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Improvement of Malvar Wine Quality by Use of Locally-Selected *Saccharomyces cerevisiae* Strains

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Academic Editor: Ronnie G. Willaert

Received: 16 February 2016; Accepted: 10 March 2016; Published: 14 March 2016

Abstract: Malvar grape juice offers relatively little in the way of a sensory experience. Our interest lies in the use of locally-selected yeast strains in experimental fermentations to improve the sensory characteristics of Malvar wines. Two locally-selected strains of *Saccharomyces cerevisiae* were used as starter cultures in vinifications and compared with spontaneous fermentations of the same cultivar musts. Wine quality was investigated by their principal oenological parameters, analysis of the volatile aroma components, and corroborated by an experienced taster panel. The most salient chemical attributes were its high concentrations of isoamyl acetate and hexyl acetate and the high acidity, which have been detected to be key constituents in setting the fruity and fresh character of Malvar wines. Winemakers of winegrowing areas where this grape variety is cultivated will have improved options to elaborate new white wines styles, using selected yeast strains that enhance its aromatic properties.

Keywords: sensory analysis; inoculation; spontaneous fermentation; yeast selection

1. Introduction

One of the most cultivated varieties in the winegrowing region of Madrid, with a total extension of 11,758 ha, is the white grape variety Malvar (*Vitis vinifera* L.). Part of the economic development of this area is based on wine production using grapes from this cultivar. However, Malvar grape juice offers relatively little in the way of a sensory experience, but after wine fermentation, it has a multitude of attributes [1]. Hence, the intervention of the yeasts in the fermentation process can be noteworthy. In a previous work, we characterized the enological relevance of 12 selected non-*Saccharomyces* wine yeast species isolated from spontaneous fermentations of this variety to propose their use in mixed starter cultures to improve the organoleptic properties of the Malvar wines [1]. The yeast strain *Torulaspora delbrueckii* CLI 918 was defined as a yeast strain with potential interest for its contribution to the aromatic Malvar wine profile with flowery and fruity aromas. This yeast species could be used in mixed starter cultures with *S. cerevisiae*. It is well known that ethanol-tolerant yeasts, such as *S. cerevisiae*, play an essential role in the evolution of yeast-derived and grape-derived flavor molecules [2–6]. However, not all wine yeasts strains are equal; juice from the same grapes will deliver quite different wines, depending on the choice of yeast [7] and the management of the fermentation processes [8]. Thus, revealing new data relevant to Malvar wines is necessary.

Autochthonous yeast strains selected for their use as starter cultures is a profitable approach. Thus, lately, in a challenge to enrich distinctive aromatic properties, some researchers have addressed their attention to the selection of yeasts from restricted areas [8–12]. Ecological studies of wine yeasts are essential for finding novel strains with new molecular and enological attributes. Some winemakers proclaim that wines with geographical characteristics can be obtained only with selected yeast starters originated from the same area where wines are elaborated and the use of autochthonous wine yeasts selected from each vine-growing area is widely spread [8,9].

Uninoculated fermentation is a complex microbial process accomplished by the sequential action of non-*Saccharomyces* and the different *Saccharomyces* yeast strain populations present on the skin of the grapes, winery equipment, in the musts, and in the wine [13]. On the other hand, inoculation with indigenous yeasts as a starter culture is engaged to set a dominant population of a selected strain from the beginning to the end of fermentation [14,15]. The yeast activity during a spontaneous fermentation is capricious and could contribute less desirable attributes to the wine. Moreover, risks related with natural fermentations include both sluggish or arrested fermentations and the propagation of contaminant yeasts [13,14]. To keep away of these problems, commercially available wine yeast exhibits great diversity in degrees of robustness to dryness but, unfortunately, the most resilient strains do not necessarily deliver optimal sensory characteristics to the wine [7,15]. In addition, the use of commercial starters could disguise the distinctive properties that characterize some local wines [7,15]. Thus, understanding how yeasts influence the principal properties of wine aroma, flavor, and color provided the basic steps for selection of autochthonous yeast strains for use as starter cultures and control of the alcoholic fermentation as a new commercial option for wine makers. Furthermore, the use of selected native yeast strains in starter cultures is rather preferred since they are better acclimatized to the environmental circumstances and may ensure the maintenance of the typical sensory characteristics of the wines of a certain region [11].

The goal of this study is the evaluation of Malvar wines made with indigenous and selected yeast strains of *S. cerevisiae* in order to evaluate the effect, to devise the use of these yeast strains to make wine, and to determine the most remarkable chemical and sensory characteristics of such wines belonging to the Appellation of Origin "Vinos de Madrid" (Madrid, Spain). Moreover, no investigations have been carried out to improve its enological characteristics using selected or autochthonous strains of *S. cerevisiae* selected in this area.

2. Experimental Section

2.1. Yeast Strains and Vinification Procedure

The yeast strains utilized in this study were two *S. cerevisiae* yeast strains (coded as CLI 889 and CLI 892) previously isolated among 18 different genetic profiles obtained by PFGE with different occurrence in the Madrid winegrowing region. These were selected and characterized in our laboratories based on some established and desirable enological criteria, such as high fermentation performance, resistance to ethanol, low production of hydrogen sulfide and sulfur dioxide, and volatile-derived, among others [16] (see Supplementary Material). Fermentations were conducted at the IMIDRA's experimental cellar. Must obtained showed 240 g/L of reducing sugars, 170 mg/L of yeast absorbable nitrogen (YAN), 900 mg/L of total amino acids, the pH value was 3.61, and the titratable acidity (expressed as g/L of tartaric acid) was 5.78. Musts were carefully racked, homogenized, and dislodged statically (at 4 °C) adding 0.01 g/L of pectolytic enzymes (Enozym Altair, Agrovin, Spain) and 50 mg/L of sulfur dioxide (SO_2). This must was divided into nine stainless steel vats of 100 L coded as A1, A2, and A3 (those inoculated with CLI 889); B1, B2, and B3 (those inoculated with CLI 892), and the spontaneous fermentations as S1, S2, and S3. Triplicate fermentations were carried out at a controlled temperature of 18 °C. Musts were inoculated with a final concentration of 10^6 cells/ml of pure selected yeast. Fermentation kinetic was controlled by monitoring daily the density. When its value was the same during two consecutive days, residual sugars were analyzed by

