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Original Research

Preliminary Study on Yeasts Associated with the Production of "Tostado"—a Traditional Sweet Wine from Galicia (NW Spain)

David Castrillo[1,*], Pilar Blanco[1]

[1]Estación de Viticultura e Enoloxía de Galicia (EVEGA-AGACAL), Ponte San Clodio s/n, 32428 Leiro, Ourense, Spain
*Correspondence: david.castrillo.cachon@xunta.gal (David Castrillo)
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Abstract

Background: Tostado is a traditional sweet wine from the Designations of Origins (DOs) of Ribeiro and Valdeorras in Galicia (NW Spain). The harvested grapes are air-dried and pressed to increase the concentrations of sugars, acids, and flavour compounds. Therefore, knowledge of the yeasts involved in fermentation under these conditions is essential to guarantee the quality and uniqueness of the valuable, distinctive, and expensive Tostado wines. **Methods**: *Saccharomyces* and non-*Saccharomyces* yeasts were identified using Wallerstein Laboratory (WL) Nutrient Agar and lysine plating, followed by polymerase chain reaction (PCR) amplification, enzymatic digestion, and sequencing. *Saccharomyces cerevisiae* isolates were further characterised at the strain level using mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP). Statistical analyses were also performed, including different diversity indices, Similarity Percentage (SIMPER) analysis, principal component analysis (PCA), neighbor-joining clustering, parsimony–phylogram, and network plot. In addition, the total acidity, volatile acidity, reducing sugars, and alcoholic strength by volume of the Tostado wines were analysed. **Results**: A wide diversity of autochthonous yeasts was found, which were predominantly species of oenological relevance, such as *Lachancea thermotolerans*, *Starmerella bacillaris*, *Hanseniaspora uvarum*, *Debaryomyces hansenii*, *Torulaspora delbrueckii*, *Pichia* spp., and *Saccharomyces cerevisiae* from the must and paste stages of Tostado wine. In addition, 19 different *S. cerevisiae* strains were identified. This high yeast diversity, which changed from the early stages of fermentation, could contribute to the distinctive characteristics observed in Tostado wine. **Conclusions**: Characteristic and differentiating chemical and microbiological profiles were found as early as the pre-fermentation stages, which adds value to these special wines that have rarely been studied.

Keywords: sweet wine; Tostado; Ribeiro DO; Valdeorras DO; non-*Saccharomyces*; oenological yeasts

1. Introduction

The Ribeiro Designation of Origin (DO) is one of the oldest DOs in the Iberian Peninsula and one of the most important in the region of Galicia because of its socioeconomic impact. Tostado is a naturally sweet wine produced mainly within the Ribeiro DO on a very small scale but with a high value by preserving the origin and the most ancient winemaking tradition. Tostado is a relevant manifestation of Ribeiro's cultural and oenological heritage [1]. The selected Tostado wine was highly appreciated for centuries, was the celebratory wine of the well-to-do peasants, priests, and noblemen, and later had numerous mentions among the emigrants, according to the news of the Society of the Galician press in America [2]. Therefore, the centuries-old tradition of Tostado wine could be comparable to the French *Vin de Paille*, the Austrian *Strohwein*, the Italian *Vin Santo*, or Falanghina sweet *Passito*, which are wines made from raisins [3]. The organoleptic properties of Tostado wine, similar to other native sweet wines made from raisins, differ in their volatile compounds because of their highly valued distinctiveness and contribute to their increasing sales [4].

Tostado wines are scarce and expensive due to their low yield (a maximum of 0.4 L/kg of raisins) and their costly traditional production process. Thus, Tostado elaboration involves the natural raisining of native grapes under covered premises, at least three months of drying, and controls to avoid losses. The must of raisined grapes should have a minimum sugar content of 300 g/L. In addition, Tostado wine matures in wooden vats (for a minimum of six months) and bottles (for at least three months) with a minimum actual alcoholic strength of 13% vol. [1]. For all these reasons, Tostado wine is characterised by its homogeneity and well-defined sensory characteristics in its visual appearance (amber colour), olfactory (aroma of dried fruits, citrus, and raisins), and taste (natural sweetness, body, persistence, and freshness) [5]. This differentiation makes them highly appreciated wines with high value-added and unique characteristics.

Previous studies on Tostado wine are limited. The microbiology of wines is known to have a decisive influence on their quality and can mitigate the unfavourable effects of climate change [6,7]. Some authors have studied the fermentation kinetics and chemical and sensory characteristics of Tostado wine, the influence of the yeast used (commercial or spontaneous) and the fermentation temperature [5,8–10]. However, they have not focused on the yeast population present during spontaneous fermentation.

Ribeiro's Tostado wine is mainly made from the white variety Treixadura. However, previously, in the region of Valdeorras (also in Galicia), Tostado was made from white grapes of the Godello variety. Traditional red grape varieties have also been used. Currently, there is interest in recovering this tradition by diversifying the Galician wine market into a high-quality product. To this end, other local grape varieties have been proposed, such as the Garnacha Tintorera Red variety, which is suitable for the raisining process [11–13].

Few publications exist on this sweet wine owing to the particularities of Tostado mentioned above; however, its distinctive character and high value-added justify further research.

2. Materials and Methods

2.1 Samples

The samples used in this study were collected at different steps during the elaboration of Tostado wine: Grape (TG), must (TM), paste (TP), and Tostado wine (TW). The paste of Tostado wine is the fermentation stage in which the must have been released from the dried grapes and is fermented together with the skin and pulp in a pasty mass because of the high concentration of sugars and low proportion of must. Samples were taken in triplicate from a winery belonging to the Ribeiro DO and EVEGA cellar from 2011 to 2018. All elaborations were conducted following the indications of the regulatory council. Representative samples of each phase (TM and TP during fermentation) were collected in sterile 100 mL bottles closed with sterile-venting membrane screw caps [14] and transported to the laboratory, while wine samples were used directly from the bottle when the wine was finished.

2.2 Yeast Isolation and Identification

Microbiological analyses of the four matrices (TG, TM, TP, and TW) were performed. Decimal dilutions were prepared, and 100 µL of each dilution was plated in duplicate on solid medium WL nutrient agar (Scharlau Microbiology). The plates were incubated at 28 °C for 48 h until the colonies appeared. After incubation, visible colonies were counted. Dilutions containing 100–300 viable colony forming units were used to isolate 25 colonies from the Tostado must and 30 from the Tostado paste. Colonies were isolated based on their morphological characteristics (shape, size, and colour). Pure cultures of the selected colonies were observed under an optical microscope (Eclipse NIKON, Tokyo, Japan) to identify cell morphology. Repetition and re-isolation of the colonies were performed, and their morphology was observed on a WL plate under the microscope. Additionally, the isolates were cultured on a lysine medium (Scharlau Microbiology) to differentiate them into *Saccharomyces* and non-*Saccharomyces* yeast types (negative and positive growth, respectively).

