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Do Non-*Saccharomyces* Yeasts Work Equally with Three Different Red Grape Varieties?

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Abstract: The present study aimed to investigate the oenological changes induced by non-*Saccharomyces* yeasts in three red grape varieties from the Rioja Qualified Designation of Origin. Pilot plants fermentation of three different varieties, were conducted following early inoculations with *Metschnikowia pulcherrima* and with mixed inoculum of *Lachancea thermotolerans-Torulaspora delbrueckii* from La Rioja and compared to a wine inoculated with *Saccharomyces cerevisiae*. The microbiological and physicochemical characteristics of vinifications were analysed. Results showed that most of the variations due to inoculation strategies were observed in Tempranillo just after the alcoholic fermentation, probably because of the better adaptation of the inocula to the must's oenological properties. Finally, after the malolactic fermentation the inoculation with the mix of *Lachancea thermotolerans* and *Torulaspora delbrueckii* caused more changes in Tempranillo and Grenache wines while the early inoculation with *Metschnikowia pulcherrima* had more effects on Grenache wines. Therefore, the study was aimed to identify the fermentation effects of each inoculation strategy by using different non-*Saccharomyces* yeasts and different grape varieties.

Keywords: *Metschnikowia pulcherrima*; *Lachancea thermotolerans*; *Torulaspora delbrueckii*; Grenache; Graciano

1. Introduction

Grapes hold a diverse microbial population consisting of bacteria and yeasts that meet the microorganisms located in the winery facilities after the harvest. During the initial stages of the spontaneous alcoholic fermentation (AF), this pool of microbes achieves a balance until *Saccharomyces* (*S.*) *cerevisiae* becomes the main yeast in the fermentative process.

Early AF is characterized by a diverse yeast population, with low frequency of detection of *S. cerevisiae*, but with a high presence of non-*Saccharomyces* yeasts. The presence of unknown microbiota makes it a risky and unpredictable practice. Therefore, the inoculation of commercial *S. cerevisiae* strains has been widespread in the modern wine industry all over the World. Indeed, the non-*Saccharomyces* yeasts have not been well-regarded by oenologists and these have tended to make efforts to avoid their involvement in AF [1]. These traditional and conservative oenological practices have led to a homogenization and globalization of winemaking, a sameness in the taste and flavours of finished wines [2].

A general strategy to increase the diversification of wines has made oenology return to its origins of natural and diverse microbial populations. For this purpose, the employment of non-*Saccharomyces* yeast species has shown promising results. This new trend has triggered the studies and published

results of non-*Saccharomyces* yeasts which has led to some of them being used as commercial culture starters [1].

The use of mixed starter cultures of selected non-*Saccharomyces* combined with *S. cerevisiae* to avoid any stuck fermentations is thought to be a solution for ensuring AF completion, while various organoleptic characteristics involved in the quality of the final products are improved [2,3]. Furthermore, mixed cultures composed of more than one non-*Saccharomyces* species in combination with *S. cerevisiae* have been employed with the aim of simulating this complex yeast community present in spontaneous AF [4,5]. In general terms, the early inoculation of *Metschnikowia* (*M.*) *pulcherrima* has been aimed to improve flavour of wines [6]. In the case of *Lachancea* (*L.*) *thermotolerans*, the objective is the increase of lactic acid that would have also an impact in the aromatic profile of wines [7]. Moreover, *Torulaspora* (*T.*) *delbrueckii* has been initially employed for reducing the alcohol after the AF and for improving the aroma profile of wines [8].

The current study aims to describe the oenological effects of the sequential early inoculation of a pure culture of *M. pulcherrima* and a mixed culture of *L. thermotolerans* and *T. delbrueckii* in the vinification of Tempranillo, Grenache and Graciano grape varieties. With this purpose, the impact dependent on the specific grape variety in semi-industrial conditions was analysed. To this end, the kinetics of AF, implantation rate, variation of the oenological, colour and aromatic parameters after AF and clustering after malolactic fermentation (FML) were individually performed for each grape variety.

2. Material and Methods

2.1. Grapes and Initial Must Samples of the Three Varieties

Grapes of the three red grape varieties from the D.O.Ca. Rioja, Tempranillo, Grenache and Graciano were employed to perform this study. These grape varieties were chosen for being important in the region where this study was developed but also, they are very present in international winemaking areas. When the grapes had reached an average probable alcohol by volume (APBV) of approximately 13%, around 225 k of each one were individually harvested, crushed and destemmed (Figure S1).

Samples of the three must were physicochemical characterized. APBV, pH and total acidity were analysed according to official ECC methods [9]. Malic acid was determined also by the official method [9]. by an enzymatic method carried out with an automated clinical chemistry analyser (Miura One, TDI, Madrid, Spain). The yeast assailable nitrogen (YAN) was measured following the protocol described by Aerny [10].

The three musts were also microbiologically characterised by plating the appropriate dilution on Chloramphenicol Glucose Agar (CGA 05% yeast extract, 20% glucose, 005% chloramphenicol, 17% agar,) plates, incubated at 28 °C for 48 h. Ten yeast colonies were isolated from each plate containing between 30 and 300 colony forming units per millilitre (CFU/mL). DNA was then extracted from fresh culture following the protocol determined by López et al. [11]. Then, a partial region of the 26S rDNA gene was amplified with PCR using the primers and conditions established by Cocolin et al. [12]. PCR amplicons were purified and sequenced by Macrogen Inc. (Seoul, South Korea). The sequences were compared to the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) [13]. The identification was considered appropriate if gene sequences showed identities of at least 98%.

2.2. Yeast Species

This study was performed with four oenological yeast species, *M. pulcherrima*, *L. thermotolerans*, *T. delbrueckii* and *S. cerevisiae* (VRB commercial yeast from Lallemand Bio S.L., Toronto, Canada). *M. pulcherrima* and *S. cerevisiae* were pure cultures while *L. thermotolerans* and *T. delbrueckii* (*L&T*) were combined in percentages of 30% and 70%, respectively, following the natural combination of the two species observed in other studies of non-*Saccharomyces* population in Rioja red wines [14,15]. All these yeasts were selected in the Rioja Qualified Designation of Origin (D.O. Ca. Rioja) from Spain, and

they are in the last stage of the selection program. Furthermore, they are stored in the Instituto de Ciencias de la Vid y del Vino (ICVV) collection. These yeast were identified by Macrogen Inc. with the amplified region D1 of the 26S rRNA gene using the primers NL1GC and LS2 [16].