enzymatic methodology (Roche diagnostics, Darmstadt, Germany). Fermentation was considered to be completed when residual sugars concentration was less than 2 g/L. After fermentation, the wines were clarified by cold settling using 3 mL/L of colloidal silica (Silisol, Agrovin, Alcázar de San Juan, Ciudad Real, Spain) and 2 mg/L of gelatin fining (Vinigel, Agrovin, Alcázar de San Juan, Ciudad Real, Spain). After three months of cold stabilization at 4 °C, wines were filtered (0.6 µm) and bottled. Then, they were subjected to chemical analysis.

2.2. Yeast Isolation, Identification, and Typification

Samples were taken from every vat during the vinification process at different density values, initial (D1) = 1090 g/L, D2 = 1085–1070 g/L, D3 = 1060–1050 g/L, D4 = 1030–1025 g/L, D5 = 1010–1000 g/L and D6 = 990 g/L (<2 g/L of residual sugars of the fermentations). Thus, fifty-four 100 mL sterile plastic flasks were filled with the Malvar must/wine from different parts of the vessels, kept under refrigeration (4 °C), and transported to the laboratory. Aliquots of tenfold dilution of the samples were spread onto YGC agar plates (Laboratorios Conda, Madrid, Spain). The plates were incubated for 3–4 days at 26 °C. After yeast colony counting, 30 colonies were randomly selected from each fermentation sample for their identification and subsequent monitoring of the implantation rate.

DNA extraction and quantification from isolates was performed as stated by Cordero-Bueso *et al.* [17]. Identification was carried out by the amplification of the ITS1-5.8S-ITS2 r DNA region and subsequent RFLP analysis using endonucleases *Cfo*I, *Hinf*I, *Hae*II, and *Dde*I [18]. Those isolated and identified different to *S. cerevisiae* were not analyzed in this study. In order to monitor the yeast strain dominance during the fermentation processes, two methodologies were used, karyotyping by pulsed field gel electrophoresis (PFGE) and amplification by PCR of microsatellite regions. The molecular karyotype was obtained following the protocol proposed by Rodríguez *et al.* [14]. Microsatellite reaction mix and amplification protocols were identical as those used by Vaudano and García-Moruno [19]. Amplified products were scattered on an agarose gel (2.5% *w*/*v*) with a final concentration of 5 µL/mL of ethidium bromide, in 1× TBE buffer at 100 V for 90 min. DNA fragment sizes were resolved by comparison with a molecular ladder marker of 100 bp (Promega, Madison, WI, USA). Fragment differentiation and allele size determination was carried out by single capillary automatic electrophoresis (CE) in ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. Enological Parameters of the Fermentation Assays

Enological parameters measurements were performed as stated in Cordero-Bueso *et al.* [1]. Alcoholic titer was measured by near-infrared reflectance, the fermentative capacity was calculated as the difference between the initial and final sugar content. Fermentation velocity (V_F) (or alcohol production expressed as grams of sugar consumed daily) was measured checking daily the sugar percentage lost during the fermentation. In addition, V_{50} amount of sugar daily transformed by the yeasts when 50% of the sugar content had been used up was evaluated. Free and total sulfur dioxide, pH, titratable acidity, and volatile acidity were performed according to standard methods in the enological sector (OIV methods, Official Methods established by the European Union). Glycerol and 2,3-butanodiol compounds were determined using the Feuilles verts 588 (FV) method also suggested by the OIV.

2.4. Determination of Carboxylic Acids of the Malvar Wines

Carboxylic acids were quantified by ionic chromatography using Dionex DX 500 (Salt Lake City, UT, USA) equipment with a CD20 conductivity detector. Standard stock solutions of the organic acids (Panreac, Barcelona, Spain) were prepared by dissolution of the acids or the salts with deionized water. After filtering (0.22 µm) and dilution (1:20) with sterilized water, duplicate wine samples were injected into the chromatograph equipped with an IonPac ICE-AS6 capillary column. A concentration of 0.4 mM heptafluorobutyric acid (HFBA) (FlukaChemie AG, Buchs, Switzerland) was used as eluent

at flow rate of 1.0 mL/min in isocratic mode. Anion-Ice micro-membrane was used as suppressor column and tetrabutylamonium hydroxide (Riedel-de Haën, Seelze, Germany) as a regenerator with a flow rate of 5 mL/min. The working conditions were as follow; temperature at 25 °C, 25 µL of injection volume, and 10 µs FS of the detector conductivity.

2.5. Determination of the Volatile Fraction of the Fermentations

Six major volatile compounds of the Malvar wines obtained were settled by gas chromatography coupled to a flame ionization detector (GC-FID) and 19 minor volatiles by gas chromatography combined with mass spectrometry (GC-MS). Major volatiles (acetaldehyde, acetoin, ethyl acetate, and the higher major alcohols, 1-propanol, isobutanol, and the isoamylic alcohols (2-methyl-1-butanol and 3-methyl-1-butanol)) were determined after steam distillation using a Hewlett Packard Series II (Palo Alto, CA, USA) gas chromatograph with a flame ionization detector. At the same time, minor volatiles were extracted after a liquid–liquid process using a mixture of ether hexane (1:1 v/v) as extractant. The organic phases were concentrated under a stream of N_2 and injected into a Hewlett-Packard 6890 gas chromatograph (Agilent, Avondale, PA, USA) fitted to a mass spectrometer detector HP Mass Selective 5973 (Agilent, Avondale, PA, USA). The reference Gil *et al.* [20] shows a complete description of both methods.

