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Original Research

Characterization of Indigenous Non-*Saccharomyces* Yeast Strains with Potential Use in Winemaking

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Abstract

Background: The vineyard is a great reservoir of autochthonous yeast strains whose composition is defined by different regional (edaphology, orography or climatology) and anthropological factors (cultivation systems or cultural practices). Most of this yeast diversity corresponds to non-*Saccharomyces* strains, some of which have potential use in winemaking. **Methods**: The oenological potential of 29 different native non-*Saccharomyces* strains belonging to 4 species (*Lachancea thermotolerans*, *Torulaspora delbrueckii*, *Starmerella bacillaris* and *Metschnikowia* spp.) was evaluated, using the autochthonous *Saccharomyces cerevisiae* XG3 strain as a control. Microfermentations with pure culture of each strain were performed in duplicate and the basic parameters and major volatiles of wines were analysed following official methodology. The best strain within each species was selected using a quantification matrix including the relevant oenological characteristics. **Results**: The fermentative ability of non-*Saccharomyces* was lower than *S. cerevisiae* in all cases, but with differences among species. *L. thermotolerans* and *T. delbrueckii* showed higher fermentation rates than *Starm. bacillaris*, whereas *Metschnikowia* spp. presented a low fermentative power. At chemical level all non-*Saccharomyces* strains reduced the alcoholic content, the higher alcohols and the volatile acidity of wines and increased the content of glycerol, with differences among strains within a given species. *T. delbrueckii* and *L. thermotolerans* increased the total acidity of wines. The latter and *Metschnikowia* spp. strains produced lactic acid, which decreased the wine pH in the case of *L. thermotolerans*. According to their oenological traits the best rated strains of each species were Lt93, Td315, Mf278 and Sb474. In addition, the data obtained in pure fermentations were correlated to those chemical and aromatic compounds obtained with these non-*Saccharomyces* strains in sequential fermentations. **Conclusions**: Autochthonous strains of non-*Saccharomyces* yeast species contribute distinctive chemical characteristics to the wines. The correlations observed between wines fermented with the different non-*Saccharomyces* indigenous strains in pure and sequential fermentations suggest that their contribution to wine properties remains stable regardless of must composition or winemaking techniques.

Keywords: non-*Saccharomyces*; autochthonous yeasts; chemical composition; Pearson correlation; *Lachancea thermotolerans*; *Torulaspora delbrueckii*; *Starmerella bacillaris*; *Metschnikowia*

1. Introduction

Nowadays, there is a global trend towards differentiation and typicity of quality wine which is of great importance in commercial terms and as a generator of socio-economic value. However, in a context where wine regions are facing challenges such as the consequences of climate change, competition, market evolution or the development of new products, they require the use of new tools. In the last decades, different biological approaches as alternative strategies have been proposed to enhance complexity and aromatic profile of wines as well as to solve technical problems on analytical parameters such as ethanol or acidity [1–3]. Among these tools, indigenous non-*Saccharomyces* yeasts have been used to improve the quality and distinctiveness of wines associated with a given region [4–6].

In addition, non-*Saccharomyces* yeasts have shown other characteristics of oenological relevance such as antimicrobial activity, bioprotective action, and changes in the production of compounds such as ethanol, glycerol, or lactic acid. In particular, *Torulaspora delbrueckii* has shown capacity to increases the concentrations of desired aroma compounds of wine, to reduce the final ethanol content, to act against spoilage yeasts and to decrease levels of acetic acid and biogenic amines [1,7,8]. *Lachancea thermotolerans* is characterized by a low production of volatile acidity; it shows a high production of lactic acid, sometimes decreasing wine pH during fermentation, has relevant potential to reduce ethanol content and improves the production of 2-phenylethanol and glycerol [3,9,10]. Other studies have shown that the presence of *Starmerella bacillaris* increased the level of glycerol, and, remarkably, reduced acetaldehyde and total SO_2 in wines [2,3,11,12]. On the other hand, *Metschnikowia* spp. promoted the formation of higher alcohols and esters, reduced volatile phenols, and showed enzymatic activities involving aromatic and colour precursors, and potential antimicrobial activity against spoilage yeasts and fungi [3,13,14].

Most of these properties of non-*Saccharomyces* yeasts are strain-dependent; thus, the knowledge of the oenological potential of autochthonous strains is a valuable aid to

their application. There is a great interest in the elaboration of wines with specific characteristics linked to a production area. This tendency has led to a rediscovery of the fermentation directed by native yeasts to obtain differentiated quality wines [4,15]. In this sense, it is known that continued use of commercial strains can lead to the loss of complexity and the wines standardization, together with the decrease of yeast species diversity in the winemaking environments. Therefore, despite the availability of commercial cultures, the use of native non-*Saccharomyces* but also indigenous *Saccharomyces cerevisiae* strains can confer a specific aromatic imprint to the wine due to the metabolic interaction between *S. cerevisiae*/non-*Saccharomyces* strains in mixed starter cultures [9,16–19] contributing uniqueness to the wines in preserving the regional character from a given territory as a biogeographic or microbial terroir [4–6,20,21]. However, not all native strains show the desired oenological aptitudes. In this context, it is necessary to search for the oenological aptitudes of a wide range of strains. This study evaluated the potential of 29 different autochthonous strains within *Metschnikowia* spp., *L. thermotolerans*, *T. delbrueckii* and *Starm. bacillaris* species from the yeast culture collection maintained at Estación de Viticultura e Enoloxía de Galicia (EVEGA-AGACAL). Each strain was evaluated in pure fermentations and the results of the fermentative ability, and the chemical composition and aromatic profile of the resulting wines were used to select the best rated strains from each species. In addition, the results were correlated with those obtained in sequential fermentations of the selected strains with the autochthonous strain *S. cerevisiae* XG3 and with the commercial strain EC1118 to confirm the contribution of a given strain to wines.

2. Materials and Methods

2.1 Origin of Yeast Strains and Sampling Design

Yeast strains used in this study were obtained from the yeast culture collection maintained at EVEGA-AGACAL. The autochthonous yeast strains were isolated from grapes and musts from organic and conventional vineyards in Galicia over several years, identified as explained in [22] and stored at EVEGA. Within this yeast collection, 29 non-*Saccharomyces* strains belonging to *Metschnikowia* spp. (10 strains), *Starm. bacillaris* (10 strains), *L. thermotolerans* (6 strains) and *T. delbrueckii* (3 strains), were chosen due to the oenological interest of these species in recent literature [2,3,23,24]. *Metschnikowia pulcherrima* and *Metschnikowia fructicola* are two genetically very close species with a very similar oenological potential that are difficult to identify, so they have been considered in the same group [13]. Depending on the availability of indigenous strains isolated from each of the relevant species mentioned above, between 3 and 10 isolates were evaluated (Table 1). The use of strains isolated from different Denominations of Origin (DOs) and from different plots and cultivation systems within the same species allowed to explore different oenological behaviours to select the most appropriate ones. In addition, the *S. cerevisiae* XG3 strain, previously tested and selected at EVEGA, was used as a control [25,26].

2.2 Yeast Fermentation

The original culture of each yeast strain was streaked on WL nutrient agar medium to isolate single colonies. Then, a single colony was used for pre-inoculum growth (for 24 h at 28 °C) with the aim of obtaining sufficient biomass of fresh growth. The number of cells in the preinocula was quantified by measurement of the optical density at 600 nm in a spectrophotometer. Microfermentations were carried out in duplicate, using 75 mL of pasteurized (10 min at 100 °C) white grape must in 100 mL sterilized bottles closed with sterile-venting membrane screw caps. The strains in pure culture were inoculated in flame-sterile conditions at a concentration of 5×10^6 CFU/mL in the pasteurised must. White thawed must (obtained from a mix of traditional white grape cultivars from Galicia) with the following characteristics was used: probable alcohol content: 11.5 %vol., total acidity: 6.3 g tart. L^{-1}, malic acid: 4.1 g·L^{-1}, tartaric acid: 3.1 g·L^{-1}, sugars: 188 g·L^{-1}, probable alcoholic strength: 11.5 %vol., soluble solids: 19.6 °Brix, and total SO_2: 15 mg·L^{-1}. The chemical analyses of the must after pasteurization remained constant; only malic acid (4.2 g·L^{-1}), tartaric acid (3.0 g·L^{-1}) and total SO_2 (8 mg·L^{-1}) parameters varied slightly.

Fermentations were carried out at a controlled temperature of 17 °C ± 1 °C for 31 days. The fermentative ability was monitored by daily measurement of weight loss caused by CO_2 evolution. At the end of fermentation, the presence of the added strain in each assay was confirmed by spreading 100 μL of wine on WL medium to assess colony morphology.

2.3 Chemical Analysis

Wines were analysed in the chemistry laboratory of EVEGA immediately after the end of fermentation. General parameters (total acidity, volatile acidity, lactic acid, malic acid, glucose + fructose, glycerol, alcohol content and pH) were determined by Fourier transform infrared spectrometry (FTIR) using a Wine Scan FT120 analyser (FOSS Electric, Barcelona, Spain) calibrated according to [27]. In addition, the ethanol yield was calculated as the ethanol production (g·L^{-1}) per sugar consumption (g·L^{-1}).

Volatile compounds including higher alcohols (methanol, propanol, isobutanol, 1-butanol, 2-methyl-1-butanol and 3-methyl-1-butanol), acetaldehyde and ethyl acetate were quantified in an Agilent model 7890A (Palo Alto, CA, USA) chromatograph with flame ionization detector (FID) as described by [28].

2.4 Statistical Analysis

Significant differences between samples were tested by variance analysis (ANOVA) and Tukey tests ($p <$

Table 1. Strains origin. Origin of the autochthonous yeast strains used in this study: species and strain, location (Denomination of Origin), year of isolation, grape variety, vineyard farming system and fermentation stage.

