study of dry weight, residual sugars (glucose + fructose), and volatile acidity (as g/L of acetic acid); and Section II (sequential culture), the other duplicate from Section I, was inoculated with 10^6 cells/mL *S. cerevisiae* CLI 889. In this case, Falcon tubes hermetically sealed and fitted with air locks ensured anaerobic conditions. The fermentation process was conducted at 20 °C with shaking at 130 rpm and was monitored daily until constant weight. Then, wine analyses were carried out.

Dry cell weight measurements were performed on samples from sections I and II. The wine samples were centrifuged (10,000 rpm, 5 min) and the pellets were washed with deionized water twice. Finally, dry weight was determined by filtering through a 0.45 µm pore size membrane filter (Millipore). Filters were heat-dried at 105 °C until constant weight was obtained.

2.3. Analitycal Determination of Wines

The concentration of glucose, fructose, glycerol, ethanol, and organic acids (malic, lactic, and acetic acids) was determined using a Waters 600E HPLC system (Waters, Milford, MA) at the end of fermentation. The HPLC was equipped with a Waters 2414 refractive index (RI) and Waters 2996 photodiode array detector (PDA) on a Rezex RHM−Monosaccharide H+ (8%) column (300 × 7.8 mm, Phenomenex, Torrance, CA, USA). The column was maintained at 65 °C, and 5 mM H_2SO_4 was used as mobile phase at a flow rate of 0.6 mL/min. In wine samples at 96 h, only residual sugars (glucose + fructose) and volatile acidity (as g/L of acetic acid) were measured with a multi analyzer LISA 200 (TDI, Barcelona, Spain), using enzymatic kits (TDI, Barcelona, Spain).

Quantification of major volatile compounds of wines was achieved using the gas chromatography coupled to flame ionization detector (GC−FID) technique. The GC system employed was an Agilent 6850 with a FID detector equipped with a column DB-Wax (60 m × 0.32 mm × 0.5 µm film thickness) from J&W Scientific (Folsom, CA, USA). The extraction and analysis methodologies of volatile analytes were performed following the procedure described by Ortega et al. [44]. Identification and quantification of the 32 individual major volatiles was performed using commercial pure standards. Calibration curves were drawn for each standard at 6 different concentration levels. Each standard was prepared in a synthetic wine solution (5 g/L of tartaric acid, dissolved in 13% of ethanol solution (*v/v*), at pH 3.4 adjusted with NaOH). The obtained coefficients of regression (R^2) were > 0.990 [32,45].

2.4. Statistical Treatment of Data

The data were analyzed with SPSS Statistics 25 software (SPSS Inc., Chicago, IL, USA). Analysis of variance was carried out by ANOVA Tukey's test to examine significant differences between samples. Thus, a principal component analysis (PCA) was used to study the contribution of oenological and aromatic variables to the differences between Malvar wines.

3. Results

The genetic identification of the 33 non-*Saccharomyces* strains from vineyards and cellars of D.O. "Vinos de Madrid" allowed them to be classified into 13 species belonging to 10 different genera. The initial strain selection was designed to include species frequently isolated from the winemaking environment. Moreover, our strategy for ethanol reduction was the use of one non-*Saccharomyces* strain that exhibited a low ethanol yield but consumed enough sugars to affect the ethanol concentration (Section I) and be compatible with *S. cerevisiae* in order to ensure the completion of fermentation (Section II).

3.1. Section I: Pure Culture of Non-Saccharomyces Strains

In this section of the work, we studied fermentative kinetics of non-*Saccharomyces* strains and the control strain (*S. cerevisiae* CLI 889) in pure cultures. The fermentative profiles during the first 96 h permitted the division of the strains into three different groups: A, B, and C (Figure 1). Group A was represented by four non-*Saccharomyces* species: *Wickerhamomyces anomalus* (two strains), *Candida stellata* (one strain), *Lachancea thermotolerans* (one strain), and *Hanseniaspora guilliermondii* (two strains), which showed similar CO_2 released to *S. cerevisiae* CLI 889 control strain. Group B included all *Torulaspora delbrueckii* and *Hanseniaspora valbyensis* strains studied in this work together with the other three *L. thermotolerans* strains. These strains showed less fermentative capacity than the control, with a CO_2 loss between 2.2−1.2 g against above 3 g liberated by *S. cerevisiae* CLI 889. Nine different non-*Saccharomyces* species were represented within group C. This latter group had 19 of

33 non-*Saccharomyces* strains studied, wherein their fermentation kinetics presented the lowest CO_2 liberation observed during the first 96 h.

