Growth of Non-Saccharomyces Native Strains under Different Fermentative Stress Conditions

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Abstract: The selection of yeast strains adapted to fermentation stresses in their winegrowing area is a key factor to produce quality wines. Twelve non-Saccharomyces native strains from Denomination of Origin (D.O.) “Vinos de Madrid” (Spain), a warm climate winegrowing region, were tested under osmotic pressure, ethanol, and acidic pH stresses. In addition, mixed combinations between non-Saccharomyces and a native Saccharomyces cerevisiae strain were practised. Phenotypic microarray technology has been employed to study the metabolic output of yeasts under the different stress situations. The yeast strains, Lachancea fermentati, Lachancea thermotolerans, and Schizosaccharomyces pombe showed the best adaptation to three stress conditions examined. The use of mixed cultures improved the tolerance to osmotic pressure by Torulaspora delbrueckii, S. pombe, and Zygosaccharomyces bailii strains and to high ethanol content by Candida stellata, S. pombe, and Z. bailii strains regarding the control. In general, the good adaptation of the native non-Saccharomyces strains to fermentative stress conditions makes them great candidates for wine elaboration in warm climate areas.

Keywords: non-Saccharomyces; stress; fermentation; mixed culture; phenotypic microarray technology; climate change

1. Introduction

Viticulture and wine elaboration are agricultural sectors greatly influenced by climate change [1]. The general influence of temperature increases has the immediate oenological consequence of an increased sugar content and, consequently, ethanol content. In addition, wines present a reduced acidity and increased pH, which implies a risk of undesired microbial proliferation and changes in the wine color, taste, and aroma [2–4]. While the external environment is continuously changing, the yeasts are exposed simultaneously to numerous stress conditions (oxidative, osmotic, and ethanol stress, among others). Yeast cells possess systems to respond to stress conditions including the rapid synthesis of protective molecules and the activation of signal transduction pathways that induce secondary events as the reactivation of enzyme activities and the transcription of genes encodes factors with protective functions [5]. Therefore, a biotechnological approach to mitigate product depreciation due to climate change could be the selection of yeast strains that are able to respond to these stress situations without important viability loss [6].

Typically, the inoculation of a S. cerevisiae strain to carry out the vinification is a common practise in winemaking. However, many other species of yeasts belonging to non-Saccharomyces genera present oenological aptitude of interest. This group not only contributes to alcoholic fermentation but also are helpful to solve specific oenological problems [7,8], to modulate wine aroma [7,9] and to control the activity of undesired microbes [10,11]. Some non-Saccharomyces species are now commercially available as T. delbrueckii, L. thermotolerans, M. pulcherrima, S. pombe, and Pichia kluveri [12]. The yeast species T. delbrueckii is probably considered the most suitable non-Saccharomyces yeast for

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wine elaboration [13]. It presents a good fermentative capacity and has positive aspects as low acetic acid and ethanol production, high glycerol and desirable aroma compounds release [13–15]. The fission yeast *S. pombe* has been recognized to improve some of the sensory parameters in wine, especially those linked to acidity control and wine color stability [16,17]. Two other genera abundantly found to be associated with grapes are *Hanseniaspora* and *Candida*, where *C. stellata* exhibit a positive impact in winemaking due to its fructophilic character, high glycerol, and extracellular enzyme production [18]. On the other hand, *Hanseniaspora* species are generally related to the increase of almost all acetate esters and some species stabilize the color of wine [19]. Most of non-*Saccharomyces* yeasts produce small changes in wine composition, while others prevent the addition of chemical compounds [20]. Thus, the main advantage in the use of *L. thermotolerans* is its inherent production of lactic acid, which avoid the addition of tartaric acid; in the same way, *M. pulcherrima* possess an antimicrobial effect by the secretion of the natural compound, pulcherrimin [21]. However, as long as non-*Saccharomyces* yeasts can confer beneficial characteristics to the resulting wines, most of them have a lesser fermentative capacity than *S. cerevisiae*. Consequently, controlled mixed fermentations that use more than one selected yeast strain play an increasingly important role in wine elaboration [22,23].

In the present work, phenotypic microarray technology was applied to test the growth capacity of microorganisms under different stressors using small volumes of culture. Twelve native non-*Saccharomyces* strains and one *S. cerevisiae* were studied alone or in mixed culture for better understanding of the behavior of these yeasts under different fermentative stress conditions given in a warm climate area (central Spain). Then, two non-*Saccharomyces* (*S. pombe* CLI 1085 and *C. stellata* CLI 920) and *S. cerevisiae* CLI 519 strains were assayed at higher volume under high osmotic pressure to analyze their fermentative kinetic and physicochemical changes associated with this stress situation.