Species	Strain	Denomination of origin	Year	Grapevine variety	Production system	Fermentation stage
Saccharomyces cerevisiae	ScXG3					
Lachancea thermotolerans	Lt3	Monterrei	2015	Treixadura	Organic	Final
	Lt18	Monterrei	2015	Treixadura	Organic	Initial
	Lt93	Monterrei	2015	Mencía	Organic	Initial
	Lt132	Monterrei	2015	Mencía	Organic	Initial
	Lt262	Ribeiro	2014	Brancellao	Organic	Initial
	Lt205	Monterrei	2013	Treixadura	Organic	Final
Torulaspora delbrueckii	Td336	Ribeira Sacra	2015	Mencía	Conventional	Must
	Tdm5	Rías Baixas	2013	Albariño	Organic	Must
	Td315	Ribeiro	2014	Treixadura	Conventional	Final
Starmerella bacillaris	Sb295	Ribeira Sacra	2015	Mencía	Organic	Must
	Sb306	Ribeira Sacra	2015	Mencía	Organic	Must
	Sb326	Ribeira Sacra	2015	Mencía	Organic	Initial
	Sb333	Ribeira Sacra	2015	Mencía	Conventional	Must
	Sb404	Rías Baixas	2015	Albariño	Organic	Initial
	Sb405	Rías Baixas	2015	Albariño	Organic	Initial
	Sb304	Ribeiro	2014	Treixadura	Conventional	Final
	Sb472	Rías Baixas	2015	Treixadura	Organic	Initial
	Sb474	Rías Baixas	2015	Treixadura	Organic	Final
	Sb494	Rías Baixas	2015	Treixadura	Organic	Initial
Metschnikowia fructicola	Mf278	Ribeiro	2015	Treixadura	Conventional	Initial
Metschnikowia pulcherrima	Mp114	Monterrei	2015	Mencía	Organic	Initial
	Mp131	Monterrei	2015	Mencía	Organic	Initial
	Mp176	Ribeiro	2015	Treixadura	Organic	Initial
	Mp193	Ribeiro	2015	Brancellao	Organic	Initial
	Mp205	Ribeiro	2015	Brancellao	Organic	Initial
	Mp294	Ribeira Sacra	2015	Mencía	Organic	Must
	Mp325	Ribeira Sacra	2015	Mencía	Organic	Initial
	Mp385	Rías Baixas	2015	Albariño	Organic	Initial
	Mp468	Rías Baixas	2015	Treixadura	Organic	Must

0.05) using SPSS software (version 18.0, PASW Statistics, Chicago). The data obtained from the basic chemical and aromatic analyses of the wines were assessed in a quantification matrix, evaluating each of the oenological characteristics.

Strain evaluation for each species was based on the weighted scoring method of the different parameters among all wines made with all different strains (of all species equally). The reference values established by the regulatory councils of the Galician Denominations of Origin for quality white wines were used. Therefore, the scores are comparable between strains of different species; however, it is more appropriate to compare strains within a given species because there are parameters such as the presence of reducing sugars that will penalise species with lower fermentative capacity. A maximum value of ten points was established for the positive range which was calculated by applying the following function: f = 10 × value of the parameter/highest value of the parameter found among all the strains. All the wines ranged total acidity concentrations between 4 and 9 $g \cdot L^{-1}$ (in accordance with the requirements of the DO Ribeiro); a higher total acidity value was considered positive (within these limits), i.e., it was awarded a higher score. Conversely, the altering parameters or those whose lower concentration was considered more positive were weighted inversely, i.e., with respect to a minimum score of 0 points: volatile acidity, ethyl acetate, acetaldehyde and presence of reducing sugars. For volatile acidity, the lowest value was considered the best value = 0.36 $g \cdot L^{-1}$, with none of the wines exceeding the highest concentration allowed by the DO Ribeiro (0.8 $g \cdot L^{-1}$). However, wines that exceeded 0.6 $g \cdot L^{-1}$ of volatile acidity were penalised. Ethyl acetate was penalised above 120 $mg \cdot L^{-1}$ (maximum value allowed by DO Rías Baixas). Acetaldehyde was penalised for amounts above 40 $mg \cdot L^{-1}$. In the case of pH, the optimum value was pH = 3.3. The minimum alcoholic strength was 9° (according to DO Ribeiro). The total score was calculated as the arithmetic sum of the

scores assigned to each parameter. In addition, a weighting was added for each parameter, giving a higher relative score according to its relative importance depending on the standards required by these regulatory councils. The best rated native strain for each species was selected and its oenological potential was further evaluated in sequential fermentation with autochthonous strain *S. cerevisiae* XG3 and with a commercial strain (*S. cerevisiae* EC1118) as reported in [29,30]. In addition, Principal Component Analysis (PCA) was used to separate the wines according to basic chemical and volatile composition. Pearson's correlation analysis (r coefficient) was applied to determine the correlation of chemical compounds between different wines fermented with native non-*Saccharomyces* strains in pure and sequential fermentations, using Past software (version 4.07b, Øyvind Hammer, Oslo). Previously, data were standardized using the function f = (x-mean)/standard deviation, to guarantee the equity in those chemical compounds with different values or units.

3. Results & Discussion

3.1 Microbiological Quantification of the Pre-Inoculum

The mean value of the estimated number of cells in the pre-inoculum for all strains was $10^{5.5\pm0.1}$ cells mL^{-1} (Fig. 1). These values justify a correct and homogeneous inoculation of each strain ensuring the correct fermentation implantation in pure culture [31].

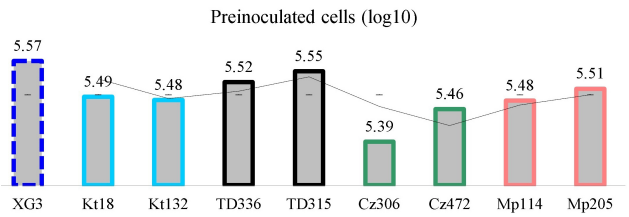

Fig. 1. Estimated number of cells in the pre-inoculum. Number of cells in preinocula of some strains checked by measurement of the optical density at 600 nm.

3.2 Kinetics of Fermentations

Fermentation kinetics is shown in Fig. 2. As expected, *S. cerevisiae* XG3 began to ferment within the first 2–3 days and showed a higher fermentation speed than non-*Saccharomyces* vinifications. The fermentative ability of autochthonous non-*Saccharomyces* strains in pure culture varied among species and strains, with *L. thermotolerans* and *T. delbrueckii* standing out for their high fermentation rates, but lower speed than *S. cerevisiae*, as reported by other works [8–10,12,32,33]. *Starm. bacillaris* strains showed a lower fermentation ability than strains from *L. thermotolerans* and *T. delbrueckii*, although this species has been described as tolerant to relative high ethanol contents and can survive up to the middle-end phase of the fermentation process [12,34]. Regarding *Metschnikowia* spp. strains, they exhibited low to moderate fermentative power, as this species is not able to tolerate ethanol concentrations over 4–5% (v/v), so it naturally disappears when this content of ethanol is exceeded during alcoholic fermentation [13]. *M. fructicola* and *M. pulcherrima* presented similar fermentation kinetics and fermentation yields over time. The poor or slower fermentative activity of non-*Saccharomyces* species confirmed the need to add a *S. cerevisiae* strain to successfully complete fermentation. However, the slower fermentation kinetics could be positive for a better retention of volatile compounds and for energy savings during the tanks cooling [3,35]. Unsurprisingly, the control (pasteurised must sample w/o inoculum) showed no evidence of fermentation or contamination.

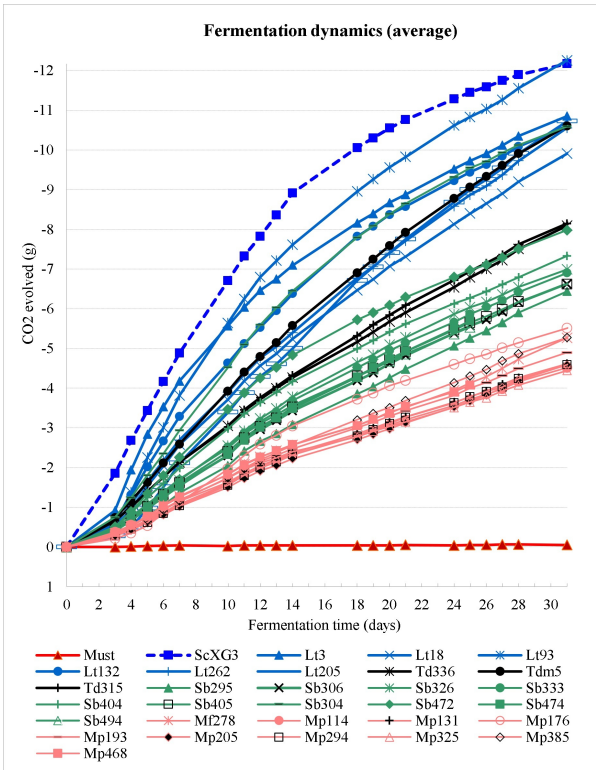

Fig. 2. Fermentative yield of 30 autochthonous strains. Fermentative ability in pure culture of indigenous non-*Saccharomyces* strains and *S. cerevisiae* XG3. Lt, *L. thermotolerans*; Mf, *M. fructicola*; Mp, *M. pulcherrima*; Sb, *Starm. bacillaris*; Td, *T. delbrueckii;* Sc, *S. cerevisiae*.

3.3 Chemical Analyses

Fermentations with different non-*Saccharomyces* strains influenced the chemical characteristics of wines. Basic chemical parameters and aromatic composition of wines are summarized in Tables 2,3, respectively. The results evidenced a significant effect of species and strains

Table 2. Basic chemical compounds. Chemical characteristics of wines elaborated with different autochthonous non-*Saccharomyces* and *S. cerevisiae* strains in pure culture and significance according to one-way ANOVA.