Yeast identification at the species level was performed by polymerase chain reaction (PCR) amplification of the 5.8S rRNA gene and two (non-coding) internal ribosomal ITS1 and ITS2 spacers (using the ITS1 and ITS4 primers), and subsequent digestion of the obtained product with *Hinf* I, *Hae* III, and *Cfo* I restriction enzymes [15]. PCR amplification and sequencing of the D1/D2 region of the 26S rDNA gene were used to confirm yeast identification. The D1 and D2 domains were amplified using NL-1 and NL-4 primers. PCR products and digestion fragments were separated on a 2.7% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer. Isolates identified as *Saccharomyces* were characterised at the strain level using mitochondrial DNA restriction patterns (mtDNA-RFLPs) [16]. The digestion reaction mix contained 16 µL of total DNA, 2 µL of FastDigest enzymes *Hinf* I (Thermo Fisher Scientific, Madrid, Spain), and 2 µL of 10× FastDigest Green buffer (Thermo Fisher Scientific, Madrid, Spain) and was incubated at 37 °C for 2 h. Restriction fragments were separated by electrophoresis on 0.8% (*w/v*) agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). After staining the gel with ethidium bromide (0.5 µg/mL), the banding pattern was visualised by UV light using a Molecular Imager® Gel DocTM XR+ imaging system (BIO-RAD, Madrid, Spain). Sequence similarities were obtained using GenBank BLASTN (search services), and the identified yeast strains were compared by the obtained banding patterns.

2.3 Chemical Analysis of Tostado Wines

The wines were analysed in the chemistry laboratory of EVEGA, immediately after fermentation. The following chemical parameters were determined in the resulting Tostado wines: total acidity (g tartaric acid; Titrator), volatile acidity (g acetic acid; Volumetry), reducing sugars (glucose + fructose; Enzymatic), and alcoholic strength by volume (%vol.; Electronic densimetry) using Fourier transform infrared spectrometry (FTIR) with a Wine Scan FT120 analyzer (FOSS Electric, Barcelona, Spain), calibrated as outlined by OIV [17].

2.4 Statistical Analysis

An analysis of variance (ANOVA) was performed for each determined parameter using SPSS (version 18.0, IBM Corp., Armonk, NY, USA) to detect statistically significant differences between the analysed Tostado wines.

All other statistical analyses were performed using PAST (version 14.4, PAleontological STatistics, Oslo, Norway) software. For the 19 different *Saccharomyces cerevisiae* strains identified, a mapping or matrix plot was performed as a unified representation of the data obtained in the agarose gels, assigning a 0 or 1 depending on the presence or absence of bands between 200 and 610 base pairs. In addition, principal component analysis (PCA) was performed to determine the variance–covariance between the different must, wine, or Tostado paste strains in each group.

A phenogram (neighbor-joining clustering) using Jaccard's index and a cladogram (parsimony–phylogram) were also performed to evaluate the genetic clustering. Finally, a network plot was constructed using the Dice similarity index using scale nodes with n °edges and, in turn, with scale edges by similarity. In this way, traces of greater intensity or thickness are shown as the greater similarity or distance measure of the interconnections between different strains of *S. cerevisiae* at the neural network level.

On the other hand, biodiversity indices (Shannon-Wiener index; (H'), Simpson's diversity index (1 - dominance) and equitability (E)) were also carried out as indicated in Castrillo *et al*. [18], interpolating the results by the type of sample (TM, TP, and TW) in each case. The Shannon index was calculated using both yeast species richness (S) and frequency (k). The Shannon index can be used to determine the frequency of species in an ecosystem. In this case, the formula for the Shannon index (1) was adapted as in (2) by substituting S with k:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i \quad (1)$$

$$H' = -\sum_{i=1}^{k} p_i \ln p_i \quad (2)$$

where p_i is the proportion of individuals of species i, and S and k are the total number of species and distribution among the species, respectively. Finally, Similarity Percentage (SIMPER) analysis with the Bray–Curtis similarity measure was performed to assess the species mainly responsible for the observed difference in yeast diversity in the samples [19].

3. Results and Discussion

3.1 Fermentation Kinetics

Fermentation kinetics is a crucial phase in the production of wines made from raisins, where the microbiota of the must and cellar residential yeasts play an important role [3,8]. Table 1 shows the number of colony forming units (CFU/mL) of the yeasts detected in the analysed samples. Similar to other studies, the highest number of CFU or viable cells was detected in the samples of Tostado paste, followed by the must, and the wines showed the least viable cells [3,20]. This is consistent with the fermentative state; as it reaches the tumultuous phase, the number of viable yeast cells increases (Fig. 1). CFU/mL was sufficiently high to guarantee fermentation without contamination. In addition, the fermentation process was measured with Tostado musts from red and white grape varieties. In the latter case, it was possible to evaluate how the volume (10 L, 50 L, and 100 L) influenced the fermentation.

Table 1. Colony forming units (CFU/mL) count of yeasts detected in the different must, paste, and Tostado wine samples.

Sample	N° of viable yeasts (log10 CFU/mL)
TM1 (2014)	6.22
TM2 (2014)	6.16
TP1 (2015)	7.33
TP2 (2015)	6.05
TPr (2018)	6.49
TPw 10L (2018)	7.31
TPw 50L (2018)	7.22
TPw 100L (2018)	7.00
TW Caiño Longo (2015)	3.94
TW 2011 to 2013	Countless *
TW 2014	2.88
TW 2015	3.88

The most representative mean values of the two replicates for each dilution ranged from 0 to –5. * Uncountable owing to the presence of fungi. TM, Tostado must; TP, Tostado paste; TW, Tostado wine; r, red grapes (wine); w, white grapes (wine).

In the case of highly concentrated Tostado musts, only wines of acceptable quality are produced through spontaneous fermentation at low temperatures [8]. Since fermentation is an exothermic process, the temperature varied slightly between 15.0 and 17.5 °C in the different phases, with it higher during the tumultuous and final phases of fermentation. However, these temperatures were sufficiently low and optimal to guarantee the preservation of the aromas and desirable sensory attributes in the final toasted wines. In general, the number of viable yeast populations (log CFU/mL) was equal to or higher than that found in traditional wines (organic and conventional) produced in the same region and years [21].