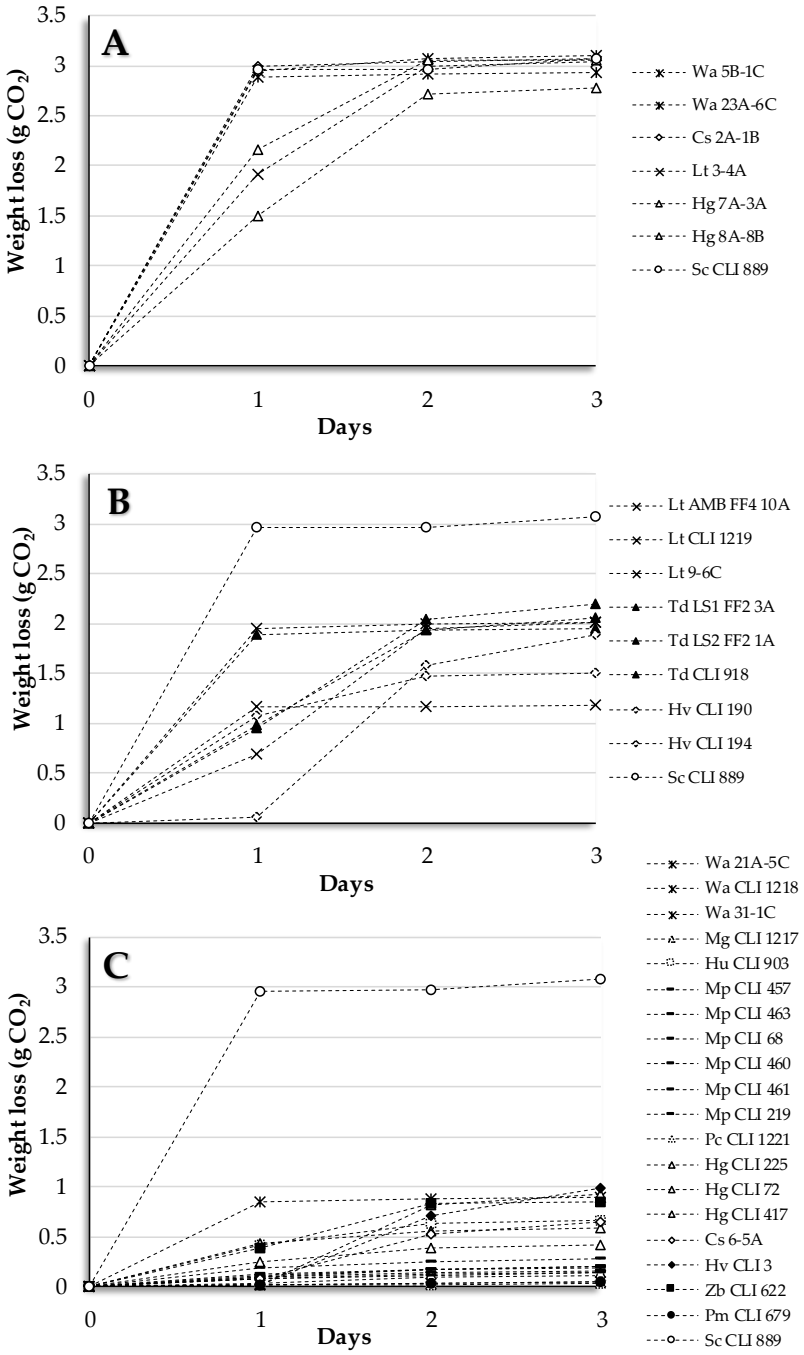

Figure 1. Release of CO_2 in aerobic conditions. (**A**) Six non-*Saccharomyces* strains showed similar CO_2 released to Sc CLI 889 control strain (white circles). (**B**) Eight non-*Saccharomyces* strains showed less CO_2 released than the control (white circles). (**C**) Nineteen non-*Saccharomyces* strains showed values below 1 g of CO_2 liberated from the control (white circles). Wa, *W. anomalus*; Cs, *C. stellata*; Lt, *L. thermotolerans*; Hg, *H. guilliermondii*; Sc, *S. cerevisiae*; Td, *T. delbrueckii*; Hv, *H. valbyensis*; Mg, *M. guilliermondii*; Hu, *H. uvarum*; Mp, *M. pulcherrima*; Pc, *P. carsonii*; Hv, *H. vineae*; Zb, *Z. bailii*; Pm, *P. membranifaciens*.

3.2. Section II: Sequential Culture of Non-*Saccharomyces*/*S. cerevisiae* Strains

After 96 h, *S. cerevisiae* CLI 889 was sequentially inoculated into all fermentations in Section I. A total of 8 days were needed by yeast strains to complete the fermentation process (Sections I and II).

Yeast isolates with fermentation behavior showing in group A (Section I) did not exhibit an increase on the CO_2 release, producing similar amounts of ethanol at the end of fermentation—all of these wines were about 13% (*v/v*).

When sequential fermentations finished, some strains combinations produced wines with ethanol concentration similar to the control (13%, *v/v*), and thus they were discarded as low-ethanol cultures. These sequential combinations that were not selected included the strains CLI 679, CLI 1218, 31-1C, CLI 457, CLI 72, CLI 219, CLI 461, CLI 463, CLI 1221, and CLI 903; all of them were classified into group C (Section I). Another group of non-*Saccharomyces*/*S. cerevisiae* fermentations, including CLI 918, CLI 194, CLI 1219, AMB FF4 10A, CLI 190, and LS1 FF2 3A strains from group B (Section I), and CLI 225, CLI 622, CLI 417, 6-5A, and CLI 3 strains from group C (Section I), increased by between 7% and 10% in terms of ethanol concentration, but high amounts of residual sugars were not fermented, and thus these combinations were not selected either; most of them belonged to group B (Section I) in which CO_2 liberated was lower than the control with values between 1.18 and 2.19 g. Finally, four non-*Saccharomyces*/*S. cerevisiae* sequential inoculations produced wines with decreased ethanol proportions compared with the control, and the residual sugars values were suitable for dry wines (<5 g/L residual sugar) (Table 2).