### 2. Materials and Methods

#### 2.1. Yeast Strains

Twelve non-*Saccharomyces* yeast strains (Table 1) isolated from spontaneous fermentations in Malvar grape variety have been included in this study. The biotechnological potential for elaboration of high-quality wines from D.O. “Vinos de Madrid” have helped to their selection [24,25]. The *S. cerevisiae* CLI 519 from IMIDRA collection was considered as control and selected on its good adaptative capacity to stress conditions inherent to wine fermentation in warm climates such as the Madrid winegrowing region (central Spain) [26].

#### Table 1. Yeast strains tested in this study isolated from spontaneous fermentations of grape variety Malvar. Yeast abbreviation included in brackets.

| Yeast Code | Yeast Species                      |
|------------|-----------------------------------|
| CLI 920    | *Candida stellata* (Cs)           |
| CLI 1220   | *Lachancea fermentati* (Lf)       |
| CLI 1219   | *Lachancea thermotolerans* (Lt)   |
| CLI 460    | *Metschnikowia pulcherrima* (Mp)  |
| CLI 1217   | *Meyerozyma guilliermondii* (Mg)  |
| CLI 679    | *Pichia membranaefaciens* (Pm)    |
| CLI 2465   | *Pichia toletana* (Pt)            |
| CLI 1221   | *Pricomycyes carsonii* (Pc)       |
| CLI 519    | *Saccharomyces cerevisiae* (Sc)    |
| CLI 1085   | *Schizosaccharomyces pombe* (Sp)   |
| CLI 918    | *Torulaspora delbrueckii* (Td)    |
| CLI 1218   | *Wickerhamomyces anomalus* (Wa)   |
| CLI 622    | *Zygosaccharomyces bailii* (Zb)   |

#### 2.2. Phenotypic Microarray Analysis

Phenotypic microarray (PM) technology enables growth of microorganism or consortia in 96-well plate, each with a different substrate, stressor, or nutrient by providing
a phenotypic characterization of the cultures \[27,28\]. This technology is commercially available through Biolog, Inc. (Hayward, CA, USA). The system allows for culturing in small volumes where cells growing up under aerobic or anaerobic conditions and their metabolic output is colorimetrically measured on a microplate reader or Omnilog unit (Biolog, Hayward, CA, USA). By comparing growth on plates, phenotypic variation between yeast strains can be detected \[28,29\]. PM technology uses a reporter system, which utilizes a redox-sensitive tetrazolium dye that can be reduced to a soluble purple formazan product, correlates with an increase in the metabolic rate of a cell, which is oxidizing a carbon source. Therefore, the presence of a chemical compound that can oxidize by cell have the effect of increasing the cell metabolism and reducing the tetrazolium dye leads to a change from colorless into purple; this change is irreversible and cumulative. Instead, if the cell cannot oxidize a chemical, its metabolic rate does not rise, and no change of color takes place \[30\]. The cumulative amount of formazan is measured spectrophotometrically at 590 nm and is directly proportional to the number of metabolically active cells \[31\]. For PM analysis, each 96-well contained growth medium with a final concentration of 0.67\%(w/v) yeast nitrogen base (YNB) and 6\%(w/v) glucose in a total assay volume of 120 µL, supplemented with 2.6 µL of yeast nutrient mixture \[26\] and 0.2 µL dye D (Biolog). The final volume of growth medium was 30 µL using sterile distilled water, and inhibitory reagents were added as appropriate to maintain this volume. Thus, a stock solution of 80\%(w/v) sorbitol adjusted to generate 20\% and 30\%(w/v) concentrations in a final volume of 120 µL. For ethanol, 5\%(v/v), 8\%(v/v) and 13\%(v/v) were used to induce ethanol stress. Assays with pH used media YNB modified with phosphoric acid and then sterilized by filtration. Cell suspensions from inoculums were adjusted to a transmittance of 62 \%(around 5 × 10⁶ cells/mL) and 90 µL of a mixture of these cells and IFY buffer (Biolog) was inoculated to each well in the 96-well plate.

The Omnilog reader photographs the plates every 15 min, reflecting the metabolic output from dye conversion in each well. Finally, the percentage of redox signal intensity was determined by dividing the redox signal intensity under each stress condition and their non-stressed condition after 25 h incubation.

2.3. Small-Scale Fermentations

Sterile flasks of 120 mL were filled with YNB + glucose (87.5 g/L) medium (as control) and YNB + glucose (87.5 g/L) + 30% sorbitol medium (as stress condition) and were fermented at 30 °C. The fermenters were inoculated with 10⁶ cells/mL of S. cerevisiae CLI 519 (p-Sc), S. pombe CLI 1085 (p-Sp) and C. stellata CLI 920 (p-Cs) in pure cultures. Mixed fermentation trials were simultaneously inoculated with the same concentration (10⁶ cells/mL) of non-Saccharomyces cultures and S. cerevisiae strain. These pure and mixed cultures were tested as culture standard conditions (p-Sc, p-Sp, p-Cs, m-Sc/Sp, m-Sc/Cs) as for high osmotic pressure studies (p-Sc30%, p-Sp30%, p-Cs30%, m-Sc/Sp30%, m-Sc/Cs30%). The fermentation process was monitored daily by weight loss \[32\]. When the weight was constant, the fermentation was considered finished. Finally, samples were centrifuged and analyzed by HPLC.

