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Selection Process of a Mixed Inoculum of Non-*Saccharomyces* Yeasts Isolated in the D.O.Ca. Rioja

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Abstract: The use of non-*Saccharomyces* yeasts in sequential fermentations with *S. cerevisiae* has been proposed to improve the organoleptic characteristics involved in the quality of wine. The present study set out to select a non-*Saccharomyces* inoculum from the D.O.Ca. Rioja for use in winemaking. Strains included in the study belonged to *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Zygosaccharomyces bailii*, *Williopsis pratensis*, *Debaryomyces hansenii*, *Pichia kluyveri*, *Sporidiobolus salmonicolor*, *Candida* spp., *Cryptococcus* spp. and two mixed inocula of *Lachancea thermotolerans*-*Torulaspora delbrueckii* in a 30/70 ratio. In the first stage of the process, SO₂ resistance and presence of enzymatic activities related to wine aroma and wine color and fining (esterase, esterase-lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, β-glucosidase, pectinase, cellulose, xylanase and glucanase) were studied. In the later stages, selection criteria such as fermentative behavior, aroma compound production or influence on phenolic compounds were studied in laboratory scale vinifications. Taking into account the results obtained in the different stages of the process, a mixed inoculum of *Lachancea thermotolerans*-*Torulaspora delbrueckii* in a 30/70 ratio was finally selected. This inoculum stood out for its high implantation capacity, the production of compounds of interest such as glycerol and lactic acid and the consequent modulation of wine acidity. Given these characteristics, the selected inoculum is suitable for the production of quality wines.

Keywords: selection; non-*Saccharomyces*; winemaking; *Lachancea thermotolerans*; *Torulaspora delbrueckii*



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1. Introduction

Spontaneous fermentations are complex microbial processes performed by the sequential action of indigenous non-*Saccharomyces* and *Saccharomyces* yeasts. The high diversity of species involved in the process gives rise to wines with more complex profiles. However, these fermentations are often associated with extended lag phases and long fermentation times, high residual sugar content and organoleptic defects [1]. These disadvantages, together with the lack of predictability of the process, have led winemakers to seek alternatives to spontaneous fermentations. Currently, the most extended practice in winemaking involves the inoculation of the must with selected *S. cerevisiae* strains in the form of active dry yeasts (ADY). This technique has some advantages, such as the rapid and complete fermentation of the must and great reproducibility of the final product [2]. However, this practice also leads to less complexity and a certain standardization of the wines obtained as a consequence of the reduction of the diversity of the microbial populations involved in the fermentation [3,4].

To avoid homogeneity of the wines inoculated with *S. cerevisiae* commercial strains, the use of selected non-*Saccharomyces* yeasts in mixed fermentations with *S. cerevisiae* has been proposed. This practice offers some of the advantages of spontaneous fermentations such as higher organoleptic complexity, while allowing greater control of the process,

avoiding stuck fermentations or possible defects in the wine caused by the indigenous microbiota [4–6].

In the past, non-*Saccharomyces* yeasts were not considered to be very relevant and were even viewed as spoilage yeasts because of their high production of compounds that could be negative for wine quality, such as acetic acid, ethyl acetate, acetaldehyde or acetoin [7]. However, the recent literature has shown that, through appropriate selection programs, non-*Saccharomyces* yeast strains can have a positive influence on wine characteristics [8–13].

Among the non-*Saccharomyces* yeasts that have been studied to improve wine characteristics is *Torulaspota delbrueckii*, which has been reported to decrease volatile acidity [14] or increase glycerol content [15]. This yeast can modulate the aromatic properties of wines producing high levels of fruity esters, thiols and terpenes [16–18]. Another non-*Saccharomyces* yeast that has been proposed for use in winemaking is *Lachancea thermotolerans*. Fermentations with this yeast usually lead to a higher total acidity, which is generally attributed to the production of lactic acid [19,20]. The production levels of lactic acid can vary between strains [21,22]. According to the literature, a low production of acetic acid is another feature of this yeast that can contribute to wine quality [7,23].

Technological criteria, such as the SO₂ resistance of the yeast strains, are usually taken into account in the selection processes. SO₂ is an additive widely used in winemaking, so the resistance of the yeasts to this compound is a relevant characteristic when carrying out the selection process. This is a highly variable trait, varying not only between species but also between yeast strains [24]. In recent years, the qualitative properties of yeasts have been considered of great importance, since they can influence the chemical composition and sensory characteristics of wine [25]. These characteristics include the ability to produce enzymes that can influence the aroma and color of wine or the release of compounds of interest derived from yeast metabolism. The activity of enzymes related to aroma allows the release of aromatic compounds to the wine. As an example, β-glucosidase enzymes catalyze the release of terpenes from their precursors [26,27]. Other enzymes, such as carbohydrases, allow the improvement of color extraction in red wines during maceration due to the degradation of structural polysaccharides [28]. Yeast metabolism can lead to the release of compounds that contribute to the quality of the wine such as glycerol, higher alcohols or esters [29–31]. As has been shown in the literature, many of the yeast features that can influence the quality of wine are strain dependent [4,32,33], which highlights the importance of considering metabolic diversity within species, at the strain level.

Many wineries and wine-growing regions have developed programs for the selection of indigenous yeasts, including isolates from the winery's own ecosystem or from its vineyards. This trend is based on the idea that yeasts isolated from these enological environments are better adapted to the ecology and technological characteristics of the musts of the region from which they come [34,35]. The use of autochthonous yeasts can be a useful tool to improve the characteristics of the typical wines of a region due to their adaptation to the environment and an ability to highlight the peculiarities of the product [36–39].

In the current study, the selection process of non-*Saccharomyces* strains isolated in the Rioja winegrowing region in Spain for use in winemaking is explained. For this study, selection criteria such as SO₂ resistance, enzymatic activities or influence on wine aroma and phenolic compounds were taken into account in order to choose the most suitable strain for its use in making red wine.

2. Materials and Methods

2.1. Yeast Isolates

Non-*Saccharomyces* yeast isolates included in the selection program were part of the Instituto de Ciencias de la Vid y el Vino (ICVV) collection, composed of more than 500 strains belonging to more than 30 different species. The selection process started with ninety-seven non-*Saccharomyces* wine yeast strains belonging to ten different genera.

This initial list included species that had shown good behavior as inocula in previous studies (*Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri* and *Torulasporea delbrueckii*), and species that had not been previously considered but that had been isolated in different D.O.Ca. Rioja enological ecosystems in previous works [40–42] (*Candida* spp. and *Cryptococcus* spp. genera, *Debaryomyces hansenii*, *Sporidiobolus salmonicolor*, *Williopsis pratensis* and *Zygosaccharomyces bailii*). In addition, during the process, two spontaneously developed mixed inocula in two of the studied cultures were detected and included in the study. Furthermore, a commercial strain of *Saccharomyces cerevisiae* (Uvaferm VRB, Danstar Ferment AB, Fredericia, Denmark) was used as a fermentative reference or as part of the inoculum in sequential inoculations.

2.2. Genetic Characterization of the Strains at Clonal Level

In the species in which several isolates were included, they were identified at a clonal level. Thus total of seventy-one strains were characterized by Random Amplification of Polymorphic DNA ((RAPD)-PCR) using the primers M13, M14, Coc, OPA02 and OPA09, following the methodology described by Renault et al. [23] with some modifications [24].

2.3. SO₂ Resistance

Resistance of the non-*Saccharomyces* strains to different concentrations of SO₂ (20, 40, 80 and 120 mg/L) was studied in tubes with 5 mL of buffered YPD (1% yeast extract, 2% dextrose, 2% peptone) following the procedure described in González-Arenzana et al. [43]. The resistance to SO₂ was determined by contrasting the growth of the studied strains in the presence of SO₂ with the growth of the same strains without SO₂. Measures of growth were conducted by reading the absorbance at 600 nm.

2.4. Enzymatic Characterization

The presence of enzymatic activities related to wine aroma (esterase, esterase-lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase and β -glucosidase) and wine color and fining (pectinase, cellulose, xylanase, glucanase, leucine arylamidase, valine arylamidase and cystine arylamidase) was studied in the non-*Saccharomyces* strains. Enzymatic activities were measured using an API-ZYM test system and different specific media plates as described in Escribano et al. [44]. To carry out the API-ZYM test, yeast cultures were suspended in distilled water until the suspensions reached a turbidity of 5–6 McFarland. 65 μ L of the culture suspensions were inoculated in each cupule of the API ZYM test strip.

2.5. Vinifications

2.5.1. Pure 100 mL Vinifications

In this assay alcoholic fermentations were performed in sterile, 100 mL Erlenmeyer flasks containing pasteurized (10 min at 70 °C) Viura grape juice (206.45 g/L initial reducing sugar concentration, 0.93 g/L malic acid, 2.36 total acidity expressed as g/L of tartaric acid, 150 mg/L yeast assimilable nitrogen (YAN) and pH 3.56). The fermentation vessels were sealed with Müller valves filled with H₂SO₄. Yeasts were precultured in a YPD liquid medium at 25 °C for 48 h before inoculation of the flasks with 10⁶ cells/mL. All the fermentations were carried out in duplicate without SO₂ addition at 20 °C and 70 rpm in an orbital incubator. Fermentation activity was measured daily as weight loss from CO₂ release, and fermentations were assessed as stuck when no further weight loss was observed for three consecutive days. Enological parameters and fermentative aromatic compounds were analyzed in final wines and results were compared with those obtained in fermentations performed with a commercial strain of *Saccharomyces cerevisiae*.

2.5.2. Sequential 2000 mL Vinifications

Vinifications of the following assay were performed in microscale fermenters [45] containing 2000 mL of red Tempranillo must and 500 g of pomace obtained after mechanical

treatment of the grapes, in a conditioned chamber at 25 °C. Assays were carried out in duplicate with unsterilized and unsulfited must (14.5% probable degree, 2.26 g/L malic acid, 4.9 total acidity expressed as g/L of tartaric acid, 181 mg/L YAN and pH 3.73). Vinifications were conducted with nine strains belonging to six different species [*T. delbrueckii* (T18 and T19), *M. pulcherrima* (M28 and M29), *L. thermotolerans* (L54 and L57), *Z. bailii* (Z71), *W. pratensis* (W87) and *C. zeylanoides* (C342)] and two mixed inocula of *L. thermotolerans* and *T. delbrueckii* in a 70/30 ratio (LT1 and LT2) in sequential inoculations with *S. cerevisiae*. Non-*Saccharomyces* yeasts were pre-cultured in YPD liquid medium at 25 °C for 48 h before inoculation of flasks with 10^6 cells/mL. Three days after non-*Saccharomyces* inoculation, a commercial *S. cerevisiae* strain was inoculated at a density of 1×10^6 cells/mL. Fermentation activity was measured daily as Brix decrease. Wines were pressed 14 days after inoculation, sulfited at 30 mg/L and left to settle for a week. Enological parameters and fermentative aromatic compounds were analyzed in final wines and results were compared with those obtained in fermentations performed only with a commercial strain of *Saccharomyces cerevisiae*.