Table 2. Oenological parameters and cell dry weight for the best non-*Saccharomyces*/*S. cerevisiae* sequential combinations to reduce ethanol concentration in wines.

Parameters	Yeast Culture				
	Wa 21A-5C(S)	**Mp CLI 68(S)**	**Mg CLI 1217(S)**	**Mp CLI 460(S)**	**Sc CLI 889(P)**
Malic acid (g/L)	0.66 ± 0.12 ^{a}	0.60 ± 0.02 ^{a}	0.64 ± 0.02 ^{a}	0.45 ± 0.09 ^{a}	0.55 ± 0.02 ^{a}
Lactic acid (g/L)	2.10 ± 0.26 ^{a}	2.48 ± 0.30 ^{a}	2.50 ± 0.23 ^{a}	2.22 ± 0.53 ^{a}	2.31 ± 0.02 ^{a}
Acetic acid (g/L)	0.77 ± 0.08 ^{a}	0.86 ± 0.01 ^{ab}	0.78 ± 0.01 ^{ab}	0.71 ± 0.16 ^{a}	0.43 ± 0.00 ^{ac}
Glucose (g/L)	2.45 ± 0.30 ^{a}	2.80 ± 0.54 ^{a}	2.04 ± 0.39 ^{a}	2.97 ± 0.53 ^{a}	2.70 ± 0.01 ^{a}
Fructose (g/L)	1.06 ± 0.10 ^{a}	2.72 ± 0.60 ^{b}	0.69 ± 0.04 ^{ac}	1.93 ± 0.29 ^{ab}	1.01 ± 0.02 ^{a}
Glycerol (g/L)	7.83 ± 0.31 ^{a}	8.32 ± 0.10 ^{a}	7.06 ± 0.61 ^{ab}	9.30 ± 0.90 ^{ac}	7.60 ± 0.02 ^{a}
Alcohol degree (%)	12.05 ± 0.12 ^{a}	11.75 ± 0.05 ^{a}	11.77 ± 0.32 ^{a}	12.16 ± 0.26 ^{a}	13.00 ± 0.01 ^{b}
Dry weight (mg)	4.35 ± 0.07 ^{a}	3.73 ± 0.08 ^{a}	4.77 ± 0.57 ^{ab}	3.28 ± 0.61 ^{a}	2.95 ± 0.01 ^{ac}

Data are means ± standard deviation (n = 2). Data with different letters $^{(a,b,c)}$ within each row are significantly different (Tukey test; $p < 0.05$). (S), sequential culture; (P), pure culture.

3.3. Yeast Strain Sequential Combinations Selected as Low-Ethanol Producers

In order to reduce the ethanol content in wines, the selected non-*Saccharomyces* yeast strains were *W. anomalus* 21A-5C, *Metschnikowia pulcherrima* CLI 68, *Meyerozyma guilliermondii* CLI 1217, and *M. pulcherrima* CLI 460 used in sequential combination with *S. cerevisiae* CLI 889. These co-cultures produced wines with between 0.8% (*v/v*) and 1.3% (*v/v*) lower ethanol concentrations in Malvar wines, showing significant differences from the control (Table 2).

Sequential cultures inoculated with 21A-5C, CLI 68, and CLI 460 produced more glycerol than the control, highlighting *M. pulcherrima* CLI 460 strain with values significantly higher than the control (Table 2). There were no significantly differences in malic and lactic acid content, and fermentations with sequential combinations generated more acetic acid than the amount produced by the *S. cerevisiae* control (Table 2). Regarding dry weight, all sequential fermentations presented greater values compared with the control; in particular, sequential culture of *M. guilliermondii* CLI 1217 was 1.6-fold higher, showing significant differences (Table 2).

To find the aromatic composition of these wines, we studied 32 volatile compounds classified in alcohols, esters, acids, and aldehydes/ketones (Table 3). Sequential inoculation produced Malvar wines with greater total concentration of higher alcohols. The amounts of isoamyl alcohol (harsh,

bitter) and β-phenylethyl alcohol (flowery, roses) were significantly higher in wines produced in sequential culture, increasing the total concentration of alcohols. The ethyl isovalerate and isoamyl acetate ester concentration responsible for fruity and sweet aromas were significantly different in wines generated from sequential inoculations. Regarding volatile acids, isobutyric acid and hexanoic acid were the main compounds responsible for the total concentration of volatile acids in all wines. The sequential culture *W. anomalus* 21A-5C/*S. cerevisiae* CLI 889 produced the highest concentration of the ketone acetoin. Finally, sequential cultures with *M. pulcherrima* strains (CLI 68 and CLI 460) and the control showed higher amounts of γ-butyrolactone, related to sweet aroma in wines.

Table 3. Major volatile compounds (mg/L) of wines produced in the Section II (sequential culture of non-*Saccharomyces* strains + *S. cerevisiae* CLI 889 and a control, *S. cerevisiae* CLI 889 pure culture).