2.3. Inoculation Procedure and Alcoholic Fermentation

The must of each variety were put into nine 30 L tanks that were kept at 25 °C to carry out the AF (Figure S1). When the tanks were filled, potassium metabisulphite was added to the samples to achieve a total SO_2 concentration of 50 mg/L.

After this, the 27 tanks were inoculated with the different yeasts following three different inoculation strategies. For each variety, three out of the nine tanks (n = 3) made up the control sample (C) and were inoculated with the commercial *S. cerevisiae* starter culture VRB^{TM} following the producer's instructions, another three made up the sample early inoculated with *M. pulcherrima* (n = 3) (M) and the last three (n = 3) the sample early inoculated with a 30/70 mixture of *L. thermotolerans* and *T. delbrueckii* (*L&T*). The non-*Saccharomyces* yeasts had been pre-cultured in YPD liquid medium at 25 °C for 48 h with orbital shaking until the stationary phase. The concentration of cells/mL was counted with the Neubauer chamber. *M. pulcherrima* pure culture was inoculated in a concentration of 10^6 cells/mL counted while the mixed culture contained 3×10^5 cells/mL of *L. thermotolerans* and 7×10^5 cells/mL of *T. delbrueckii*. Three days later, all the 27 tanks were inoculated with the *S. cerevisiae* starter culture VRB^{TM} at a concentration of 1×10^6 cells/mL.

The kinetics of AF was monitored by daily determination of the Brix degree and density decrease. Samples for implantation control were taken under aseptic conditions at three different moments. The first one was three days after harvest and initial inoculation with *Saccharomyces* and non-*Saccharomyces* yeasts (day 3). The second one was at the fourth day (day 4) when the 27 tanks had been inoculated with *S. cerevisiae* VRB^{TM}. Eventually, the third control of implantation was performed one week after the first inoculation (day 7) (Figure S1). At these three moments, serial dilutions were carried out and the samples were microbiologically characterized as described above (Section 2.1). With the sequencing results, the percentage of each species composing each replicate was determined.

When the 27 wines had reached about 990 g/L density, they were pressed and fermented to dryness. The AF was complete when reducing sugars were lower than 2 g/L. Then, the wines were characterized by measuring the alcohol by volume (ABV), pH, total acidity, volatile acidity, colour intensity and hue according to official ECC methods [9]. Moreover, the malic and lactic acids, glycerol and acetaldehyde contents were determined by an enzymatic method carried out by an automated clinical chemistry analyser (Miura One) and tartaric acid by the Rebelein method [17]. Furthermore, total anthocyanins were measured by decolouring using SO_2 [18] and total phenolics were determined as the total polyphenol index by spectrophotometric absorbance at 280 nm after dilution of samples. Ionized anthocyanins were determined according to Glories [19] and the polymerization index was calculated according to Ruiz [20].

2.4. Analytical Techniques

The analysis of fermentative volatile or aromatic compounds after the AF was performed using the method described by Ortega et al. [21] with some modifications. The extraction was carried out by 4 mL of sample, 9 mL of $(NH_4)_2SO_4$ saturated solution, 40 µL of internal standard solution (2-butanol, 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone, 2-octanol, and heptanoic acid, 40 mg of each of them in 100 mL of ethanol) and 300 µL of dichloromethane in tubes. The tubes were shaken for 1 h at 32 × g and then centrifuged at 3220× *g* for 10 min. Once the phases were separated, the dichloromethane phase was recovered. Two µL was injected onto a Hewlett-Packard (Palo Alto, California, CA, USA) 6890 series II gas chromatograph. Separation was carried out with a DB-Wax capillary column (60 m × 0.32 mm I.D. × 0.5 µm film thickness; J&W Scientific, Folsom, CA, USA).

2.5. Malolactic Fermentation

After AF, the wines were drawn off the lees and transferred to 15 L containers that were inoculated with the commercial LAB *Uvaferm alpha*® (Lallemand Bio S.L., Toronto, Canada) to carry out the MLF, at a temperature of 20 °C. The evolution of the fermentation was controlled by periodic determination of the malic acid content (g/L). After this, the wines were sulphited again and bottled. One month after MLF had ended, the wines were again analysed in terms of oenological and colour parameters, including the parameters described above for the AF (Section 2.2).

2.6. Statistical Treatment

The statistical analysis of physicochemical data consisted of two multivariate analysis performed with discriminant analysis and classification by a hierarchical cluster. The analysis of the discriminate capacity of the oenological parameters was assessed for each replicate (n = 3) of must and wines after AF. The hierarchical cluster was built with the averages of every oenological and colour parameter assessed by triplicates (n = 3) for the oenological parameters of samples after FML. Both analyses were carried out by using the statistical package IBM SPSS Statistic 20.0 (Chicago, IL, USA). Raw data of replicates employed for statistical analysis could be consulted in the Spreadsheet S1.

3. Results

3.1. Musts Physicochemical Characterization

Results of the statistical canonical discriminant analysis (CDA) of oenological parameters of must samples of Tempranillo, Grenache and Graciano are shown in Figure 1. The 100% of the variability between the three musts was explained by two possible canonical functions (F). F1 explained over 96.1% of variability and F2 3.9%, with both being significant.