2.6. Microbial Analysis and Implantation Controls of the Seeded Yeasts

Samples for microbial studies were taken at different times throughout the fermentations. In pure fermentations samples for implantation controls were taken under aseptic conditions on day 7 (tumultuous fermentation) and at the end of fermentation (day 14). In sequential fermentations samples were taken daily under aseptic conditions. Serial dilutions were carried out and the samples were seeded onto plates containing a chloramphenicol glucose agar medium (0.5% yeast extract, 20% glucose, 0.05% chloramphenicol, 17% agar). The plates were incubated at 25 °C for 48 h. Ten colonies from each sample were randomly selected from plates containing between 30 and 300 colony forming units per milliliter (CFU/mL).

The extraction of DNA for the identification of the isolates was conducted from the fresh yeast culture, using the method suggested by López et al. [46]. Domains D1/D2 of the 26S rDNA gene were amplified by PCR using primers and conditions described by Kurtzman and Fell [47]. The PCR products were purified and then sequenced at the facilities of Macrogen Inc. (Amsterdam, The Netherlands) in order to determine which species of yeast each isolate belonged to (according to data retrieved from GenBank: www.ncbi.nlm.nih.gov/blast/blast.org, accessed on 10 February 2017). The identification was considered correct when gene sequences showed identities of at least 98%.

2.7. Analysis of Enological Parameters in Wines

Wines were characterized by measuring the alcohol strength, pH, total acidity and volatile acidity according to official European Economic Community EEC methods [48]. Moreover, the malic and lactic acids, glycerol, acetaldehyde and residual sugars were determined by an enzymatic method carried out in a MIURA One enological analyzer (TDI, Barcelona, Spain). Total anthocyanins were measured by decoloring using SO₂ [49].

2.8. Analysis of Aromatic Compounds

The analysis of fermentative aromatic compounds in wine was performed by gas chromatography using the method described by Ortega et al. [50] with minor modifications [51]. Extracted samples were injected onto a 6890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an automatic injector and a Hewlett-Packard FID detector. 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). Identification of compounds was carried out by comparison of their retention times with those of pure reference standards using a Hewlett-Packard GCD Series II Gas Chromatograph Electron Ionization Detector.

2.9. Analysis of Stilbenes

In stilbene analysis, a previous purification step was conducted by solid phase extraction (SPE) [52] with PCX SPE cartridges (500 mg, 6 mL; Bond Elut Plexa, Agilent, Palo Alto, CA, USA) placed in a Gilson GX-271 Spec (Gilson Inc., Middleton, WI, USA). The fraction obtained from the SPE step was analyzed by HPLC-DAD using an Agilent 1260 Infinity system following the methodology adapted from Portu et al. [53]. Wine samples were injected into a LiCrospher R 100 RP-18 reversed-phase column (Agilent) with pre-column LiCrospher R 100 RP-18 (Agilent), both thermostated at 40 °C. Flow rate was at 0.500 mL/min and injection volume was 20 mL. Solvent A was water/acetonitrile/formic acid (100:10:0.1, *v/v/v*) and solvent B was acetonitrile. The linear solvent gradient was: 0 min, 0% B; 20.8 min, 16% B; 32.8 min, 16% B; 49.4 min, 42% B; 60 min, 0% B.

2.10. Statistical Analysis

Analysis of variance (ANOVA) was carried out for all the parameters analyzed for the different assays with the IBM® SPSS® Statistics version 23 (company, city, state abbrev if USA, country). Significant differences were established by using the Tukey post hoc test ($p \leq 0.05$).

3. Results

3.1. Clonal Characterization of Non-Saccharomyces Strains

In order to evaluate clonal diversity among the most representative non-*Saccharomyces* yeasts included in the study, genetic characterization was carried out in 71 isolates of five different species (*T. delbrueckii*, *M. pulcherrima*, *L. thermotolerans*, *Z. bailii* and *W. pratensis*). RAPD analysis allowed the differentiation of 17 clones among the 21 isolates of *T. delbrueckii*, 13 clones among the 16 isolates of *M. pulcherrima*, 15 clones among the 16 isolates of *L. thermotolerans* and 7 clones among the seven isolates of *Z. bailii* and the 11 isolates of *W. pratensis*. Results obtained showed high clonal diversity among the studied species [43].

3.2. SO₂ Resistance

Resistance to SO₂ was evaluated after 24 and 48 h in the same isolates in which clonal diversity was assessed. The percentage of isolates in each species able to grow at the same level as the control in different concentrations of SO₂ at 24 h is shown in Figure 1. Strains of *T. delbrueckii* and *Z. bailii* showed the best adaptation at 24 h. At that timepoint, 52.4% of *T. delbrueckii* isolates were able to grow at a similar level as the control in media containing 40 mg/L of SO₂, and 19.0% were able to grow with a concentration of 80 mg/L. In contrast, isolates of *M. pulcherrima* showed less tolerance to this compound. 56.3% of the isolates were not able to grow in the medium with a dose of 20 mg/L of SO₂ and the remaining isolates only tolerated that concentration. Isolates of *L. thermotolerans* also tolerated low levels of SO₂, but the proportion tolerating at least 20 mg/L was higher than in *M. pulcherrima*, with 81.3% of the isolates. Results showed better adaptation of the strains at 48 h, but the ability to develop rapidly in presence of SO₂ during vinification is an advantage for the yeast to establish in the medium. For that reason, growth at 24 h was considered as the selection criterion for SO₂ tolerance.

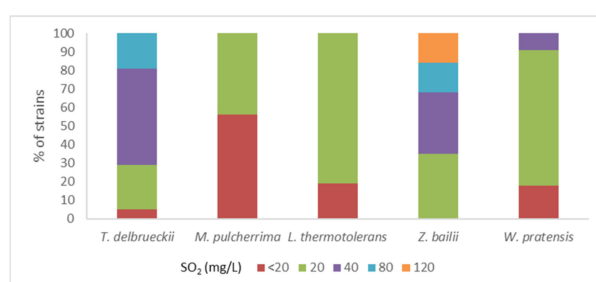


Figure 1. Distribution of strains within each species according to the maximum level of SO₂ which allowed a strain growth similar to control at 24 h [54].

3.3. Screening of Enzymatic Activities

Enzymatic activities were studied in non-*Saccharomyces* strains belonging to ten different genera and species (*Candida* spp., *Cryptococcus* spp., *Debaryomyces hansenii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Sporidiobolus salmonicolor*, *Torulasporea delbrueckii*, *Williopsis pratensis* and *Zygosaccharomyces bailii*). After determination of the enzymatic activities present in the strains, they were classified into different profiles related with wine aroma and color and fining in order to facilitate the selection of the most suitable strains depending on the type of wine or the desired characteristics to be modulated. Profiles related to aroma were named with an “A” and profiles related to color and fining were named with a “C” after the initials of the species or genera.

Enzymatic profiles related to aroma (Table 1) within each species were mainly differentiated by esterase, esterase-lipase and β -glucosidase activities. The β -glucosidase breaks down the terpenes combined with the sugars to release them, thus enhancing the varietal aroma of the wine. Lipases contribute to an increase in the concentrations of free fatty acids and esterases catalyze the synthesis of esters that contribute to the secondary aroma of wines. Esterase and esterase-lipase were the most widespread activities among the studied strains. All the strains of *Cryptococcus* spp. genera and *W. pratensis*, *S. salmonicolor*, *P. kluyveri* and *M. pulcherrima* species showed esterase activity. Similarly, esterase-lipase was detected in all the strains of *Candida* spp., *Cryptococcus* spp., *W. pratensis*, *S. salmonicolor*, *P. kluyveri*, *M. pulcherrima* and *L. thermotolerans*. On the other hand, lipase activity was scarcely detected and only was found in some strains of *S. salmonicolor* and *L. thermotolerans*.

Table 2 shows enzymatic profiles found in each species/genera based on the enzymes involved in color and clarity of wine. The degradation of structural polysaccharides by enzymes such as pectinase, cellulase, xylanase, and glucanase, can result in greater extraction of substances that enhance color and improve clarification and filterability during winemaking. Regarding these enzymes, the activity most frequently detected among the strains was pectinase. Nevertheless, in many species there were profiles that did not show any of the carbohydrase activities. The proteolytic enzymes (leucine arylamidase, valine arylamidase, cystine arylamidase) increase the amino acid content, improving the production of aromatic compounds in the wine [55,56] and they are also involved in reducing protein instability in wines [57]. Within this group of aminopeptidases, *W. pratensis* showed the three activities studied in nine out of ten strains. As happened with carbohydrases, there were also some strains that did not show any of the aminopeptidase activities. Profile LC6 of *L. thermotolerans* stood out for being the only one in which all the activities of these groups of enzymes were detected (the four carbohydrases and the three aminopeptidases).

Table 1. Specific enzymatic profiles related to wine aroma.

Species/Profiles	N	Enzymatic Activities						
		Esterase	Esterase-Lipase	Lipase	LeucineA	ValineA	CystineA	β -Glucosidase
<i>Candida</i> spp. (C)								
CA 1	5	+	+	–	+	+	–	–
CA 2	3	+	+	–	+	–	–	–
CA 3	1	–	+	–	+	+	–	–
CA 4	1	–	+	–	+	+	–	+
CA 5	2	+	+	–	+	+	–	+
<i>Cryptococcus</i> spp. (CC)								
CCA 1	2	+	+	–	+	–	–	+
CCA 2	1	+	+	–	+	–	–	+
CCA 3	2	+	+	–	+	–	–	–
<i>Debaryomyces hansenii</i> (D)								
DA 1	1	–	–	–	–	–	–	–
DA 2	1	+	+	–	–	–	–	–
DA 3	1	+	+	–	–	–	–	–

Table 1. Cont.