Compound	Wa 21A-5C(S)	Mp CLI 68(S)	Mg CLI 1217(S)	Mp CLI 460(S)	Sc CLI 889(P)
1-Propanol	n.q.	n.q.	n.q.	n.q.	3.69 ± 0.13 ^{a}
1-Butanol	1.81 ± 0.14 ^{a}	0.50 ± 0.05 ^{b}	0.46 ± 0.05 ^{b}	0.48 ± 0.12 ^{b}	0.40 ± 0.10 ^{b}
Isobutanol	31.96 ± 1.31 ^{a}	33.51 ± 4.11 ^{a}	30.51 ± 1.28 ^{a}	49.46 ± 0.24 ^{b}	26.30 ± 0.95 ^{a}
Isoamyl alcohol	118.13 ± 1.88 ^{a}	114.56 ± 5.92 ^{ab}	122.03 ± 0.33 ^{a}	106.47 ± 0.53 ^{b}	91.37 ± 3.14 ^{c}
(Z)-3-Hexen-1-ol	0.12 ± 0.00 ^{a}	0.04 ± 0.00 ^{b}	0.05 ± 0.00 ^{c}	0.04 ± 0.00 ^{b}	0.20 ± 0.10 ^{d}
1-Hexanol	0.49 ± 0.00 ^{a}	0.26 ± 0.00 ^{b}	0.23 ± 0.03 ^{b}	0.22 ± 0.00 ^{b}	0.88 ± 0.05 ^{c}
Metionol	0.09 ± 0.00 ^{a}	0.37 ± 0.00 ^{ab}	0.32 ± 0.00 ^{ab}	0.53 ± 0.20 ^{b}	0.61 ± 0.10 ^{b}
Benzyl alcohol	0.17 ± 0.00 ^{a}	0.19 ± 0.00 ^{b}	0.27 ± 0.00 ^{c}	0.13 ± 0.00 ^{d}	0.15 ± 0.06 ^{e}
β-Phenylethyl alcohol	27.55 ± 0.05 ^{a}	21.27 ± 2.02 ^{b}	18.93 ± 1.04 ^{b}	23.03 ± 1.40 ^{ab}	10.53 ± 0.29 ^{c}
∑ Alcohols	**181.48 ± 3.37** ^{a}	**171.86 ± 11.99** ^{a}	**173.94 ± 0.01** ^{a}	**181.51 ± 1.83** ^{a}	**134.13 ± 4.92** ^{b}
Ethyl butyrate	0.21 ± 0.03 ^{a}	0.30 ± 0.01 ^{a}	0.30 ± 0.04 ^{a}	0.29 ± 0.03 ^{a}	0.31 ± 0.05 ^{a}
Ethyl isovalerate	1.35 ± 0.07 ^{a}	0.81 ± 0.01 ^{b}	0.98 ± 0.10 ^{b}	0.90 ± 0.06 ^{b}	0.28 ± 0.05 ^{c}
Ethyl isobutyrate	n.q.	n.q.	n.q.	n.q.	2.60 ± 0.37 ^{a}
Isoamyl acetate	2.07 ± 0.02 ^{a}	1.97 ± 0.04 ^{a}	2.80 ± 0.00 ^{b}	1.96 ± 0.17 ^{a}	0.99 ± 0.05 ^{c}
Ethyl hexanoate	0.03 ± 0.00 ^{a}	0.21 ± 0.00 ^{b}	0.13 ± 0.03 ^{ab}	0.20 ± 0.05 ^{b}	0.70 ± 0.06 ^{c}
Ethyl-3-hydroxybutyrate	0.16 ± 0.00 ^{a}	0.57 ± 0.00 ^{b}	0.68 ± 0.00 ^{c}	0.47 ± 0.00 ^{d}	0.32 ± 0.06 ^{e}
Hexyl acetate	0.05 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}	0.06 ± 0.00 ^{a}	0.07 ± 0.00 ^{a}	0.07 ± 0.05 ^{a}
2-Phenylethyl acetate	0.31 ± 0.01 ^{a}	0.28 ± 0.01 ^{a}	0.24 ± 0.09 ^{a}	0.39 ± 0.06 ^{a}	0.76 ± 0.09 ^{b}
Diethyl succinate	n.q.	0.09 ± 0.00 ^{a}	0.05 ± 0.00 ^{a}	0.26 ± 0.08 ^{b}	6.57 ± 0.13 ^{c}
Ethyl octanoate	0.06 ± 0.00 ^{a}	0.17 ± 0.00 ^{b}	0.21 ± 0.00 ^{b}	0.18 ± 0.04 ^{b}	0.51 ± 0.06 ^{c}
Ethyl lactate	1.71 ± 0.56 ^{a}	8.23 ± 1.24 ^{b}	1.73 ± 0.39 ^{a}	5.93 ± 1.27 ^{bc}	3.32 ± 0.11 ^{ac}
∑ Esters	**5.98 ± 0.63** ^{a}	**12.68 ± 1.19** ^{b}	**7.19 ± 0.45** ^{a}	**10.63 ± 1.14** ^{b}	**16.43 ± 1.08** ^{c}
Isobutyric acid	4.86 ± 0.05 ^{a}	4.99 ± 0.34 ^{a}	3.25 ± 0.07 ^{b}	4.62 ± 0.04 ^{a}	2.89 ± 0.05 ^{b}
Butyric acid	0.22 ± 0.00 ^{a}	0.29 ± 0.01 ^{a}	0.25 ± 0.00 ^{a}	0.40 ± 0.14 ^{a}	0.23 ± 0.06 ^{a}
Isovaleric acid	1.82 ± 0.03 ^{a}	1.25 ± 0.04 ^{b}	1.29 ± 0.10 ^{b}	0.76 ± 0.01 ^{c}	0.74 ± 0.06 ^{c}
Hexanoic acid	0.90 ± 0.01 ^{a}	2.97 ± 0.65 ^{abc}	2.03 ± 0.60 ^{ac}	5.01 ± 0.76 ^{b}	3.11 ± 0.34 ^{bc}
Octanoic acid	0.41 ± 0.02 ^{a}	1.88 ± 0.09 ^{b}	1.26 ± 0.16 ^{c}	1.45 ± 0.15 ^{c}	2.18 ± 0.05 ^{b}
Decanoic acid	0.09 ± 0.00 ^{a}	0.18 ± 0.06 ^{a}	0.07 ± 0.00 ^{a}	0.14 ± 0.05 ^{a}	0.73 ± 0.09 ^{b}
∑ Acids	**8.30 ± 0.11** ^{a}	**11.57 ± 0.13** ^{bc}	**8.15 ± 0.60** ^{a}	**12.37 ± 1.14** ^{c}	**9.88 ± 0.65** ^{ab}
Diacetyle	0.58 ± 0.01 ^{a}	0.46 ± 0.17 ^{a}	0.51 ± 0.02 ^{a}	0.48 ± 0.04 ^{a}	0.63 ± 0.09 ^{a}
Furfural	n.q.	0.07 ± 0.00 ^{a}	0.04 ± 0.00 ^{a}	0.08 ± 0.00 ^{a}	0.07 ± 0.05 ^{a}
Benzaldehyde	0.01 ± 0.00 ^{a}	0.04 ± 0.00 ^{ab}	0.06 ± 0.00 ^{b}	0.05 ± 0.01 ^{b}	0.09 ± 0.05 ^{c}
Phenylacetaldehyde	n.q.	n.q.	n.q.	n.q.	0.52 ± 0.05 ^{a}
Acetoin	5.19 ± 0.46 ^{a}	1.48 ± 0.01 ^{b}	3.71 ± 0.00 ^{c}	2.30 ± 0.43 ^{b}	0.20 ± 0.10 ^{d}
∑ Aldehydes/Ketones	**5.78 ± 0.47** ^{a}	**2.04 ± 0.15** ^{b}	**4.32 ± 0.02** ^{c}	**2.91 ± 0.45** ^{bd}	**1.51 ± 0.34** ^{be}
γ-Butyrolactone	0.98 ± 0.00 ^{a}	6.78 ± 0.13 ^{b}	1.64 ± 0.00 ^{c}	5.41 ± 0.07 ^{d}	9.40 ± 0.10 ^{e}