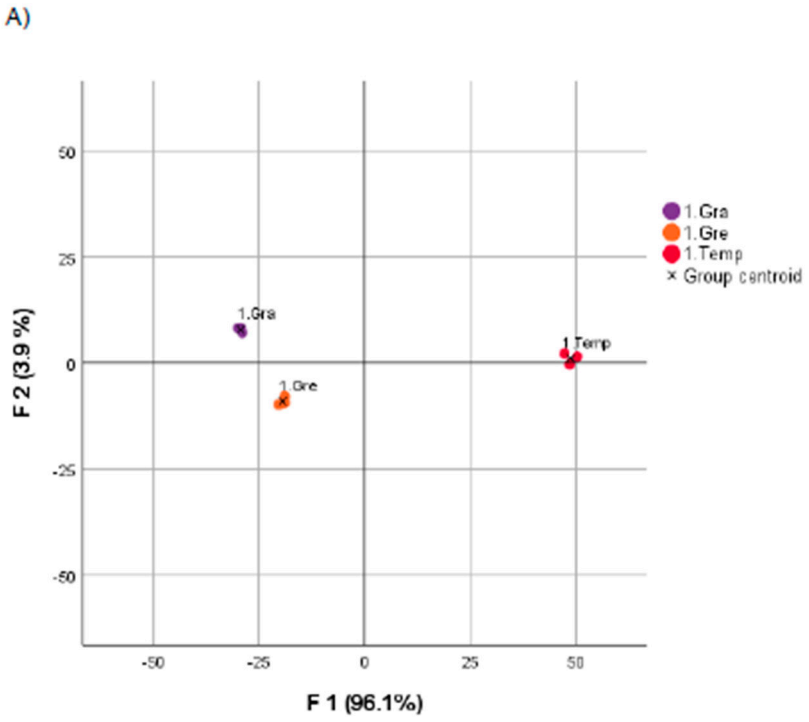


Figure 1. *Cont.*

B)

Standardized canonical coefficients

Oenological parameters	F 1	F 2
NFA	2.792	0.784
APBV (%)	- 4.247	-0.804
pH	2.056	-0.130
Total acidity (acetic acid g/l)	- 1.045	0.802
Malic acid (g/l)	0.444	1.198

Figure 1. (**A**) Canonical discriminant analysis of control initial must (1.) of Tempranillo (Temp), Grenache (Gre) and Graciano (Gra). (**B**) Standardized canonical coefficients of the two main discriminant functions (F1 and F2) obtained for oenological parameters.

All five analysed parameters contributed to the separation along F1, but APBV loading was the most dominant. F2 was also employed by the statistical software to construct the graph being mainly loaded by the malic acid content. The Tempranillo must sample was separated along F1 from the other two varieties. Grenache must was placed in the negative part of the F2 axis, and Graciano must on the positive F2 axis. Tempranillo had low APBV and the high pH and malic acid content while the Graciano must also was characterised also by low ABPV and high total acidity (data shown in Spreadsheet S1). The Grenache must had high APBV and low malic acid content.

3.2. Control of Yeast Populations and AF Kinetics in Each Grape Variety

3.2.1. Tempranillo

Results of the yeast population found in Tempranillo are shown in Figure 2A. The initial indigenous yeast population of Tempranillo must (day 0) was composed of 50% *Hanseniaspora* (*H.*) *uvarum*, 31% *S. cerevisiae* and the 19% remaining *T. delbrueckii* and *Cryptococcus* (*Cr.*) *laurenti*. The control sample of Tempranillo (C) before the second *S. cerevisiae* inoculation (day 3), was 30% *S. cerevisiae* and 70% *M. pulcherrima*. One day later (day 4), it was 77% *S. cerevisiae* and 23% *M. pulcherrima* and after a week (day 7) it was 100% *S. cerevisiae*. For samples early inoculated with *M. pulcherrima* (M), at day 3, the yeast community was 60% *M. pulcherrima* and 30% *S. cerevisiae*, 7% *H. uvarum* and 3% *L. thermotolerans*. One day later (day 4), the yeast community was composed of 70% *S. cerevisiae* and 30% *M. pulcherrima.* Eventually, after a week (day 7), all the identified yeasts were *S. cerevisiae*. In the case of Tempranillo grapes initially inoculated with *L. thermotolerans* and *T. delbrueckii* (*L&T)*, three days after their inoculation (day 3, the yeast community was 87% *T. delbrueckii* and 13% *L. thermotolerans*. One day after *S. cerevisiae* was inoculated (day 4), it reached 40% of the yeast community and 47% *T. delbrueckii* and 13% *L. thermotolerans* was found. Finally, a week after the first inoculation (day 7), all identified yeasts were *S. cerevisiae.*