2.6. Sensory Analysis

Sensory evaluations were done under ISO standards [21–23] related to methodology and sensory analysis vocabulary (ISO 8586-1:1993), selection and formation of tasters (ISO 11035:1994), and tasting room (ISO 8589:2007). Two sensory analyses were performed in only one session by eleven skilled judges. Malvar wine sample positions were randomized every time and the sensory profile was defined using 13 descriptors previously described by Lozano *et al.* [24] and chosen by the taster panel in a previous session as stated in the ISO 11035:1994 rules and according to their importance in Malvar wines.

The first wine tasting was carried out by filling in a blank official tasting scorecard used in the Appellation of Origin "Vinos de Madrid". Penalizing scores were used; thus, the better quality wines obtained a lower score. Six variables (appearance, aroma quality, aroma intensity, taste intensity, taste quality, and harmony) were selected for estimation of wine quality, and a scale of seven categories (excellent: 0–7, very good: 8–23, good: 24–44, correct: 45–52, ordinary: 53–78, defective: 79–90, eliminated > 90) like those proposed by Vilanova *et al.* [25]. The total scores given by the eleven tasters for each parameter corresponding to the sensorial characteristics of wine were then statistically analyzed. After the first wine tasting, all judges were also asked to evaluate the sensory profile of the samples on a 0–5 point scale of intensity filling in a second official tasting scorecard containing the aroma descriptors mentioned above. Scale zero (0) implied that the descriptor was not perceived, while a score of five (5) was equal to the highest perception.

2.7. Statistical Analysis

One-way ANOVA and Tukey's test were accomplished to emphasize the effects of yeast strains on sensorial descriptors. Discriminant analysis was performed to point up any differences due to yeast strain. Principal component analysis (PCA) was carried out with 20.0 SPSS (Inc. Chicago, IL, USA) for Windows statistical package (significance level $p = 0.05$).

3. Results and Discussion

3.1. Fermentation Kinetics

Figure 1 shows the average of the fermentative kinetics and yeast population evolution of Malvar musts. A lag phase of two days was observed in all fermentations and proceeding to dryness between eight and nine days in the case of the inoculated fermenters, and between 10 and 11 days in the

spontaneous fermentations. Although different kinetic behaviors were found, all strains were able to finish the fermentation consuming over 98.5% of initial sugar. Differences on sugar consumption in the middle stages of the fermentations have been observed. In spite of the similar profile obtained for the inoculated fermentations during the first days (until the third day) and at the completion of fermentation (from the sixth day), the strain CLI 892 showed higher fermentation rate than the CLI 889 strain and spontaneous fermentations, which showed the same rate during this period of time.

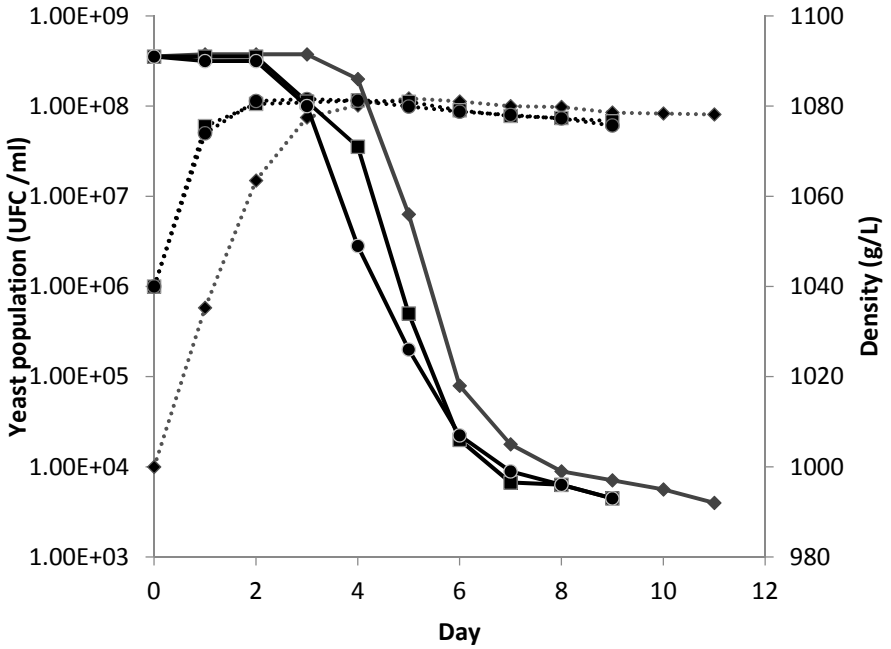

Figure 1. Evolution of the total yeast population (dashed lines) and must density (continuous lines) during three wine fermentations performed with Malvar musts using the following inoculation procedures: no inoculation (♦); inoculation with yeast strain CLI 889 (■); and inoculation with yeast strain CLI 892 (●). Data correspond to mean values obtained from triplicate experiments.

3.2. Implantation Rate

A total of 1620 colonies were isolated from the different stages of the fermentations of Malvar among the nine fermenters. Molecular identification using ITS-5.8S amplification and restriction analysis, and comparing the restriction profiles with those obtained by Esteve-Zarzoso *et al.* [18], showed that from first day of inoculated fermentations all isolates belonged to *S. cerevisiae*, while in the uninoculated musts, 131 of 540 colonies were identified as non-*Saccharomyces* in the initial and middle stages of the spontaneous fermentations.