2.4. Analytical Determinations

Chemical parameters as ethanol, glucose, acetic acid, glycerol and trehalose related to yeasts’ fermentative ability and their response to stressful conditions were analyzed in samples by HPLC. The Dionex Ultimate 3000 HPLC system (Thermo Scientific, Waltham, MA, USA) was equipped with a RefractoMax 520 refractive index (RI) detector and a Rezex ROA-Organic Acid H⁺ column (300 × 7.8 mm) at room temperature. Five mmol/L sulfuric acid was used as mobile phase, at a flow rate of 1 mL/min and a sample volume of 10 µL.

2.5. Data Statistical Analysis

Analysis of variance was carried out by ANOVA Tukey’s test to examine significant differences in fermentative parameters produced by mixed cultures of C. stellata CLI 920 and
S. pombe CLI 1085 strains regarding their respective controls (pure cultures of S. cerevisiae with or without stress factor). The data were analyzed with SPSS Statistics 25 Software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Fermentative Stress Tolerance of Non-Saccharomyces Yeast Strains in Aerobic (AE) and Anaerobic (AN) Conditions

The 12 native non-Saccharomyces strains from IMIDRA Collection were exposed to stress conditions present during the fermentation process: osmotic pressure, alcohol content, and pH. The resistance results are expressed in redox signal intensity units, which is the percentage of Biolog growth in a strain in comparison with this value for the same strain in non-stressed conditions.

The metabolic output of non-Saccharomyces strains has been analyzed in AE and AN condition in standard YNB culture medium for 96 h. Their metabolic activities are represented in Figure S1 (Supplementary Materials). All strains grew in both conditions at different percentages although a higher response is usually observed under aerobic situations. The species P. toletana, P. carsonii, S. pombe, and T. delbrueckii showed a parallel growth in both culture situations. Instead, the larger differences between AE and AN status were observed in the development of M. pulcherrima, W. anomalus and Z. bailii yeast strains.

3.1.1. Tolerance of Wine Yeast to Osmotic Stress

Sorbitol, a non-fermentable carbon source by yeasts, was employed to induce osmotic stress. In the presence of 20% sorbitol (Figure 1a), the strains L. fermentati (formerly Zygosaccharomyces fermentati), L. thermotolerans, and S. pombe exhibited the highest metabolic output between 100% and 125% under aerobic conditions. In anaerobiosis, the growth ratio of non-Saccharomyces is similar to in the presence of oxygen in general, though remain high by L. fermentati and L. thermotolerans. Strains C. stellata and P. toletana showed an opposed tendency over others and their growth is not extremely high, behave better on AN condition.

The osmophilic strains, L. fermentati, L. thermotolerans and S. pombe presented the highest resistance in AE and AN again at 30% sorbitol. The yeast strains that showed the worst adaptation to fermentative stress in this trial have been M. guilliermondii, P. membranaefaciens, P. carsonii, and W. anomalus with percentages between 27% and 48% (Figure 1b). As the same case observed before, C. stellata and P. toletana are better adapted to osmotic stress in AN condition. The strains M. pulcherrima and Z. bailii exhibited intermediate values around 50% with very similar behavior in AE and AN environment.

![Figure 1](image-url). Metabolic output expressed as redox signal intensity of non-Saccharomyces yeast strains under high osmotic pressure. (a) Growth ratio under stress situations generated by 20% of sorbitol medium in aerobic and anaerobic conditions; (b) Growth ratio under stress situations generated by 30% of sorbitol medium in aerobic and anaerobic condition.
3.1.2. Tolerance of Wine Yeast to Ethanol

The resistance of yeasts to alcohol degree was tested at concentrations between 5% to 13% of ethanol to determine tolerant and sensitive yeast strains (Figure 2). The growth response of non-Saccharomyces strains was analyzed in 5% ethanol medium, and it can be observed that all of them grew well at this ethanol content, especially in the presence of oxygen (Figure 2a). Several yeast species showed growth ratios above 100%, as in case of C. stellata, L. fermentati, L. thermotolerans, P. toletana, S. pombe, T. delbrueckii, and Z. bailii. In contrast, P. membranaefacien, P. carsonii, and W. anomalus presented the highest sensibility to 5% of ethanol (Figure 2a). When ethanol content was increased at 8% (Figure 2b), the development capacity of strains was reduced, being more affected the strains, M. pulcherrima and P. toletana. In this trial, P. carsonii and S. pombe grew better under anaerobic conditions. The growth capacity of strains was considerably reduced in the presence of 13% of ethanol, remaining very low or even non-existent in AN condition. Only Z. bailii reached values of 25% in Biolog units (Figure 2c).