Species/Profiles	N	Enzymatic Activities						
		Esterase	Esterase-Lipase	Lipase	LeucineA	ValineA	CystineA	β-Glucosidase
<i>Lachancea thermotolerans</i> (L)								
LA 1	11	+	+	−	+	+	−	−
LA 2	1	+	+	−	+	−	−	−
LA 3	1	−	+	−	+	+	−	−
LA 4	1	+	+	+	+	+	−	−
LA 5	1	+	+	+	+	+	−	+
LA 6	1	+	+	−	+	+	+	−
<i>Metschnikowia pulcherrima</i> (M)								
MA 1	5	+	+	−	+	−	−	−
MA 2	2	+	+	−	+	−	−	+
MA 3	1	+	+	−	−	+	−	−
MA 4	5	+	+	−	+	+	−	+
MA 5	2	+	+	−	+	−	−	+
MA 6	1	+	+	−	+	+	−	+
<i>Pichia kluyveri</i> (PK)								
PKA 1	4	+	+	−	+	−	−	−
<i>Sporodiobulus salmonicolor</i> (SP)								
SPA 1	4	+	+	−	+	+	−	−
SPA 2	2	+	+	+	+	+	−	−
<i>Torulaspora delbrueckii</i> (T)								
TA 1	1	+	+	−	−	−	−	−
TA 2	5	−	−	−	−	−	−	−
TA 3	8	+	+	−	+	−	−	−
TA 4	3	−	−	−	+	−	−	−
TA 5	1	+	+	−	+	−	+	−
<i>Williopsis pratensis</i> (W)								
WA 1	9	+	+	−	+	+	+	−
WA 2	1	+	+	−	+	+	−	−
<i>Zygosaccharomyces bailii</i> (Z)								
ZA 1	1	+	+	−	+	−	−	−
ZA 2	1	+	−	−	+	−	−	−
ZA 3	1	+	+	−	+	−	−	+
ZA 4	2	−	+	−	+	−	−	−
ZA 5	2	+	+	−	+	−	−	−

Presence (+) or absence (−) of each activity; N: number of strains in each enzymatic profile.

Table 2. Specific enzymatic profiles related to color and fining.

Species/Profiles	N	Enzymatic Activities						
		Pectinase	Cellulase	Xylanase	Glucanase	LeucineA	ValineA	CystineA
<i>Candida</i> spp. (C)								
CC 1	3	+	−	−	−	+	+	−
CC 2	3	+	−	−	−	+	−	−
CC 3	6	−	−	−	−	+	+	−
<i>Cryptococcus</i> spp. (CC)								
CCC 1	2	−	+	−	+	+	−	−
CCC 2	1	+	+	−	+	+	−	−
CCC 3	2	+	−	−	−	+	−	−
<i>Debaryomyces hansenii</i> (D)								
DC 1	1	+	−	−	+	−	−	−
DC 2	1	+	+	−	+	−	−	−
DC 3	1	+	−	−	−	−	−	−

Table 2. Cont.

Species/Profiles	N	Enzymatic Activities						
		Pectinase	Cellulase	Xylanase	Glucanase	LeucineA	ValineA	CystineA
<i>Lachancea thermotolerans</i> (L)								
LC 1	9	–	+	+	+	+	+	–
LC 2	2	–	+	–	+	+	+	–
LC 3	1	–	–	–	+	+	+	–
LC 4	2	–	–	–	–	+	+	–
LC 5	1	+	–	–	–	+	+	–
LC 6	1	+	+	+	+	+	+	+
<i>Metschnikowia pulcherrima</i> (M)								
MC 1	5	+	–	–	–	+	–	–
MC 2	2	+	+	–	–	–	–	–
MC 3	2	+	–	–	–	+	+	–
MC 4	1	+	+	–	–	+	–	–
MC 5	2	–	+	–	–	+	+	–
MC 6	1	–	–	–	+	+	+	–
<i>Pichia kluyveri</i> (PK)								
PKC 1	4	+	–	–	–	+	–	–
<i>Sporodiobolus salmonicolor</i> (SP)								
SPC 1	4	+	–	–	–	+	+	–
SPC 2	2	–	–	–	–	+	+	–
<i>Torulaspora delbrueckii</i> (T)								
TC 1	5	+	–	–	–	–	–	–
TC 2	1	–	–	–	–	–	–	–
TC 3	2	–	–	–	–	+	–	–
TC 4	2	+	+	+	–	+	–	–
TC 5	7	+	–	–	–	+	–	–
TC 6	1	+	–	–	–	+	–	+
<i>Williopsis pratensis</i> (W)								
WC 1	9	–	–	–	–	+	+	+
WC 2	1	–	–	–	–	+	–	–
<i>Zygosaccharomyces bailii</i> (Z)								
ZC 1	2	+	+	–	–	+	–	–
ZC 2	3	+	–	–	–	+	–	–
ZC 3	2	–	–	–	–	+	–	–

Presence (+) or absence (–) of each activity; N: number of strains in each enzymatic profile.

3.4. Fermentative Behavior and Aromatic Compound Production of Non-Saccharomyces Strains in Pure 100 mL Vinifications

Taking into account results obtained in the previous steps of the selection process, twenty-four strains belonging to eight different species (three strains of *Candida zeylanoides*, one strain of *Cryptococcus uzbekistanensis*, *Debaryomyces hansenii* and *Zygosaccharomyces bailii*, five strains of *Metschnikowia pulcherrima* and *Williopsis pratensis* and four strains of *Lachancea thermotolerans*, and *Torulaspora delbrueckii*) were selected to continue the study. Since *Pichia kluyveri* and *Sporodiobolus salmonicolor* strains were not able to grow in liquid medium, they were eliminated from the study.

The establishment of the inoculated yeasts was total in most cases, with the exception of musts inoculated with *W. pratensis*, two of the strains of *C. zeylanoides* and the strain of *Cr. uzbekistanensis*. As these yeasts were not able to develop in the must, they were eliminated from the selection process and data corresponding to them were not included in the results.

Analysis of fermentative features showed that *S. cerevisiae* was the only yeast capable of consuming all the sugar (Table 3). *C. zeylanoides* showed the highest levels of acetic acid and acetaldehyde among the yeasts studied, ahead of *Z. bailii*, which was the next species

with the highest values. *L. thermotolerans* and *Z. bailii* showed the ability to increase the acidity of the wine due to the production of lactic acid and to the production of malic acid, respectively. The highest levels of glycerol were also found in *Z. bailii*, followed by those detected in *L. thermotolerans* and *M. pulcherrima*.

Table 3. Enological traits of wines fermented by different non-*Saccharomyces* yeast species and statistical analysis [58].

Parameters	Yeast Species						
	<i>D. hansenii</i>	<i>C. zeylanoides</i>	<i>M. pulcherrima</i>	<i>T. delbrueckii</i>	<i>L. thermotolerans</i>	<i>Z. bailii</i>	<i>S. cerevisiae</i>
Residual sugars (g/L)	201.0 e	151.1 d	111.2 cd	47.2 b	75.6 bc	96.6 c	0.08 a
% sugar (72 h)	00.0 a	10.5 ab	15.5 b	24.5 bc	23.0 b	12.0 ab	39.5 c
Ethanol (% v/v)	0.11 a	2.99 b	5.31 c	9.83 e	8.22 de	6.33 cd	12.60 f
Glycerol (g/L)	0.55 a	3.10 b	4.80 de	4.05 c	4.65 d	5.60 f	5.20 ef
pH	3.54 b	3.45 b	3.46 b	3.47 b	3.26 a	3.25 a	3.43 b
Total acidity (g/L)	2.30 a	3.30 b	3.50 b	3.25 b	5.70 d	4.40 c	3.55 b
Volatile acidity (g/L)	0.12 ab	0.41 e	0.05 a	0.22 c	0.26 cd	0.34 de	0.20 bc
Acetaldehyde (mg/L)	0.00 a	45.0 b	11.5 a	7.50 a	17.5 a	21.5 ab	19.5 a
Malic acid (g/L)	0.90 bc	0.95 c	0.90 bc	0.71 a	0.74 ab	1.26 d	0.69 a
Lactic acid (g/L)	0.13 a	0.13 a	0.12 a	0.11 a	3.81 b	0.09 a	0.09 a

Different letters mean significant differences between species ($p \leq 0.05$).

The aromatic composition of wines was determined by analyzing a total of 30 aromatic compounds. Table 4 shows concentrations of the main groups of aromatic compounds, ethyl acetate, acetoin and diacetyl. Results showed a higher concentration of higher alcohols in *M. pulcherrima* and *S. cerevisiae*. *D. hansenii* and *C. zeylanoides*, both with low fermentation power, produced the lowest concentrations of higher alcohols. The presence of these compounds in concentrations below 350 mg/L contributes to the aromatic complexity of the wine, while levels above 400 mg/L can be negative, since they produce pungent odors [59]. *M. pulcherrima* showed the highest values of 2-phenylethanol and also reached the highest levels of 2-phenylethyl acetate (data not shown). This rose-scented compound contributes positively to the bouquet of wine [60]. Higher levels of ethyl acetate were observed in *M. pulcherrima* than in the rest of the species. *M. pulcherrima* also showed elevated levels of diacetyl and acetoin. Together with *M. pulcherrima*, *C. zeylanoides* was the only yeast that exceeded the limit value of 150 mg/L for acetoin, above which it can provide unpleasant buttery aromas [61]. Small differences were also detected in other aromatic compounds among the different species, but especially between *S. cerevisiae* and non-*Saccharomyces* yeasts (sum of acetates, esters and acids) [58].

Table 4. Concentrations of aromatic compounds (mg/L) in wines made with different non-*Saccharomyces* yeast species and statistical analysis [58].

Parameters	Yeast Species						
	<i>D. hansenii</i>	<i>C. zeylanoides</i>	<i>M. pulcherrima</i>	<i>T. delbrueckii</i>	<i>L. thermotolerans</i>	<i>Z. bailii</i>	<i>S. cerevisiae</i>
∑ higher alcohols	11.2 a	62.0 ab	272 e	178 cd	215 cde	130 bc	264 de
∑ acetates	0.00 a	0.05 a	0.21 a	0.09 a	0.20 a	0.19 a	1.10 b
∑ esters	0.01 a	0.03 a	0.30 bc	0.46 d	0.36 cd	0.20 b	0.71 e
∑ Acids	0.79 ab	0.96 ab	0.67 a	3.57 c	2.20 b	1.10 ab	9.10 d
Ethyl acetate	0.22 a	2.30 a	269 c	33.4 ab	51.7 b	31.1 ab	22.7 ab
Acetoin	69.7 ab	272 d	184 c	4.50 a	99.3 b	23.4 ab	5.20 a
Diacetyl	0.04 a	0.38 a	2.10 b	0.30 a	0.40 a	0.10 a	0.05 a

Different letters mean significant differences between species ($p \leq 0.05$).