All colonies identified as *S. cerevisiae* were characterized by PFGE, showing five different karyotypes. Isolates from inoculated vessels with the yeast strain CLI 889 showed two different karyotype patterns during the early and middle phases of the fermentations, but at the end of the fermentation process only the karyotype (A) corresponding to the inoculated yeast strain was found (Figure 2), while colonies of *S. cerevisiae* from the three vessels inoculated with the yeast strain CLI 892 showed one unique profile (B), throughout the entire fermentation process and corresponding to the inoculated yeast (Figure 2). The implantation rate at the end of the fermentation 100% fit the profile of the inoculated strain in both tanks. While in the uninoculated vessels, 90% of the population was represented by a single karyotype (A) as showed Figure 2. Dominance or competitiveness of a starter yeast strain could have an impact on the sensorial quality of wine by dominating its aromatic profile or eliminating the collaborative role of natural *S. cerevisiae* populations [13].

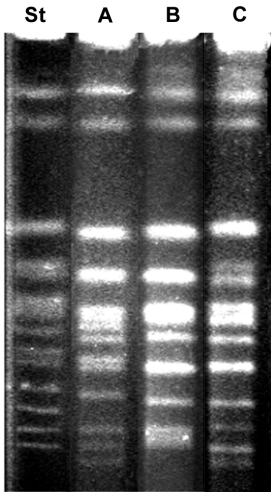

Figure 2. Electrophoretic karyotypes of the majority at the end of the fermentation *Saccharomyces cerevisiae* yeast strain isolated from the spontaneous fermentations (**A**) and selected inoculated yeast strains CLI 889 (**B**) and CLI 892 (**C**) The chromosomes of the standard *S cerevisiae* YNN 295 (Bio-Rad) were used as a reference (St).

The microsatellite PCR analysis of the *S. cerevisiae* strains, CLI 892 and CLI 889 showed that both strains are homozygous for the three alleles fingerprinted. The sizes of the alleles were as follows: SCPTSY7 (235 bp and 268 bp), SC8132X (215 bp and 209 bp), and YOR267C (416 bp and 451 bp), respectively. In the case of the strain isolated from spontaneous fermentations (S) allele sizes were as follows: SCPTSY7 (292 bp and 292 bp), SC8132X (212 bp and 310 bp), and YOR267C (308 bp and 389 bp). The comparison of these profiles with the five obtained by PFGE during the last phases of the fermentation of Malvar musts enabled that the number of patterns obtained and implantation rates during all fermentation processes in the different tanks were identical.

3.3. Principal Enological Parameters of the Fermentation Assays

Table 1 shows the means and standard deviations of the principal enological parameters, including organic acids of Malvar must fermentations carried out with the selected strains and uninoculated fermentations. Fermentative velocity (V_F) was higher in spontaneous fermentations, although the V_{50} was higher in the must inoculated with the yeast strain CLI 892. This finding is in agreement with the fact that those *S. cerevisiae* naturally present in must are better adapted to the fermentation conditions and environment than allochthonous inoculated yeasts [10]. It is interesting to point out that volatile acidity and acetic acid content were significantly lower in the uninoculated musts than the inoculated ones.

Regarding carboxylic acids, these compounds may contribute favorably to the organoleptic properties of young white wines. Our results showed that the total amount of these compounds was similar in wines from the CLI 892 strain and spontaneous fermentation, and higher than those obtained in wines from the CLI 889 strain. The excessive production of glycerol during wine fermentations for its positive sensory attributes gives rise to an increase in acetic acid concentration [26]. According to Erasmus *et al.* [27] it is, therefore, conceivable that different yeast strains experiencing the same fermentation conditions will respond by producing different concentrations of glycerol and acetic acid. The wine yeast strain isolated from the Malvar spontaneous fermentation (S), which produces low concentrations of acetic acid and conducts fermentations efficiently, seems to be a great candidate for the future production of high-quality Malvar wines in the Madrid winegrowing region, alone, or using mixed starter cultures.

Table 1. Enological parameters and organic acid concentrations of fermented wines (average and standard deviation (SD), Fisher's test (F, and significance (Sig) factors according to one-way ANOVA, test of comparison of means (Tukey): 2 and 11 degrees of freedom; the characters a, b, and c mean significant differences at $p \leqslant 0.05$. V_{50} = fermentation velocity consumption of 50% of the sugar content; V_F = fermentation velocity (% of daily sugar consumption).