Strain	Total acidity ($g_{tartaricacid}$ L^{-1})	Volatile acidity ($g_{acetic\ acid}$ L^{-1})	Lactic acid ($g{\cdot}L^{-1}$)	Malic acid ($g{\cdot}L^{-1}$)	Glucose + fructose ($g{\cdot}L^{-1}$)	Glycerol ($g{\cdot}L^{-1}$)	Alcohol content (% vol)	Ethanol yield (g/g)	pH
ScXG3	**6.8 ± 0.0^{a}**	**0.60 ± 0.03^{a}**	**0.1 ± 0.0^{a}**	**3.5 ± 0.1^{a}**	**0.2 ± 0.1^{a}**	**3.8 ± 0.3^{a}**	**10.05 ± 0.07^{a}**	**0.42 ± 0.00^{a}**	**3.66 ± 0.01^{ab}**
Lt3	7.9 ± 0.1^{ab}	0.39 ± 0.06	1.7 ± 0.6	2.6 ± 0.3	6.1 ± 7.6	5.6 ± 1.0	9.12 ± 0.40	0.39 ± 0.00	3.58 ± 0.09
Lt18	7.4 ± 0.1^{ab}	0.38 ± 0.01	0.8 ± 0.3	2.85 ± 0.2	20.3 ± 10.7	5.6 ± 0.3	8.08 ± 0.11	0.38 ± 0.02	3.58 ± 0.07
Lt93	8.0 ± 0.0^{a}	0.43 ± 0.01	2.6 ± 1.0	2.3 ± 0.3	0.8 ± 0.9	6.1 ± 0.2	9.02 ± 0.11	0.38 ± 0.01	3.53 ± 0.08
Lt132	7.6 ± 0.6^{ab}	0.40 ± 0.04	1.5 ± 1.1	2.5 ± 0.5	9.8 ± 5.3	5.5 ± 0.2	9.12 ± 0.54	0.40 ± 0.01	3.65 ± 0.06
Lt262	6.9 ± 0.1^{b}	0.36 ± 0.03	0.5 ± 0.0	3.0 ± 0.0	6.5 ± 8.3	4.9 ± 0.5	8.71 ± 0.22	0.38 ± 0.01	3.68 ± 0.04
Lt205	7.3 ± 0.0^{ab}	0.36 ± 0.00	0.9 ± 0.0	2.8 ± 0.0	0.6 ± 0.0	5.2 ± 0.0	8.75 ± 0.0	0.37 ± 0.00	3.58 ± 0.00
Lt	**7.5 ± 0.4^{b}**	**0.39 ± 0.03^{b}**	**1.3 ± 0.8^{b}**	**2.6 ± 0.3^{b}**	**7.4 ± 8.5^{a}**	**5.5 ± 0.5^{a}**	**8.8 ± 0.4^{ab}**	**0.38 ± 0.01^{b}**	**3.60 ± 0.07^{a}**
Td336	7.1 ± 0.0	0.36 ± 0.01	0.1 ± 0.0	3.2 ± 0.0^{a}	37.5 ± 0.4^{b}	5.1 ± 0.4^{b}	7.75 ± 0.03^{a}	0.41 ± 0.00	3.59 ± 0.02
Tdm5	7.7 ± 0.5	0.61 ± 0.12	0.1 ± 0.0	3.9 ± 0.2^{b}	2.5 ± 2.6^{a}	3.3 ± 0.2^{a}	9.25 ± 0.07^{b}	0.39 ± 0.01	3.63 ± 0.07
Td315	7.1 ± 0.1	0.39 ± 0.03	0.1 ± 0.0	3.1 ± 0.0^{a}	31.8 ± 5.9^{b}	5.7 ± 0.4^{b}	8.08 ± 0.30^{a}	0.41 ± 0.00	3.64 ± 0.01
Td	**7.3 ± 0.4^{ab}**	**0.45 ± 0.13^{ab}**	**0.1 ± 0.0^{a}**	**3.4 ± 0.4^{a}**	**23.9 ± 17.0^{ab}**	**4.7 ± 1.1^{a}**	**8.4 ± 0.7^{bc}**	**0.40 ± 0.01^{bc}**	**3.62 ± 0.04^{a}**
Sb295	6.8 ± 0.3	0.50 ± 0.01	0.1 ± 0.0	3.5 ± 0.1	55.5 ± 4.7	16.7 ± 0.5^{b}	6.60 ± 0.36	0.39 ± 0.01	3.85 ± 0.01^{b}
Sb306	6.9 ± 0.1	0.49 ± 0.03	0.1 ± 0.0	3.4 ± 0.0	44.0 ± 1.2	14.5 ± 0.4^{b}	7.34 ± 0.24	0.40 ± 0.01	3.76 ± 0.00^{ab}
Sb326	6.5 ± 0.0	0.41 ± 0.30	0.1 ± 0.0	3.4 ± 0.4	67.4 ± 40.7	16.7 ± 2.3^{b}	6.22 ± 1.70	0.41 ± 0.03	3.82 ± 0.06^{ab}
Sb333	6.9 ± 0.4	0.62 ± 0.08	0.1 ± 0.0	3.3 ± 0.1	47.7 ± 11.2	16.1 ± 1.1^{b}	6.92 ± 0.32	0.39 ± 0.01	3.78 ± 0.04^{ab}
Sb404	6.2 ± 0.4	0.54 ± 0.04	0.1 ± 0.0	3.0 ± 0.5	29.8 ± 14.4	13.4 ± 1.8^{ab}	7.84 ± 0.67	0.39 ± 0.00	3.71 ± 0.06^{ab}
Sb405	6.5 ± 0.4	0.37 ± 0.21	0.1 ± 0.0	3.6 ± 0.6	66.9 ± 40.4	16.6 ± 2.8	6.25 ± 1.63	0.41 ± 0.03	3.85 ± 0.05^{b}
Sb304	7.1 ± 0.1	0.50 ± 0.04	0.1 ± 0.0	3.2 ± 0.1	0.3 ± 0.1	7.0 ± 0.3^{a}	9.70 ± 0.57	0.41 ± 0.02	3.58 ± 0.04^{a}
Sb472	6.5 ± 0.2	0.53 ± 0.09	0.1 ± 0.0	3.1 ± 0.1	41.1 ± 18.7	15.2 ± 1.9^{b}	7.38 ± 0.52	0.40 ± 0.02	3.77 ± 0.10^{ab}
Sb474	6.8 ± 0.2	0.39 ± 0.25	0.1 ± 0.0	3.5 ± 0.4	65.9 ± 40.4	17.0 ± 3.0^{b}	6.35 ± 1.66	0.41 ± 0.03	3.92 ± 0.11^{b}
Sb494	6.5 ± 0.4	0.49 ± 0.04	0.1 ± 0.0	3.3 ± 0.1	47.8 ± 0.4	15.9 ± 0.4^{b}	6.91 ± 0.08	0.39 ± 0.01	3.77 ± 0.08^{ab}
Sb	**6.6 ± 0.3^{a}**	**0.48 ± 0.13^{ab}**	**0.1 ± 0.0^{a}**	**3.3 ± 0.3^{a}**	**46.6 ± 26.3^{b}**	**14.9 ± 3.2^{b}**	**7.1 ± 1.2^{c}**	**0.40 ± 0.02^{bc}**	**3.78 ± 0.10^{b}**
Mf278	6.7 ± 0.4	0.42 ± 0.01^{a}	0.6 ± 0.1^{b}	1.6 ± 0.0^{ab}	78.5 ± 10.6	11.6 ± 1.1^{abc}	4.16 ± 0.51	0.30 ± 0.01	3.69 ± 0.07
Mp114	6.8 ± 0.6	0.50 ± 0.02^{a}	0.3 ± 0.0^{ab}	2.0 ± 0.1^{ab}	86.5 ± 2.1	12.4 ± 0.2^{abc}	3.61 ± 0.03	0.28 ± 0.00	3.68 ± 0.01
Mp131	6.3 ± 0.3	0.52 ± 0.03^{a}	0.3 ± 0.0^{ab}	1.6 ± 0.4^{a}	83.5 ± 7.8	12.9 ± 1.1^{bc}	3.71 ± 0.36	0.28 ± 0.01	3.72 ± 0.00
Mp176	7.2 ± 0.0	0.73 ± 0.00^{b}	0.3 ± 0.1^{ab}	2.7 ± 0.0^{cd}	97.5 ± 2.1	10.1 ± 0.1^{a}	3.65 ± 0.11	0.31 ± 0.00	3.78 ± 0.05
Mp193	6.8 ± 0.1	0.46 ± 0.01^{a}	0.4 ± 0.0^{ab}	2.0 ± 0.3^{abc}	83.5 ± 2.1	10.8 ± 0.6^{ab}	4.10 ± 0.08	0.31 ± 0.01	3.64 ± 0.03
Mp205	7.1 ± 0.1	0.47 ± 0.04^{a}	0.4 ± 0.1^{ab}	2.3 ± 0.1^{bcd}	88.0 ± 1.4	11.5 ± 0.2^{abc}	3.94 ± 0.13	0.31 ± 0.01	3.69 ± 0.01
Mp294	6.6 ± 0.1	0.44 ± 0.00^{a}	0.2 ± 0.1^{a}	2.8 ± 0.3^{d}	92.5 ± 17.7	11.3 ± 0.4^{abc}	3.66 ± 0.20	0.31 ± 0.04	3.64 ± 0.03
Mp325	7.2 ± 0.1	0.44 ± 0.02^{a}	0.6 ± 0.1^{b}	1.8 ± 0.1^{ab}	95.5 ± 0.7	13.2 ± 0.4^{bc}	3.52 ± 0.06	0.30 ± 0.00	3.71 ± 0.01
Mp385	6.5 ± 0.3	0.58 ± 0.16^{ab}	0.4 ± 0.1^{ab}	2.0 ± 0.1^{ab}	83.8 ± 3.9	11.3 ± 0.4^{abc}	3.99 ± 0.27	0.30 ± 0.03	3.63 ± 0.12
Mp468	7.5 ± 0.5	0.54 ± 0.00^{ab}	0.4 ± 0.0^{ab}	2.2 ± 0.0^{abcd}	88.0 ± 1.4	11.8 ± 0.4^{abc}	3.68 ± 0.05	0.29 ± 0.01	3.69 ± 0.02
Mp	**6.8 ± 0.4^{a}**	**0.51 ± 0.10^{ab}**	**0.4 ± 0.1^{a}**	**2.1 ± 0.4^{b}**	**87.7 ± 7.8^{c}**	**11.6 ± 1.0^{b}**	**3.8 ± 0.3^{d}**	**0.30 ± 0.02^{c}**	**3.68 ± 0.06^{ab}**
Sig.	***	*	***	***	***	***	***	***	***

Different letters in **bold** in the same column indicate significant differences among species for that parameter according to Tukey's test. Letters in *italics* in the same column indicate significant differences among strains within a given species at $p < 0.05$ according to Tukey's test. Sig.: the notations * and *** indicate significant differences between wines from different species at $p < 0.05$ and $p < 0.001$, respectively.

Table 3. Major volatile compounds. Concentration of major volatile compounds determined in the wines produced with different autochthonous non-*Saccharomyces* and *S. cerevisiae* strains in pure culture and significance according to one-way ANOVA.