![Graphs showing metabolic output as redox signal intensity of non-Saccharomyces yeast strains under different concentrations of ethanol in AE and AN: (a) 5% of ethanol; (b) 8% of ethanol; and (c) 13% of ethanol.](image)

Figure 2. Metabolic output as redox signal intensity of non-Saccharomyces yeast strains under different concentrations of ethanol in AE and AN: (a) 5% of ethanol; (b) 8% of ethanol; and (c) 13% of ethanol.

3.1.3. Tolerance of Wine Yeast to Acidic pH

In general, there are not major differences for the strains between AE and AN condition as a whole (Figure 3). The strains with a good metabolic output were L. fermentati (96–90%), L. thermotolerans (110–100%) and S. pombe (90–71%); by contrast, P. membranaefacien, P. carsonii in AN, and W. anomalus were more sensitive to stress caused by low pH (Figure 3), with values below 40%. 

3.2. Phenotypic Response to Stress Conditions (30% Sorbitol and 13% Ethanol) in Mixed Cultures under Anaerobic Fermentation

The phenotypic response is studied for two stress situations, osmotic pressure at 30% of sorbitol and ethanol concentration at 13%. Mixed cultures are fermented in AN and 1:1 proportion of *S. cerevisiae* together with each of the twelve non-*Saccharomyces* included in this work.

In pure cultures exposed to high osmotic pressure (Figure 4a), control culture of *S. cerevisiae* has undergone a large increase in its metabolic rate after 5 h of fermentation, completing the entire process only overtaken by pure cultures of *L. fermentati* (p-Lf) and *L. thermotolerans* (p-Lt). These two non-*Saccharomyces* strains had already shown their osmophilic character before (Figure 1a,b). The combinations between *S. cerevisiae* and *S. pombe* (m-Sc/Sp), *T. delbrueckii* (m-Sc/Td) and *Z. bailii* (m-Sc/Zb) (Figure 4b) presented a metabolic output upper than control in the presence of 30% sorbitol.

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**Figure 3.** Metabolic output expressed as redox signal intensity of non-*Saccharomyces* yeast strains at low pH in aerobic (AE) and anaerobic (AN).

**Figure 4.** Metabolic rate as redox signal intensity of non-*Saccharomyces* strains and *S. cerevisiae* under high osmotic stress induced by 30% sorbitol in: (a) pure culture and (b) mixed culture 1:1 with *S. cerevisiae*. Pure culture of *S. cerevisiae* (p-Sc) was considered as control in both cases.
Similar behavior has been observed when the stress factor is the 13% of ethanol (Figure 5). The *S. cerevisiae* strain showed better adaptation to high levels of ethanol in the medium, only overtaken by *Z. bailii* (Figure 5a). However, there is a rise in the growth response when *S. cerevisiae* is associated with the non-*Saccharomyces*, *Z. bailii* (m-Sc/Zb) and *S. pombe* (m-Sc/Sp) (Figure 5b). The metabolic output was improved in the association of *S. cerevisiae* with the rest of non-*Saccharomyces* since their growth was higher than pure culture of non-*Saccharomyces* alone (Figure 5a). The non-conventional species that showed good resistance to ethanol were *Z. bailii* (p-Zb), *L. fermentati* (p-Lf) and *M. guilliermondii* (p-Mg) in pure culture (Figure 5a).

**Figure 5.** Metabolic rate as redox signal intensity of non-*Saccharomyces* strains and *S. cerevisiae* under alcohol degree stress induced by 13% ethanol in: (a) pure culture and (b) mixed culture 1:1 with *S. cerevisiae*. Pure culture of *S. cerevisiae* (p-Sc) was considered as control in both cases.

3.3. Small Scale Fermentations with *Candida stellata* CLI 920 and *Schizosaccharomyces Pombae* CLI 1085 in Mixed Culture with *Saccharomyces cerevisiae* CLI 519 under Osmotic Stress

Microvinifications were carried out in a synthetic medium with the non-*Saccharomyces* species, *C. stellata* and *S. pombe* inoculated at 50% together with *S. cerevisiae* in a control medium and in a high osmotic pressure medium generated with 30% of sorbitol. The fermentative kinetic in standard medium (Figure 6a) indicates that the fermentation process finished from 47 h, except for pure culture of *S. pombe* (p-Sp). The control p-Sc and both mixed cultures presented a kinetic pattern very similar. By contrast, the non-*Saccharomyces* pure cultures (p-Cs and p-Sp) presented a slow fermentation, especially for p-Sp was more long-delayed and incomplete.
Figure 6. Fermentative kinetic of pure and mixed cultures of *S. cerevisiae*, *C. stellata*, and *S. pombe* in: (a) YNB + glucose medium and, (b) YNB + glucose + 30% sorbitol medium.

Figure 6b corresponds to cited combinations but under high osmotic pressure media. The worst adapted strain was *S. pombe* p-Sp, being all others very similar. *S. cerevisiae* p-Sc has shown a good adaptation to osmotic pressure again, but it is worth highlighting the similar fermentative behavior of p-Cs to the control p-Sc.