Parameters	S	CLI 889	CLI 892	F	Sig
Alcoholic degree % (*v*/*v*)	12.50 ± 0.08 [a]	12.54 ± 0.06 [a]	12.70 ± 0.02 [b]	9.69	0.0132
Fermentative capacity	12.00 ± 0.07 [a]	12.61 ± 0.01 [c]	12.50 ± 0.01 [b]	186.53	0.0000
V_{50}	18.00 ± 0.04 [b]	15.45 ± 0.64 [a]	20.30 ± 0.2 [c]	128.70	0.0000
V_F	6.7 ± 0.3 [b]	5.40 ± 0.42 [a]	4.9 ± 0.7 [a]	10.27	0.0115
pH	3.81 ± 0.01 [b]	3.77 ± 0.01 [a]	3.79 ± 0.02 [b]	7.94	0.0206
Free SO_2 (mg/L)	6.0 ± 0.3 [a]	9.5 ± 0.71 [b]	10.0 ± 1.0 [b]	26.82	0.0010
Total SO_2 (mg/L)	15.0 ± 2.7	16.1 ± 1.56	14.0 ± 2.0	0.72	0.5230
Volatile acidity (g/L; acetic acid)	0.14 ± 0.01 [a]	0.23 ± 0.03 [b]	0.31 ± 0.04 [c]	25.04	0.0012
Titratable acidity (g/L; tartaric acid)	4.90 ± 0.07 [a]	5.14 ± 0.06 [b]	5.20 ± 0.07 [b]	16.93	0.0034
Citric acid (g/L)	0.31 ± 0.01 [b]	0.30 ± 0.00 [a,b]	0.29 ± 0.01 [a]	4.00	0.0787
Malic acid (g/L)	2.82 ± 0.05 [b]	2.58 ± 0.01 [a]	2.72 ± 0.09 [b]	27.44	0.0010
Lactic acid (g/L)	0.59 ± 0.06 [b]	0.55 ± 0.02 [b]	0.45 ± 0.04 [a]	8.36	0.0184
Acetic acid (g/L)	0.17 ± 0.01 [a]	0.32 ± 0.02 [b]	0.39 ± 0.03 [c]	69.14	0.0001
Succinic acid (g/L)	0.29 ± 0.01 [a,b]	0.28 ± 0.00 [a]	0.31 ± 0.01 [b]	5.55	0.0433
Reducing sugar (g/L)	1.5 ± 0.3	1.55 ± 0.07	1.3 ± 0.2	1.17	0.3730
Glycerol (g/L)	7.1 ± 1.6 [a,b]	7.85 ± 0.92 [b]	4.40 ± 1.5 [a]	5.13	0.0503
2,3-butanodiol (mg/L)	373.5 ± 67.5 [a]	386.8 ± 16.3 [a]	518.4 ± 91.2 [b]	7.86	0.0211

3.4. Aromatic Profile of Malvar Wines Fermented with Different Yeast Strains

Table 2 shows the average and standard deviations of the volatile compounds detected in the different fermentations. From all the volatile compounds identified, those presented at concentrations higher than their OTH (OAV higher than 1) are mainly considered as aroma-contributing compounds, and indicated in bold in Table 2.

Table 2. Data (Mean ± S.D.) of volatile composition related to the uninoculated fermentations (S) and inoculated fermentations with two locally-selected *S. cerevisiae* yeast strains (CLI 889 and CLI 892). Odor descriptors (ODE) and odor thresholds (OTH) described in the literature are included. Thresholds were calculated in a 10%–12% water/ethanol mixture. Odor activity values were also calculated. F = Fisher's test; Sig, one-way ANOVA analysis, test of comparison of means (Tukey): 2 and 11 degrees of freedom; the characters a, b, and c mean significant differences at $p \leqslant 0.05$.

Compound (mg/L)	S	CLI 889	CLI 892	F	Sig	ODE	OTH (mg/L)	OAV *		
								S	CLI 889	CLI 892
Acetaldehyde	50.75 ± 8.85 [a]	69.61 ± 8.19 [b]	60.35 ± 10.30 [ab]	3.18	0.1142	Pleasant, fruity	0.0025 [2]	**20.30**	**27.84**	**24.14**
Acetoin	tr [a]	2.4±1.03 [b]	1.27±0.73 [ab]	8.14	0.0195	Flowery, wet	150.0 [1]	<0.1	<0.1	<0.1
Ethyl acetate	68.71 ± 1.01 [a]	69.28 ± 4.41 [a]	64.65 ± 10.66 [a]	0.43	0.6700	Fruit, solvent	12.26 [1]	**5.60**	**5.65**	**5.27**
1-Propanol	35.02 ± 1.83 [b]	36.63 ± 1.41 [b]	25.80 ± 2.58 [a]	25.63	0.0012	Alcohol, ripe fruit	306.0 [1]	0.11	0.11	<0.1
Isobutanol	37.39 ± 0.58 [b]	39.50 ± 1.53 [b]	23.52 ± 2.63 [a]	70.62	0.0001	Fusel, alcohol	40.00 [1]	0.93	0.98	0.58
Isoamylic alcohols	175.23 ± 1.74 [b]	182.20 ± 2.99 [c]	156.38 ± 3.25 [a]	71.47	0.0001	Bitter, harsh	30.00 [1]	**5.84**	**6.07**	**5.21**
Σ Higher major alcohols	247.65 ± 0.66 [b]	258.13 ± 5.63 [b]	205.71 ± 8.45 [a]	66.86	0.0001					
1-Hexanol	0.81 ± 0.01 [a]	0.95 ± 0.00 [a]	1.00 ± 0.06 [a]	0.42	0.6754	Green grass	8.00 [1]	0.1	0.11	0.12
2-Phenylethanol	10.79 ± 0.29 [b]	9.29 ± 0.85 [a]	9.99 ± 0.82 [ab]	3.46	0.100	Roses	14.00 [1]	0.77	0.66	0.71
Σ Higher minor alcohols	11.59 ± 0.27 [b]	10.22 ± 0.83 [a]	10.99 ± 0.80 [ab]	3.03	0.1233					