Strain	∑ Higher alcohols	1-butanol	2-methyl-1-butanol	3-methyl-1-butanol	Isobutanol	Propanol	Methanol	Acetaldehyde	Ethyl acetate
	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)
ScXG3	**185.0 ± 28.3^{a}**	**3.4 ± 0.6^{a}**	**24.1 ± 3.4^{a}**	**110.7 ± 15.7^{a}**	**20.3 ± 8.6^{b}**	**26.8 ± 2.5^{a}**	**22.5 ± 0.7**	**8.5 ± 2.1**	**4.5 ± 2.1**
Lt3	136.5 ± 57.3	2.9 ± 3.6	23.7 ± 4.9	73.2 ± 35.1	19.1 ± 10.0	24.8 ± 12.1	25.5 ± 4.9	24.5 ± 12.0	4.7 ± 1.0
Lt18	162.5 ± 4.9	4.9 ± 0.3	27.4 ± 2.1	87.7 ± 3.0	20.8 ± 1.6	29.8 ± 0.4	21.2 ± 1.2	23.8 ± 0.3	3.2 ± 0.1
Lt93	159.4 ± 46.2	2.7 ± 3.3	24.8 ± 2.8	88.2 ± 34.2	21.4 ± 8.1	28.1 ± 8.0	25.5 ± 6.4	33.5 ± 6.4	4.3 ± 1.0
Lt132	116.0 ± 62.2	5.9 ± 0.0	14.1 ± 13.2	49.4 ± 50.9	22.1 ± 2.1	32.2 ± 4.2	23.5 ± 2.1	18.5 ± 3.5	8.7 ± 6.2
Lt262	154.9 ± 4.5	4.6 ± 0.4	25.6 ± 0.5	85.4 ± 5.9	17.3 ± 3.4	28.3 ± 2.7	22.0 ± 0.0	25.5 ± 4.9	4.8 ± 1.1
Lt205	162.0 ± 0.0	4.7 ± 0.0	25.0 ± 0.0	84.0 ± 0.0	15.2 ± 0.0	33.0 ± 0.0	21.0 ± 0.0	15.0 ± 0.0	4.0 ± 0.0
Lt	**148.0 ± 34.11^{ab}**	**4.3 ± 1.9^{a}**	**23.4 ± 6.3^{a}**	**77.9 ± 25.7^{b}**	**19.3 ± 4.8^{b}**	**29.3 ± 5.5^{a}**	**23.1 ± 3.2**	**23.5 ± 7.5**	**4.9 ± 2.6**
Td336	116.5 ± 3.5	1.5 ± 0.7	11.0 ± 0.0	73.0 ± 1.4	17.5 ± 0.7	13.5 ± 0.7^{a}	21.0 ± 0.0	30.5 ± 2.1	6.5 ± 0.7^{a}
Tdm5	131.0 ± 0.0	1.5 ± 0.7	13.0 ± 1.4	58.0 ± 9.9	17.0 ± 0.0	32.5 ± 2.1^{b}	21.0 ± 1.4	16.5 ± 14.8	6.0 ± 0.0^{a}
Td315	122.0 ± 22.6	2.0 ± 0.0	11.0 ± 1.4	74.5 ± 12.0	16.5 ± 2.1	13.5 ± 0.7^{a}	22.5 ± 0.7	10.5 ± 2.1	10.0 ± 0.0^{b}
Td	**123.2 ± 12.2^{bc}**	**1.7 ± 0.5^{ab}**	**11.7 ± 1.4^{b}**	**68.5 ± 10.7^{bc}**	**17.0 ± 1.1^{b}**	**19.8 ± 9.9^{a}**	**21.5 ± 1.0**	**19.2 ± 11.4**	**7.5 ± 2.0**
Sb295	80.5 ± 0.7	-	3.3 ± 0.6	11.3 ± 1.1	46.0 ± 0.7	19.7 ± 0.5	15.0 ± 8.5	16.5 ± 6.4	8.5 ± 3.5
Sb306	81.5 ± 0.7	-	3.9 ± 0.3	12.8 ± 0.5	39.2 ± 4.9	25.6 ± 5.3	24.5 ± 2.1	27.5 ± 16.3	11.5 ± 2.1
Sb326	43.0 ± 0.0	-	4.0 ± 0.0	6.0 ± 0.0	11.7 ± 0.0	20.1 ± 0.0	26.0 ± 0.0	20.0 ± 0.0	7.0 ± 0.0
Sb333	93.0 ± 2.8	-	4.6 ± 0.1	11.7 ± 0.2	46.1 ± 0.5	31.3 ± 2.4	22.0 ± 1.4	32.5 ± 20.5	12.0 ± 1.4
Sb404	77.5 ± 4.9	-	4.6 ± 0.1	12.5 ± 1.0	33.4 ± 2.7	26.8 ± 0.9	21.0 ± 1.4	22.5 ± 6.4	6.5 ± 4.9
Sb405	84.3 ± 10.3	0.4 ± 0.1	7.5 ± 4.7	23.4 ± 15.5	33.7 ± 4.3	19.8 ± 14.1	23.0 ± 2.8	23.0 ± 11.3	6.5 ± 4.9
Sb304	60.0 ± 24.0	-	4.1 ± 0.1	9.2 ± 4.5	21.2 ± 13.4	24.9 ± 6.8	23.5 ± 3.5	17.5 ± 3.5	8.5 ± 2.1
Sb472	61.0 ± 29.7	-	3.8 ± 0.6	9.9 ± 4.0	22.3 ± 20.2	23.4 ± 8.5	24.5 ± 4.9	36.0 ± 33.9	9.5 ± 2.1
Sb474	139.0 ± 58.0	6.2 ± 0.9	15.4 ± 14.4	52.1 ± 53.0	32.5 ± 26.6	33.7 ± 10.3	22.0 ± 1.4	20.5 ± 0.7	6.5 ± 4.9
Sb494	84.5 ± 0.7	-	10.1 ± 7.1	14.6 ± 0.6	38.6 ± 3.3	26.5 ± 1.4	20.0 ± 1.4	22.0 ± 7.1	10.0 ± 0.0
Sb	**81.02 ± 30.2^{c}**	**3.3 ± 3.3^{a}**	**6.1 ± 5.4^{b}**	**16.4 ± 18.2^{d}**	**33.1 ± 14.2^{b}**	**25.2 ± 6.8^{a}**	**22.1 ± 3.9**	**23.7 ± 12.2**	**8.6 ± 3.1**
Mf278	110.2 ± 0.6^{a}	0.9 ± 0.2	8.4 ± 1.5^{c}	43.9 ± 6.4^{ab}	47.0 ± 7.1^{a}	9.9 ± 0.5^{bcd}	18.0 ± 1.4^{ab}	21.5 ± 2.1^{bc}	12.0 ± 1.4^{a}
Mp114	116.5 ± 2.1^{ab}	1.0 ± 0.1	7.5 ± 0.7^{bc}	50.0 ± 2.8^{ab}	51.0 ± 0.0^{ab}	8.0 ± 1.4^{abc}	19.5 ± 0.7^{ab}	10.5 ± 6.4^{ab}	40.5 ± 34.6^{ab}
Mp131	109.0 ± 8.5^{a}	1.0 ± 0.0	8.0 ± 0.0^{bc}	42.0 ± 1.4^{a}	49.0 ± 5.7^{ab}	10.0 ± 1.4^{bcd}	20.5 ± 0.7^{ab}	10.5 ± 0.7^{ab}	201.0 ± 41.0^{bc}
Mp176	104.5 ± 0.7^{a}	0.0 ± 0.0	8.3 ± 0.6^{bc}	34.9 ± 2.3^{a}	46.6 ± 2.8^{a}	14.7 ± 0.9^{d}	21.0 ± 0.0^{ab}	13.0 ± 2.8^{ab}	330.0 ± 39.6^{c}
Mp193	122.0 ± 24.0^{ab}	0.0 ± 0.0	6.5 ± 0.7^{abc}	51.5 ± 14.8^{ab}	54.5 ± 7.8^{ab}	9.0 ± 0.0^{abcd}	20.5 ± 0.7^{ab}	8.5 ± 0.7^{a}	55.0 ± 9.9^{ab}
Mp205	104.0 ± 17.0^{a}	0.0 ± 0.0	7.0 ± 0.0^{bc}	38.0 ± 5.7^{a}	49.5 ± 7.8^{ab}	8.5 ± 3.5^{abc}	29.5 ± 13.4^{ab}	8.5 ± 2.1^{a}	45.5 ± 44.5^{ab}
Mp294	117.0 ± 15.6^{ab}	0.0 ± 0.0	7.0 ± 0.0^{bc}	39.5 ± 4.9^{a}	58.5 ± 9.2^{ab}	11.5 ± 2.1^{bcd}	33.5 ± 0.7^{b}	13.0 ± 1.4^{ab}	89.5 ± 74.2^{ab}
Mp325	99.0 ± 8.5^{a}	1.0 ± 0.0	7.5 ± 0.7^{bc}	41.0 ± 1.4^{a}	42.0 ± 5.7^{a}	7.0 ± 0.0^{ab}	19.0 ± 1.4^{ab}	10.0 ± 1.4^{a}	36.0 ± 1.4^{ab}
Mp385	159.0 ± 1.4^{b}	-	5.7 ± 0.1^{ab}	67.3 ± 5.6^{b}	72.9 ± 6.4^{b}	13.3 ± 1.1^{cd}	15.5 ± 3.5^{a}	45.0 ± 4.2^{d}	133.0 ± 100^{ab}
Mp468	108.0 ± 0.0^{a}	-	4.2 ± 0.0^{a}	36.2 ± 0.0^{a}	64.5 ± 0.0^{ab}	3.4 ± 0.0^{a}	12.0 ± 0.0^{a}	26.0 ± 0.0^{c}	67.0 ± 0.0^{ab}
Mp	**114.9 ± 18.4^{bc}**	**0.5 ± 0.5^{b}**	**6.9 ± 1.3^{b}**	**44.4 ± 10.4^{cd}**	**53.5 ± 10.1^{a}**	**9.5 ± 3.3^{b}**	**20.9 ± 6.9**	**16.6 ± 11.4**	**100.9 ± 100.9**
Sig.	***	***	***	***	***	***	ns	ns	***

The values are mean ± standard deviation of two independent experiments. Sig.: the notations *** and ns indicate significance at $p < 0.001$ and not significant, respectively. Different letters in **bold** in the same column indicate significant differences among species for that parameter according to Tukey's test. Letters in *italics* in the same column indicate significant differences among strains within a given species according to Tukey's test.