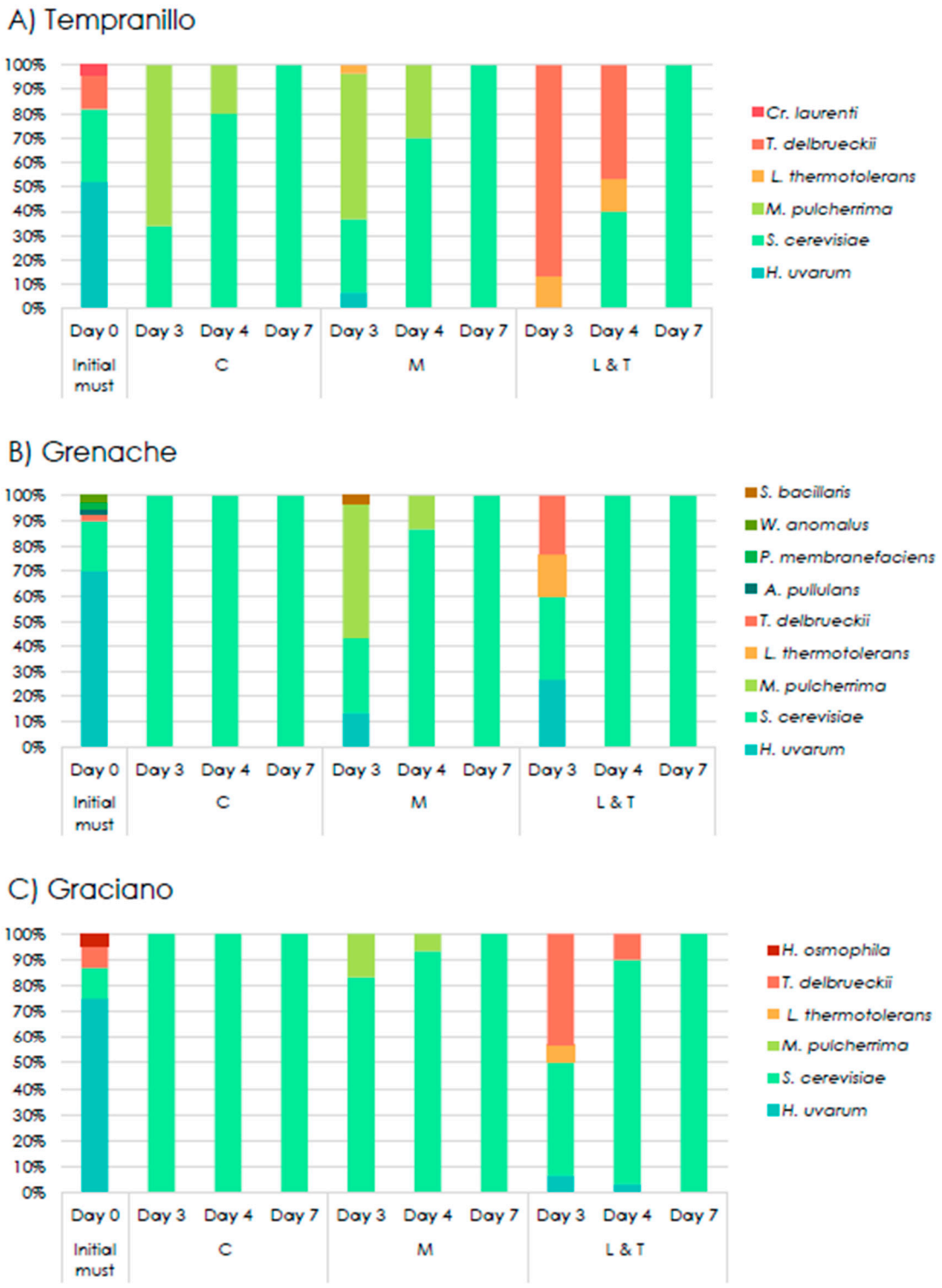


Figure 2. Percentage of yeast species in (**A**)Tempranillo, (**B**) Grenache and (**C**) Graciano, initial must (day 0), and control (C) samples and samples early inoculated with *M. pulcherrima* (M) and with a mix of *L. thermotolerans* and *T. delbrueckii* (*L&T*) during days 3, 4 and 7.

Considering the AF completed when the Brix degree had values between five and seven, the control AF of Tempranillo was completed in six days and the other two (M and *L&T*) lasted a day longer (Figure 3A).

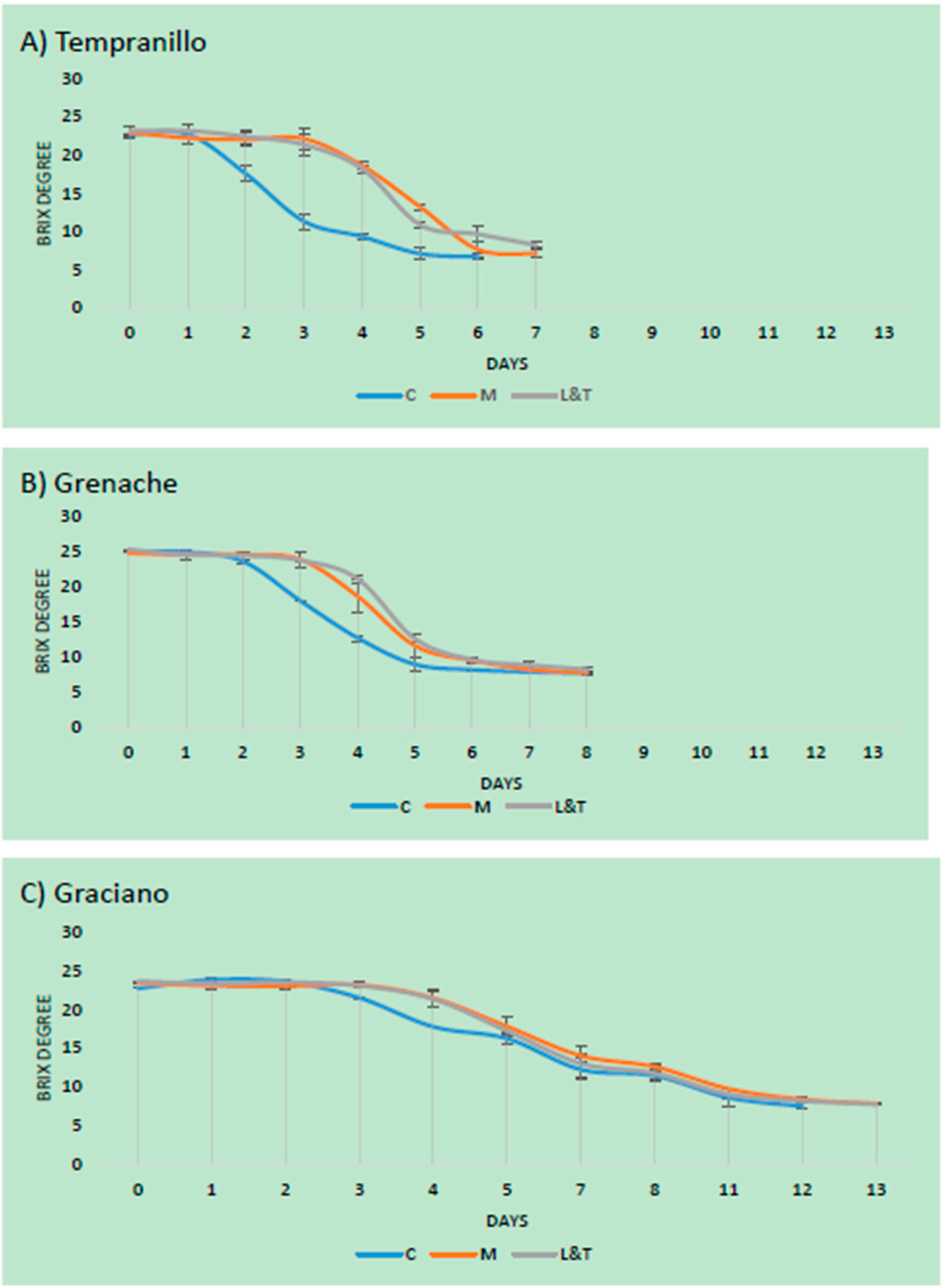


Figure 3. Brix degree measurement during alcoholic fermentation of control (C) samples and samples early inoculated with *M. pulcherrima* (M) and with a mix of *L. thermotolerans* and *T. delbrueckii* (*L&T*); (**A**)Tempranillo, (**B**) Grenache and (**C**) Graciano.