Fermentations of white grape varieties using tanks with different volumes of must showed some differences. Even when starting from similar densities and CFU/mL, the higher the volume of fermentation, the higher the speed or kinetics of the fermentation. On the other hand, the red Tostado wine that had a high concentration of initial sugars in the must (44.1 ºBrix) and the lowest concentration of viable cells (6.49 CFU/mL), maintained a reduced fermentation speed, with a minimal decrease of 2 ºBrix after two weeks of fermentation. The low fermentation temperature could cause slow fermentation in Tostado wines compared to high temperatures of up to 28 °C [8]. However, because the temperature was similar in all cases, the fermentation volume factor was more influential, although because heat is generated during fermentation, the volume is directly related to the temperature increase in the fermentation tank. Moreover, musts with high sugar content (over 300 g/L), acids, polyphenols, metal ions, and other by-products can contribute to the stagnation of fermentation; therefore, it may be advisable to inoculate commer-

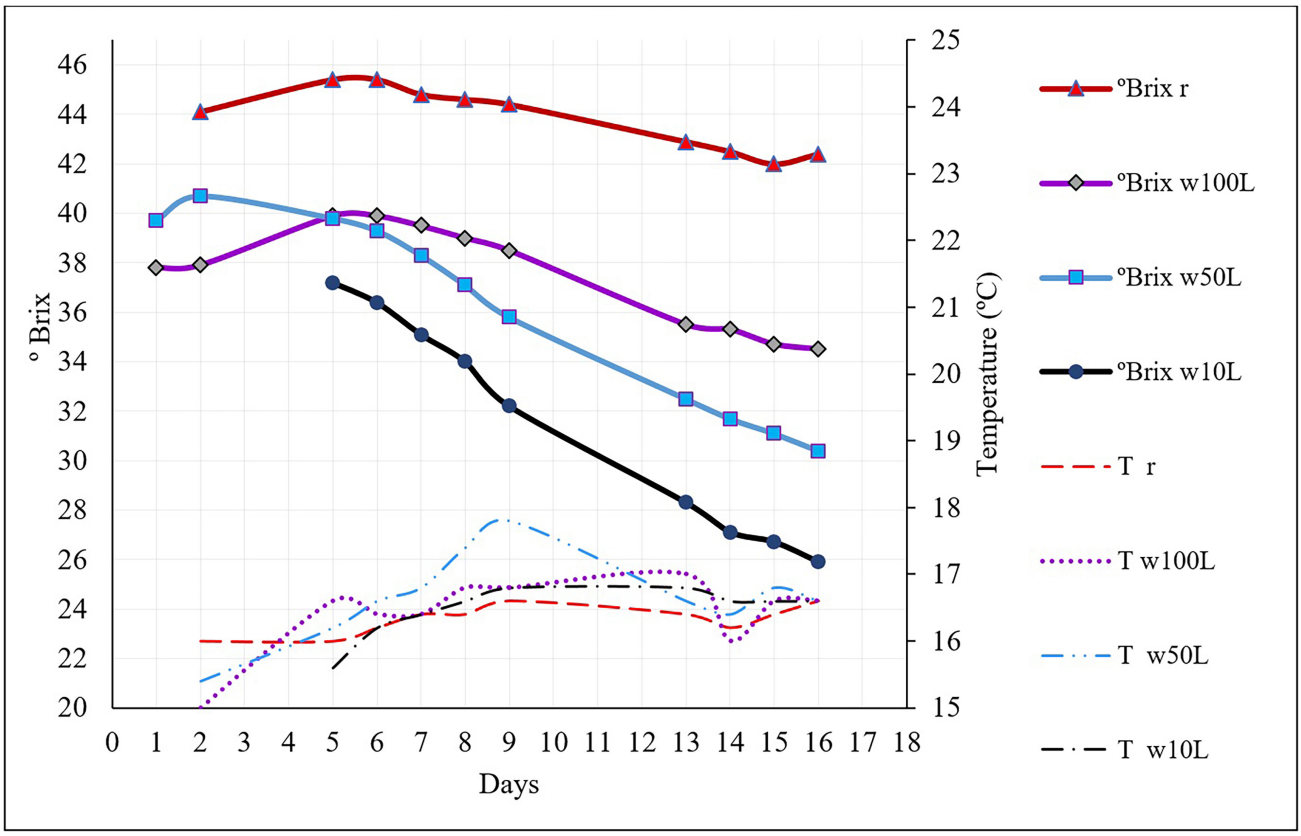

Fig. 1. Fermentation dynamics. Evolution of sugar concentration (Brix) and temperature (°C) throughout fermentation of Tostado wine. T, temperature; r, red grapes (wine); w, white grapes (wine); 10 L, 50 L, and 100 L: volumes of 10, 50, and 100 litres, respectively.

cial strains of active dry yeast (ADY), culture starters, or those selected in the winery from pure *S. cerevisiae* culture or in co-fermentation/sequential fermentation with other indigenous non-*Saccharomyces* species, such as *Lachancea thermotolerans*, *Starmerella bacillaris*, or *Torulaspora delbrueckii* [3,14].

Regarding the evolution of the yeast population during fermentation, the amount of viable yeast during the initial stage of fermentation was higher in white wines (6.8–7.0 log10 CFU/mL) than in red ones (6.1 log10 CFU/mL) (**Supplementary Fig. 1**). It also increased in the tumultuous phase and as the fermentation volume increased (7.1 to 7.5 log10 CFU/mL in 10 L and 100 L respectively). It should be noted that approximately the same number of different yeast species was identified for all wines except for the 100 L white wine, which showed higher yeast diversity. This was because a higher fermentation volume increased the diversity of minority species such as *Metschnikowia* spp. and *Pichia* spp. In addition, higher diversity was found in the initial phase than in the tumultuous fermentation phase. This is explained by the fact that, in the final stages of Tostado fermentation, only the most ethanol-tolerant yeasts, such as *Starm. bacillaris*, *T. delbrueckii*, and the different strains of *S. cerevisiae* survived. Finally, there was no distinction in the number of isolates between the red and white wine types, as the toasted process was the same regardless of the variety (there was a release of must and continuous contact of the microbiology of the skins with the must and paste at all stages). However, there was a higher proportion of *Candida* spp. in red wine.

3.2 Yeast Diversity

As the number of samples studied varied according to the availability of each year, four stages of the Tostado winemaking process were considered: Grapes (TG), must (TM), paste (TP), and wine (TW). Therefore, biodiversity indices were calculated using the set or total data available at each stage to provide a global approach. Approximately 230 representative isolates were analysed, considering all samples from different years.

Genetic characterisation of the isolated yeasts allowed the identification of 16 yeast species, including *S. cerevisiae*. In addition, 29 different strains were identified within *S. cerevisiae*. These yeasts were stored in an EVEGA yeast collection.

As biodiversity indices are sensitive to species richness and frequency, different indices may be more suitable for different types of data or research questions. The different indices showed that the fermentation stage factor (TG, TM, TP, and TW) influenced yeast diversity in terms of both species richness (S) and frequency (k) in the whole set of samples (Table 2).