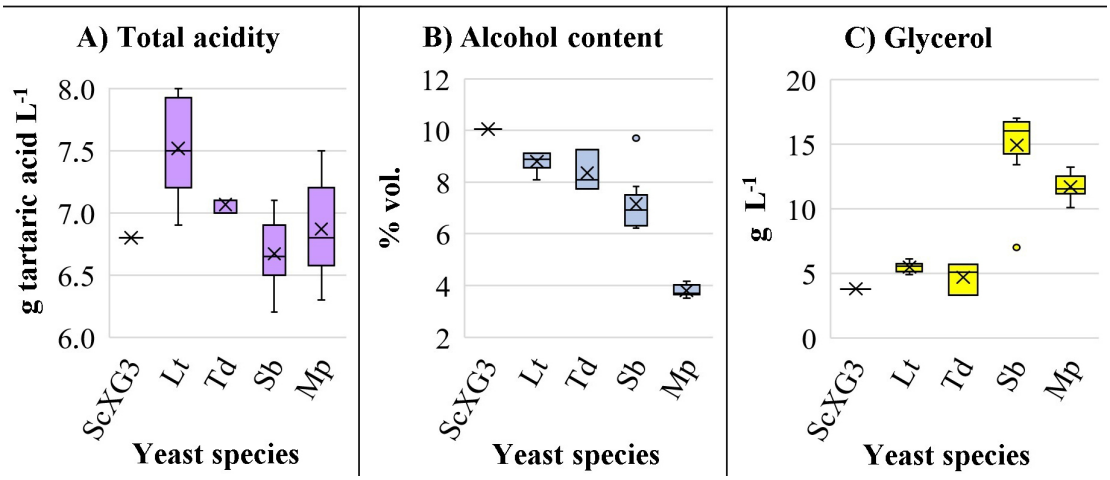

Fig. 3. Total acidity, alcohol content and glycerol. Box and whisker chart of three relevant basic chemical compounds: (A) alcohol content, (B) total acidity, and (C) glycerol of non-*Saccharomyces* strains compared to *S. cerevisiae* XG3.

in almost all the parameters determined. *T. delbrueckii* and *L. thermotolerans* increased the total acidity and lowered the volatile acidity of wines compared with the control wines (fermented with *S. cerevisiae* XG3) (Fig. 3A). This ability has been widely reported for *L. thermotolerans* [36–38], but the results are less clear for *T. delbrueckii* [39]. To a lesser extent *Metschnikowia* spp. and *Starm. bacillaris* also decreased the volatile acidity as found in other studies [13,36]. *L. thermotolerans*, especially Lt93 strain, also increased the lactic acid ratio, as well as *Metschnikowia* spp. but the *M. pulcherrima* strains did so to a lesser extent than *L. thermotolerans*. Accordingly, the pH was lower in the *L. thermotolerans* wines but not in all the wines fermented with *Metschnikowia* spp. Besides, *L. thermotolerans* strains reduced the amount of malic acid in the wine compared to *S. cerevisiae*. This ability is of special interest in warm regions to mitigate the acidity reduction in wines because of climate change [10,29,40].

Moreover, the alcoholic degree of non-*Saccharomyces* wines was lower than control in all species and strains (Fig. 3B). For instance, a reduction of 1.25% v/v in the case of *L. thermotolerans,* 1.69% v/v with *T. delbrueckii*, and 2.90% v/v with *Starm. bacillaris* was observed, although in most cases there was residual sugars in the wines. Accordingly, the wines from these species presented lower ethanol yields than control wines obtained with ScXG3. Concerning to alcoholic strength and sugar consumption by the different non-*Saccharomyces* in single fermentations, results are consistent with the literature, but some strains such as Lt93 reached higher ethanol yield (ethanol production ($g \cdot L^{-1}$)/sugar consumption ($g \cdot L^{-1}$), g/g) than most of the studies consulted [36,41,42]. As expected, *Metschnikowia* spp. did not complete fermentation either; in this case, an average alcohol content of only 3.8% v/v (ethanol yield 0.30 g/g) was achieved and a high glucose + fructose content was remained in wine as reported by other authors [18,43]. *L. thermotolerans* and *T. delbrueckii* produced wines with a higher ethanol concentration and consumed more sugars in the must compared to *Starm. bacillaris* and *M. pulcherrima*. Most of the strains of *Starm. bacillaris* has a strong fructophilic character, adapted to sweet wine fermentations and during fermentation ethanol yield from sugar consumed is lower as compared with *S. cerevisiae* [12].

Finally, all non-*Saccharomyces* strains increased the concentration of glycerol in the wine, especially *Starm. bacillaris* and *Metschnikowia* spp. (Fig. 3C). Particularly, with some strains of *Starm. bacillaris* the content of glycerol was very high (17.0 in strain Sb474), as described by other authors [2,11,12,36]. This compound contributes unctuousness, volume, slight sweetness and silky mouthfeel to wines [12,44,45].

Yeast strains modulate the aroma of wine during fermentation. In particular, higher alcohols are the major compounds that also contribute to the generation of secondary metabolites whose biosynthesis is species dependent but not always is strain dependent [46]. Accordingly, all the yeast strains/species used in this study influenced the fermentative aroma composition of wines. Other major volatile compounds include acetaldehyde and ethyl acetate, both are undesirable at high concentrations imparting aroma like pungent and nail-polish, respectively. However, below 0.5 $mg \cdot L^{-1}$, acetaldehyde may contribute pleasant notes of bruised apple, sherry and nutty; in the case of ethyl acetate below 7.5 $mg \cdot L^{-1}$ imparts pineapple and fruity notes [47]. The concentration of major volatile compounds of wines fermented with pure cultures of different yeast strains is shown in Table 3. The results showed significant differences among species for almost all volatile compounds analysed, including the sum of higher alcohols. However, at strains level within a given species, significant differences were only found among *Metschnikowia* spp. strains for all parameters (except 1-butanol) and among *T. del-*

brueckii strains for propanol and ethyl acetate. The content of higher alcohols ranged between 45 and 185 mg·L^{-1} in wines from Sb326 and ScXG3, respectively (Fig. 4). These concentrations are lower than 300 mg·L^{-1}, above which the aromas become undesirable, although the wines from pure culture fermentations achieved lower content of higher alcohols than those obtained for the same strains tested in sequential fermentations [29,30]. In addition, all the yeast species evaluated produced lower concentrations of these volatiles in the wines than *S. cerevisiae*, being *L. thermotolerans* the non-*Saccharomyces* species that produced the highest concentration of higher alcohols, in some cases doubling the concentration generated by *Starm. bacillaris*. Some studies corroborate the high production of higher alcohols in fermentations performed with *L. thermotolerans*, *M. pulcherrima* and *Starm. bacillaris*; however, variations up to ± 30% (increments or reductions) compared to the *S. cerevisiae* controls are found [3,13,19]. These results suggest that the production of higher alcohols and other chemical compounds is not only influenced by the strain effect, but may also be influenced by factors such as interactions with *S. cerevisiae*, other non-*Saccharomyces* or grape must characteristics [34,48]. In our study, the differences were mainly due to the concentration of 3-methyl-1-butanol and isobutanol. *S. cerevisiae* was the species with the highest concentration of 3-methyl-1-butanol. However, the content of isobutanol was, compared to *S. cerevisiae*, two-fold higher in the wines made with *Metschnikowia* spp. and *L. thermotolerans* wines and increased by more than 50% with *Starm. bacillaris* in most strains as reported in some studies [11,43].

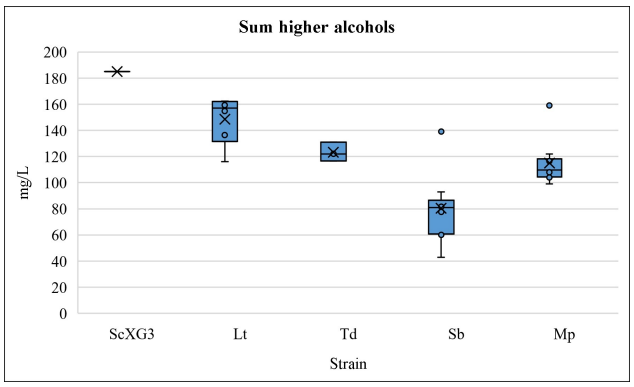

Fig. 4. Higher alcohols content. Box and whisker chart of higher alcohols content comparing the non-*Saccharomyces* strains to *S. cerevisiae* XG3. The sum of higher alcohols included 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, isobutanol (2-methyl-1-propanol) and propanol.

On the other hand, the high levels of ethyl acetate in wines fermented with some strains of *Metschnikowia* spp. are noteworthy. However, other strains obtained very low non-altering concentrations of this compound, which reinforces the argument that its production is strain-dependent; therefore, the strain selection is very important [23,36].

3.4 Selection of Autochthonous Strains According to their Oenological Traits

The data from the chemical analyses (Tables 2,3) of the wines were included in a quantification matrix (Table 4), evaluating each of the desired oenological characteristics. Lt93 was characterised by high total acidity (+1.2 g·L^{-1}; +17.6%), lactic acid (+2.5 g·L^{-1}), fermentation yield (only 0.8 g glucose + fructose and alcohol production –0.93 %vol.) and higher alcohols production (159.4 mg·L^{-1}; –16.0%) compared to the control ScXG3 as found in similar studies [9,36,38]. Within the group, Lt93 stood out from the other *L. thermotolerans* as the strain with the best fermentation performance and the highest production of lactic acid and glycerol. Td315 was characterized by a better balance of positive parameter values, higher glycerol concentration than ScXG3 (+1.9 g·L^{-1}; +150%) and lower acetaldehyde concentration than the other *T. delbrueckii* strains. These are desirable values for a strain among the most widespread commercial non-*Saccharomyces* species [8,49]. Sb474 highlighted because its wines had the lowest volatile acidity and highest glycerol concentration of all *Starm. bacillaris* wines. Furthermore, Sb474 showed very little variation between the two replicates in terms of fermentation development (CO_2 evolution). This homogeneity can be favourable in fermentation with *S. cerevisiae* in aspects such as chemical-aromatic modulation, cell interaction, temperature, or oxygen availability [48,50,51]. Mf278 was noted for a high glycerol concentration, higher lactic acid and alcohol content as reported by other authors [3,13] and it was the only *Metschnikowia* strain with very low (and acceptable) ethyl acetate concentration. The high concentration of ethyl acetate can be a limiting attribute, as it can spoil the quality of the resulting wine.

According to these values, the best rated strains within *L. thermotolerans*, *T. delbrueckii*, *Starm. bacillaris* and *Metschnikowia* spp. species were Lt93, Td315, Sb474 and Mf278, respectively. These strains were evaluated in sequential fermentation with the autochthonous *S. cerevisiae* strain XG3 [29] and with the commercial strain *S. cerevisiae* EC1118 [30].