Table 2. *Cont.*

Compound (mg/L)	S	CLI 889	CLI 892	F	Sig	ODE	OTH (mg/L)	OAV *		
								S	CLI 889	CLI 892
Isobutyl acetate	0.16 ± 0.00 [b]	0.18 ± 0.00 [c]	0.13 ± 0.01 [a]	37.75	0.0004	Sweet fruit	1.60 [1]	0.1	0.11	<0.1
Isoamyl acetate	7.66 ± 0.18 [b]	9.37 ± 0.54 [c]	6.19 ± 0.08 [a]	67.73	0.0001	Banana	0.030 [1]	**255.33**	**312.33**	**206.33**
Hexyl acetate	0.14 ± 0.01 [b]	0.19 ± 0.02 [b]	0.06 ± 0.05 [a]	11.07	0.0097	Fruity, green, pear	0.020 [3]	**7.00**	**9.50**	**3.00**
Phenylethyl acetate	0.56 ± 0.03 [b]	0.56 ± 0.07 [b]	0.38 ± 0.04 [a]	13.14	0.0064	Pleasant, flowery	0.250 [1]	**2.24**	**2.24**	**1.52**
Σ Higher alcohol acetates	8.38 ± 0.20 [b]	10.10 ± 0.62 [c]	6.70 ± 0.04 [a]	61.06	0.0001					
Ethyl butyrate	0.33 ± 0.01 [ab]	0.39 ± 0.00 [b]	0.27 ± 0.06 [a]	10.24	0.0116	Acid fruit	0.020 [1]	**16.5**	**19.50**	**13.5**
Ethyl hexanoate	0.60 ± 0.05 [b]	0.52 ± 0.07 [ab]	0.39 ± 0.13 [a]	4.16	0.0735	Green apple	0.014 [1]	**42.86**	**37.14**	**27.86**
Ethyl octanoate	1.01 ± 0.25 [a]	1.10 ± 0.11 [a]	0.73 ± 0.42 [a]	1.34	0.3314	Sweet, soap	0.005 [1]	**202.00**	**220.00**	**146.00**
Ethyl decanoate	0.08 ± 0.06 [b]	0.23 ± 0.02 [c]	tr [a]	28.09	0.0009	Pleasant, soap	0.200 [1]	0.40	**1.15**	<0.1
Σ Fatty acid esters	2.01 ± 0.37 [a]	2.22 ± 0.19 [a]	1.40 ± 0.0.58 [a]	3.21	0.1127					
Ethyl lactate	tr [a]	2.24 ± 1.01 [b]	tr [a]	14.57	0.005	Lactic	0.157 [2]	<0.1	**14.26**	<0.1
Diethyl succinate	0.03 ± 0.01 [a]	0.09 ± 0.04 [b]	0.05 ± 0.01 [ab]	4.67	0.0599	Apple, fruity	0.20 [2]	0.15	0.45	0.25
Isobutyric acid	3.01 ± 0.02 [a]	3.02 ± 0.03 [a]	2.91 ± 0.01 [a]	0.94	0.4429	acid, fatty	0.230 [1]	**13.09**	**13.13**	**12.65**
Butyric acid	1.90 ± 0.04 [a]	1.93 ± 0.21 [a]	1.31 ± 1.14 [a]	0.81	0.4876	Cheese	0.173 [1]	**10.98**	**11.15**	**7.57**
Isovaleric acid	0.08 ± 0.06 [a]	0.26 ± 0.07 [b]	0.02 ± 0.01 [a]	14.60	0.005	Blue cheese	0.033 [1]	**2.42**	**7.87**	0.60
Σ SCFA	6.67 ± 0.07 [a]	6.65 ± 0.15 [a]	6.08 ± 1.06 [a]	0.88	0.4640					
Hexanoic acid	4.97 ± 0.23 [a]	4.29 ± 0.36 [a]	4.54 ± 0.11 [ab]	5.27	0.0477	Cheese	0.420 [1]	**11.83**	**10.21**	**10.80**
Octanoic acid	6.11 ± 0.20 [c]	5.45 ± 0.50 [b]	5.07 ± 0.20 [a]	17.91	0.0030	Rancid, harsh	0.500 [1]	**12.22**	**10.90**	**10.14**
Decanoic acid	3.70 ± 0.77 [a]	3.68 ± 0.32 [a]	2.85 ± 0.21 [a]	2.89	0.1319	Fatty	1.00 [1]	**3.70**	**3.68**	**2.85**
Σ MCFA	14.78 ± 1.20 [b]	13.39 ± 1.15 [ab]	12.46 ± 0.35 [a]	4.23	0.0713					
4-Vinylguaiacol	0.47 ± 0.18 [ab]	0.53 ± 0.04 [b]	0.29 ± 0.09 [a]	3.33	0.1062	Pleasant, phenolic	1.10 [1]	0.42	0.48	0.26

tr = traces; 1 = thresholds from Gil *et al.* [20]; 2 = thresholds from Duarte *et al.* [28]; 3 = threshold from Falqué *et al.* [29]; SCFA Small Chain Fatty Acids; MCFA Medium Chain Fatty Acids; * in bold, compounds with OAV > 1.

In all cases, achieved acetaldehyde levels were higher than the threshold proposed by Duarte *et al.* [28]. Among these values the highest values corresponds to the inoculated musts, while in the spontaneous fermentations the values were slightly lower (Table 2). Only free acetaldehyde has flavor relevance; at low levels it provides fruity flavors, while high concentrations (>200 mg/L) contribute "flatness" in wines [20]. All of the studied Malvar wines can be treated as correct due to their mean content being within the range previously studied in other non-oxidized white wines [30]. High concentration of acetoin is mostly related to non-*Saccharomyces* fermentations [1,8] but in our study, high concentrations were only detected in the inoculated fermentations. On the other hand, ethyl acetate concentrations showed higher values than the threshold proposed (OAVs, from 5.2 to 5.6) over all Malvar wines (Table 2). Ethyl acetate may confer with pleasant and fruity fragrances to the global wine aroma at concentrations lower than 150 mg/L.

Quantitatively, the largest group of volatile compounds present in wines were higher alcohols. Many of these compounds are strongly correlated with dislikable aromas in wines, but even though significant differences were found among the uninoculated and inoculated wines, the values obtained in wines fermented by the CLI 892 strain were significantly lower than the other two wines (Table 2). The amount of higher alcohols ranged from 205.71 ± 8.45 to 258.13 ± 5.63 mg/L, this variation principally is due to the isoamyl alcohol produced, which represent in all cases close to 60% of the total higher alcohols (Table 2). Discrete concentrations of fusel alcohols contribute to the wine's aromatic complexity. With respect to the higher minor alcohols, this group of volatiles had no odor activity contribution.