The chemical compounds analyzed at final of fermentation are shown in Table 2. Yeasts consume glucose content in p-Sc and mixed cultures when the culture medium has not fermentative stress element. However, pure cultures of non-*Saccharomyces* finished the fermentation with elevated amount of glucose under the same situation, highlighting p-Sp (45.8 g/L of glucose); both showed significant differences (*p* > 0.05) respect to their control (p-Sc) (Table 2). Only p-Sp was unable to consume the glucose portion under stressed condition (30% sorbitol), affecting to its ethanol production and pH values at final of fermentation. The highest ethanol content was produced by *C. stellata* in pure culture under osmotic stress. This data is in contrast with its behavior under standard conditions where p-Cs finished with 13.53 g/L of glucose.

Table 2. Fermentative parameters produced by pure and mixed cultures with *S. cerevisiae*, *S. pombe* and *C. stellata* in YNB + glucose medium (control) and YNB + glucose + 30% sorbitol (stressful medium). Data are means ± standard deviation (*n* = 3). Asterisks (*) denoted significant differences regarding the controls, p-Sc, and p-Sc30% (Tukey test; *p* < 0.05).

| Type of Culture | Ethanol (g/L) | Glucose (g/L) | Acetic Acid (g/L) | Glycerol (g/L) | Trehalose (g/L) | pH  |
|-----------------|--------------|---------------|-------------------|---------------|-----------------|-----|
| p-Sc            | 43.79 ± 0.20 | 0.02 ± 0.00   | 0.73 ± 0.10       | 4.29 ± 0.08   | 0.53 ± 0.01     | 2.51 ± 0.02 |
| p-Sp            | 12.03 ± 4.58 * | 45.77 ± 8.15 * | 0.02 ± 0.01 *     | 1.50 ± 0.76 * | 0.59 ± 0.01 *   | 2.99 ± 0.10 * |
| p-Cs            | 34.27 ± 1.95 * | 13.53 ± 1.95 * | 0.00 ± 0.00 *     | 3.64 ± 0.26   | 0.72 ± 0.01 *   | 2.55 ± 0.02 |
| m-Sc/Sp         | 44.09 ± 1.17 | 0.00 ± 0.00   | 0.69 ± 0.07       | 4.18 ± 0.11   | 0.56 ± 0.02 *   | 2.51 ± 0.01 |
| m-Sc/Sp         | 44.54 ± 0.29 | 2.54 ± 0.02   | 0.68 ± 0.10       | 4.23 ± 0.13   | 0.57 ± 0.00 *   | 2.55 ± 0.02 |
| p-Sc30%         | 33.81 ± 0.17 | 0.02 ± 0.01   | 1.16 ± 0.01       | 11.41 ± 0.11  | 0.92 ± 0.00     | 2.46 ± 0.00 |
| p-Sp30%         | 8.99 ± 2.42 * | 35.96 ± 4.52 * | 0.10 ± 0.00 *     | 6.83 ± 0.72 * | 0.84 ± 0.00 *   | 2.87 ± 0.10 * |
| p-Cs30%         | 36.95 ± 0.35 | 0.00 ± 0.00   | 0.00 ± 0.00       | 9.90 ± 0.59 * | 0.96 ± 0.02 *   | 2.48 ± 0.01 |
| m-Sc/Sp30%      | 34.19 ± 0.17 | 0.02 ± 0.00   | 1.15 ± 0.06       | 11.59 ± 0.08  | 0.93 ± 0.01     | 2.49 ± 0.01 |
| m-Sc/Sp30%      | 34.63 ± 0.06 | 0.03 ± 0.01   | 1.02 ± 0.01       | 11.00 ± 0.03  | 0.93 ± 0.01     | 2.56 ± 0.03 |

Glycerol and trehalose contents are consistent with the conditions of study, a higher production is observed under high osmotic pressure. Mixed cultures secreted high amount of glycerol with similar values to the control of *S. cerevisiae*. The non-*Saccharomyces*, *S. pombe* was the strain that produced a lesser quantity of glycerol (Table 2).

There are some differences concerning to acetic acid contents (Table 2). Mixed cultures presented high concentration similar to the control of *S. cerevisiae*; these values are more elevated under osmotic stress situations. By contrast, significant differences are observed in both non-*Saccharomyces* pure cultures regarding to the control, showing very low values (0.10 g/L in p-Sp30% and 0.00 g/L in p-Cs30%).
4. Discussion

According to the hypothesis proposed by Ungar [33], climate change lacks daily relevance that may encourage people to obtain knowledge about the topic. For winemakers, weather phenomena have become very important since it is related to the annual course of the vineyard operations and to the quality of final products, even for their place in the market [1]. A key factor to reach the biotechnological success with wine yeasts is to know their tolerance to environmental stress conditions. There is a correlation between fermentative capacity and high tolerance to stress [34] and stress tolerance is a good criterion for selecting oenologically interesting yeasts [35]. The actual work is a first screening in synthetic media of native non-*Saccharomyces* yeasts resistance to stresses currently caused by global warming in wine elaboration, in order to select the best yeast strains as strategy to mitigate its effects on wine quality. Thus, twelve autochthonous non-*Saccharomyces* strains and one *S. cerevisiae* strain from grapes and musts of cellars belonging to D.O. “Vinos de Madrid” has been employed alone or combined in fermentations since these strains are adapted to conditions associated with their specific winegrowing area [24,36].