Table 2. Biodiversity indices at each stage of Tostado winemaking: yeast species or strain richness (S), Shannon-Wiener index (H'), Simpson's index (1 - dominance), and equitability.

	Total	TG	TM	TP	TW
	Non-*Saccharomyces* yeasts by species richness (S)				
$H' = -\Sigma^S p_i \ ln(p_i)$	2.61	1.43	2.25	1.88	1.10
$E = H/log_2(S)$	0.94	0.89	0.98	0.97	0.99
$1 - D = 1 - \Sigma^S \ (pi)^2$	0.92	0.72	0.89	0.84	0.67
S*	16	5	10	7	3
	Non-*Saccharomyces* yeasts by frequency species (k)				
$H' = -\Sigma^k p_i \ ln(p_i)$	1.84	1.63	1.44	1.65	0.50
$E = H/log_2(k)$	0.66	0.91	0.54	0.69	0.36
$1 - D = 1 - \Sigma^k \ (pi)^2$	0.77	0.78	0.66	0.74	0.22
	Saccharomyces cerevisiae strains				
$H' = -\Sigma^S p_i \ ln(p_i)$	3.04	-	1.79	2.44	2.02
$E = H/log_2(S)$	0.90	-	0.97	0.87	0.88
$1 - D = 1 - \Sigma^S \ (pi)^2$	0.94	-	0.83	0.88	0.83
S*	29	-	6	5	10

* Yeast species or strain richness (S = the total number of yeasts or strains found) and proportion of yeasts (k) found in grapes (TG), must (TM), paste (TP), and wine (TW) in Tostado winemaking.

Thus, the values of the biodiversity indices were similar in both non-*Saccharomyces* isolates and *S. cerevisiae*, except for the Shannon index, which was slightly higher in *S. cerevisiae* (H' = 2.61 and H' = 3.04, respectively). However, although they were sufficiently high, biodiversity was observed to be cumulative, as some species or strains differed in each fermentation (year). Therefore, the diversity values in grapes, musts, and organic and conventional wines from traditional Galician wineries and their vineyards were similar to those found in previous studies for the same species richness [21,22] and other regions worldwide [23–25].

Nonetheless, it is important to note a significant particularity: Variation in the yeast population during the winemaking stages. The presence of *S. cerevisiae* and the high diversity of non-*Saccharomyces* in the pre-fermentative stages marks a difference compared to standard wines. In addition, the values of total diversity and the fermentative phase that showed the highest values in the indices (TM and TP) were not very different, indicating that diversity during the key fermentative process in forming aromas and desirable sensory elements was optimal. Considering that most of the species identified in these phases have oenological potential (Fig. 2) and the sufficiently high diversity values found in the indices (Table 2), we can affirm that there is a high differentiation of yeasts in Tostado wines with changes in each fermentation (year). Considering the richness and frequency of non-*Saccharomyces* species, the Shannon-Wiener index showed the lowest values in the final phase of the Tostado wine (H'_{TW} = 1.10). Indeed, the diversity of grapes (H'_{TG} = 1.43) was not as low as that found in our previous studies of healthy grapes (not raisins) [26]. This was expected because the non-fermentative yeasts present

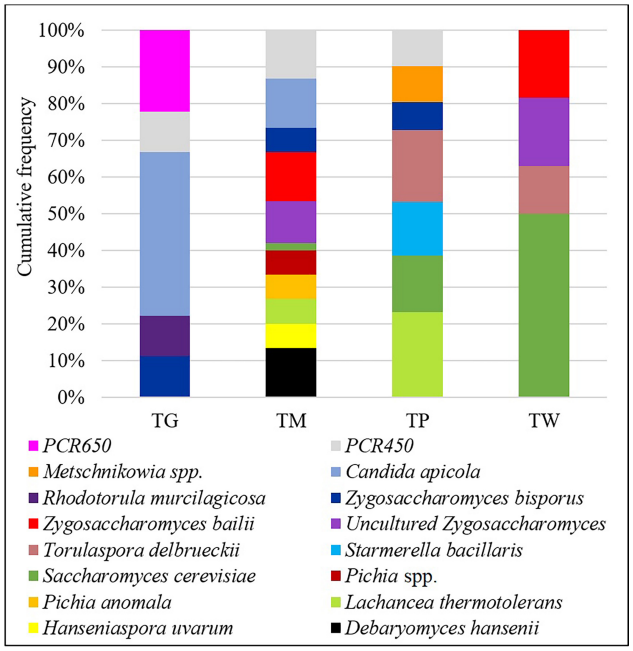

Fig. 2. Yeast species richness. The proportion of yeast species present in the different phases of Tostado wine elaboration. PCR, polymerase chain reaction.

in the skin and green parts of the bunches and the plant share a niche with the weakly fermentative yeasts in the raisin grape phase, as they begin to have availability of sugars due to microfissures, damage to the skin or insects that allow their growth [27]. This release increased in the must phase (H'_{TM} = 2.25), allowing the growth of colonies of weakly fermentative and fermentative yeast species to a greater extent than would be found in wine made through con-

ventional winemaking. Subsequently, in the fermentative phases of Tostado wine production, the population of non-fermentative and weakly fermentative yeasts decreased in favour of fermentative species (H'_{TP} = 1.88) until the final phase of Tostado wine mentioned above, mainly because of the high concentration of alcohol present in Tostado wines (up to 15% vol.).

On the other hand, biodiversity indices varied and generally decreased when species frequency was considered.

The main difference was found in the lower value of H'_{TM} = 1.44. This suggests that, despite the higher species richness of non-*Saccharomyces* in the must, compared to the fermentative phases of the Tostado paste, this diversity was reduced when considering their proportion. Furthermore, as estimated, both equitability and the Simpson index were high when only species richness (presence or absence) was considered. Conversely, when considering species frequency, these values showed greater dominance.

However, when studying *S. cerevisiae* strains exclusively, some peculiarities were found. As expected, the diversity of *S. cerevisiae* strains was slightly higher in the paste or fermentative phase (H'_{TP} = 2.44) than in the must phase (H'_{TM} = 1.79). The large amount of sugars and nutrients in the paste favours the growth of multiple strains of *S. cerevisiae* that compete with the rest of the yeast species because of their high fermentative capacity and ethanol tolerance. Nevertheless, not all of these strains would be in most of the fermentation processes, as diversity decreased during fermentation until the final stage of the wine (H'_{TW} = 2.02). This indicates a replacement of the yeast population in which some strains give way to others, possibly due to factors such as decreased nutrient concentration, competition, ethanol tolerance, temperature, dominance, or killer factor [27–30]. Furthermore, the equitability or even distribution values of strains (E_{TM} = 0.97, E_{TP} = 0.87, E_{TW} = 0.88) and dominance (D_{TM} = D_{TW} = 0.167, D_{TP} = 0.119) supported this deduction. The lower dominance (higher 1 - D value) in the paste indicates fewer dominant strains (greater strain diversity). However, the equitability was the same as in the wines, indicating an even distribution of strains.