In terms of the number of components analyzed, esters represent the largest group (Table 2). Higher alcohol acetates are an important group of fermentative aromas, which are normally linked to fruity descriptors, but in a wine can significantly modify the global aroma. The fruity character

associated to the aroma of Madrid white wines is mainly related to fruit notes such as banana, green apple, or pear [20,31,32], produced by the acetates of higher alcohols and fatty acids.

Significant differences were detected among the different fermentations for isoamyl acetate, hexyl acetate, and phenyl ethyl acetate (Table 2). The major ester in the Malvar wines analyzed was isoamyl acetate which was present in high concentrations and above of the OTH and OAV in all fermentations. Ethyl esters of fatty acids showed important variations in the concentrations, but in the majority of the cases, they showed higher values than the OTH and OAVs values proposed (Table 2). The real contribution of ethyl lactate and diethyl succinate to the white wines of the "D.O. Vinos de Madrid" has been previously described as insignificant [20,32]. Interestingly, Malvar wines fermented by the yeast strain CLI 889 showed a high concentration of ethyl lactate. It is now widely understood that ester concentration is conditioned by several factors, such as yeast strain, fermentation temperature, aeration degree and sugar content [33–35]. In our experimental conditions, in spite of the use of the same must under the same conditions, the different amounts of esters found can be attributed to the yeast strain used.

Fatty acids have been described with cheese, harsh, fatty, and rancid notes [20]. All compounds in the different fermentations reached the OTH and OAVS values proposed (Table 2), with the exception of the isovaleric acid in the fermentations carried out by CLI 892. Supposing that the presence of fatty acids is frequently associated to off flavors, they play an important role in the aromatic equilibrium in wines because they are antagonistic to the hydrolysis of the analogous esters [20].

High concentrations of vinyl phenols can be responsible for strong pharmaceutical odors in white wines [18,36], but at low or moderate concentrations they could be linked with grassy, herbaceous, or pleasant spicy aromas. Accordingly, Grando et al. [37] pointed out that 4-vinylguaiacol was the principal responsible compound for the spicy aroma of Gewürztraminer's wines. In our study, 4-vinylguaiacol was detected among the uninoculated and inoculated white wines. Statistically significant differences were observed when using Malvar juices fermented spontaneously or inoculated with the selected yeast strains.

PCA was performed to disclose the compounds that differentiated best among the uninoculated and inoculated Malvar wines (Figure 3). Hence, only yeast volatile fermentation constituents enabled a suitable discrimination between inoculated and spontaneous fermentation wines.

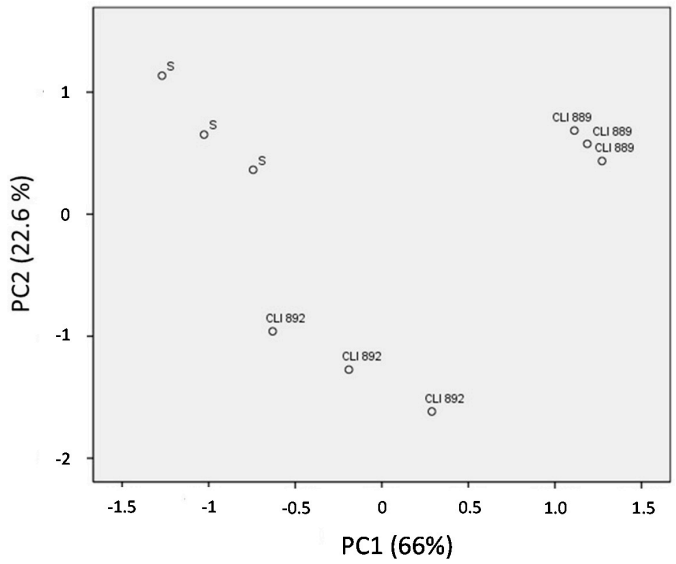

Figure 3. Principal components analysis of volatile composition data. Projection of the Malvar wines fermented spontaneously (S), and with CLI 892 and CLI 889 *S. cerevisiae* strains in the dials formed by the PC1 (22.6%) and PC2 (66%).

The PCA explained the 88.63% of the total variance. Musts fermented with the strain CLI 889 shaped a clear group, which was associated with the esters ethyl octanoate, isoamyl acetate, and ethyl lactate, as long as fermentation inoculated with CLI 892 was located in a different dial of the PCA plot and most associated with those compounds contained in the PC2 (ethyl hexanoate, 4-vinylguaiacol, ethyl butyrate, and ethyl acetate). The uninoculated wines showed a cluster mostly associated with those compounds of the PC1, but none was nearly correlated with any of the inoculated fermentation wines (data not shown). Thus, taking into account that fermentations were conducted in the same must, if the yeast starter dominates on native yeast population, the wine will exhibit singular aroma and sensory profiles of the each yeast starter involved [14]. Confirming this, PCA analysis and data of Table 2 showed that by comparison with inoculated wines with the yeast strains CLI 889 and CLI 892, uninoculated fermentation wines showed a high variability in the composition of volatile compounds that also contributes to wine aroma.

3.5. Sensory Analysis of Wines

After the first sensory analysis using a penalizing system on all of the wines, attributes related to the appearance, taste intensity, taste quality, and harmony were not statistically different. However, differences ($p < 0.05$) were found within the different Malvar wines in terms of their aroma intensity, and quality. The ratings obtained were; uninoculated 15.73 $\pm$ 3.20, inoculated with the yeast strain CLI 889 13.30 $\pm$ 1.92, and 16.40 $\pm$ 4.55 using the strain CLI 892. Thus, judges considered that wine made by inoculation of CLI 889 seem to have the better quality. On the other hand, the sensory analysis using the different selected descriptors reached that some descriptors were statistically influenced by the yeast strain (Figure 4).