Data are means ± standard deviation (n = 2). Data with different letters $^{(a,b,c,d,e)}$ within each row are significantly different (Tukey test; $p < 0.05$). n.q., not quantifiable. (S), sequential culture; (P), pure culture.

A PCA analysis was performed to cluster wines from sequential combinations and the control according to their oenological and aromatic composition. In the score plot for the first two principal components, PC1 and PC2 explain 75.9% of the total variance (Figure 2). PC1 was mainly determined by ethyl hexanoate (0.986), ethyl octanoate (0.950), decanoic acid (0.944), 2-phenylethyl acetate (0.916), total esters (0.916), 1-propanol (0.916), γ-butyrolactone (0.903), and alcohol degree (0.831); this component allowed us to differentiate the control *S. cerevisiae* fermentation from those fermentations conducted by sequential non-*Saccharomyces*/*S. cerevisiae* combinations. The principal constituents for PC2 were total volatile acids (0.903), butyric acid (0.840), hexanoic acid (0.810), glycerol (0.780), fructose (0.773), and isobutanol (0.740).

PC2 differentiated the sequential fermentations among them, showing clearly two groups. One group formed by sequential inoculations with *W. anomalus* 21A-5C/*S. cerevisiae* CLI 889 and *M. guilliermondii* CLI 1217/*S. cerevisiae* CLI 889, mostly related to dry weight, isoamyl alcohol, acetoin, total aldehydes/ketones, isoamyl acetate, ethyl isovalerate, and β-phenylethyl alcohol in the loadings plot (Figure 2B). Another group contained the sequential cultures with *M. pulcherrima* strains (CLI 68 and CLI 460), mainly classified by total acids, butyric acid, ethyl lactate, hexanoic acid, glycerol, fructose, and isobutanol.