The PCA using all chemical compounds analysed allowed a clear separation of wines elaborated with different strains of non-*Saccharomyces* species in pure culture (Fig. 5A). The first two components, PC1 and PC2, explained 60.5% of the variance. *L. thermotolerans* strains were located in the positive first quadrant, characterized by higher concentrations of 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, total acidity and lactic acid. In contrast, wines made with *Starm. bacillaris* were plotted on the negative side of PCA (except for the Sb474 and Sb304 strains), with a high content of glycerol. *Torulaspora* wines appeared close to the centre, between Lt and Sb strains,

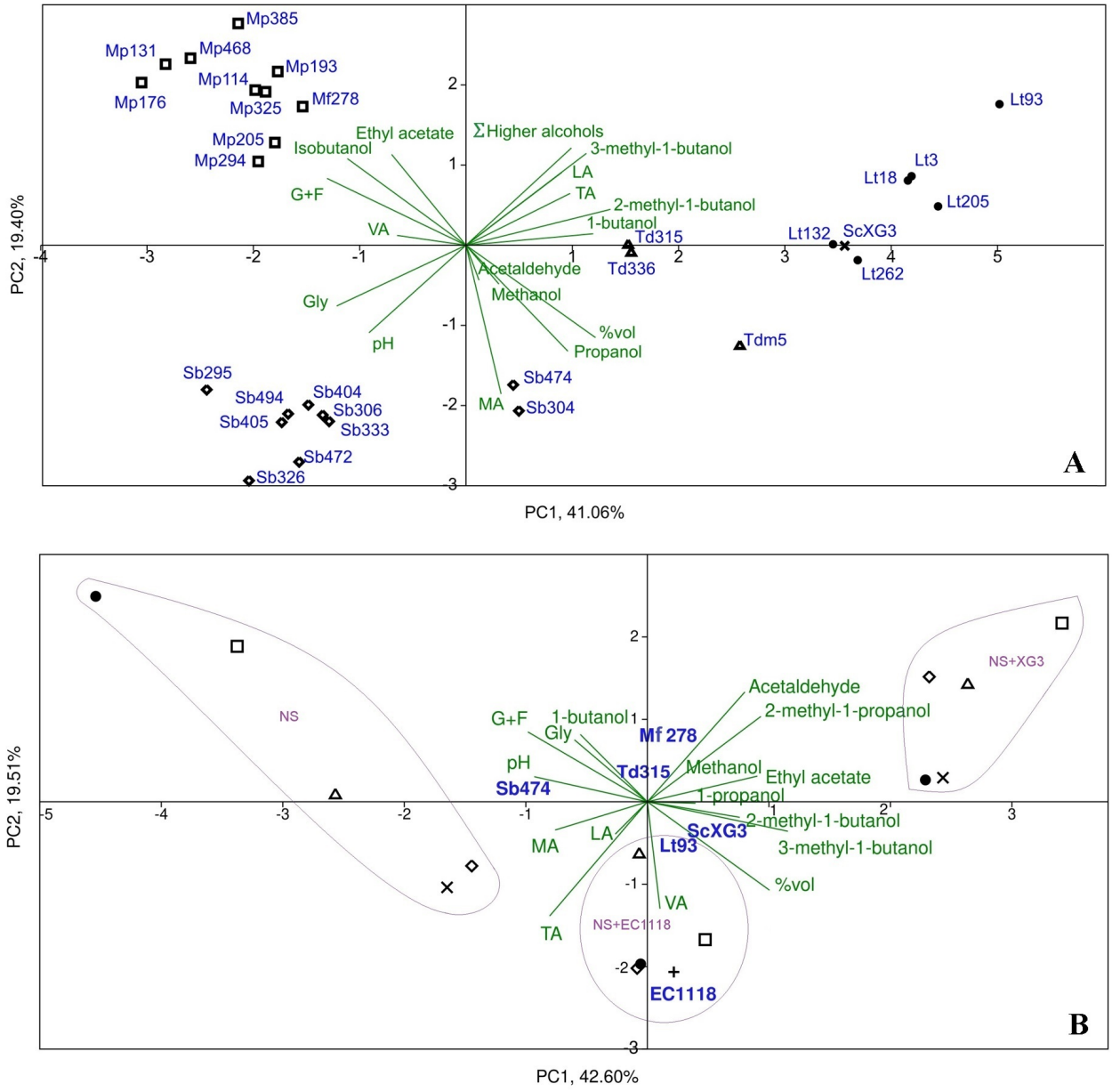

Fig. 5. Principal component analysis (PCA). (A) Pure cultures of autochthonous non-*Saccharomyces* strains: *Metschnikowia* spp. (square), *L. thermotolerans* (dot), *Starm. bacillaris* (diamond) and *T. delbrueckii* (triangle). Chemical parameter: %vol., alcohol content; G+F, reducing sugars (glucose + fructose); Gly, glycerol; LA, lactic acid; MA, malic acid; TA, total acidity; VA, volatile acidity. (B) Pure cultures of autochthonous non-*Saccharomyces* strains (NS), sequential fermentation with *S. cerevisiae* autochthonous XG3 (x-cross) and commercial EC1118 (plus). Biplot for the first two components (PC) for basic parameters and volatile compounds: ScXG3-fermentation with *S. cerevisiae* ScXG3; Lt93, Sb474, Td315 and Mf278-fermentations with each of these non-*Saccharomyces* strains and sequential inoculation with *S. cerevisiae* ScXG3 (NS+XG3) and *S. cerevisiae* EC1118 (NS+EC1118).

Table 4. Quantification matrix of the pure culture of autochthonous strains. Scores obtained by indigenous non-*Saccharomyces* strains in different parameters of oenological interest and final punctuation. A higher score means a better value for a given non-*Saccharomyces* strain, according to the quality of the wine pondered with the requirements of the regulatory councils, regardless of the net values.

Parameter	ScXG3	Lt3	Lt18	Lt93	Lt132	Lt262	Lt205	Td336	Tdm5	Td315	Sb295	Sb306	Sb326	Sb333	Sb404	Sb405	Sb304	Sb472	Sb474	Sb494	Mf278	Mp114	Mp131	Mp176	Mp193	Mp205	Mp294	Mp325	Mp385	Mp468	Weighting**
Higher alcohols	10.0	7.4	8.8	8.6	6.3	8.4	8.8	6.3	7.1	6.6	4.4	4.4	2.3	5.0	4.2	4.6	3.2	3.3	7.5	4.6	6.0	6.3	5.9	5.6	6.6	5.6	6.3	5.4	8.6	5.8	1.1
Acetaldehyde	8.1	4.6	4.7	2.6	5.9	4.3	6.7	3.2	6.3	7.7	6.3	3.9	5.6	2.8	5.0	4.9	6.1	2.0	5.4	5.1	5.2	7.7	7.7	7.1	8.1	8.1	7.1	7.8	0.0	4.2	1
Methanol	6.7	7.6	6.3	7.6	7.0	6.6	6.3	6.3	6.3	6.7	4.5	7.3	7.8	6.6	6.3	6.9	7.0	7.3	6.6	6.0	5.4	5.8	6.1	6.3	6.1	8.8	10.0	5.7	4.6	3.6	0.8
Ethyl acetate	9.6	9.6	9.7	9.6	9.3	9.6	9.7	9.5	9.5	9.2	9.3	9.0	9.4	9.0	9.5	9.5	9.3	9.2	9.5	9.2	9.0	6.6	–6.8	–17.5	5.4	6.2	2.5	7.0	–1.1	4.4	1.1
Total acidity (g tart L^{-1})	8.5	9.9	9.3	10.0	9.5	8.6	9.1	8.9	9.6	8.9	8.5	8.6	8.1	8.6	7.8	8.1	8.9	8.1	8.5	8.1	8.4	8.5	7.9	9.0	8.5	8.9	8.3	9.0	8.1	9.4	1.2
Volatile acidity (g acetic L^{-1})	2.0	6.7	6.8	6.1	6.5	7.1	7.1	7.1	1.6	6.7	5.2	5.3	6.4	1.3	4.6	6.9	5.2	4.7	6.7	5.3	6.2	5.2	4.9	–0.5	5.7	5.6	6.0	6.0	4.1	4.6	1.3
Lactic acid	0.4	6.5	3.1	10.0	5.8	1.9	3.5	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	2.3	1.2	1.2	1.2	1.5	1.5	0.8	2.3	1.5	0.4	1
Malic acid	9.0	6.7	7.3	5.9	6.4	7.7	7.2	8.2	10.0	7.9	9.0	8.7	8.7	8.5	7.7	9.2	8.2	7.9	9.0	8.5	4.1	5.1	4.1	6.9	5.1	5.9	7.2	4.6	5.1	5.6	1
Reducing sugars*	10.0	9.4	7.9	9.9	9.0	9.3	9.9	6.2	9.7	6.7	4.3	5.5	3.1	5.1	6.9	3.1	10.0	5.8	3.2	5.1	1.9	1.1	1.4	0.0	1.4	1.0	0.5	0.2	1.4	1.0	1.2
Glycerol	2.2	3.3	3.3	3.6	3.2	2.9	3.1	3.0	1.9	3.4	9.8	8.5	9.8	9.5	7.9	9.8	4.1	8.9	10.0	9.4	6.8	7.3	7.6	5.9	6.4	6.8	6.6	7.8	6.6	6.9	1.1
Alcoholic strength (%vol.)	10.0	9.1	8.0	9.0	9.1	8.7	8.7	7.7	9.2	8.0	6.6	7.3	6.2	6.9	7.8	6.2	9.7	7.3	6.3	6.9	4.1	3.6	3.7	3.6	4.1	3.9	3.6	3.5	4.0	3.7	1
pH	8.1	8.3	8.3	8.4	8.1	8.1	8.3	8.3	8.2	8.2	7.7	7.9	7.8	7.9	8.0	7.7	8.3	7.9	7.6	7.9	8.0	8.1	8.0	7.9	8.2	8.0	8.2	8.0	8.2	8.0	1
Sum	84.6	88.9	83.5	91.3	86.1	83.1	88.2	74.9	79.9	80.3	75.9	76.9	75.5	71.5	76.0	77.3	80.3	73.0	80.6	76.3	67.5	66.4	51.6	35.5	67.1	70.3	67.1	67.2	51.2	57.7	
Weighted score	89.8	95.3	89.9	97.8	92.2	89.6	95.0	80.7	84.9	86.0	81.4	82.0	80.3	75.6	81.2	82.6	85.9	77.9	86.4	81.6	72.6	70.8	54.4	35.3	71.4	74.1	70.2	71.7	54.8	62.2	

Values are the average of two fermentations. * Glucose + fructose. ** Weighting factor used for each parameter according to its relative importance on the total wine quality.

due to their higher alcohol content. In the opposite, on the negative side of PC2 and in the positive side of PC1 *Metschnikowia* wines were characterized by higher content of sugars, ethyl acetate and isobutanol.

In addition, the results obtained with the best rated strains in pure fermentations were compared with those obtained with the same non-*Saccharomyces* strains in sequential fermentations. PCA results also shown a proper separation of wines elaborated with non-*Saccharomyces* strains in pure culture and in sequential fermentations (Fig. 5B). In this case, the first two components, PC1 and PC2, explained 62.1% of the variance. Wines made with non-*Saccharomyces* in pure culture (NS) were in left side. On the opposite side were grouped the wines produced by sequential fermentation with ScXG3. However, wines fermented in sequential fermentation with the commercial ScEC1118 strain (NS+EC1118) were plotted in the middle of the PCA. In addition, strains from the sequential fermentations, especially those from the NS+EC1118 group, clustered more closely than those from the pure culture, indicating greater homogeneity due to the influence of *S. cerevisiae* strains on the resulting wines while retaining their differentiation. Some authors have proposed different mechanisms in the relationships between *S. cerevisiae* and non-*Saccharomyces* [18,51,52]. In this sense, the NS+XG3 group showed the highest concentration of volatile compounds, which have confirmed that native strains can improve the chemical and sensory characteristics, and differentiation of wines in accordance with numerous recent studies [3,30,53–55]. In contrast, compounds accounting for acidity in the biplot are concentrated in the area of NS+EC1118 wines. On the other hand, some chemical compounds such as glycerol do not point to the species that produce the highest concentration in the biplot, which indicates that they do not depend on the type of fermentation but rather depends on the species and strain.