In addition to a comparison between species, differences in analytical parameters of strains within the same species were studied (data not shown) [58]. Species in which different strains were included were *T. delbrueckii* (four strains), *L. thermotolerans* (four strains) and *M. pulcherrima* (five strains). Less significant differences were found among

strains within these species than those found among the different species. In *T. delbrueckii* significant differences between genotypes were detected only in four parameters: malic acid consumed, volatile acidity and concentration of 1-butanol and isovaleric acid. The four clones of *L. thermotolerans* were differentiated mainly by the acidity parameters (see Table S4 in Supplementary Materials). Lactic acid production, characteristic of *L. thermotolerans*, varied widely among the clones studied. One of the parameters that showed significant differences between clones in the three species studied was volatile acidity. In addition, in *M. pulcherrima* and *L. thermotolerans*, significant differences were observed in ethyl acetate and acetoin, which can also give rise to aromatic defects. These results underline the importance of strain selection for their use in winemaking.

3.5. Influence of Sequential Inoculations with Non-Saccharomyces/*S. cerevisiae* on Wine Aroma and Composition of Anthocyanins and Stilbenes in 2000 mL Vinifications

As it was expected, the previous assay showed that none of the non-Saccharomyces strains included in the study was able to complete fermentation. For that reason, in this stage of the selection process sequential fermentations with a commercial strain of *S. cerevisiae* were performed in order to ensure complete fermentation of the must.

After analyzing results obtained in the previous steps of the study, nine strains belonging to six different species [*T. delbrueckii* (T18 and T19), *M. pulcherrima* (M28 and M29), *L. thermotolerans* (L54 and L57), *Z. bailii* (Z71), *W. pratensis* (W87) and *C. zeylanoides* (C342)] and two mixed inocula of *L. thermotolerans* and *T. delbrueckii* in a 70/30 ratio (LT1 and LT2) were selected to evaluate their influence on wine quality in sequential inoculations with *S. cerevisiae*. Results were compared with those obtained in wines fermented only with *S. cerevisiae*. During the development of the study, it was observed that two of the *L. thermotolerans* strains (L54 and L57) developed in the must together with *T. delbrueckii*, both in a 30/70 ratio. Due to the interest that this combination of strains could have in winemaking, it was decided to include the mixed inocula in the following assays.

Control of the presence of the inoculated yeasts showed that on day four (24 h after inoculation of *S. cerevisiae*), *Z. bailii*, *W. pratensis*, *C. zeylanoides* and one strain of *M. pulcherrima* were not present in the medium. The rest of the yeasts (the other *M. pulcherrima* strain and the two *L. thermotolerans* strains) disappeared in the next two days and only the two *T. delbrueckii* clones remained in the medium until day six. Regarding the *L. thermotolerans*/*T. delbrueckii* mixed cultures, it was observed that they maintained percentages similar to initial proportions (30/70) until *S. cerevisiae* was inoculated (Figure 2). After that, population of the mixed inocula decreased until they disappeared in seven days.

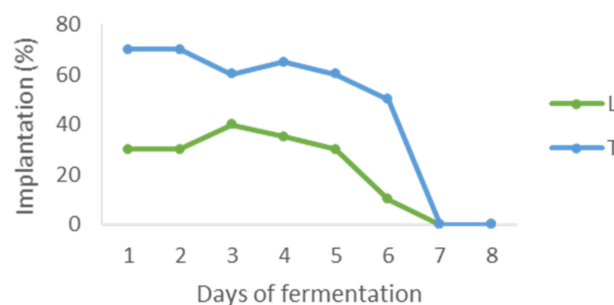


Figure 2. Presence of the yeasts of the mixed inoculum LT1 during the fermentation (L: *Lachancea thermotolerans*, T: *Torulaspora delbrueckii*).

Regarding analytical characteristics of wines after alcoholic fermentation (see Table S5 in Supplementary Materials), in general, wines corresponding to sequential inoculations with non-Saccharomyces yeasts showed an increase in glycerol and acetaldehyde levels, lower volatile acidity and a higher consumption of malic acid compared to wines fermented with *S. cerevisiae* alone.

Table 5 shows aromatic compounds that presented significant differences in wines sequentially fermented with different non-*Saccharomyces* yeasts and *S. cerevisiae*. The wines sequentially inoculated with *T. delbrueckii* and *L. thermotolerans* exhibited a significant increase in the concentration of higher alcohols compared to those fermented only with *S. cerevisiae*. Levels of isobutanol were higher in wines made with *L. thermotolerans* and strains T18 and M28 of *T. delbrueckii* and *M. pulcherrima* than in wines fermented only with *S. cerevisiae*. Wines inoculated with *T. delbrueckii* showed higher concentrations of 2-phenylethanol, 1-butanol and methionol ((3-methylthio)propionol) than the wines fermented only with *S. cerevisiae*. In *M. pulcherrima* samples, high levels of methionol were also found and *T. delbrueckii* and *L. thermotolerans* showed concentrations of 1-propanol higher than those detected in *S. cerevisiae*. No significant differences were found in ester and acid content in the final wines (data not shown). Regarding acetates, wines made with *W. pratensis* showed the highest levels, but the differences were only significant with respect to strain L54 of *L. thermotolerans*. The highest levels of diacetyl were detected in *T. delbrueckii*, except for one of the strains of *L. thermotolerans*, which showed the highest value of this compound. Diacetyl levels remained below this threshold value in all samples. In wines inoculated with *L. thermotolerans* the levels of acetoin and ethyl lactate were the highest. Acetoin is a compound with a relatively high detection value (150 mg/L) [61] that was not exceeded in any of the samples.

With regard to total anthocyanins concentration, the wines inoculated only with *S. cerevisiae* reached a total anthocyanin content of 221 mg/L. The wines obtained by sequential inoculation with *W. pratensis*, *L. thermotolerans*, and the mixed inocula LT did not show significant differences in anthocyanin content with respect to *S. cerevisiae*. On the other hand, in wines inoculated with *M. pulcherrima*, *T. delbrueckii*, *Z. bailii* and *C. zeylanoides*, a significant increase in anthocyanin content was observed compared to wines fermented with *S. cerevisiae* alone (Figure 3).

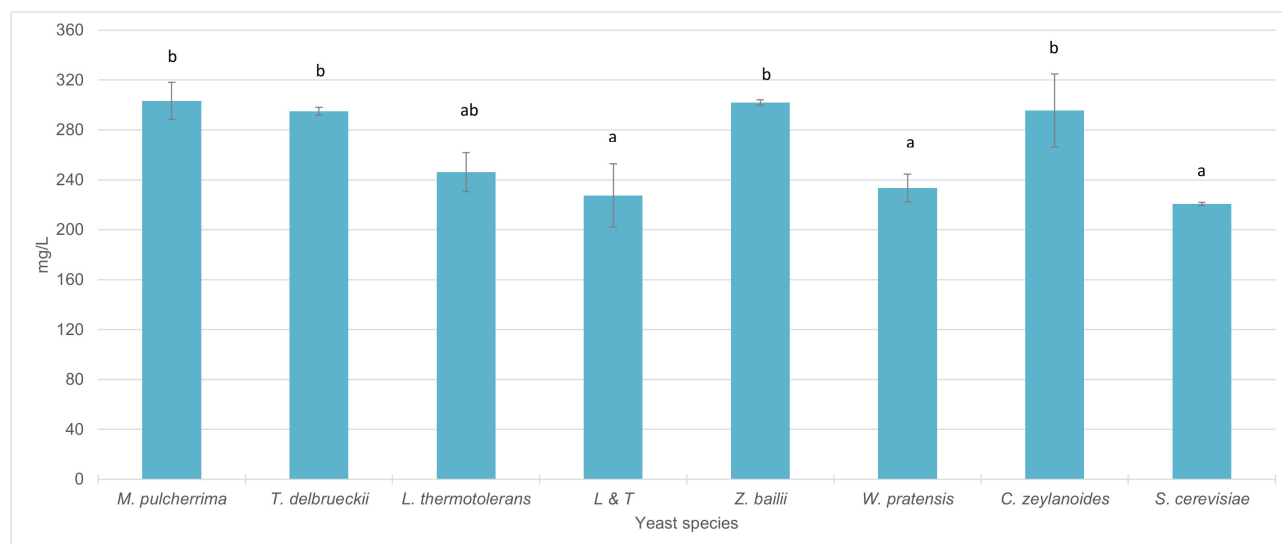


Figure 3. Total anthocyanin content (mg/L) in wines sequentially fermented with different yeast species. (Average of the different strains in each species included in the study). Different letters mean significant differences between the samples ($p \leq 0.05$).

Figure 4 presents the total stilbene content in wines inoculated with the different yeasts included in this part of the study. Vinifications with *S. cerevisiae* exhibited the lowest level of total stilbenes. In wines inoculated with *C. zeylanoides*, *T. delbrueckii* and *Z. bailii*, the increase in total content of stilbenes was significant with respect to vinifications with *S. cerevisiae*. In the rest of the genera, significant differences were not observed respect to *S. cerevisiae*.

Table 5. Aromatic compounds (mg/L) with significant differences in wines sequentially fermented with different non-*Saccharomyces* yeasts and *S. cerevisiae* at the end of alcoholic fermentation [62].