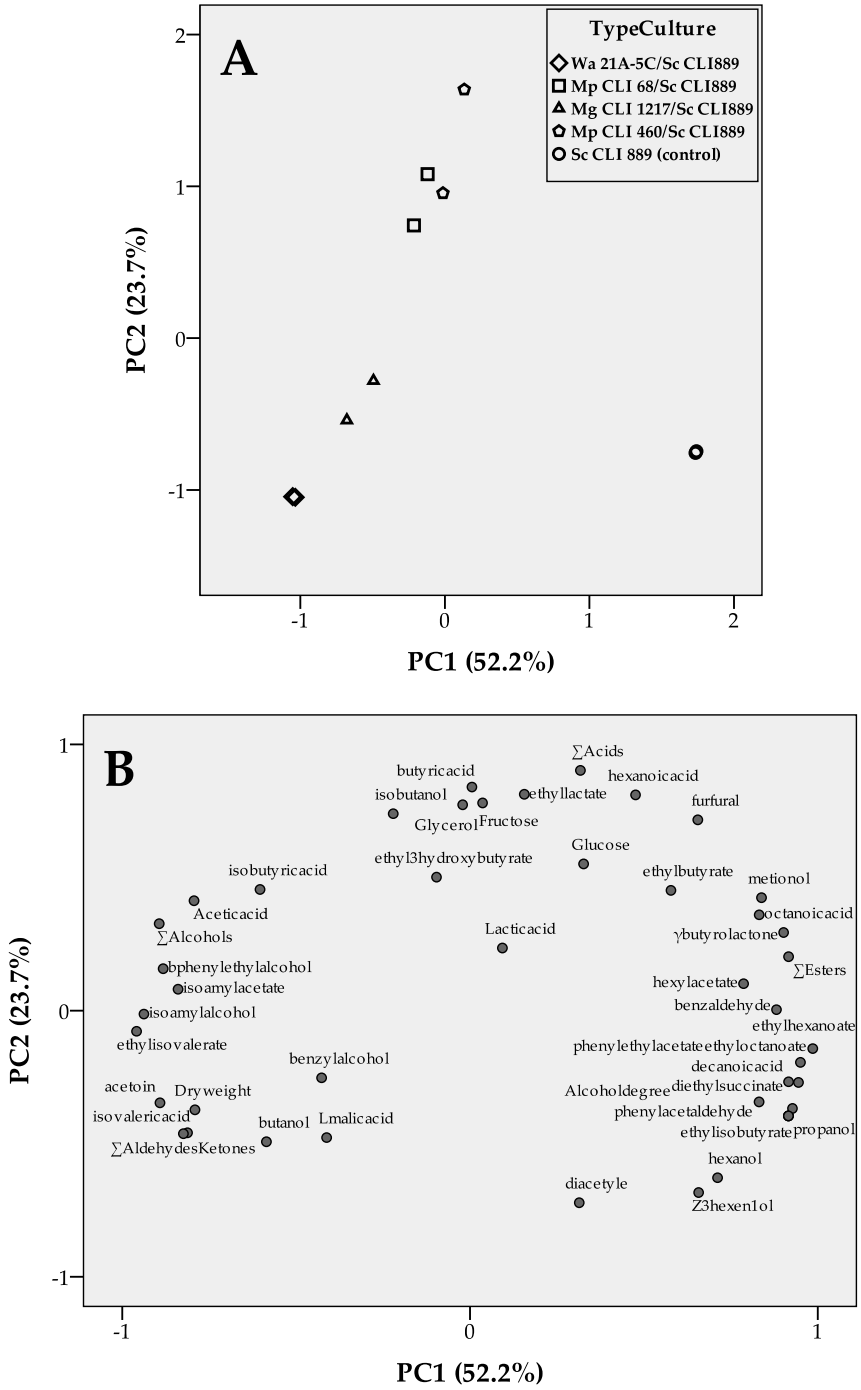

Figure 2. Principal component analysis (PCA) score plot (**A**) and loadings plot (**B**) using main fermentation parameters and 32 volatile compounds.

4. Discussion

A combination of quality, health, and economic reasons will force wine producers to find efficient strategies that enable the production of wines with lower ethanol content without detriment on sensory properties. In this work, the strategy employed for this purpose was the use of sequential combinations between non-*Saccharomyces* and *S. cerevisiae* yeast strains. It is well documented that non-*Saccharomyces* strains are often unable to consume all sugar present in a grape must [13,46]. Hence, sequential culture application would allow the completion of fermentation using one *S. cerevisiae* strain in a second instance [40,47–49]. The successful trials will be carried out by non-*Saccharomyces* strains with a low ethanol yield or those that are able to aerobically metabolize sugars without the simultaneous production of ethanol, prior to *S. cerevisiae* inoculation [2,50]. Regarding aeration regimen, some authors have suggested the use of aerobic yeasts in order to oxide sugars at early stages of winemaking and therefore decrease ethanol production [30,51,52]. After *S. cerevisiae* inoculation, researchers have favored anaerobic conditions to increase the ethanol yield of *Saccharomyces* strain and to avoid excessive oxidation of wine. The fermentation procedure programmed in this work was found to have positive results with other authors [50,51,53].