3.2.2. Grenache

The yeast population identified in Grenache are shown in Figure 2B. The initial Grenache must was composed of 70% *H. uvarum*, 20% *S. cerevisiae*, and 10% of *M. pulcherrima*, *Pichia (P.) membranaefaciens*, *Aureobasidium (A.) pullulans* and *Williopsis (W.) anomalous (day 0)*. In control (C) samples analysed three, four and seven days after the first inoculation of *S. cerevisiae*, all yeast isolates belonged to this species. Samples of Grenache inoculated with *M. pulcherrima* (M) were composed of 53% *M. pulcherrima*, 30% *S. cerevisiae* and 21% of *H. uvarum* and *Starmerella* (*St.*) *bacillaris* at day 3. One day later (day 4), 87% was *S. cerevisiae* and 13% *M. pulcherrima* and a week after the first inoculation (day 7), the entire yeast community was identified as *S. cerevisiae*. Grenache inoculated with *L. thermotolerans* and *T. delbrueckii*

(*L&T*) were composed of 27% *H. uvarum*, 33% *S. cerevisiae*, 23% *T. delbrueckii* and 17% *L. thermotolerans* at day 3. The other two checks of implantation (days 4 and 7) showed that 100% of the yeast community was *S. cerevisiae*. In the case of the Grenache variety (Figure 3B), the control sample either ended AF in six days and the others in seven days. The kinetics of the AF control sample were quicker than the AF of the samples early inoculated with the non-*Saccharomyces* yeasts.

3.2.3. Graciano

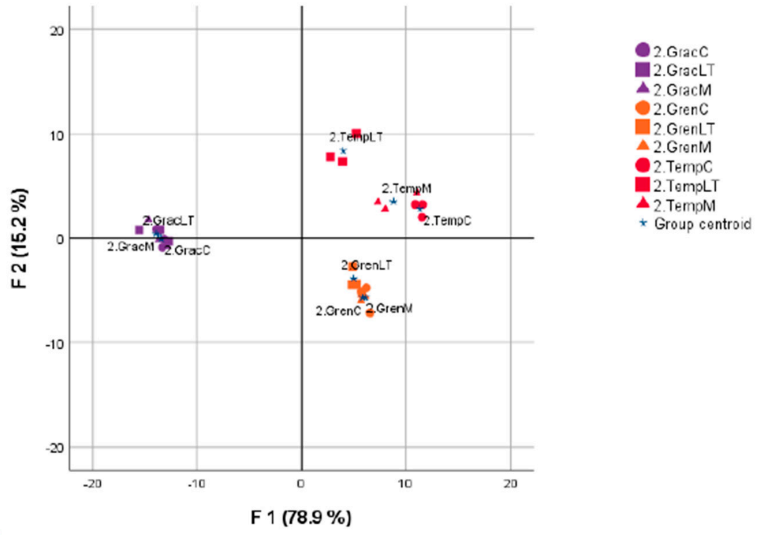
The species identified in Graciano samples are shown in Figure 2B. The initial Graciano must (day 0) had 75% *H. uvarum*, 12% *S. cerevisiae*, and 13% of *T. delbrueckii* and *H. osmophila*. Control samples were composed of 100% *S. cerevisiae* at each sampling checked. The samples inoculated with *M. pulcherrima* (M) at the third day had 17% *M. pulcherrima* and 83% *S. cerevisiae.* One day later (day 4), *S. cerevisiae* was 93% and one week later (day 7) all the samples were composed of *S. cerevisiae*. The samples inoculated with *L. thermotolerans* and *T. delbrueckii* (*L&T*) three days later (day 3) were made up of 7% *H. uvarum*, 43% *S. cerevisiae*, 43% *T. delbrueckii* and 6% *L. thermotolerans*. One day later (day 4), samples had 87% *S. cerevisiae* and one week later (day 7), they were composed solely of *S. cerevisiae*.

The Graciano AF kinetics (Figure 3C) of control samples and samples early inoculated with non-*Saccharomyces* yeasts were similar regardless of the inoculated yeasts used and took thirteen days to complete.

3.3. Characterisation of Wines

The statistical CDA of the oenological parameters of the samples of Tempranillo, Grenache and Graciano wines after AF, are shown in Figure 4. The variability between the samples (n = 3) was explained by four possible canonical functions (F) with statistical significance. F1 explained over 78.9% of variability and F2 15.2%, both explaining the 94.1% of the variance. Four out of the five assessed parameters contributed to the separation along F1, but the pH was the most influencer. F2 was mainly loaded by the total acidity, F3 and F4 (not included in the graph) were loaded by the volatile acidity. The three samples of Graciano wines stayed close in the negative part of axis F1 and Grenache and Tempranillo wines were separated by the axis 2. The three Grenache wines were clustered together. The sample of Tempranillo early inoculated with *L. thermotolerans* and *T. delbrueckii* (LT) was separated from the other two types of Tempranillo wines (control –C- and inoculated with *M. pulcherrima* –*M*-).

Results of statistical CDA of the colour parameters of the samples of Tempranillo, Grenache and Graciano after AF, are shown in Figure 5. The variability between the samples (n = 3) was explained by four possible canonical functions (F) with statistical significance. F1 explained over 89.2% of variability and F2 8.3%, both explaining the 98.5% of the variance. The six colour parameters analysed contributed to the separation along F1 and F2, but the most important one was the total polyphenol index. F3 and F4 (not included in the graph) were loaded by the hue and the colour intensity, respectively. The three samples of Graciano wines stayed together in the positive part of axis F1 and Grenache and Tempranillo wines were in negative part of axis F1 and separated by the axis 2. The three Grenache wines were clustered together in the positive part of axis F2. The samples of Tempranillo were placed in the negative part of both axis and the samples early inoculated with *L. thermotolerans* and *T. delbrueckii* (LT)were separated from the other two types of Tempranillo wines (control –C- and inoculated with *M. pulcherrima* –*M*-).