Parameters	Yeast Strains											
	T18	T19	M28	M29	L54	L57	LT1	LT2	Z71	W87	C342	SC
1 Propanol	40.3 cde	37.7 bcd	32.3 abc	30.7 ab	48.6 e	44.5 de	34.7 abc	41.4 cde	32.3 abc	30.0 ab	26.7 a	28.1 a
1 butanol	1.06 bc	1.26 c	1.03 abc	0.92 abc	1.01 abc	1.00 abc	0.99 abc	1.04 abc	0.81 ab	0.87 ab	0.96 abc	0.69 a
Isobutanol	50.6 bc	45.1 abc	53.1 bc	47.8 abc	59.7 c	59.8 c	43.3 abc	44.5 abc	38.0 ab	36.6 ab	37.0 ab	31.5 a
2 phenyl ethanol	98.9 c	81.2 bc	65.9 ab	64.1 ab	54.8 a	63.0 ab	54.1 a	62.1 ab	52.9 a	48.2 a	53.6 a	47.8 a
1 hexanol	1.86 ab	1.78 ab	1.86 ab	1.57 ab	2.11 b	1.93 ab	1.86 ab	1.97 ab	1.83 ab	1.56 ab	1.45 a	1.82 ab
Benzyl alcohol	0.28 b	0.27 ab	0.23 ab	0.26 ab	0.26 ab	0.23 ab	0.22 ab	0.18 a	0.19 ab	0.20 ab	0.24 ab	0.20 ab
Methionol	4.44 e	3.49 cde	4.03 de	3.40 bcde	1.57 a	2.49 abcd	2.30 abcd	2.61 abcd	2.21 abc	1.75 abc	1.90 abc	1.72 ab
∑ higher alcohols	197 d	171 bcd	158 abcd	149 abcd	169 bcd	173 cd	138 abc	154 abcd	128 abc	119 ab	122 abc	112 a
Isoamyl acetate	2.24 ab	1.56 ab	2.21 ab	1.79 ab	1.36 a	2.08 ab	2.14 ab	2.65 ab	2.55 ab	3.27 b	2.50 ab	2.13 ab
2 phenylethyl acetate	0.30 bc	0.19 abc	0.17 ab	0.16 abc	0.14 a	0.22 abc	0.19 abc	0.25 abc	0.24 abc	0.33 c	0.19 abc	0.13 a
∑ acetates	2.64 ab	1.75 ab	2.38 ab	2.95 ab	1.50 a	2.30 ab	2.33 ab	2.90 ab	2.80 ab	3.60 b	2.69 ab	2.26 ab
Ethyl propionate	0.31 d	0.26 d	0.11 ab	0.10 ab	0.11 ab	0.14 bc	0.16 bc	0.18 c	0.12 abc	0.11 ab	0.10 ab	0.07 a
Octanoic acid	1.47 abc	1.28 ab	2.04 abc	1.72 abc	0.88 a	1.37 abc	1.76 abc	1.75 abc	2.16 bc	2.55 bc	2.39 bc	2.57 c
Ethyl lactate	1.65 ab	1.46 ab	2.65 bcd	2.50 bc	16.5 g	10.6 f	4.25 e	3.88 cde	2.83 bcde	3.24 cde	4.11 de	0.48 a
Acetoin	9.84 cd	11.3 d	2.66 a	2.83 a	17.0 e	15.6 e	8.75 cd	7.55 bc	4.72 ab	3.93 a	4.30 ab	2.37 a
Diacetyl	2.46 e	2.33 de	1.37 abc	1.12 abc	3.46 f	1.68 cd	1.45 bc	1.11 abc	0.88 ab	0.83 ab	0.97 ab	0.69 a
Butyrolactone	1.78 c	1.57 abc	1.36 abc	1.18 a	1.62 abc	1.67 abc	1.58 abc	1.75 bc	1.52 abc	1.47 abc	1.40 abc	1.20 ab

Different letters mean significant differences between species ($p \leq 0.05$).

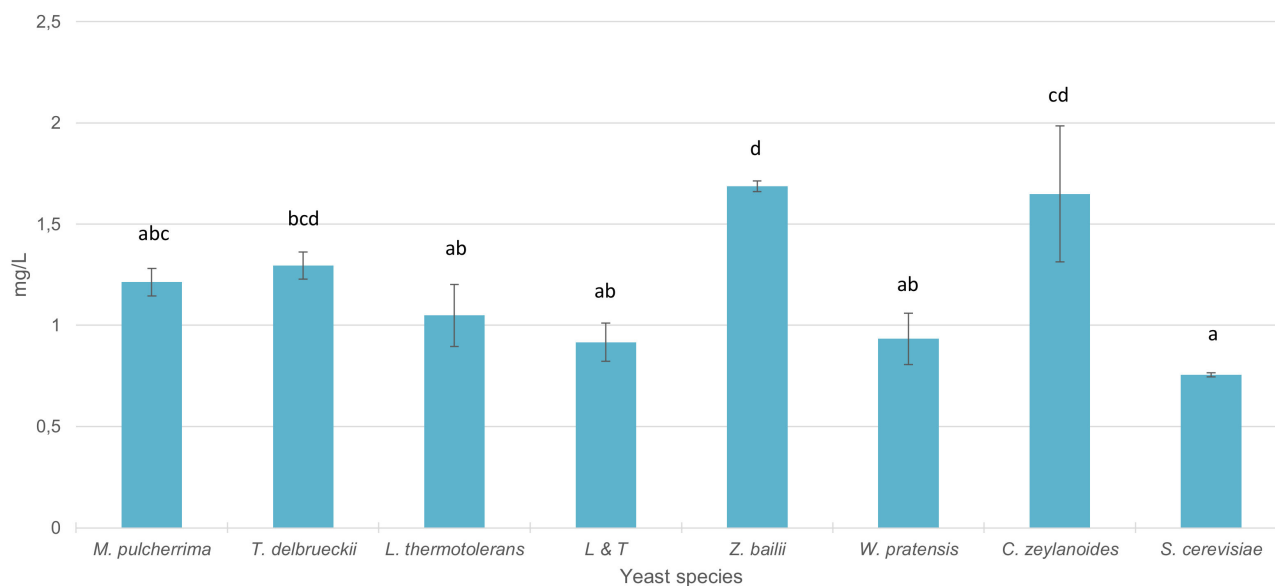


Figure 4. Total stilbene content (mg/L) in wines sequentially fermented with different yeast species. Different letters mean significant differences between the samples ($p \leq 0.05$).

4. Discussion

4.1. Clonal Characterization and SO₂ Resistance

A wide clonal diversity was found among the non-*Saccharomyces* isolates included in this work. As revealed in various studies, non-*Saccharomyces* yeasts show high intraspecific diversity, which involves the production of different enzymes and compounds during vinification, as well as different enological behavior [28,33,63,64]. This diversity was also reflected in the results obtained in SO₂ resistance, which showed great differences between strains belonging to the same species. Growth respect to a control without SO₂ was evaluated after 24 and 48 h. Nevertheless, growth in the presence of SO₂ at 24 h was taken into account as a selection criterion. Given the great competition between microorganisms that occurs in industrial vinifications, the ability to grow rapidly in the presence of SO₂ represents an advantage for the yeast to establish itself in the medium. Results obtained at this stage confirm the importance of clonal characterization in the selection of yeasts for their use as inoculums in winemaking. For the following selection steps, representative clones were chosen and extremely sulfur sensitive strains were eliminated.

4.2. Screening of Enzymatic Activities

Among the enzymatic activities related to aroma, esterase and esterase-lipase were the most widespread activities and were found in strains of all the studied species. These activities are involved in the formation of esters, compounds that provide fruity aromas to wine. Results are in agreement with those obtained in the study carried out by Comitini et al. [7], in which the presence of esterase activity was observed in a large number of strains of different non-*Saccharomyces* yeast genera. On the other hand, in the present study, lipase activity was scarcely detected and only 33% of the *S. salmonicolor* strains and 12% of *L. thermotolerans* showed this activity. These enzymes cause the degradation of lipids, releasing fatty acids. Another activity that allowed the differentiation of the enzymatic profiles was β -glucosidase. This activity was more widespread in strains of *Cryptococcus* spp. and *M. pulcherrima*. Fernández et al. [65] indicated that β -glucosidase activity was mainly related to the species *M. pulcherrima*. Mendes Ferreira et al. [66] showed the potential of this species for the release of terpenes due to its high β -glucosidase activity, which coincides with the results obtained in the present work.

Enzymatic profiles related to aroma shown in Table 1 within each species were mainly differentiated by esterase, esterase-lipase and β -glucosidase activities. For that reason, the strains that would be chosen based on their aromatic potential would be the ones included in the profiles that had all three enzymatic activities: CA5 of *Candida* spp., CCA1 of *Cryptococcus* spp., LA5 of *L. thermotolerans*, MA4 of *M. pulcherrima* and ZA3 of *Z. bailii*; and the ones included in the profiles with two of the activities: DA2 of *D. hansenii*, PKA1 of *P. kluyveri*, SPA2 of *S. salmonicolor*, TA5 of *T. delbrueckii*, WA1 of *W. pratensis*, and ZA5 of *Z. bailii*.

Concerning enzymatic activities related to color and fining, pectinase was the activity most detected within the group of carbohydrases. In their study, Maturano et al. [67] found higher pectinase activity in *T. delbrueckii* compared to *S. cerevisiae*. Activities of the group of carbohydrases favor the degradation of grape structural polysaccharides improving must extraction, clarification, wine filterability, and extraction of substances responsible for color and aroma during maceration [28]. The proteolytic enzymes aminopeptidases are involved in the reduction of proteic instability in wine [57]. Profiles that had more enzymatic activities related with color and limpidity of wine were: CC1 in *Candida* spp., CCC2 in *Cryptococcus* spp., DC2 in *D. hansenii*, LC6 in *L. thermotolerans*, MC4 and MC6 in *M. pulcherrima*, PKC1 in *P. kluyveri*, SPC1 in *S. salmonicolor*, TC4 and TC6 in *T. delbrueckii*, WC1 in *W. pratensis* and ZC1 in *Z. bailii*. Profile LC6 of *L. thermotolerans* showed all the carbohydrase and aminopeptidase activities studied, which means great potential to improve wine characteristics.

All the strains previously indicated passed to the next selection stage due to their potential to improve the aroma, color, clarity and stability of red wines. Once the strains with the potential capacity to improve the characteristics of the wines had been chosen, their fermentative behavior under different conditions was studied.

4.3. Fermentative Behavior and Aromatic Compound Production of Non-Saccharomyces Strains in Pure 100 mL Vinifications

As expected, an increase in acidity levels was observed in wines inoculated with *L. thermotolerans* and *Z. bailii*. Other authors have described the production of lactic acid by *L. thermotolerans* [20], giving rise to pH drops and increases in total acidity values. Due to this feature, characteristic of *L. thermotolerans*, the use of this yeast in vinifications has been proposed as a tool to adjust the acidity of wines [20,68]. Regarding the increase in acidity levels in *Z. bailii* samples, it was related to the production of malic acid by this yeast. The production of this compound has been described by other authors in *Z. rouxii* [69]. The highest levels of glycerol were found in *Z. bailii*, followed by those detected in *L. thermotolerans* and *M. pulcherrima*, although differences were not significant respect to *S. cerevisiae*. This compound, which provides softness, sweetness and complexity to the wine, has been detected in higher concentrations in vinifications with non-Saccharomyces yeasts compared to *S. cerevisiae* [64]. Other authors have found a high production of glycerol in *L. thermotolerans* and *C. zemplinina* [70,71].