Several yeast members that constitute wine fermentation microbiota as *Saccharomyces*, *Hanseniaspora*, *Pichia*, *Candida*, *Starmerella*, *Torulaspora*, and *Lachancea* are facultative anaerobes microorganisms that can grow and survive under these conditions [37]. Nevertheless, most of non-*Saccharomyces* yeasts have more oxygen demands and low fermentative power in comparison with *S. cerevisiae* [38–42]. In the present work, the non-*Saccharomyces* strains grew with a similar profile independently of the presence of oxygen without any stress factor, but *M. pulcherrima*, *W. anomalus*, and *Z. bailii* showed larger oxygen dependence since their metabolic output was noticeably higher in aerobiosis. The yeast species *M. pulcherrima* has demonstrated in previous studies [39,42–44] that its capacity to contribute actively to the outcome of the fermentation is directly related to oxygen availability, being considered an obligate aerobic microorganism. In fact, this non-*Saccharomyces* species has displayed a fully respiratory glucose metabolism [41,44]. In the same way, Contreras et al. [45] observed that *Z. bailii* AWRI 1578 presented the capacity to metabolize sugars without the collateral production of ethanol with a determined aeration regime, producing wines with lower alcohol content in sequential culture with *S. cerevisiae*. In addition, a fully aerobic or weakly fermentative metabolism has been described in *W. anomalus* and it is known for film formation covering bulk wines in unfilled vessels [46,47].

One of the first stress conditions supported in the fermentation process by yeasts is produced by an elevated concentration of osmotically active substances of the grape must, mainly glucose and fructose. Yeast cells respond to such hyperosmotic shock through the modification of their cell wall and cytoskeleton and the synthesis and accumulation of compatible compounds, which help to reestablish an osmotic equilibrium [6]. In the present work, 20% and 30% of sorbitol generate the hyperosmotic scenario by yeasts. In general, the growth percentage in the non-*Saccharomyces* strains is similar with or without oxygen under osmotic stress created by the different contents of sorbitol. In the work of Krantz et al. [48], they excluded oxygen from the culture and noted that the stress response did not differ qualitatively from that under aerobic conditions and, particularly, encompasses the oxidative stress response genes. They observed an almost perfect coincidence between genes showing up- or downregulated expression after osmotic stress under aerobic and anaerobic conditions. In our study, the most resistant non-*Saccharomyces* strains to osmotic pressure have been *L. fermentati* CLI 1220, *L. thermotolerans* CLI 1219, *S. pombe* CLI 1085 in AE and AN. These results agree with those obtained previously [26] where the non-*Saccharomyces*, *L. thermotolerans* and *S. pombe* strains presented high tolerance under 30% sorbitol. Accordingly, a broad study about the genus *Lachancea* [49] presented three yeast species mainly associated with grape must and wine fermentation processes: *L. fermentati*, *L. thermotolerans* and *L. lanzarotensis*. These species demonstrated an optimal growth on the 50% D-glucose supplemented medium, suggesting to their increased osmotic tolerance. To mitigate such stress, *L. thermotolerans* strains showed the ability to assimilate glycerol while *L. fermentati* and *L. lanzarotensis* strains displayed variability in glycerol
assimilation [49]. For its part, the application of S. pombe has been suggested in warm viticulture area characterized by grape juices with high sugar content, pH values close to 4 and low malic acid concentration [50,51]. In addition, the cell wall of S. pombe presents a big thickness and a particular presence of galacto-mannoproteins in the outer layer that give them structural strength enough to resist high osmotic pressures [17,52].