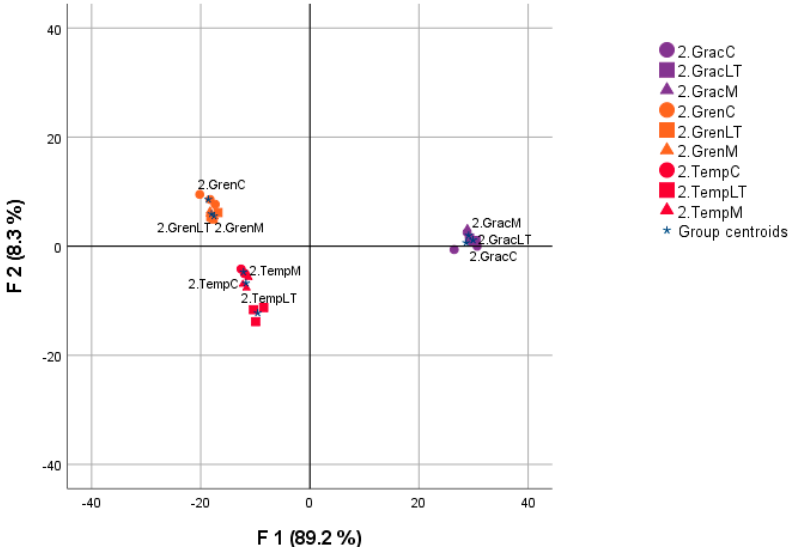
A)

B)

Standardized canonical coefficients

Oenological parameters	F 1	F 2	F3	F4
pH	0.690	0.755	-0.224	0.343
Total acidity (acetic acid g/l)	-0.481	0.978	-0.402	0.115
Volatile acidity (acetic acid g/l)	-0.116	-0.217	0.636	0.835
Lactic acid (g/l)	0.167	0.141	1.044	-0.254

Figure 4. (**A**) Canonical discriminant graph of oenological parameters in control samples (C), samples early inoculated with *M. pulcherrima* (M) and samples early inoculated with a mix of *L. thermotolerans* & *T. delbrueckii* (LT), after alcoholic fermentation (2.) of Tempranillo (Temp), Grenache (Gre) and Graciano (Gra) (**B**) Standardized canonical coefficients of the four main discriminant variables in functions (F1 and F2) for oenological parameters.



(**A**)

Figure 5. *Cont.*

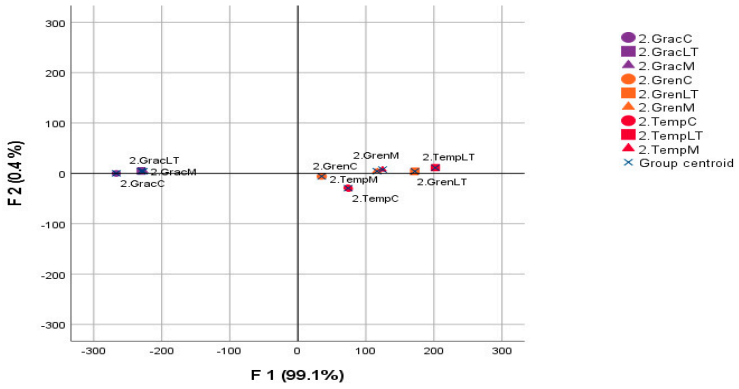
Standardized Canonical Coefficients.				
Colour Parameters	F 1	F 2	F3	F4
Colour intensity	1.380	−0.203	−0.164	−1.787
Hue	−0.136	0.303	1.255	0.080
Anthocyanins (mg/l)	0.658	−1.461	0.716	1.017
Total poliphenol index	−1.616	1.773	0.002	0.267
Ionization index	0.509	0.668	0.551	0.722
Polymerization index	−0.052	−0.246	−0.230	0.499

(**B**)

Figure 5. (**A**) Canonical discriminant graph of colour parameters in control samples (C), samples early inoculated with *M. pulcherrima* (M) and samples early inoculated with a mix of *L. thermotolerans* & *T. delbrueckii* (LT), after alcoholic fermentation (2.) of Tempranillo (Temp), Grenache (Gre) and Graciano (Gra). (**B**) Standardized canonical coefficients of the four main discriminant variables in functions (F1 and F2) for colour parameters.

The statistical CDA of the aromatic compounds of samples of Tempranillo, Grenache and Graciano after AF, are shown in Figure 6. The variability between the samples (n = 3) was explained by four possible canonical functions (F) with statistical significance. F1 explained over 99.1% of variability and F2 0.4%, explaining the 99.5% of the variance. 12 alcohols and six esters out of the 34 aromatic compounds measured, contributed to the separation along F1 that was mainly loaded by propanol-1 compound and F2 by the hexyl acetate contents. The F3 by 2-phenylacetate and F4 by ethyl-3-hidroxibutyrate although not included in the graph. The three samples of Graciano wines stayed together in the negative part of axis F1, being separated the control sample (C) of the other two samples. Grenache and Tempranillo wines were separated by the axis 1 but in the positive part. In this case, wines were separated, being the control samples of Tempranillo and Grenache very close while the samples of both varieties but early inoculated with *L. thermotolerans* and *T. delbrueckii* (LT) were quite distant.

The MLF of each wine was completed without problems (data no shown). Six months after completion of MLF and bottling, the wines were analytically analysed in the colour and oenological parameters described for samples after AF and the hierarchical cluster built with the average data is shown in Figure 7.