Analysis of aromatic composition showed interesting results in *M. pulcherrima*. This yeast produced high levels of higher alcohols and contributed positively to the aroma, since the concentration remained below 400 mg/L, above which these compounds can cause organoleptic deviations. *M. pulcherrima* also showed the highest concentrations of 2-phenylethanol and 2-phenyl acetate (data not shown). High production of 2-phenylethanol was found in *M. pulcherrima* in studies carried out by other authors [72–74]. Regarding production of esters, Rodríguez et al. [75] observed high concentrations of these compounds in *M. pulcherrima*. On the other hand, *M. pulcherrima* showed the highest levels of ethyl acetate, a compound that can give rise to off odors. These results are in agreement with those obtained in previous studies [11]. Besides, in wines inoculated with *M. pulcherrima* acetoin concentration exceeded the threshold above which unpleasant buttery aromas may appear.

The study of differences within clones of *M. pulcherrima*, *T. delbrueckii* and *L. thermotolerans* revealed variations in some important parameters such as volatile acidity, which can

cause aromatic deviations in wine. In *L. thermotolerans*, clones were differentiated mainly by acidity parameters. Lactic acid production, a trait of great interest in this species, varied widely among the clones studied. Different studies have pointed out the importance of intraspecific heterogeneity in non-*Saccharomyces* yeasts [32,76,77].

4.4. Influence of Sequential Inoculations with Non-*Saccharomyces*/*S. cerevisiae* on Wine Aroma and Composition of Anthocyanins and Stilbenes in 2000 mL Vinifications

The previous phases in the selection program showed that none of the non-*Saccharomyces* strains was able to complete fermentation. For that reason, sequential fermentations of the non-*Saccharomyces* strains with *S. cerevisiae* were performed in order to validate the behavior of these non-*Saccharomyces* in the conditions in which they will be used.

The enological parameters studied showed higher glycerol levels in wines sequentially inoculated with *M. pulcherrima*, *T. delbrueckii* and the two mixed inocula compared to those inoculated only with *S. cerevisiae* (data not shown). Previous studies have shown the ability of different non-*Saccharomyces* yeasts to produce high concentrations of glycerol [64,70,71].

Aromatic compound analysis revealed a differentiation of samples inoculated with *T. delbrueckii* and *L. thermotolerans*. Wines sequentially inoculated with these yeasts showed an increase in the concentration of higher alcohols compared to those fermented only with *S. cerevisiae*. As occurred in the previous stage, the levels remained below the concentration limit that can cause aromatic deviations. In *T. delbrueckii* and one of the *L. thermotolerans* strains (L54) the highest levels of diacetyl were detected. This compound can give rise to unpleasant buttery aromas in wine that are detected when the concentration reaches the threshold value of 8 mg/L [61]. Diacetyl levels remained below this threshold value in all samples. On the other hand, acetoin showed the highest values in *L. thermotolerans*, but they also remained below the threshold value. In wines sequentially inoculated with *L. thermotolerans* concentrations of ethyl lactate were significantly higher than those found in wines inoculated only with *S. cerevisiae* and inoculated with other non-*Saccharomyces* yeasts. In their study, Del Fresno et al. [78] only detected ethyl lactate in fermentations with *L. thermotolerans*, which is explained by the ability of this yeast to produce lactic acid and the dependence of ethyl lactate formation on the levels of lactic acid present in the medium. As can be observed, *T. delbrueckii* and *L. thermotolerans*, the two species that remained the longest in the tanks, showed the most notable differences in the aromatic profile. These results highlight the importance of the implantation ability of the selected inoculum to achieve the modulation of the characteristics of the wine.

Regarding parameters related with wine color, total anthocyanin content was significantly higher than that observed for wines inoculated only with *S. cerevisiae* in *M. pulcherrima*, *T. delbrueckii*, *Z. bailii* and *C. zeylanoides*. In their work, Chen et al. [79] observed higher concentration of total anthocyanins in sequential fermentations with *M. pulcherrima* and *T. delbrueckii* compared to fermentations carried out only with *S. cerevisiae*. On the other hand, Belda et al. [80] noticed increases in color intensity and total polyphenol index (TPI) in mixed fermentations with *M. pulcherrima* compared to fermentations only with *S. cerevisiae*.

Total stilbene content showed higher values in vinifications sequentially inoculated with non-*Saccharomyces* yeasts respect to those found in vinifications inoculated only with *S. cerevisiae*, and differences were significant in *C. zeylanoides*, *Z. bailii* and *T. delbrueckii*. Several studies have shown the influence that different yeast strains can have on stilbene levels in wine [81,82]. The presence of enzymatic activities such as β -glucosidase can influence the resveratrol content. Gaensly et al. [83] observed that yeast strains with this enzymatic activity favored the hydrolysis of the transpiceid into *trans*-resveratrol.

4.5. Selection of the Inoculum for Red Winemaking

The review of the characteristics observed during the selection program in *M. pulcherrima* and the mixed inoculum *L. thermotolerans*/*T. delbrueckii* was conducted in order to choose the most suitable option for its use in winemaking. The high implantation and the ability to remain in the tanks for a longer time of the mixed inoculum *L. thermotolerans*/*T. delbrueckii* were important features to be taken into account in an inoculum intended for

its use in industrial vinifications. On the other hand, the release by the two species of the mixed inoculum of compounds that improve the quality of the wine, such as glycerol and lactic acid, was another important criterion in the selection. The previous results, coupled to the fact that there is no inoculum on the market composed of these two non-*Saccharomyces* species together, led to the decision to select the mixed inoculum composed of *T. delbrueckii* and *L. thermotolerans* (70/30) for its use in winemaking in order to improve the characteristics of red wines.

5. Conclusions

This study shows the different steps carried out for the selection of a non-*Saccharomyces* yeast inoculum from the Rioja winegrowing region. Some advantages over fermentations performed only with *S. cerevisiae* have been proven, which demonstrates the potential of non-*Saccharomyces* yeasts for use as inocula in winemaking. Differences in important parameters such as SO₂ resistance, enzymatic activities or production of compounds have been observed between strains, highlighting the relevance of clonal screening in selection processes. On the other hand, during the development of the selection program it is important to consider the type of wine yeasts are destined to produce and the specific characteristics that want to be modulated in order to choose the most suitable inoculum. Taking into account all the mentioned points, the mixed inoculum of *L. thermotolerans*/*T. delbrueckii* was selected in the final stage for its use in winemaking. Given its characteristics, such as the high implantation capacity, the production of compounds of interest and the modulation of wine acidity, this inoculum is suitable for the production of quality wines.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/fermentation7030148/s1>, Table S1: Yeast isolates of each species and place of isolation, Table S2: Percentage (%) of strains growing at the same level as control at 48 h after inoculation, with different dosages of SO₂, Table S3: Maximum level of SO₂ which allowed a similar growth to control at 24 h and enzymatic activities of the twenty-four strains selected for the vinification trials, Table S4: Average oenological traits of wines (with significant differences) fermented by different *Lachancea thermotolerans* (Lt) strains and statistical analysis, Table S5: Analytical parameters (mg/L) with significant differences produced by different non-*Saccharomyces* yeasts at the end of alcoholic fermentation.

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References

1. Varela, C.; Siebert, T.; Cozzolino, D.; Rose, L.; McLean, H.; Henschke, P.A. Discovering a chemical basis for differentiating wines made by fermentation with “wild” indigenous and inoculated yeasts: Role of yeast volatile compounds. *Aust. J. Grape Wine Res.* **2009**, *15*, 238–248. [CrossRef]
2. Romano, P.; Capece, A.; Jespersen, L. *Taxonomic and Ecological Diversity of Food and Beverage Yeasts*; Springer: Berlin/Heidelberg, Germany, 2006; ISBN 4535283230.
3. Rainieri, S.; Pretorius, I.S. Selection and improvement of wine yeasts. *Ann. Microbiol.* **2000**, *50*, 15–31.
4. Romano, P.; Fiore, C.; Paraggio, M.; Caruso, M.; Capece, A. Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* **2003**, *86*, 169–180. [CrossRef]