3.5 Correlation Analysis

A correlation (Pearson) study (Table 5) was performed to show possible correlations between chemical compounds, strains, and fermentation groups (pure and sequential cultures). Regarding correlations between strains in single fermentations, the results showed some differences. As expected, the correlations between *S. cerevisiae* control strains in pure culture fermentations showed high r-values with each other ($r > 0.9$; $p < 0.05$ in all compounds, except for those related to acidity where no significant differences were found). Moreover, the correlations were higher between the same strain ScXG3 under different conditions (pure and sequential fermentation controls with different musts, temperatures, and fermentation volumes). Furthermore, comparing all the strains with each other in pure culture, the highest correlations were found in the higher alcohols, except for Mf278. However, in the case of basic chemical compounds the opposite occurred, especially in acidity-related compounds, Mf278 obtained the highest correlations.

On the other hand, the main correlations with significant differences ($p < 0.05$) between the groups of sequential fermentations (NS+ScXG3 and NS+EC1118) occurred in the following pairwise: in all overall compounds and in volatile acidity (r = 0.885), 3-methyl-1-butanol (r = 0.893), ethyl acetate (r = 0.880) and glycerol (r = 0.985). Furthermore, the compounds 2-methyl-1-propanol (r = 0.868; p = 0.056) and reducing sugars (r = 0.852; p = 0.067) showed correlation with significance close to p = 0.05. Similarly, some correlations with significant differences ($p < 0.05$) were found between pure culture fermentations and sequential fermentations in the overall major volatile compounds, glycerol, and ethyl acetate. Lt93 and Td315 are the non-*Saccharomyces* strains that showed the highest correlation in all tests performed ($r > 0.8$; $p < 0.05$) in most of the comparisons between pure and sequential culture. Sb474 showed medium correlation (Sb474+ScXG3: r = 0.56, p = 0.03; Sb474+EC1118: r = 0.50, p = 0.17). In contrast, Mf278 barely showed any correlation (r = 0.33–0.46; $p > 0.05$) with sequential fermentations. This suggests that the contribution of the selected non-*Saccharomyces* strains can be stable regardless of other variables such as must type (chemical composition, variety), winemaking techniques (temperature, fermentation volume) or *S. cerevisiae* strain (indigenous or commercial) but not for all parameters and it depends on species and strain. Furthermore, in the overall comparisons (in the compound groups), many correlations were observed between the sequential fermentations with each other including Mf278 and Sb474 strains, indicating a stronger influence of the *S. cerevisiae* strain (**Supplementary Figs. 1,2,3** of the supplementary material). Moreover, the number of these correlations was observed more frequently in the sequential fermentations with ScXG3, suggesting a greater influence of the indigenous strains on the preservation of the chemical profile, especially in the aromatic parameters analysed. However, correlations of the different native non-*Saccharomyces* strains between pure culture and sequential fermentation showed some singularities for some specific parameters (data not shown). Lt93 showed a higher correlation with the native strain ScXG3 in sequential fermentation, although no significant differences were found for some basic chemical parameters such as acidity or sugar content. As for Td315, the pure culture showed correlation with both sequential fermentations. Moreover, for volatile compounds, correlation was also observed between the two sequential fermentations with Td315 ($r > 0.9$; $p = 0.038$), although in the comparison with Td315+ScXG3 no correlation was found for some basic chemical parameters, such as sugar content. The influence of the pure culture with Sb474 showed a correlation mainly between the sequential fermentation with ScXG3, but no significant differences were found between both sequential fermentations (except for acidity) and

Table 5. Pearson analysis. Correlation between different chemical compounds and the type of fermentation (pure strains or sequential fermentation with *S. cerevisiae* (indigenous strain XG3 or commercial EC1118 strain) (r: Pearson correlation factor).

Basic compounds				Mayor volatile compounds				All compounds				
r overall	NS+XG3	NS+EC1118	Pure	**r overall**	NS+XG3	NS+EC1118	Pure	**r overall**	ScXG3		ScXG3*	EC1118
NS+XG3		0.01119	0.90839	NS+XG3		0.00097	0.00053	ScXG3			3.60E-11	3.64E-08
NS+EC1118	0.49859		0.63378	NS+EC1118	0.93621		0.00948	ScXG3+	0.97971			2.96E-07
Pure	0.01879	0.10017		Pure	0.54862	0.56474		EC1118	0.99466		0.99028	
r G+F	NS+XG3	NS+EC1118	Pure	**r 1-propanol**	NS+XG3	Pure		**r overall**	Lt93		Lt93+XG3	Lt93+EC1118
NS+XG3		0.0669	0.86208	NS+XG3		0.22280		Lt93			1.95E-08	0.00023
NS+EC1118	0.85186		0.71677	Pure	0.66277			Lt93+XG3	0.95816			0.00281
Pure	0.10854	–0.22434						Lt93+EC1118	0.93407		0.89319	
r TA	NS+XG3	NS+EC1118	Pure	**r 2-methyl-1-propanol**	NS+XG3	NS+EC1118	Pure	**r overall**	Td315		Td315+XG3	Td315+EC1118
NS+XG3		0.57074	0.67839	NS+XG3		0.05638	0.80942	Td315			1.64E-05	1.27E-05
NS+EC1118	–0.34405		0.14339	NS+EC1118	0.86806		0.86528	Td315+XG3	0.87850			0.00084
Pure	–0.2554	0.75108		Pure	0.15025	0.10601		Td315+EC1118	0.97139		0.92935	
r VA	NS+XG3	NS+EC1118	Pure	**r 3-methyl-1-butanol**	NS+XG3	NS+EC1118	Pure	**r overall**	Sb474		Sb474+XG3	Sb474+EC1118
NS+XG3		0.04615	0.45604	NS+XG3		0.04109	0.04054	Sb474			0.02962	0.17303
NS+EC1118	0.88474		0.65738	NS+EC1118	0.89342		0.06032	Sb474+XG3	0.56086			0.00186
Pure	–0.44208	–0.27251		Pure	-0.89438	-0.86188		Sb474+EC1118	0.49744		0.90720	
r Gly	NS+XG3	NS+EC1118	Pure	**r Acetaldehyde**	NS+XG3	NS+EC1118	Pure	**r overall**	Mf278		Mf278+XG3	Mf278+EC1118
NS+XG3		0.00219	0.00969	NS+XG3		0.33377	0.42275	Mf278			0.08303	0.38415
NS+EC1118	0.98503		0.00230	NS+EC1118	0.55288		0.00079	Mf278+XG3	0.46191			0.00484
Pure	0.95960	0.98456		Pure	0.47147	0.99246		Mf278+EC1118	0.33108		0.87118	
r MA	NS+XG3	Pure		**r Ethyl acetate**	NS+XG3	Pure		**r overall**	ScXG3	Lt93	Td315	Sb474	Mf278
								ScXG3		9.51E-09	2.68E-06	0.03651	0.21111
NS+XG3		0.17011		NS+XG3		0.04923		Lt93	0.95456		3.03E-05	0.03232	0.19956
								Td315	0.89596	0.85019		0.00033	0.00457
Pure	0.72009			Pure	0.87960			Sb474	0.52564	0.53608	0.78386		5.51E-07
								Mf278	0.33056	0.33860	0.66932	0.91768	

Chemical parameter: G+F, reducing sugars (glucose + fructose); Gly, glycerol; MA, malic acid; TA, total acidity; VA, volatile acidity. ScXG3: control in pure culture, ScXG3*: control in sequential fermentation. The *p*-value (significance) is shown above the diagonal and the r-value (Pearson's correlation factor) is shown below the diagonal. NS (autochthonous non-*Saccharomyces*).

surprisingly neither between glycerol (parameter with especially high content in *Starm. bacillaris* species). That is, the amount of glycerol is higher in pure culture than in sequential fermentations. This did not happen with the Mf278 strain in which the highest correlations between pure and sequential culture were found only with ScXG3 (not in Mf278+EC1118) in glycerol, acidity, and aromatic compounds, but not for the rest of the non-volatile compounds together. Furthermore, high correlations were found between both sequential fermentations. In this sense, as expected according to the PCA results (Fig. 5B), the correlation between the chemical composition of the pure culture and the sequential fermentations is lower as the effect of the *S. cerevisiae* strain on the chemical composition is significant. Therefore, these autochthonous strains could be used as starters in sequential fermentations to obtain wines with singular properties. The results of the Pearson's test showed some correlations between the strain in pure culture and the sequential fermentation despite the influence of other factors, such as *S. cerevisiae* strain or grape juice characteristics. So, the results suggested that some properties are going to remain stable in the selected strains during sequential fermentations, which maintain the chemical properties.

The results of the PCA and Pearson test showed that the different non-*Saccharomyces* strains influenced the chemical profile of wines, through the formation of different compounds. Sequential inoculation of strain Mf278 increased the production of positive aroma compounds with respect to the indigenous control strain *S. cerevisiae* ScXG3 alone; it should be taken into consideration that ScXG3 has been selected in previous studies for its optimal winemaking behaviour [26,56,57]. Differences could be due to the fermentation process, and it should be considered for the use of these strains in future studies. *T. delbrueckii* and *Starm. bacillaris* strains were the worst qualified; however, several authors obtained better wines than their controls made with pure *S. cerevisiae* following a co-inoculated strategy with these species and *S. cerevisiae* [1,18,42,58]. In fact, there are already commercial strains of *T. delbrueckii* and to a lesser extent of *Starm. bacillaris* that can be used due to their desirable oenological characteristics such as increased glycerol concentration, acidity, low ethanol and esters production [12,59].

All these results are consistent with those found in the previous literature and allow us to affirm that some indigenous strains of non-*Saccharomyces* yeasts, used by sequential inoculation, can contribute to the typicity and differentiation of wines and even increase the contribution of aromatic compounds with positive sensory evaluation [1,3,55]. In this sense, the differentiation and correlations obtained between the different strains and fermentations show that the choice of yeast strain affects the chemical profile of the wines, but certain distinctive parameters are maintained in sequential fermentation.