Furthermore, the SIMPER analysis identified that the species contributing most to the differences in the fermentative process of the Tostado wine had oenological importance (Table 3), with the most relevant (>5%) being *L. thermotolerans*, *S. cerevisiae*, *Starm. bacillaris*, *Candida apicola*, *Metschnikowia* spp., and *T. delbrueckii*. Rationally, the species with the highest fermentative power were predominant in the final stages of fermentation, which agrees with our previous studies [14,21,22]. It is also important to note that the majority of species found in previous studies on grapes and musts, such as *Aureobasidium* spp. or *Cryptococcus* spp. (non-fermentative yeasts common in grapes and freshly pressed musts), were not even found in the grapes or musts of Tostado [3,22,27], particularly in comparison with the same DO Ribeiro, where the rest of the yeast species were more typical of the DOs Ribeira Sacra and Rías Baixas with greater diversity. This could be due to the release of the must during raisining, which favours the growth of weakly fermentative species.

3.3 Occurrence of Non-Saccharomyces Species

As depicted in Fig. 2, 15 different non-*Saccharomyces* species were identified, with the most abundant species richness and frequency found in TM and TP, as suggested by the biodiversity indices. In addition, two species identified with 450 bp and 650 bp PCR could not be sequenced because of their fragile survival in the laboratory, although their genetic profile in the enzymatic digestion allowed us to consider them as different species from the rest.

However, although not all species have winemaking potential (for example, *Rhodotorula mucilaginosa*) and fail to thrive in the early stages of fermentation, most of them could benefit Tostado winemaking differentiation. For instance, weakly fermentative species, such as *Hanseniaspora uvarum*, *Zygosaccharomyces* spp., *Pichia anomala*, or *C. apicola*, which were majority in grapes and must, have recently been proposed in mixed or sequential fermentations for contributing aromatic precursors to the musts, or potential cultures starters such as enzymes in winemaking [31,32]. *Debaryomyces hansenii* has been studied for several decades as a model to understand salt and osmotic tolerance and is now also a candidate in the era of green biotechnology in bioreactors [33]. However, there has recently been growing interest in the role of *D. hansenii* as a co-culture partner of *S. cerevisiae* during grape must fermentation. This is due to its oenological potential to enhance the aroma-active composition of wine, which is attributed to its enzymatic compounds, such as β-glucosidases [34–36]. On the other hand, during fermentation (TP), non-*Saccharomyces* species such as *Metschnikowia* spp., *L. thermotolerans*, *Starm. bacillaris*, and *T. delbrueckii* have been widely reported as having high oenological potential due to the formation of desirable aromatic compounds, such as esters, acids, terpenes, higher alcohols, acetates, phenols, thiols, and lactones [37–40]. Finally, it is worth noting that the presence of some species, such as *Zygosaccharomyces* spp., in the wines (TW) should be considered for the stability of the most artisanal unfiltered wines. All this yeast diversity indicates that there is a presence of non-*Saccharomyces* from the initial stages of fermentation (pre-fermentative in the raisining of healthy grapes and pressing of musts) and throughout the entire process (with longer kinetics than conventional wines), which allows a greater release of compounds and influence of these yeasts than in standard wines. Microbiology in Tostado wines is a key factor that is much more relevant than in industrial wineries that use only common strains of commercial *S. cerevisiae* during fermentation. If fermentation is carried out using control of densities, temperatures, sulphur dioxide, aeration

Table 3. Similarity percentage (SIMPER) analysis.

Yeast species	Average dissimilarity	Contribution %	Cumulative %	Mean TM	Mean TP
Lachancea thermotolerans	8.20	11.42	11.42	0.07	0.23
Saccharomyces cerevisiae	8.11	10.70	22.12	0.00	0.15
Starmerella bacillaris	8.01	10.22	32.34	0.00	0.14
Zygosaccharomyces spp.	7.89	10.13	42.47	0.14	0.00
Debaryomyces hansenii	7.54	9.38	51.85	0.13	0.00
Candida apicola	7.41	9.10	60.95	0.13	0.00
Metschnikowia spp.	5.98	7.44	68.39	0.00	0.10
Torulaspora delbrueckii	5.34	7.12	75.51	0.00	0.09
Hanseniaspora uvarum	4.45	5.66	81.17	0.06	0.00
Pichia spp.	4.09	5.47	86.64	0.06	0.00

Contribution (>5%) of the different yeast species to the total similarity or dissimilarity between pairs and groups of samples of the oenologically most relevant phases in Tostado fermentation (group 1: TM and group 2: TP) using a Bray–Curtis distance/similarity measure.

or oxygen levels and hygienic practices to prevent the proliferation of spoilage yeasts, all this could result in greater complexity and differentiation of Tostado wines. Moreover, co-inoculation with the osmotolerant *Starm. bacillaris* and *Z. bailii* with *S. cerevisiae* have been suggested as a solution for sweet wine winemaking (made from raisin grapes) with higher ethanol and lower acetic acid contents [3].

3.4 Occurrence and Genetic Evaluation of S. Cerevisiae Strains

Saccharomyces yeast cannot grow on a lysine medium because they cannot metabolise lysine as the sole nitrogen source [41]. For example, in 2014 wines, the lysine test showed 25% of non-*Saccharomyces* yeast species (lysine positive). However, in 2015 wines, the percentage of non-*Saccharomyces* (lysine positive) increased to 76% of yeast species. It should be noted that, in general, yeast diversity in 2015 was higher than in 2014, influenced by climatological factors [21,42]. This is a major difference from conventional industrial wines, where most of the yeasts in the wines are *S. cerevisiae* and, in many cases, are the same or include very few strains. Overall, a great diversity of strains within *S. cerevisiae* species was found in Tostado wines.

From the results of the lysine agar and considering all vintages, 120 strains of *S. cerevisiae* were selected. Of these, more than 80 representative isolates were analysed by mtDNA-RFLP, obtaining differentiated genetic profiles (Fig. 3). Genetic characterisation of the yeast isolates allowed the identification of 29 strains of *S. cerevisiae*.