(**A**)

Figure 6. *Cont.*

Standardized Canonical Coefficients				
Aromatic Compounds	**F1**	**F2**	**F3**	F4
Propanol-1	22.50	1.130	1.052	0.426
1-Butanol	5.032	0.673	0.317	−0.075
Isobutanol	5.265	3.127	−1.571	−0.358
Amyl alcohols	9.093	−0.583	1.999	−2.660
2-Phenylethanol	−19.06	1.024	−1.460	3.235
1-Hexanol	−18.62	−3.436	0.393	−0.780
Benzyl alcohol	4.95	−1.644	0.809	−0.204
Methionol	20.36	0.404	0.566	−1.000
Cis-3-hexanol	16.92	−1.313	−0.198	−0.318
Isoamyl acetate	−19.78	−7.869	−2.783	−0.868
Hexyl acetate	0.879	9.344	−0.553	1.507
2-phenylacetate	21.38	−0.245	2.894	−0.175
Ethyl propionate	6.045	2.237	−0.493	−0.493
Ethyl-3-hidroxibutyrate	19.44	3.535	−0.014	3.737
Ethyl isobutyrate	−13.88	−1.270	0.264	0.838
Ethyl butyrate	−7.602	−2.158	1.274	−0.366
Ethyl hexanoate	5.535	2.814	0.109	1.004
Ethyl octanoate	−14.04	−1.859	0.277	−2.057

(**B**)

Figure 6. (**A**) Canonical discriminant graph of aromatic compounds in control samples (C), samples early inoculated with *M. pulcherrima* (M) and samples early inoculated with a mix of *L. thermotolerans* & *T. delbrueckii* (LT), after alcoholic fermentation (2.) of Tempranillo (Temp), Grenache (Gre) and Graciano (Gra) (**B**) Standardized canonical coefficients of the four main discriminant variables in functions (F1 and F2) for aromatic compounds.

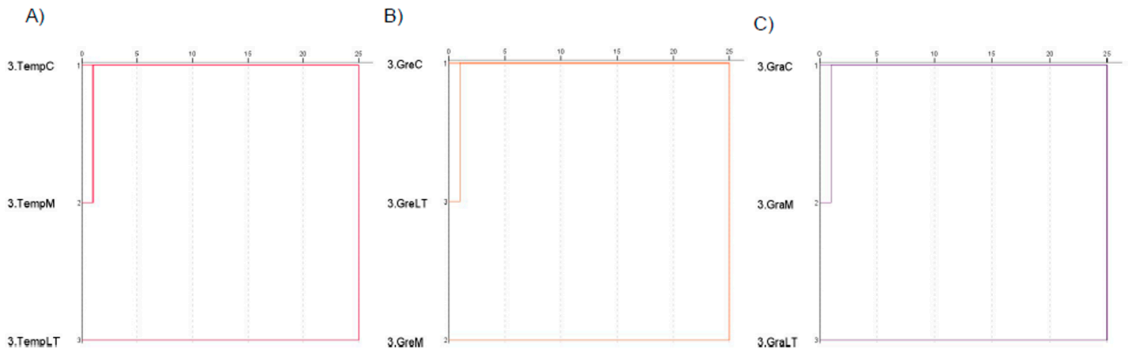


Figure 7. Hierarchical clusters assessed with average oenological and colour parameters of control samples (C), samples early inoculated with *M. pulcherrima* (M) and samples early inoculated with a mix of *L. thermotolerans* & *T. delbrueckii* (LT), after the malolactic fermentation (3.) of (**A**) Tempranillo (Temp); (**B**) Grenache (Gre); and (**C**) Graciano (Gra).

Tempranillo and Graciano grapes inoculated early with *L. thermotolerans* and *T. delbrueckii* (LT) were separated from the control samples and from the samples inoculated early with *M. pulcherrima* (M) that were clustered together. In contrast, in Grenache wines after MLF the samples inoculated early with *M. pulcherrima* (M) were separated from the other two samples that stayed clustered together.

4. Discussion

This study was focused on individual pilot plant vinifications of Tempranillo, Grenache and Graciano inoculated with non-*Saccharomyces* yeast inocula for responding if every non-*Saccharomyces* yeast would cause similar physicochemical and aromatic profiles in different grape varieties. The initial

must of three grape varieties musts were separated according to parameters of APBV and acidity. Moreover, their indigenous yeast communities were also different. These initial differences fitted with a standard winemaking of non-sterile grapes [22].

4.1. Yeasts Establishment and Fermentation Kinetics

Tempranillo grapes had low APBV and high malic acid content, which was initially positive for the establishment of yeasts and bacteria populations and consequently for the evolution of AF and MLF. The presence of *S. cerevisiae* in the grape surface and must is usually low and this was corroborated in this study [23]. The initial must had *S. cerevisiae* as residual yeast and a high population of *H. uvarum*, *H. osmophila* and *T. delbrueckii* that were naturally present. The control sample inoculated only with *S. cerevisiae* had a large population of *M. pulcherrima* after three days, which might be due to an external contamination of the tanks with the *M. pulcherrima* inoculated vinification that coexisted in the experimental winery. Nonetheless, indigenous *T. delbrueckii* and *H. osmophila* were not detected and AF proceeded without problems; it was rapid and lasted only six days. In Tempranillo samples, early inoculated with non-*Saccharomyces* and then with *S. cerevisiae*, the establishment of the different yeast species happened as it was expected, probably due to the preadaptation of the strains to the grape variety because they had been isolated from this same variety.

The microbial composition of the Grenache must was characterised by a large population of *H. uvarum*, with *S. cerevisiae* as a minority strain. A diverse indigenous population characterized the initial must. Furthermore, indigenous *M. pulcherrima* was found in Grenache grapes, although with low percentage. *S. cerevisiae* inoculated in the control sample was able to achieve total implantation in spite of the high APBV of the must, and of the ecological pressure that other initial yeast species could have exerted. Indeed, the AF was not as rapid as it was in Tempranillo. The establishment of inoculated yeast species in Grenache must sample were not so successful that the observed in Tempranillo samples, in effect, the diversity of indigenous and inoculated non-*Saccharomyces* stayed until the day 4 and after this, *S. cerevisiae* became the majority.

The Graciano must had a similar microbial composition to that observed for Tempranillo. *H. uvarum* was the most frequently detected species and *T. delbrueckii* was initially present in the must sample. Similarly, to what was observed in Grenache, the implantation of *S. cerevisiae* in the control sample was total in spite of the high acidity and low pH of the must although the AF kinetics was very slow and lasted thirteen days. Similar to the described in Grenache, the establishment of inoculated non-*Saccharomyces* species was even less successful in percentages of identification.

4.2. Discriminant Analysis of Wines after AF

4.2.1. Statistical Analysis of Oenological Parameters

In order to know how the wine samples were separated depending only on the must inoculation strategy, the statistical analysis was performed without the ABV and the malic acid content that separated the must samples in the discriminant analysis.