The increasing ethanol concentration during the alcoholic fermentation represents another stress factor by yeasts. The study of suppression of the cellular growth in the presence of ethanol is a simple and useful method by classification of a high number of strains by its ability to tolerate alcohol [53]. During the present work, the presence of 5% ethanol is not a limiting factor by the metabolic output of most non-Saccharomyces strains, though P. carsonii and W. anomalus presented lower growth values. The fermentative capacity of these two non-Saccharomyces strains (P. carsonii CLI 1221 and W. anomalus CLI 1218) was tested in previous works [25,54]. Both strains showed a fermentative kinetics studied as weight loss in CO₂ grams released, considerably less than a S. cerevisiae native strain [54]; probably, this low fermentative power can be related with their evident low ethanol tolerance. When ethanol content rises to 8%, M. pulcherrima and P. toletana viability was considerably reduced. These results are in accordance with those obtained in a previous work, where these strains presented one of the lowest tolerances to ethanol content [26]. In general, the fermentative capacity of M. pulcherrima species is considered low, with many strains reaching around 4% v/v in ethanol [55,56], although some studies have denoted the production of ethanol up 6–10% v/v [43,57]. Under 13% ethanol stress condition, the non-Saccharomyces cell survival are enhanced in the presence of oxygen, although the resistance of Z. bailii CLI 622 also highlights in an anaerobic environment. The genus Zygosaccharomyces is included in the group of spoilage yeasts for the food and drinks industries, with Z. bailii and Z. rouxii being more frequent. This group presents key physiological characteristics as ability to vigorously ferment hexose sugars such as glucose and fructose, resistance to weak-acid preservatives, extreme osmotolerance and high resistance to both ethanol and acetic acid that contribute to their spoilage capacity [58–62]. Sousa et al. [58] described a physiological strategy carried out by Z. bailii where the presence of ethanol in the medium appears to reinforce acetic acid control and plays a protective role for this yeast, inhibiting the acid uptake ad maintaining its intracellular concentration below toxic levels.

Due to climatic change, pH values are increased, affecting the global wine quality [63]. Furthermore, higher pH values hold the risk of higher microbial contamination mainly in the early stages of fermentation course. To overcome this problem, the correction of the initial pH in musts is a common practise but it may involve an additional stress factor for the survival and growth of some yeast species [64]. In our assay, we tested the yeast performance at pH 3.0 and, in general, most strains were metabolically active at this acidic pH value. It is worth noting that L. fermentati, L. thermotolerans and S. pombe were more tolerant yeast strains again. It is well known that the Lachancea and Schizosaccharomyces species have the capacity to modulate different organic acids during fermentation, varying the pH of the wine. Thus, a microbiological acidification with L. thermotolerans is a classical use of this yeast species in warm climate viticultural areas [65]. This character is due to the metabolic activity of L. thermotolerans, which is a good L-lactic producer [66]. A L. thermotolerans native strain from D.O. “Vinos de Madrid” (Spain) led to a fall to pH 2.43 because of 8.32 g/L of lactic acid production in Malvar white wines elaboration [56]. Kapsopoulou et al. [67] reported similar pH value (2.9) due to the lactic acid production (9.6 g/L) using L. thermotolerans strain in pure culture. This biological acidification can improve the overall wine quality in warm areas [68]. In the same way, L. fermentati strains has also been described as lactic acid producer [49] and proposed to sour beer or low alcohol beer elaborations [69–71]. In addition, the production of lactic acid from glucose and cellobiose has been demonstrated in S. pombe [72]. Alternatively, some authors have proposed the combined use of S. pombe and L. thermotolerans to solve problems in warm regions [73,74], since S. pombe consumes totally malic acid achieving the microbiological
stabilization objective and L. thermotolerans produces lactic acid increasing the acidity of wines from low sourness musts.

In the last years, non-Saccharomyces species are considered capable of helping to improve specific characteristics of wine quality [75–77], depending on certain yeasts species and strains used. Generally, one disadvantage of non-Saccharomyces species is their low fermentative activity and their low resistance to additives as sulfur dioxide [8]. Thus, the use of some non-Saccharomyces together with a high fermentative S. cerevisiae strain is proposed as a strategy to metabolize all sugars into ethanol [78]. In the present work, non-Saccharomyces strains were fermented in mixed combination with S. cerevisiae under two fermentative stress conditions (30% sorbitol and 13% ethanol). Over each inoculation, yeast strains are exposed to a number of stress conditions that require a period of adaptation. The time needed for the adaptation is known as the lag-phase, characterized by the absence of cellular growth. This lag-phase, typical for all newly inoculated cultures, is so another signal of a stress-induced adaptation phase [6,79]. In our work, it is possible to see that the pure cultures under osmotic or ethanol stresses presented a lag-phase longer (around 5 h) than the mixed cultures (around 2 h). The presence of S. cerevisiae in the medium seems to shorten this adaptation period.