Although 29 different *S. cerevisiae* strains were identified, detailed graphical and phylogenetic studies were performed on the 19 strains most clearly identified on the gels to reduce the subjective risk of assigning the presence or absence of bands. It should be considered that some strains remained in the winery or were repeated in wines of different vintages, such as strains 4, 5, 6, or 17; however, others were not. They likely had a wide geographical distribution and were found in wineries of different DOs from Galicia [18]. Comparing the strains of *S. cerevisiae* with these previous studies, we found that approximately 14% of the strains were genetically identical or similar to those found in other DOs from Galicia. However, this proportion increased to 22% when strains from DO Ribeiro were identified. From the matrix with the unified *S. cerevisiae* isolates, graphical statistical analyses such as PCA and phylogenetic trees were performed to understand the genetic similarity between the different strains. In addition, the presence and relationship between different types of samples were studied.

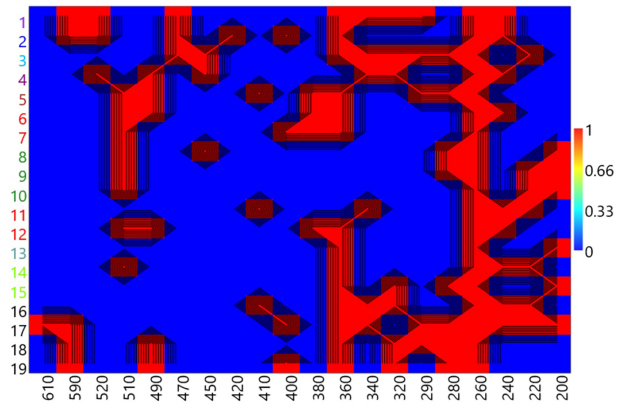

Fig. 3. Matrix plot of identified *S. cerevisiae* isolates. Mapping of 19 different *S. cerevisiae* strains generated from the presence or absence of bands between 200 and 610 bp on agarose gels.

The PCA depicted in Fig. 4 showed a clear clustering among strains of each sample type at the genetic level. This suggests that the viable *S. cerevisiae* strains or those involved in each stage of the fermentative process in Tostado winemaking are distinct.

In the same way, despite the slight differences in grouping between the cladogram and the phenogram in Fig. 5, we can observe the genetic relationship between

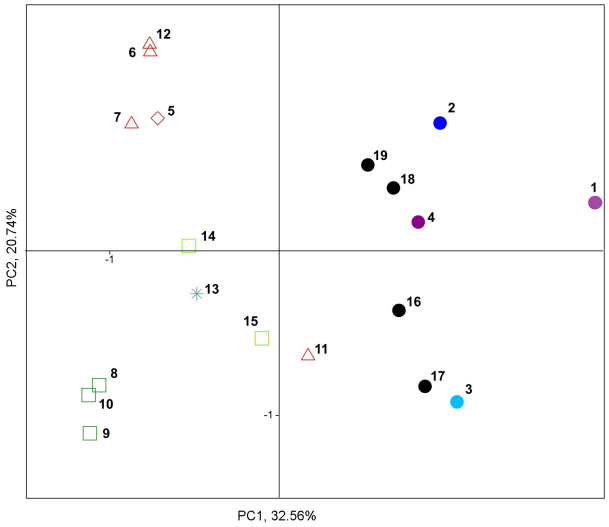

Fig. 4. Principal component analysis (PCA) of the genetic profile of *S. cerevisiae*. Principal component analysis of 19 genetically different *S. cerevisiae* strains identified in the must, paste, and Tostado wine samples. Circle: Tostado wine; diamond: in both Tostado paste and wine; triangle: Tostado paste; square: Tostado must; asterisk: in both Tostado must and wine. Different colours indicate different vintages within the same type of sample.

some of the strains. This confirms the results obtained in the PCA between strains associated with the sample type and year. For instance, strains 5, 6, 7, and 12 (TP), strains 8, 9, and 10 (TM), and strains 18 and 19 (TW) are genetically similar. Other strains, such as 5 and 13, were even found in two sample types (in both TP + TW and TM + TW, respectively), demonstrating their survival throughout the complex Tostado winemaking process. Moreover, in this regard, some strains, such as 11, indicate a genetic similarity to other *S. cerevisiae* strains found in the three types of samples analysed. The presence of prevalent strains of *S. cerevisiae* has been reported in other studies and is typical of selected commercial strains. Therefore, toasted vinification could be an optimal and useful means for the natural selection of indigenous strains of good differentiation in oenological suitability [18,43–45].

Finally, to try to clarify the phylogenetic relationships, a network plot was created (Fig. 6). Strains 6–7, 8–9–10, 11–16, and 18–19 showed the highest intensity or thickness traces in their connections indicating a higher genetic similarity between them. In addition, some strains showed or did not show linkages between them at the level of their mitochondrial genetics, i.e., a measure of the distance of interconnections at the neural network level between the 19 different *S. cerevisiae* strains studied. Considering all the above, the genetic factor is thus confirmed as a distinguishing characteristic of *S. cerevisiae*. In this regard, it can be inferred that, given that the formation of desirable volatile aromatic compounds is strain-dependent, Tostado wines could owe a portion of their distinct quality to the microbiology present throughout the winemaking and fermentation process, starting as early as the must–paste phase [46–48].

According to the above, the longer fermentation time, the long winemaking process, and the presence of a great diversity of yeasts from the raisining stage imply a long prefermentation maturation phase. This could be of great relevance in the specific sensory characteristics in the visual, aroma, and taste of Tostado wine, such as its amber colour, its nutty, citric and dry grape aroma and body, and fresh and persistent taste [5,8].

3.5 Chemical Analysis of Tostado Wines

In the chemical analysis of wines, ANOVA showed significant differences at $p = 0.01$ between the wines for the analysed overall chemical parameters (Table 4).

According to the specifications of the Regulatory Council of the DO Ribeiro in Section 2.a.8 Ribeiro Tostado [49], all analysed wines met the necessary parameters, except for residual sugars: The natural total alcoholic degree acquired remained between the maximum (20.6% vol.) and minimum (13% vol.) values, and the maximum volatile acidity (2.1 g/L expressed in acetic acid) was not exceeded. Similar Tostado wines, spontaneously fermented, have even been reported with lower concentrations of acetic acid (around 0.83 g/L) [8]. However, the minimum content of total sugars in all wines was below the required 120 g/L (expressed in glucose plus fructose). Some previous works state that in the Galician standard that regulates the production of Tostado wine, the residual sugar concentration must be higher than 70 g/L [5,8]. The observed reduction in sugar content could be attributed to an extended period of fermentation at low temperatures, which, as in a previous study, can reach up to 50 days; metabolic effects and/or spontaneous microbiota are also proposed as possible reasons for the preferred use of fructose at low temperatures [8]. This might account for the increased total acidity and the high diversity of the *S. cerevisiae* strains in the wines. Other factors, such as sugar consumption by strains with a low alcohol yield, are less likely to be the cause, given that all wines exhibited similar characteristics over several consecutive years and alcohol was produced (nor did the fermentation stop spontaneously). Results are also inconsistent with *Botrytis cinerea* infection, which has been attributed to the effect of raisin microbiota as a biocontrol agent [50,51]. Moreover, total acidity was not excessive and remained similar (6.0 to 7.2 g/L) to that found in raisins in other works [13,50,52] and in previous studies (6.5 to 8.0 g/L) that we conducted on wines from sequential fermentations of indigenous non-*Saccharomyces* and *S. cerevisiae* strains [14]. In this regard, climatic conditions affected the balance between sugars and acidity, especially in the Tostado process, with yeast strains such as *T. delbrueckii*, *Starm. bacillaris* or *L. thermotolerans* are being used to limit the undesirable effects of climate change [52–54].