The early inoculation of *S. cerevisiae*, *M. pulcherrima* and the mix of *L. thermotolerans* and *T. delbrueckii* did not provide enough changes in the oenological parameters of Graciano and Grenache wine samples, so that they appeared together regardless the inoculation strategy in the representation of the two main canonical discriminate functions. Only Tempranillo samples early inoculated with *L. thermotolerans* and *T. delbrueckii* was separated in the graph, from control wine samples and from wine samples early inoculated with *M. pulcherrima*. These Tempranillo wine samples early inoculated with the mix of *L. thermotolerans* and *T. delbrueckii* were characterized by a low pH and a high total acidity. As far as we know, this is the first time that the mixed inocula of *L&T* (30/70) has been tested in a pilot plant in three different grape varieties. Results showed that in the Tempranillo must, both yeasts achieved a total implantation maintaining a ratio of 13/87. Post AF, the inoculated wine had interesting increased

acidity parameters due to the capacity of *L. thermotolerans* to produce lactic acid [5], which could achieve balance in a grape variety generally characterised by high pH and low acidity.

4.2.2. Statistical Analysis of Colour Parameters

Analysing statistically the colour parameters of the wine samples of the three grape varieties early inoculated with *S. cerevisiae*, *M. pulcherrima* and a mix of *L. thermotolerans* and *T. delbrueckii* provided similar results to the described for oenological parameters in the later Section 4.2.1. Again, Graciano and Grenache wine samples were separated only for being different grape varieties, but not because of the three different yeast inoculation strategies. Moreover, the Tempranillo control wine samples and the samples early inoculated with *M. pulcherrima* reached high values of total polyphenol index, while samples early inoculated with a mix of *L. thermotolerans* and *T. delbrueckii* yeasts reached lower values what make them stay separated in the graph of the two main canonical functions extracted from the discriminant analysis. In one previous study, of this same mix of *L. thermotolerans* and *T. delbrueckii* was tested for oenological parameters and anthocyanins and stilbenes and similar results were described [14]. In general terms, the reduction of the total polyphenol index is not a good result for wine quality, but observing this effect only on Tempranillo that is a grape variety characterized for normal anthocyanins content, might not be so negative than if it happened in Grenache that has a low anthocyanins content [24].

4.2.3. Statistical Analysis of Aromatic Profile

Results of the aromatic profile of the three varieties inoculated with different strategies showed interesting results. For instance, wine samples were mainly separated in the graph of the two main discriminant functions by the content of propanol-1 compound that provide alcoholic and mature fruit notes. Any other aromatic compound was able to discriminate samples. Graciano wine control samples, with lower propanol-1 concentrations, were separated from samples that had been early inoculated with *M. pulcherrima* and with the mix of *L. thermotolerans* and *T. delbrueckii*, this would mean that early inoculation of non-*Saccharomyces* yeast in Graciano must samples led to a more alcoholic profile than the samples inoculated only with *Saccharomyces*. Grenache wine samples were also separated by propanol content but in this case, the samples inoculated with *S. cerevisiae* had lower concentrations than the early inoculate with *M. pulcherrima* and these ones than the early inoculated with a mix of *L. thermotolerans* and *T. delbrueckii*. Just this same result was observed for Tempranillo wine samples. Giudici et al. [25] published that the higher alcohol n-propanol was directly related with the ability of some yeast strains to metabolise methionine and threonine aminoacids and depended on their initial content in wine, what could explain why the same inoculation strategy led to different concentration of propanol in wines depending on the variety. In any case, odour threshold for propanol was established by Peinado et al. [26] in 306 mg/L that was very high comparing concentrations obtained in the current study. This means that the different concentration between wine samples observed in the current research would not probably led to a differentiation in sensory terms.

Furthermore, the three Tempranillo wine samples were slightly differenced by the hexyl acetate content. In this way, the control wine sample was the one with the highest content of hexyl acetate compared to the early inoculated with *M. pulcherrima* and with a mix of *L. thermotolerans* and *T. delbrueckii.* The hexyl acetate aromatic compound is related to apple, cherry, pear and floral aromas and the odour threshold is 1.5 mg/L [27]. Only Tempranillo samples inoculated with *S. cerevisiae* overcame this threshold so that it would be fruitier than the Tempranillo samples early inoculated non-*Saccharomyces*.

4.3. Discriminant Analysis of Wines after MLF

Aromatic composition of wines after MLF was not considered because this fermentation was seeded with one commercial strain of *O. oeni*, so that differences in aroma could probably be due to the effect of this strain but not to the different inoculation strategies. Multivariate statistical analysis of oenological and colour parameters of samples of the three varieties showed clearly that early

inoculation of Tempranillo and Grenache varieties with a mix of *L. thermotolerans* and *T. delbrueckii* caused separation of control wine samples while the early inoculation of Graciano with *M. pulcherrima* was the wine that was differenced of control Grenache wine samples.

5. Conclusions

To sum up, most of the oenological differentiations due to inoculation strategies were observed in Tempranillo wines while in Graciano and Grenache changes due to different inoculation strategies were scarce in many cases. Nevertheless, wine elaborated with different wine varieties were perfectly identified considering the grape variety. This would be indicating that one of the most important previous step in obtaining not homogenous wines is the winemaking of different grape varieties. Non-*Saccharomyces* early inoculated had been isolated from Tempranillo musts, so that a preadaptation to these grape variety properties might be expected. Therefore, changes in Tempranillo wines might be linked to the implantation or establishment rates of the inoculated yeasts. This research indicated, for the first time, that early inoculation with non-*Saccharomyces* should be carefully adjusted to the properties and features of a specific grape variety in order to increase the heterogeneity of the final product.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/6/1/3/s1, One Figure S1 and one spreadsheet S1 has been included in this submission. The Figure S1 aims to clarify the sampling with a schematic representation and the spreadsheet S1 contains every data employed in statistical analysis.

Author Contributions: R.E.-V. was in charge of the methodology and the original draft preparation. P.G. was also responsible of the methodology. I.L.-A. was part of the research equipment. R.L. was a researcher of the project and also collaborated in the review and editing of the draft. P.S. researched and was in charge of resources. A.R.G. was responsible for the finding acquisition and for the project administration, and developed the formal analysis, resources and reviewed and edited the draft and eventually L.G.-A. submitted the manuscripts after reviewing and editing the draft. All authors have read and agreed to the published version of the manuscript.

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