5. Jolly, N.P.; Augustyn, O.P.H.; Pretorius, I.S. The Role and Use of Non-*Saccharomyces* Yeasts in Wine Production. *S. Afr. J. Enol. Vitic.* **2006**, *27*, 15–39. [[CrossRef](#)]
6. Rojas, V.; Gil, J.V.; Piñaga, F.; Manzanares, P. Acetate ester formation in wine by mixed cultures in laboratory fermentations. *Int. J. Food Microbiol.* **2003**, *86*, 181–188. [[CrossRef](#)]
7. Comitini, F.; Gobbi, M.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* **2011**, *28*, 873–882. [[CrossRef](#)]
8. Benito, S.; Hofmann, T.; Laier, M.; Lochbühler, B.; Schüttler, A.; Ebert, K.; Fritsch, S.; Röcker, J.; Rauhut, D. Effect on quality and composition of Riesling wines fermented by sequential inoculation with non-*Saccharomyces* and *Saccharomyces Cerevisiae*. *Eur. Food Res. Technol.* **2015**, *241*, 707–717. [[CrossRef](#)]
9. Ciani, M.; Comitini, F.; Mannazzu, I.; Domizio, P. Controlled mixed culture fermentation: A new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res.* **2010**, *10*, 123–133. [[CrossRef](#)]
10. Lambrechts, M.G.; Pretorius, I.S. Yeast and its Importance to Wine Aroma—A Review. *S. Afr. J. Enol. Vitic.* **2000**, *21*, 97–129. [[CrossRef](#)]
11. Liu, J.; Arneborg, N.; Toldam-Andersen, T.B.; Zhang, S.; Petersen, M.A.; Bredie, W.L.P. Impact of sequential co-culture fermentations on flavour characters of Solaris wines. *Eur. Food Res. Technol.* **2017**, *243*, 437–445. [[CrossRef](#)]
12. Loira, I.; Morata, A.; Comuzzo, P.; Callejo, M.J.; González, C.; Calderón, F.; Suárez-Lepe, J.A. Use of *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* strains in mixed and sequential fermentations to improve red wine sensory quality. *Food Res. Int.* **2015**, *76*, 325–333. [[CrossRef](#)] [[PubMed](#)]
13. Medina, K.; Boido, E.; Fariña, L.; Gioia, O.; Gomez, M.E.; Barquet, M.; Gaggero, C.; Dellacassa, E.; Carrau, F. Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chem.* **2013**, *141*, 2513–2521. [[CrossRef](#)]
14. Bely, M.; Stoeckle, P.; Masneuf-Pomarède, I.; Dubourdieu, D. Impact of mixed *Torulaspora delbrueckii*-*Saccharomyces cerevisiae* culture on high-sugar fermentation. *Int. J. Food Microbiol.* **2008**, *122*, 312–320. [[CrossRef](#)] [[PubMed](#)]
15. Belda, I.; Navascués, E.; Marquina, D.; Santos, A.; Calderon, F.; Benito, S. Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 1911–1922. [[CrossRef](#)]
16. Renault, P.; Coulon, J.; de Revel, G.; Barbe, J.C.; Bely, M. Increase of fruity aroma during mixed *T. delbrueckii*/*S. cerevisiae* wine fermentation is linked to specific esters enhancement. *Int. J. Food Microbiol.* **2015**, *207*, 40–48. [[CrossRef](#)]
17. Azzolini, M.; Tosi, E.; Lorenzini, M.; Finato, F.; Zapparoli, G. Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with *Saccharomyces cerevisiae*. *World J. Microbiol. Biotechnol.* **2015**, *31*, 277–293. [[CrossRef](#)] [[PubMed](#)]
18. Belda, I.; Ruiz, J.; Beisert, B.; Navascués, E.; Marquina, D.; Calderón, F.; Rauhut, D.; Benito, S.; Santos, A. Influence of *Torulaspora delbrueckii* in varietal thiol (3-SH and 4-MSP) release in wine sequential fermentations. *Int. J. Food Microbiol.* **2017**, *257*, 183–191. [[CrossRef](#)]
19. Kapsopoulou, K.; Mourtzini, A.; Anthoulas, M.; Nerantzis, E. Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. *World J. Microbiol. Biotechnol.* **2007**, *23*, 735–739. [[CrossRef](#)]
20. Gobbi, M.; Comitini, F.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.* **2013**, *33*, 271–281. [[CrossRef](#)]
21. Vilela, A. *Lachancea thermotolerans*, the Non-*Saccharomyces* yeast that reduces the volatile acidity of wines. *Fermentation* **2018**, *4*, 56. [[CrossRef](#)]
22. Hranilovic, A.; Gambetta, J.M.; Schmidtke, L.; Boss, P.K.; Grbin, P.R.; Masneuf-Pomarede, I.; Bely, M.; Albertin, W.; Jiranek, V. Oenological traits of *Lachancea thermotolerans* show signs of domestication and allopatric differentiation. *Sci. Rep.* **2018**, *8*, 1–13. [[CrossRef](#)]
23. Ciani, M.; Beco, L.; Comitini, F. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *Int. J. Food Microbiol.* **2006**, *108*, 239–245. [[CrossRef](#)]
24. Divol, B.; Du Toit, M.; Duckitt, E. Surviving in the presence of sulphur dioxide: Strategies developed by wine yeasts. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 601–613. [[CrossRef](#)] [[PubMed](#)]
25. Krieger, S. Application of yeast and bacteria as starter cultures. In *Biology of Microorganisms on Grapes, in Must and in Wine*; König, H., Uden, G.F., Eds.; Springer: Berlin/Heidelberg, Germany, 2017; pp. 605–634.
26. Fernández-González, M.; Di Stefano, R.; Briones, A. Hydrolysis and transformation of terpene glycosides from muscat must by different yeast species terpene glycosides from muscat. *Food Microbiol.* **2003**, *20*, 35–41. [[CrossRef](#)]
27. Zoecklein, B.W.; Marcy, J.E.; Williams, J.M.; Jasinski, Y. Effect of native yeasts and selected strains of *Saccharomyces cerevisiae* on glycosyl glucose, potential volatile terpenes, and selected aglycones of White Riesling (*Vitis vinifera* L.) wines. *J. Food Compos. Anal.* **1997**, *10*, 55–65. [[CrossRef](#)]
28. Strauss, M.L.A.; Jolly, N.P.; Lambrechts, M.G.; Van Rensburg, P. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. *J. Appl. Microbiol.* **2001**, *91*, 182–190. [[CrossRef](#)]

29. Sumbly, K.M.; Grbin, P.R.; Jiranek, V. Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. *Food Chem.* **2010**, *121*, 1–16. [[CrossRef](#)]
30. Nieuwoudt, H.H.; Prior, B.A.; Pretorius, L.S.; Bauer, F.F. Glycerol in South African Table Wines: An Assessment of its Relationship to Wine Quality. *S. Afr. J. Enol. Vitic.* **2017**, *23*, 22–30. [[CrossRef](#)]
31. Aranda, A.; Matallana, E.; del Olmo, M. Levaduras. *Saccharomyces* I. Levaduras de primera fermentación. In *Microbiología del Vino*; Carrascosa, A.V., Muñoz, R., González, R., Eds.; AMV Ediciones: Madrid, Spain, 2005; pp. 273–297.
32. Renault, P.; Miot-Sertier, C.; Marullo, P.; Hernández-Orte, P.; Lagarrigue, L.; Lonvaud-Funel, A.; Bely, M. Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: Potential applications in the wine industry. *Int. J. Food Microbiol.* **2009**, *134*, 201–210. [[CrossRef](#)] [[PubMed](#)]
33. Zott, K.; Thibon, C.; Bely, M.; Lonvaud-Funel, A.; Dubourdiou, D.; Masneuf-Pomarede, I. The grape must non-*Saccharomyces* microbial community: Impact on volatile thiol release. *Int. J. Food Microbiol.* **2011**, *151*, 210–215. [[CrossRef](#)] [[PubMed](#)]
34. Schvarczová, E.; Štefániková, J.; Jankura, E.; Kolek, E. Selection of autochthonous *Saccharomyces cerevisiae* strains for production of typical Pinot Gris wines. *J. Food Nutr. Res.* **2017**, *56*, 389–397.
35. Querol, A.; Huerta, T.; Barrio, E.; Ramón, D. Dry Yeast Strain For Use in Fermentation of Alicante Wines: Selection and DNA Patterns. *J. Food Sci.* **1992**, *57*, 183–185. [[CrossRef](#)]
36. Rodríguez, M.E.; Infante, J.J.; Molina, M.; Domínguez, M.; Rebordinos, L.; Cantoral, J.M. Genomic characterization and selection of wine yeast to conduct industrial fermentations of a white wine produced in a SW Spain winery. *J. Appl. Microbiol.* **2010**, *108*, 1292–1302. [[CrossRef](#)]
37. Bae, S.; Fleet, G.H.; Heard, G.M. Lactic acid bacteria associated with wine grapes from several Australian vineyards. *J. Appl. Microbiol.* **2006**, *100*, 712–727. [[CrossRef](#)]
38. Lopes, C.A.; Rodríguez, M.E.; Sangorrín, M.; Querol, A.; Caballero, A.C. Patagonian wines: The selection of an indigenous yeast starter. *J. Ind. Microbiol. Biotechnol.* **2007**, *34*, 539–546. [[CrossRef](#)] [[PubMed](#)]
39. Tristezza, M.; Fantastico, L.; Vetrano, C.; Bleve, G.; Corallo, D.; Mita, G.; Grieco, F. Molecular and technological characterization of *Saccharomyces cerevisiae* strains isolated from natural fermentation of susumaniello grape must in Apulia, Southern Italy. *Int. J. Microbiol.* **2014**, *2014*, 897428. [[CrossRef](#)] [[PubMed](#)]
40. Ocón, E.; Garijo, P.; Sanz, S.; Olarte, C.; López, R.; Santamaría, P.; Gutiérrez, A.R. Screening of yeast mycoflora in winery air samples and their risk of wine contamination. *Food Control* **2013**, *34*, 261–267. [[CrossRef](#)]
41. Ocón, E.; Gutiérrez, A.R.; Garijo, P.; Tenorio, C.; López, I.; López, R.; Santamaría, P. Quantitative and qualitative analysis of non-*Saccharomyces* yeasts in spontaneous alcoholic fermentations. *Eur. Food Res. Technol.* **2010**, *230*, 885–891. [[CrossRef](#)]
42. Ocón, E.; Gutiérrez, A.R.; Garijo, P.; López, R.; Santamaría, P. Presence of non-*Saccharomyces* yeasts in cellar equipment and grape juice during harvest time. *Food Microbiol.* **2010**, *27*, 1023–1027. [[CrossRef](#)]
43. González-Arenzana, L.; Garijo, P.; Berlanas, C.; López-Alfaro, I.; López, R.; Santamaría, P.; Gutiérrez, A.R. Genetic and phenotypic intraspecific variability of non-*Saccharomyces* yeasts populations from La Rioja winegrowing region (Spain). *J. Appl. Microbiol.* **2017**, *122*, 378–388. [[CrossRef](#)]
44. Escribano, R.; González-Arenzana, L.; Garijo, P.; Berlanas, C.; López-Alfaro, I.; López, R.; Gutiérrez, A.R.; Santamaría, P. Screening of enzymatic activities within different enological non-*Saccharomyces* yeasts. *J. Food Sci. Technol.* **2017**, *54*, 1555–1564. [[CrossRef](#)]
45. Sampaio, T.L.; Kennedy, J.A.; Vasconcelos, M.C. Use of microscale fermentations in grape and wine research. *Am. J. Enol. Vitic.* **2007**, *58*, 534–539.
46. López, I.; Torres, C.; Ruiz-Larrea, F. Genetic typification by pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) of wild *Lactobacillus plantarum* and *Oenococcus oeni* wine strains. *Eur. Food Res. Technol.* **2008**, *227*, 547–555. [[CrossRef](#)]
47. Kurtzman, C.; Fell, J.W. *The Yeasts: A Taxonomic Study*, 4th ed.; Elsevier Science: Amsterdam, The Netherlands, 1998.
48. Commission Regulation. *Laying Down Certain Detailed Rules for Implementing Council Regulation (EC) No 479/2008 as Regards the Categories of Grapevine Products, Oenological Practices and the Applicable Restrictions*; (EC) No 606/2009 of 10 July 2009; European Commission: Brussels, Belgium, 2009; pp. 1–59.
49. Somers, T.C.; Evans, M.E. Wine quality: Correlations with colour density and anthocyanin equilibria in a group of young red wines. *J. Sci. Food Agric.* **1974**, *25*, 1369–1379. [[CrossRef](#)]
50. Ortega, C.; López, R.; Cacho, J.; Ferreira, V. Fast analysis of important wine volatile compounds. *J. Chromatogr. A* **2001**, *923*, 205–214. [[CrossRef](#)]
51. López, R.; López-Alfaro, I.; Gutiérrez, A.R.; Tenorio, C.; Garijo, P.; González-Arenzana, L.; Santamaría, P. Malolactic fermentation of Tempranillo wine: Contribution of the lactic acid bacteria inoculation to sensory quality and chemical composition. *Int. J. Food Sci. Technol.* **2011**, *46*, 2373–2381. [[CrossRef](#)]
52. Portu, J.; López-Alfaro, I.; Gómez-Alonso, S.; López, R.; Garde-Cerdán, T. Changes on grape phenolic composition induced by grapevine foliar applications of phenylalanine and urea. *Food Chem.* **2015**, *180*, 171–180. [[CrossRef](#)]
53. Portu, J.; López, R.; Ewald, P.; Santamaría, P.; Winterhalter, P.; Garde-Cerdán, T. Evaluation of Grenache, Graciano and Tempranillo grape stilbene content after field applications of elicitors and nitrogen compounds. *J. Sci. Food Agric.* **2018**, *98*, 1856–1862. [[CrossRef](#)] [[PubMed](#)]