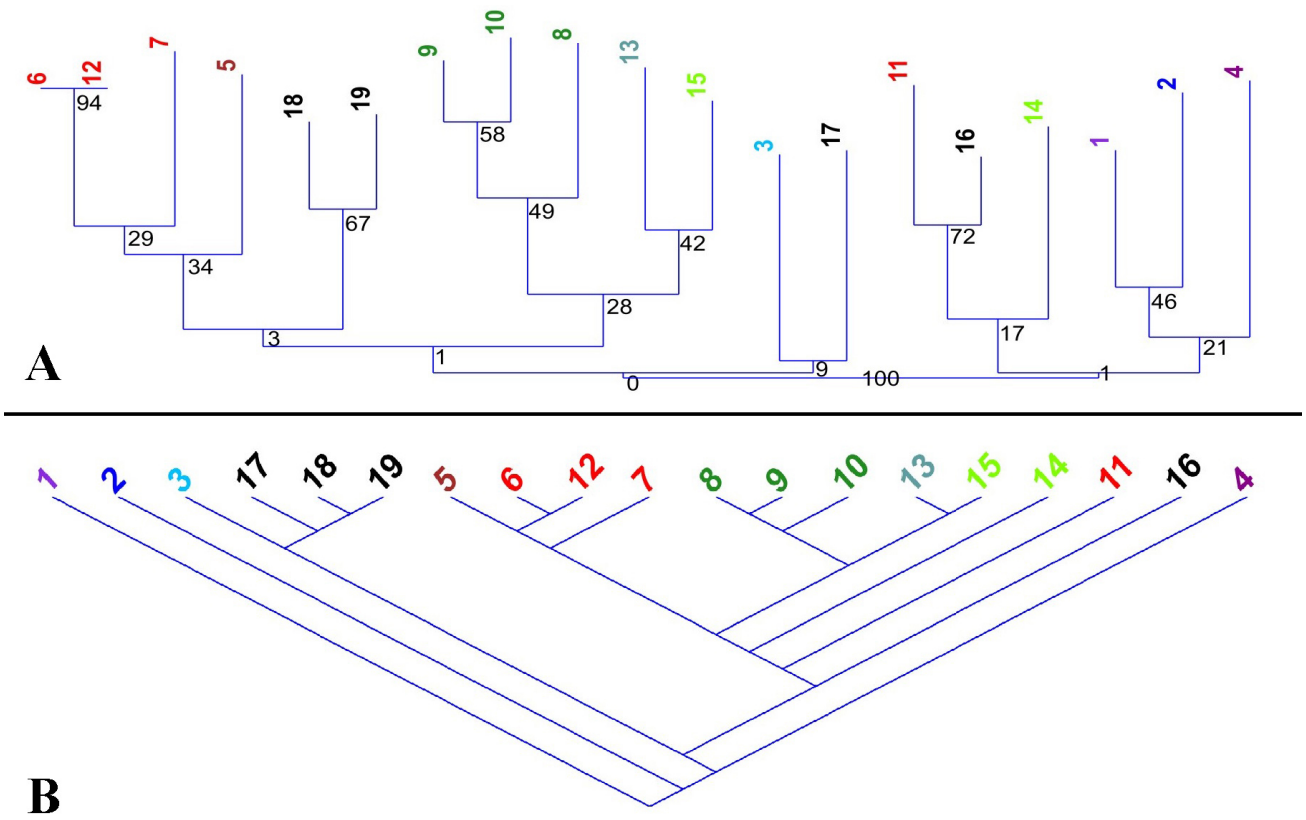

Fig. 5. Phylogenetic trees of 19 *S. cerevisiae* strains. (A) Phenogram (neighbour-joining clustering) using Jaccard's index and (B) cladogram (parsimony–phylogram) of genetic clustering of 19 genetically different *S. cerevisiae* strains identified in the must, paste, and Tostado wine samples.

Table 4. Chemical analysis of Tostado wines.

Parameter*	Caiño doce	TW 2011	TW 2012	TW 2013
Total acidity (g tartaric/L)	8.4	7.1	7.4	6.0
Volatile acidity (g acetic/L)	1.32	1.58	2.10	1.47
Reducing sugars (g/L of glucose + fructose)	59.0	78.5	95.5	83.5
Alcoholic strength by volume (%vol.)	16.4	14.2	14.6	15.2

TW, Tostado wine. *All parameters showed significant differences at $p = 0.01$ (ANOVA, analysis of variance).

Considering the most important chemical parameters, the PCA in Fig. 7 shows how Tostado wines were separated into different quadrants (with the 2011 and 2013 toasted wines being the most similar and remaining in the same quadrant). Although there are only a few samples, it is possible to detect chemical differences in the wines between vintages, even though the winemaking techniques were the same. This distinction could be largely due to the microbiology present in the different stages of winemaking. Other studies on previously macerated wines support the evidence that indigenous *S. cerevisiae* strains well adapted to climatic conditions can be used to produce quality wines versus commercial ADY [55]. Despite the limited data not allowing for significant correlations between the chemical parameters and the contributions of each yeast (as these are strain-dependent), we can provide some observations on the yeast species that may have contributed to the enological parameters of the analysed wines. Most non-*Saccharomyces* species were generic: *L. thermotolerans*, *S. bacillaris*, *H. uvarum*, and *D. hansenii*. However, in each wine, there was a predominance or exclusivity of the yeasts concerning the others: *S. cerevisiae* strains 1, 2, 3, and 4 in Caiño Longo; *Z. bisporus*, *U. zygosaccharomyces*, and *S. cerevisiae* strain 13 in TW 2011; *S. cerevisiae* strains 1 and 2 in TW 2012; PCR450 yeast in TW 2013. Therefore, it is not cautious to attribute a correspondence between a specific strain or yeast and a chemical parameter of the Tostado wine. Instead, it would be about multiple factors, including microbial interrelationships in a medium with a high sugar concentration from raisining (before the fermentation begins), resulting in a high alcohol concentration in the Tostado wine. Although during preliminary