The growth percentage of two non-Saccharomyces strains (L. fermentati CLI 1220 and L. thermotolerans CLI 1219) exceeds the S. cerevisiae values in pure culture at 30% sorbitol. A study carried out by De Kock [23] stated that a strain of L. thermotolerans produced not only glycerol but also more polyols than S. cerevisiae, which could provide more protection against osmotic stress situation. Instead, the metabolic output of Lachancea species decreased in mixed culture with S. cerevisiae. Several studies attribute this decline in L. thermotolerans to the impact of parameters as temperature [80], lack of oxygenation [42], cell-to-cell contact [81] and the secretion of toxic compounds by S. cerevisiae [82,83]. However, L. fermentati in co-culture fermentations has been poorly studied yet [49]. Three combinations presented higher growth percentage than the control when osmotic pressure stress is applied to mixed cultures: mixed cultures of T. delbrueckii, S. pombe, and Z. bailii with S. cerevisiae. The yeast species, T. delbrueckii is known as a highly resistant microorganism to stress conditions and is often isolated from fruit juices, bakery [84] and botrytized musts [85,86], but there are not so many studies about its resistance mechanisms in high-sugar ambient. Our results are in accordance with Bely et al. [14] where they observed the highest biomass presence in mixed T. delbrueckii/S. cerevisiae culture on high-sugar fermentation. As stated before, the high resistance to ethanol in Z. bailii and S. pombe has also been documented [50,61]. This resistance to ethanol could be explained the highest metabolic activity of native Z. bailii CLI 622 and S. pombe CLI 1085 strains in mixed cultures respect to the control since the presence of non-Saccharomyces and Saccharomyces at the same time during the fermentation promotes an increase in the persistence of non-Saccharomyces yeasts [87,88].

Finally, two autochthonous yeast strains previously well-studied [18,25,26,56,89] were selected to fermentations at higher volume under high osmotic pressure. The strain S. pombe CLI 1085 has shown good resistance to three stress factors tested in the current work; besides, it was a low producer of acetic acid, consumer of malic acid and producer of lactic acid [56] that are favorable characteristics in warm winemaking areas. The pure culture C. stellata CLI 920 have presented an osmotic pressure and ethanol tolerance similar to S. cerevisiae; other study showed it as high glycerol producer, its pure culture was the most appreciated by trained tasters from IMIDRA Institute and this strain was proposed by using in sweet wine elaboration [56].

It is interesting to note that C. stellata CLI 920 strain produced the highest level of ethanol under osmotic stress situations. This non-Saccharomyces species is typically associated with the fermentation of botrytized wines and wines produced from overripe grapes in cooked musts [18]. Besides, various authors have mentioned that C. stellata has better ability than S. cerevisiae to grow in high-sugar fermentations [90,91]. A strain-dependent characteristic described in S. cerevisiae [92,93] denoted that this yeast may
possess the capacity to overcome osmotic stress and to yield ethanol by fermentation of musts with high sugar proportion in winemaking. It could be possible C. stellata CLI 920 showed this ability, although more studies are needed.

After hyperosmotic shock, yeast cells produce and accumulate osmoprotectants as glycerol and trehalose [5]. This statement is in agreement with our results where these two compounds are elevated in fermentations under high osmotic pressure. However, the production of glycerol often linked to increased acetic acid yield in S. cerevisiae [94,95]. Nevertheless, it seems that many non-Saccharomyces yeasts have different metabolic responses to osmotic stress, producing low levels of acetic acid [14,96,97]. Several authors have stated an elevated acetic acid production as the main consequence of the use of S. pombe in alcoholic beverages elaboration [98–102]. The S. pombe CLI 1085 used in this study produced acetic acid values very low (0.02 g/L under control situations; 0.10 g/L under osmotic stress). This same strain produced 0.33 g/L of acetic acid in pure culture in Malvar white wines fermentation [56]. These values are similar to those reported by Du Plessis et al. [103] ranged between 0.07 g/L and 0.35 g/L acetic acid. Ciani et al. [104] described the C. stellata DBVPG 3827 strain as low acetic acid producer in both aerobic (0.12 g/L of acetic acid) and anaerobic (0.10 g/L of acetic acid) conditions regarding S. cerevisiae monoculture. This result is in agreement with our data where the acetic acid of p-Cs and p-Cs30% cultures were below detection limits. The mixed cultures completed the fermentation and had high glycerol and acetic acid levels very similar to controls (p-Sc and p-Sc30%); these fermentations seem to be dominated by S. cerevisiae strain due to the high similarity of their parameters at final of fermentation.

5. Conclusions

Most non-Saccharomyces yeasts tested have been capable of growing under different stress conditions, but better in the presence of oxygen. The most resistant species were L. fermentati, L. thermotolerans, and S. pombe under osmotic pressure, ethanol, and pH stresses. The use of mixed combinations has improved the resistance against stress situations by S. pombe, T. delbrueckii, and Z. bailii under osmotic pressure and by C. stellata, S. pombe, and Z. bailii under high ethanol content regarding S. cerevisiae pure culture. However, the mixed cultures between C. stellata and S. pombe with S. cerevisiae have shown a favorable increase of glycerol content but the adverse rise of acetic acid. Maybe one solution to the problem of excessive volatile acidity was to use a higher concentration of non-Saccharomyces strains in mixed combinations.

Therefore, the resistance presented by these non-Saccharomyces to stresses inherent to fermentation make them good candidates for winemaking in warm areas. However, more studies should be carried out to wine elaboration at a higher scale.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7030124/s1, Figure S1: Phenotypic microarray analysis (% of redox signal intensity) of non-Saccharomyces strains in aerobic (AE) and anaerobic (AN) condition after 96 h in YNB medium.

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