Several studies have evaluated the action of non-*Saccharomyces*/*S. cerevisiae* combinations in the reduction of ethanol content in wines [19–22,25–29,50,54]. In some cases, the lower ethanol yields resulted from high residual sugar at the end of fermentation [20,21,50]. By contrast, other research works have reported wines with a significant reduction in ethanol yield (0.6−1.7%, *v/v*) when using non-*Saccharomyces* and *S. cerevisiae* strains in mixed or sequential cultures. Contreras et al. [22] found that sequential inoculation of a selected *M. pulcherrima* strain (AWRI1149) with *S. cerevisiae* wine strain was the best combination for reducing the ethanol content in Chardonnay (0.9%, *v/v* lower than control) and Shiraz (1.6% *v/v* lower than control) wines. In the same way, Varela et al. [26] obtained Merlot wines fermented with *M. pulcherrima* with 1.0% less ethanol than *S. cerevisiae*-fermented wines at pilot scale. Further studies also showed ethanol reduction using immobilized selected strains of non-*Saccharomyces* yeasts followed by inoculation of free *S. cerevisiae* cells [25,47]. The sequential cultures of *M. pulcherrima* and *Starmerella bombicola* immobilized cells and *S. cerevisiae* free cells were the best for ethanol reduction with values 1.4% and 1.6% v/v, respectively [25]. In addition, ethanol lowering has been recorded in wines obtained by different *Saccharomyces* species. Using sterile Shiraz must, sequential inoculation of *M. pulcherrima* (AWRI1149) and *S. uvarum* (AWRI2846) with *S. cerevisiae* produced wines with 0.9% *v/v* less ethanol than *S. cerevisiae* alone [55]. Puškaš et al. [29] also observed that sequential cultures with *M. pulcherrima*, *S. bayanus*, and *S. cerevisiae* generated wines with 0.9% *v/v* lower ethanol than control. In the present work, the application of sequential cultures of native non-*Saccharomyces* strains (*W. anomalus* 21A-5C, *M. guilliermondii* CLI 1217, and *M. pulcherrima* CLI 68 and CLI 460) and *S. cerevisiae* CLI 889 generated a reduction of alcohol content between 0.8%−1.3% *v/v* in Malvar wines, where *M. pulcherrima* CLI 68/*S. cerevisiae* CLI 889 sequential inoculation produced the highest decrease in alcohol degree. On the other hand, *W. anomalus* has been described as low fermentative species in pure culture compared to *S. cerevisiae* [28,31]. This statement is consistent with our results where *W. anomalus* 21A-5C presented 121 g/L of residual sugars after the first 96 h (Table S1). In sequential culture with *S. cerevisiae*, previous works denoted that the presence of *W. anomalus* does not affect final alcohol contents [28]. Instead, the strain studied in this work (*W. anomalus* 21A-5C) produced wines with 0.9% *v/v* less ethanol than control, in agreement with Contreras et al. [19] who studied another strain of the *W. anomalus* species. Finally, the use of *M. guilliermondii* as a low-ethanol producer has not been well documented. Some research works have studied *M. guilliermondii* as a candidate for reducing ethanol content in wines, but none have considered its use for that purpose [19,56]. In contrast, *M. guilliermondii* CLI 1217 in sequential culture was the second-best option to decrease the ethanol concentration in Malvar wines (1.2% less ethanol than control).

Beyond ethanol, the growth of the four selected non-*Saccharomyces* affected glycerol and acetic acid concentrations in Malvar wines. Several studies have reported that the production of glycerol by yeasts leads to an increase in acetic acid concentration [57,58]. Wines produced with *M. pulcherrima* strains

CLI 68 and CLI 460 contained the greatest glycerol content (8.32 and 9.30 g/L, respectively) compared with other wines studied. The connection between *M. pulcherrima* and an increased glycerol production has been explained by the overexpression of the glycerol-3-phosphate dehydrogenase 1 (*GDP1*) gene in *S. cerevisiae* (associated with the conversion of dihydroxyacetone phosphate in glycerol-3-phosphate, an intermediate for glycerol formation). This gene is overinduced when *S. cerevisiae* coexists with *M. pulcherrima* in must fermentations [59]. Moreover, glycerol formation has been demonstrated as the best strategy, followed by yeasts for producing wines with lower ethanol content [60]. This compound is present in semi-sweet and dry wines ranging from 5 to 14 g/L, although glycerol imparts sweetness at a threshold of about 5.2 g/L in dry white wines [61]. Unlike glycerol, acetic acid imparts an objectionable character to wine at elevated concentrations. This volatile acid becomes undesirable at concentrations over 0.7–1.1 g/L, depending on the style of wine; its optimal concentration is 0.2–0.7 g/L [61]. One reason for elevated acetic acid levels is usually related to aeration, which could lead to elevated oxygen levels during fermentation [23,30,52]. However, more acetic acid was produced in Malvar wines during the anaerobic period than during aerobic fermentation in the current work, in agreement with results observed by Röcker et al. [24]. All sequential fermentation between four selected non-*Saccharomyces*/*S. cerevisiae* in this article produced wines with elevated volatile acidity (>0.7 g/L of acetic acid), significantly increased after *S. cerevisiae* inoculation (Table S1, see values of acetic acid caused by non-*Saccharomyces* fermentations). This noticeable increase could be caused by a lack of nutrition sources available for *S. cerevisiae* in the second part of fermentations [62,63]. Low YAN values (below 200 mg N/L, such as the Malvar must we studied) can also lead to elevated acetic acid concentration [64].