54. Gutiérrez, A.R.; Santamaría, P.; González-Arenzana, L.; Garijo, M.P.; Berlanas, C.; López-Alfaro, I.; López, R. SO₂ tolerance in non-*Saccharomyces* yeasts. In Proceedings of the 39th World Congress of Vine and Wine, Bento Gonçalves, Brazil, 24–28 October 2016.
55. Trinh, T.T.T.; Woon, W.Y.; Yu, B.; Curran, P.; Liu, S.Q. Effect of L-isoleucine and L-phenylalanine addition on aroma compound formation during longan juice fermentation by a co-culture of *Saccharomyces cerevisiae* and *Williopsis saturnus*. *S. Afr. J. Enol. Vitic.* **2010**, *31*, 116–124. [[CrossRef](#)]
56. Santamaría, P.; López, R.; Portu, J.; González-Arenzana, L.; López-Alfaro, I.; Gutiérrez, A.R.; Garde-Cerdán, T. Role of phenylalanine in viticulture and enology. In *Phenylalanine. Dietary, Sources, Functions and Health Effects*; Elliamson, D., Ed.; Nova Publisher: New York, NY, USA, 2015; pp. 49–70.
57. Dizy, M.; Bisson, L.F. Proteolytic activity of yeast strains during grape juice fermentation. *Am. J. Enol. Vitic.* **2000**, *51*, 155–167.
58. Escribano, R.; González-Arenzana, L.; Portu, J.; Garijo, P.; López-Alfaro, I.; López, R.; Santamaría, P.; Gutiérrez, A.R. Wine aromatic compound production and fermentative behaviour within different non-*Saccharomyces* species and clones. *J. Appl. Microbiol.* **2018**, *124*, 1521–1531. [[CrossRef](#)] [[PubMed](#)]
59. Rapp, A.; Versini, G. Influence of nitrogen compounds in grapes on aroma compounds of wines. *Dev. Food Sci.* **1995**, *37*, 1659–1694. [[CrossRef](#)]
60. Ribéreau-Gayon, P.; Dubourdieu, D.; Donèche, B.; Lonvaud-Funel, A. *Handbook of Enology*; John Wiley and Son: West Sussex, UK, 2007; Volume 2.
61. Romano, P.; Suzzi, G. Origin and production of acetoin during wine yeast fermentation. *Appl. Environ. Microbiol.* **1996**, *62*, 309–315. [[CrossRef](#)] [[PubMed](#)]
62. Escribano-Viana, R.; González-Arenzana, L.; Portu, J.; Garijo, P.; López-Alfaro, I.; López, R.; Santamaría, P.; Gutiérrez, A.R. Wine aroma evolution throughout alcoholic fermentation sequentially inoculated with non-*Saccharomyces/Saccharomyces* yeasts. *Food Res. Int.* **2018**, *112*, 17–24. [[CrossRef](#)]
63. Capece, A.; Fiore, C.; Maraz, A.; Romano, P. Molecular and technological approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine origin. *J. Appl. Microbiol.* **2005**, *98*, 136–144. [[CrossRef](#)] [[PubMed](#)]
64. Rossouw, D.; Bauer, F.F. Exploring the phenotypic space of non-*Saccharomyces* wine yeast biodiversity. *Food Microbiol.* **2016**, *55*, 32–46. [[CrossRef](#)]
65. Fernández, M.; Úbeda, J.F.; Briones, A.I. Typing of non-*Saccharomyces* yeasts with enzymatic activities of interest in wine-making. *Int. J. Food Microbiol.* **2000**, *59*, 29–36. [[CrossRef](#)]
66. Ferreira, A.M.; Clímaco, M.C.; Faia, A.M. The role of non-*Saccharomyces* species in releasing glycosidic bound fraction of grape aroma components—A preliminary study. *J. Appl. Microbiol.* **2001**, *91*, 67–71. [[CrossRef](#)]
67. Maturano, Y.P.; Rodríguez Assaf, L.A.; Toro, M.E.; Nally, M.C.; Vallejo, M.; Castellanos de Figueroa, L.I.; Combina, M.; Vazquez, F. Multi-enzyme production by pure and mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation. *Int. J. Food Microbiol.* **2012**, *155*, 43–50. [[CrossRef](#)]
68. Benito, Á.; Calderón, F.; Palomero, F.; Benito, S. Combine use of selected *Schizosaccharomyces pombe* and *Lachancea thermotolerans* yeast strains as an alternative to the traditional malolactic fermentation in red wine production. *Molecules* **2015**, *20*, 9510–9523. [[CrossRef](#)]
69. Taing, O.; Taing, K. Production of malic and succinic acids by sugar-tolerant yeast *Zygosaccharomyces rouxii*. *Eur. Food Res. Technol.* **2007**, *224*, 343–347. [[CrossRef](#)]
70. Jolly, N.P.; Varela, C.; Pretorius, I.S. Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* **2014**, *14*, 215–237. [[CrossRef](#)] [[PubMed](#)]
71. Masneuf-Pomarede, I.; Bely, M.; Marullo, P.; Albertin, W. The genetics of non-conventional wine yeasts: Current knowledge and future challenges. *Front. Microbiol.* **2016**, *6*. [[CrossRef](#)]
72. Clemente-Jimenez, J.M.; Mingorance-Cazorla, L.; Martínez-Rodríguez, S.; Las Heras-Vázquez, F.J.; Rodríguez-Vico, F. Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiol.* **2004**, *21*, 149–155. [[CrossRef](#)]
73. Padilla, B.; Gil, J.V.; Manzanares, P. Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Front. Microbiol.* **2016**, *7*, 1–20. [[CrossRef](#)]
74. Prior, K.J.; Bauer, F.F.; Divol, B. The utilisation of nitrogenous compounds by commercial non-*Saccharomyces* yeasts associated with wine. *Food Microbiol.* **2019**, *79*, 75–84. [[CrossRef](#)]
75. Rodríguez, M.E.; Lopes, C.A.; Barbagelata, R.J.; Barda, N.B.; Caballero, A.C. Influence of *Candida pulcherrima* Patagonian strain on alcoholic fermentation behaviour and wine aroma. *Int. J. Food Microbiol.* **2010**, *138*, 19–25. [[CrossRef](#)]
76. Ciani, M.; Maccarelli, F. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World J. Microbiol. Biotechnol.* **1998**, *14*, 199–203. [[CrossRef](#)]
77. van Breda, V.; Jolly, N.; van Wyk, J. Characterisation of commercial and natural *Torulaspora delbrueckii* wine yeast strains. *Int. J. Food Microbiol.* **2013**, *163*, 80–88. [[CrossRef](#)]
78. Del Fresno, J.M.; Morata, A.; Loira, I.; Bañuelos, M.A.; Escott, C.; Benito, S.; González Chamorro, C.; Suárez-Lepe, J.A. Use of non-*Saccharomyces* in single-culture, mixed and sequential fermentation to improve red wine quality. *Eur. Food Res. Technol.* **2017**, *243*, 2175–2185. [[CrossRef](#)]

79. Chen, K.; Escott, C.; Loira, I.; del Fresno, J.M.; Morata, A.; Tesfaye, W.; Calderon, F.; Suárez-Lepe, J.A.; Han, S.; Benito, S. Use of non-*Saccharomyces* yeasts and oenological tannin in red winemaking: Influence on colour, aroma and sensorial properties of young wines. *Food Microbiol.* **2018**, *69*, 51–63. [[CrossRef](#)] [[PubMed](#)]
80. Belda, I.; Conchillo, L.B.; Ruiz, J.; Navascués, E.; Marquina, D.; Santos, A. Selection and use of pectinolytic yeasts for improving clarification and phenolic extraction in winemaking. *Int. J. Food Microbiol.* **2016**, *223*, 1–8. [[CrossRef](#)] [[PubMed](#)]
81. Clare, S.S.; Skurray, G.R.; Shalliker, R.A. Effect of yeast strain selection on the concentration of cis- and trans-resveratrol and resveratrol glucoside isomers in wine. *Aust. J. Grape Wine Res.* **2005**, *11*, 9–14. [[CrossRef](#)]
82. Kostadinović, S.; Wilkens, A.; Stefova, M.; Ivanova, V.; Vojnoski, B.; Mirhosseini, H.; Winterhalter, P. Stilbene levels and antioxidant activity of Vranec and Merlot wines from Macedonia: Effect of variety and enological practices. *Food Chem.* **2012**, *135*, 3003–3009. [[CrossRef](#)]
83. Gaensly, F.; Agustini, B.C.; da Silva, G.A.; Picheth, G.; Bonfim, T.M.B. Autochthonous yeasts with β -glucosidase activity increase resveratrol concentration during the alcoholic fermentation of *Vitis labrusca* grape must. *J. Funct. Foods* **2015**, *19*, 288–295. [[CrossRef](#)]