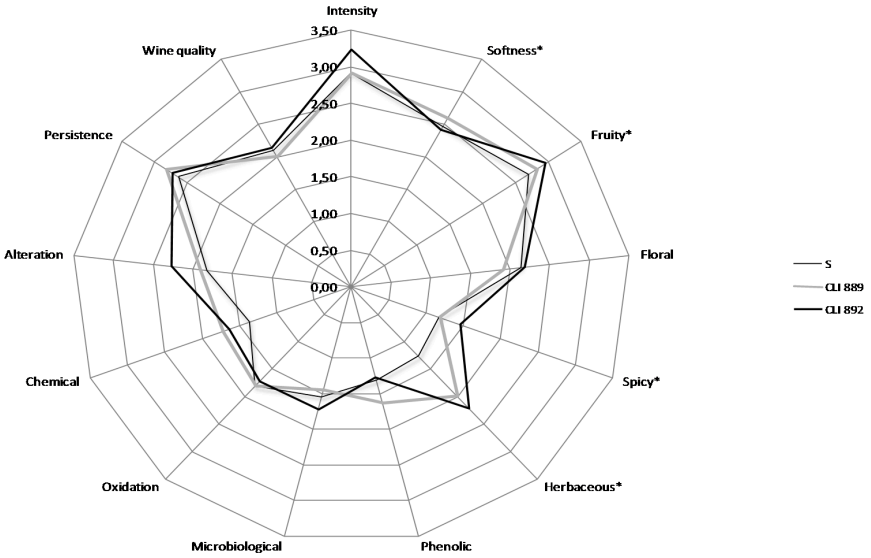

Figure 4. Polar coordinate (cobweb) graph of mean sensory scores rating of "alteration", "chemical", "herbaceous", "intensity", "floral", "fruity", "microbiological", "oxidation", "persistence", "phenolic", "softness", "spicy", and "wine quality" for wines made with the uninoculated *S. cerevisiae* (S) and locally-selected CLI 889 and CLI 892 yeast strains. In sensorial variables indicated with an asterisk (*) a difference between some trials is verified for $p \leqslant 0.05$.

The greatest softness of the spontaneously fermented wines was deserved to a lower acidity, and apparently to a large production of glycerol and as reported by Feuillat [38]. Glycerol is sweet, however its contribution to the sweet taste or palatableness of a wine remains unclear. The threshold concentration proposed by Noble and Bursick [39] is considered high (5.2 g/L) as quoted by Ugliano and Henschke [40], and the high acidity in wines is wont to interact with the remaining sweetness,

so the limit to which glycerol contributes to sweetness or mouthfeel characters is still unrevealed. Nevertheless, is in agreement with Scacco *et al.* [41], at the concentrations obtained in this work (Table 1), glycerol contributes positively to softness and viscosity of the wines. The differences concerning fruitiness cannot be considered to be related to the amount of wine flavor compounds. Substantially, wines fermented by the strain CLI 892 was characterized by a considerable content in higher alcohol acetates (10.10 $\pm$ 0.62 mg/L) with banana, pear, and herbaceous notes, followed by spontaneous fermentation with 8.38 $\pm$ 0.62 mg/L (Table 2). These compounds are usually linked to fruity descriptors; hence, it is possible to assume that the fruity descriptor in wines made with CLI 889 and CLI 892 were swayed by the lower amount of substances with a masking effect on fruity, than in the spontaneous fermentations, as reported by Campo *et al.* [42]. The spiciness was lower in wines fermented by strain CLI 892, apparently because this strain produced a lower quantity of some compounds able to influence this descriptor than the other strains, such as 4-vinylguaiacol. Sensory analysis showed that the best wines were those fermented by CLI 889, which had great gustatory and aromatic characteristics, usual in high-quality white wines. These results could be associated to a reasonable production of several fruity esters, such as isoamyl acetate, and hexyl acetate, which highlight the organoleptic properties and regional characteristic of Malvar white wines.

4. Conclusions

In the context of what is covered in this work, winemakers of the winegrowing regions where Malvar is cultivated are set to benefit from this recent development, because they will have improved options to elaborate wine. Uninoculated and locally-selected yeast strains clearly transform the fermentation and have influence over the volatile profile of Malvar white wines. In spite of wines fermented spontaneously and yeast strains CLI 889 and 892 being considered accurate by judges, the wine fermented with the strain CLI 889 was qualified as the best. The most salient chemical attributes were its high concentrations of isoamyl acetate and hexyl acetate and the high acidity, which have been detected to be key constituents in setting the fruity and fresh character of Malvar wines. Thus, this autochthonous yeast strain has been deposited at the Spanish Type Collection Culture (CECT) with the accession number CECT 13145. In addition, a new low acetic acid-producing *S. cerevisiae* yeast strain isolated from the spontaneous fermentations was characterized. Other features of its contribution to the general characteristics of these white wines are now being investigated in order to enrich the Malvar wine quality.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2311-5637/2/1/7/s1.

Acknowledgments: We are grateful to Matthew Tyndale-Tozer for revising the manuscript. This work was financed by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA- RM 2006-00012-00-00), by the Instituto Madrileño de Investigación y Desarrollo Rural Agrario y Alimentario (IMIDRA-FP07-AL2-Lev), and by the European Union's Seventh Framework Programme via the Marie Curie Action, "Co-funding of Regional, National and International Programs" to stimulate research activities without mobility restrictions, co-financed by the "Junta de Andalucía" and the European Commission under grant agreement no 291780.

Author Contributions: All authors contributed to the conception and design of experiments; Mar Gil-Díaz and Margarita García performed the experiments, Gustavo Cordero-Bueso performed the sensory analysis; all authors analyzed the data; Gustavo Cordero-Bueso and Teresa Arroyo contributed to writing the paper.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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