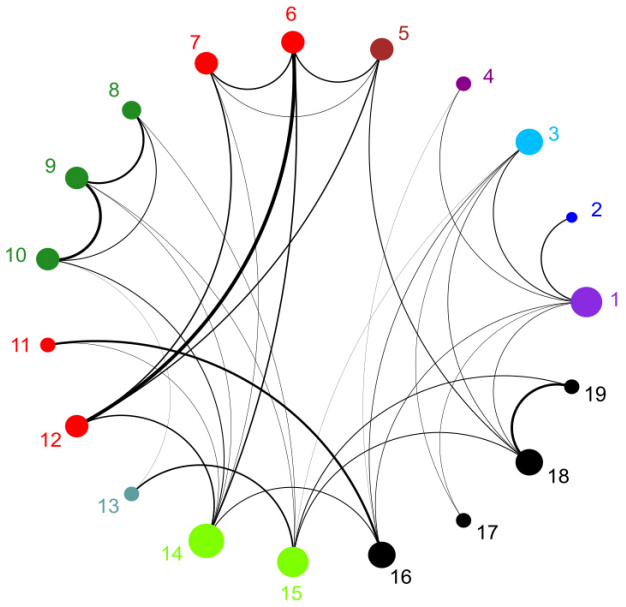

Fig. 6. Network plot of 19 *S. cerevisiae* strains. Network plot performed using the Dice similarity index, with scale nodes by n edges 362 and scale edges by the similarity between the 19 *S. cerevisiae* strains identified. Traces of greater intensity or thickness are shown as the 363 greater similarity or distance measure of the interconnections between the different strains of *S. cerevisiae* at the neural network level.

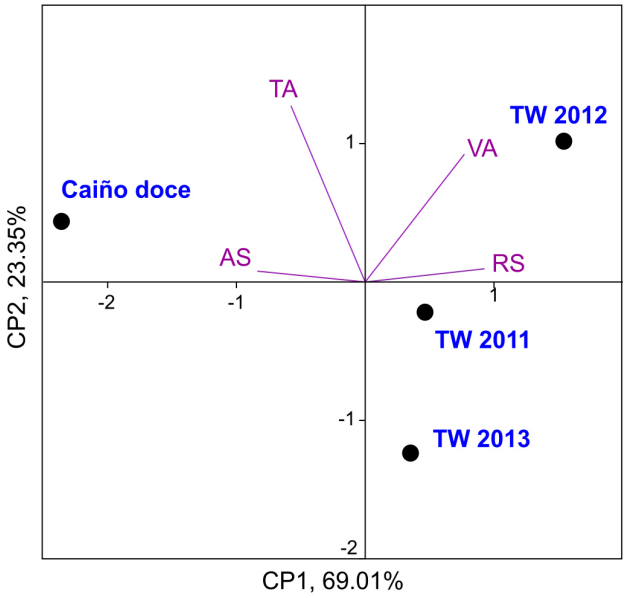

Fig. 7. PCA of the chemical analysis of Tostado wine. Principal component analysis of the most significant chemical parameters analysed in Tostado wines produced between 2011 and 2015. TA, total acidity; VA, volatile acidity; RS, reducing sugars; AS, alcoholic strength; TW, Tostado wine; CP, principal component.

tests and previous studies, we observed that the addition of *Starm. bacillaris* or *T. delbrueckii* can reduce the alcohol content and increase the total acidity of wines. Fermentation with *T. delbrueckii* also effectively limits the volatile acidity concentration in musts with a high sugar concentration, such as Tostado [14,53]. The killer strains of *T. delbrueckii* can dominate and complete fermentation in the presence of wild *Saccharomyces* yeasts despite having less fermentative vigour and a lower growth rate [56].

The oenological parameters of these wines from locally selected yeast strains showed lower levels of volatile acidity and a higher concentration of aromatic compounds than those of commercial strains. Indeed, the indigenous yeasts in the fresh must and paste might not have good oenological potential or might be stressed (due to high osmotic conditions) or suffer from stuck fermentation with the presence of spoilage yeasts resulting in low-quality Tostado wine (high residual sugar content, low alcohol content and undesirable aromas such as "solvents/chemicals") [3]. Therefore, more research is required on the chemical and sensory aspects.

Overall, "Vino Tostado" from the DO Ribeiro is so special that even the shape of the glass has been suggested as an influential factor in the perception of the smell and taste, with the G-type glass being the one recommended by tasters for consuming it [10].

4. Conclusions

This preliminary study is the first to describe the yeast population found in Tostado wine from the Ribeiro DO over several years. The findings of this study revealed that spontaneous fermentation in the production of Tostado wine exhibited a high diversity of both non-*Saccharomyces* and *S. cerevisiae* strains throughout all stages of the intricate winemaking process, from grapes, must, and paste to the wine itself. Despite the high strain diversity, only a few of them recurred over the years; a significant incidence was found in the richness, distribution, and frequency of non-*Saccharomyces* species with high oenological potential in both must and paste phases, indicating a high presence or pre-fermentative capacity that contributes desirable aromatic parameters. This yeast diversity throughout the entire fermentation process, superior to conventional wines in pre-fermentation phases, could be responsible for or contribute to increasing the complexity of the wines and, therefore, to their sensory differentiation in the distinctive quality of these special sweet wines. In addition, some strains of *S. cerevisiae* and non-*Saccharomyces* progressed favourably throughout the entire process, which could be a natural reservoir of yeasts to exploit with a high oenological potential differentiating from traditional commercial strains (LSA) with less differentiating characteristics. However, this general preliminary study does not delve into the influence of other factors that should be explored, such as the year, chromatographic chemical evaluation of aromas or sensory evaluation. Indigenous yeasts in fresh must and

paste may lead to low-quality Tostado wine due to potential yeast stress and spoilage, indicating a need for further chemical and sensory research.

Abbreviations

ADY, Active Dry Yeast; ANOVA, analysis of variance; CFU, colony forming units; DO, Designation of Origin; E, equitability; H', Shannon-Wiener index; ITS, Internal Transcribed Spacer; k, yeast species frequency; mtDNA, mitochondrial DNA; PCA, principal component analysis; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; S, yeast species richness; SIMPER, similarity percentage analysis; TAE, Tris-acetate-EDTA buffer; TG, Tostado grape; TM, Tostado must; TP, Tostado paste; TW, Tostado wine; WL, Wallerstein Laboratory Nutrient Agar.

Availability of Data and Materials

All data are available as indicated in the manuscript. The data utilized and/or examined in the present study can be obtained from the corresponding author upon a reasonable request.

Author Contributions

DC and PB designed the research. DC and PB performed the research. DC analysed the data. DC wrote the manuscript. Both authors contributed to the editorial changes in the manuscript. Both authors have read and approved the final manuscript. Both authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbe1601010.

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