For selection of low-ethanol producing wine yeast, its impact on aroma profile is of great importance. Sequential cultures in this work had an important influence on higher alcohol proportions compared with the control. High levels of these volatile compounds (>300 mg/L) can have a detrimental effect on wine aroma, while concentrations below 300 mg/L can contribute positively to aroma complexity [65,66]. All wines produced using sequential inoculations presented values of higher alcohols below 300 mg/L.

It is worth noting that isoamyl alcohol (harsh, bitter) and β-phenylethyl alcohol (flowery, roses) are increased by sequential culture with *W. anomalus* 21A-5C and *M. guilliermondii* CLI 1217 strains. The ethyl isovalerate and isoamyl acetate esters, which impart fruity (banana) and sweet aromas, were also higher in these sequential cultures. In relation with *W. anomalus* species, these results agree with other publications [28,67–70]. Rojas et al. [67] indicated that one *W. anomalus* (*P. anomala*) strain produced the highest isoamyl acetate concentration in 48 h cultures in aerobiosis conditions; moreover, the increment in acetates was also observed in sequential cultures with *W. anomalus* and *S. cerevisiae* [28,70]. In addition to increasing alcohols, as well as ethyl and acetate esters [69], Airen white wines elaborated with *W. anomalus*/*S. cerevisiae* sequential cultures were judged to be better than *S. cerevisiae* monoculture due to their higher scores for descriptors as fruity and floral, and having an intense sweet smell and longer-lasting aftertaste [68]. Nevertheless, *M. guilliermondii* has been considered as a spoilage yeast in winemaking that is able to produce large amounts of volatile phenols [71], identified with horse, stable, leather, or medicinal notes [72]; in contrast, the *M. guilliermondii* CLI 1217 strain used in sequential culture in the present work has contributed to rising amounts of fusel alcohols and some esters related to fruity and floral character in Malvar white wines.

Apart from high levels of isoamyl alcohol and β-phenylethyl alcohol previously documented by authors [22,24,73–75], sequential cultures with *M. pulcherrima* strains (CLI 68 and CLI 460) also showed an elevated proportion of isobutanol (bitter, fusel, alcohol) compared to the wine fermented solely with *S. cerevisiae*. This high isobutanol content is in good agreement with the experimental data reported previously [24,28,76]. While some reports [22,73,77] have stated that wines inoculated with *M. pulcherrima*/*S. cerevisiae* contain higher concentration of esters, other studies [16,54,74,78,79] have noted that wines fermented with these yeast species in combination have lower concentrations, as in the case of this work. Moreover, it is worth mentioning that sequential culture with *M. pulcherrima* native strains presented higher concentration of esters with fruity aroma (ethyl isovalerate, isoamyl acetate

and ethyl-3-hydroxybutyrate) than the control. On the other hand, Malvar wines elaborated with *M. pulcherrima* CLI 68 and CLI 460 strains are mostly related to volatile fatty acids. These compounds are generally associated with negative aromas in wine [80], although hexanoic, octanoic, and decanoic fatty acids impart mild and pleasant notes to wine at concentrations between 4 to 10 mg/L; however, their impact can be negative on wine at levels above 20 mg/L [81]. Thus, these fatty acids might have a positive effect on the aroma of *M. pulcherrima*/*S. cerevisiae* Malvar wines since their levels are below 20 mg/L.

Relative to wine fermented with the control, *S. cerevisiae* CLI 889, we found a higher total concentration of esters in Malvar wines using *S. cerevisiae* monoculture. This *S. cerevisiae* strain produced wines with a fruity and floral character due to the greater concentration of ethyl isobutyrate (pineapple), ethyl hexanoate (pineapple, apple), and 2-phenylethyl acetate (flowery, lilac) esters, being the perfect candidate to ferment Malvar musts, and improving the typicity of the wines produced in the area "Vinos de Madrid".

5. Conclusions

The present results indicated that sequential cultures of native non-*Saccharomyces* (*W. anomalus* 21A-5C, *M. guilliermondii* CLI 1217, and *M. pulcherrima* CLI 68 and CLI 460) with *S. cerevisiae* CLI 889 can be used as a strategy to reduce the ethanol levels in wines, whilst keeping the wine typicity of the area. These combinations could have a positive impact on glycerol content and the volatile profile of these wines, showing *W. anomalus* 21A-5C and *M. guilliermondii* CLI 1217 combinations with *S. cerevisiae* being mostly related to fruity and floral aroma compounds when compared with *M. pulcherrima* usage. However, further optimization will be required to control the acetic acid production in all sequential fermentations. Future work will focus on fermentations at a pilot scale through using the selected strains and having a second inoculation at different times, which will allow for the evaluation of the sensorial profile of the resulting wines.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/6/2/60/s1, Table S1. Volatile acidity (as g/L of acetic acid), reducing sugars (glucose + fructose, g/L), and dry weight (mg) of wines after Section I (pure culture of non-*Saccharomyces* strains and the control under aerobic conditions).

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