Therefore, we suggest further research, with different strains and even by applying more than one non-*Saccharomyces* together (in multistarter yeast inoculum or sequentially before inoculation of *S. cerevisiae*) which has been proposed to compensate the shortage of one non-*Saccharomyces* species and further enhance the wine quality [18]. This will make it possible to evaluate the positive synergistic effects on wine quality and typicity, the interaction between species and ultimately increase the knowledge of interspecific relationships. Accordingly, combinations of *T. delbrueckii, Hanseniaspora vineae*, *Hanseniaspora uvarum*, *M. pulcherrima, Zygosaccharomyces bailii* or *L. thermotolerans* were reported as a multistarter or co-inoculation fermentations improving the chemical and aromatic compositions of wines fermented only with *S. cerevisiae* [7,18,39,60].

4. Conclusions

Autochthonous strains of non-*Saccharomyces* yeast species contribute distinctive chemical characteristics to the wines. *L. thermotolerans* and *T. delbrueckii* strains influence acidity or alcohol content; *Starm. bacillaris* and *Metschnikowia* spp. increase glycerol concentration. The correlations observed between wines fermented with the different non-*Saccharomyces* indigenous strains in pure and sequential fermentations suggest that their contribution to wines properties remains stable regardless must composition or winemaking techniques. These results can help winemakers in their choice of species and strains.

Author Contributions

DC and PB designed the research study. DC and PB performed the research. DC designed and developed the chemical analysis (gas chromatography). DC analysed the data. DC and PB wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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.fbe1501001.

References

[1] Agarbati A, Canonico L, Comitini F, Ciani M. Improved *Saccharomyces cerevisiae* Strain in Pure and Sequential Fermentation with *Torulaspora delbrueckii* for the Production of Verdicchio Wine with Reduced Sulfites. Applied Sciences. 2020; 10: 6722.

[2] Englezos V, Rantsiou K, Cravero F, Torchio F, Pollon M, Fracassetti D, *et al*. Volatile profile of white wines fermented with sequential inoculation of *Starmerella bacillaris* and *Saccharomyces cerevisiae*. Food Chemistry. 2018; 257: 350–360.

[3] Binati RL, Lemos Junior WJF, Luzzini G, Slaghenaufi D, Ugliano M, Torriani S. Contribution of Non-*Saccharomyces* Yeasts to Wine Volatile and Sensory Diversity: A Study on *Lachancea thermotolerans*, *Metschnikowia* spp. and *Starmerella bacillaris* Strains Isolated in Italy. International Journal of Food Microbiology. 2020; 318: 108470.

[4] Miura T, Sánchez R, Castañeda LE, Godoy K, Barbosa O. Is Microbial Terroir Related to Geographic Distance between Vineyards? Environmental Microbiology Reports. 2017; 9: 742–749.

[5] Brandão LR, Vaz ABM, Espírito Santo LC, Pimenta RS, Morais PB, Libkind D, *et al*. Diversity and biogeographical patterns of yeast communities in Antarctic, Patagonian and tropical lakes. Fungal Ecology. 2017; 28: 33–43.

[6] Bokulich NA, Collins TS, Masarweh C, Allen G, Heymann H, Ebeler SE, *et al*. Associations among Wine Grape Microbiome, Metabolome, and Fermentation Behavior Suggest Microbial Contribution to Regional Wine Characteristics. MBio. 2016; 7: e00631-16.

[7] Zhang B, Liu H, Xue J, Tang C, Duan C, Yan G. Use of *Torulaspora delbrueckii* and *Hanseniaspora vineae* co-fermentation with *Saccharomyces cerevisiae* to improve aroma profiles and safety quality of Petit Manseng wines. LWT. 2022; 161: 113360.

[8] Benito S. The impact of *Torulaspora delbrueck*ii yeast in winemaking. Applied Microbiology and Biotechnology. 2018; 102: 3081–3094.

[9] Morata A, Loira I, Tesfaye W, Bañuelos MA, González C, Suárez Lepe JA. *Lachancea thermotolerans* Applications in Wine Technology. Fermentation. 2018; 4: 53.

[10] Benito S. The Impacts of *Lachancea thermotolerans* Yeast Strains on Winemaking. Applied Microbiology and Biotechnology. 2018; 102: 6775–6790.

[11] Nisiotou A, Sgouros G, Mallouchos A, Nisiotis C, Michaelidis C, Tassou C, *et al*. The use of indigenous *Saccharomyces cerevisiae* and *Starmerella bacillaris* strains as a tool to create chemical complexity in local wines. Food Research International. 2018; 111: 498–508.

[12] Englezos V, Giacosa S, Rantsiou K, Rolle L, Cocolin L. *Starmerella bacillaris* in winemaking: opportunities and risks. Current Opinion in Food Science. 2017; 17: 30–35.

[13] Vicente J, Ruiz J, Belda I, Benito-Vázquez I, Marquina D, Calderón F, *et al*. The Genus *Metschnikowia* in Enology. Microorganisms. 2020; 8: 1038.

[14] Ruiz J, Belda I, Beisert B, Navascués E, Marquina D, Calderón F, *et al*. Analytical Impact of *Metschnikowia pulcherrima* in the Volatile Profile of Verdejo White Wines. Applied Microbiology and Biotechnology. 2018; 102: 8501–8509.

[15] Capozzi V, Garofalo C, Chiriatti MA, Grieco F, Spano G. Microbial terroir and food innovation: the case of yeast biodiversity in wine. Microbiological Research. 2015; 181: 75–83.

[16] Varela C, Sengler F, Solomon M, Curtin C. Volatile flavour profile of reduced alcohol wines fermented with the non-conventional yeast species *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. Food Chemistry. 2016; 209: 57–64.

[17] Jolly NP, Varela C, Pretorius IS. Not Your Ordinary Yeast: Non-*Saccharomyces* Yeasts in Wine Production Uncovered. FEMS Yeast Research. 2014; 14: 215–237.

[18] Zhu X, Torija M-J, Mas A, Beltran G, Navarro Y. Effect of a Multistarter Yeast Inoculum on Ethanol Reduction and Population Dynamics in Wine Fermentation. Foods. 2021; 10: 623.

[19] Englezos V, Pollon M, Rantsiou K, Ortiz-Julien A, Botto R, Río Segade S, *et al*. *Saccharomyces cerevisiae-Starmerella bacillaris* strains interaction modulates chemical and volatile profile in red wine mixed fermentations. Food Research International. 2019; 122: 392–401.

[20] Thompson LR, Sanders JG, McDonald D, Amir A, Ladau J, Locey KJ, *et al*. A Communal Catalogue Reveals Earth's Multiscale Microbial Diversity. Nature. 2017; 551: 457–463.

[21] Vélez S, Barajas E, Blanco P, Rubio JA, Castrillo D. Spatio-Temporal Analysis of Satellite Imagery (NDVI) to Identify Terroir and Vineyard Yeast Differences According to Appellation of Origin (AOP) and Biogeographic Origin. J. 2021; 4: 244–256.

[22] Castrillo D, Rabuñal E, Neira N, Blanco P. Yeast Diversity on Grapes from Galicia, NW Spain: Biogeographical Patterns and the Influence of the Farming System. Oeno One. 2019; 53: 573–587.

[23] du Plessis HW, du Toit M, Hoff JW, Hart RS, Ndimba BK, Jolly NP. Characterisation of Non-*Saccharomyces* Yeasts Using Different Methodologies and Evaluation of Their Compatibility with Malolactic Fermentation. South African Journal of Enology and Viticulture. 2017; 38: 46–63.

[24] Ciani M, Morales P, Comitini F, Tronchoni J, Canonico L, Curiel JA, *et al*. Non-Conventional Yeast Species for Lowering Ethanol Content of Wines. Frontiers in Microbiology. 2016; 7: 642.

[25] Blanco P, Vázquez-Alén M, Garde-Cerdán T, Vilanova M. Application of Autochthonous Yeast *Saccharomyces cerevisiae* XG3 in Treixadura Wines from D.O. Ribeiro (NW Spain): Effect on Wine Aroma. Fermentation. 2021; 7: 31.

[26] Blanco P, Mirás-Avalos JM, Suárez V, Orriols I. Inoculation of Treixadura Musts with Autochthonous *Saccharomyces cerevisiae* Strains: Fermentative Performance and Influence on the Wine Characteristics. Food Science and Technology International. 2013; 19: 177–186.

[27] OIV. Compendium of International Methods of Wine and Must Analysis. Office International de la Vigne et du Vin: Paris, France. 2017.

[28] Bouzas-Cid Y, Falqué E, Orriols I, Mirás-Avalos JM. Effects of irrigation over three years on the amino acid composition of Treixadura (*Vitis vinifera* L.) musts and wines, and on the aromatic composition and sensory profiles of its wines. Food Chemistry. 2018; 240: 707–716.

[29] Castrillo D, Rabuñal E, Neira N, Blanco P. Oenological potential of non-*Saccharomyces* yeasts to mitigate effects of climate change in winemaking: impact on aroma and sensory profiles of Treixadura wines. FEMS Yeast Research. 2019; 19: foz065.

[30] Blanco P, Castrillo D, Graña MJ, Lorenzo MJ, Soto E. Evaluation of Autochthonous Non-*Saccharomyces* Yeasts by Sequential Fermentation for Wine Differentiation in Galicia (NW Spain). Fermentation. 2021; 7: 183.

[31] Comitini F, Stringini M, Taccari M, Ciani M. A Fast and Simple Method for Wild Yeast Flora Detection in Winemaking. International Journal of Wine Research. 2009; 1: 53–58.

[32] Comitini F, Gobbi M, Domizio P, Romani C, Lencioni L, Mannazzu I, *et al*. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae.* Food Microbiology. 2011; 28: 873–882.
[33] Gonzalez R, Quirós M, Morales P. Yeast respiration of sugars by non-*Saccharomyces* yeast species: a promising and barely explored approach to lowering alcohol content of wines. Trends in Food Science & Technology. 2013; 29: 55–61.
[34] Bagheri B, Zambelli P, Vigentini I, Bauer FF, Setati ME. Investigating the Effect of Selected Non-*Saccharomyces* Species on Wine Ecosystem Function and Major Volatiles. Frontiers in Bioengineering and Biotechnology. 2018; 6: 169.
[35] Schwinn M, Durner D, Wacker M, Delgado A, Fischer U. Impact of fermentation temperature on required heat dissipation, growth and viability of yeast, on sensory characteristics and on the formation of volatiles in Riesling. Australian Journal of Grape and Wine Research. 2019; 25: 173–184.
[36] Binati RL, Innocente G, Gatto V, Celebrin A, Polo M, Felis GE, *et al*. Exploring the diversity of a collection of native non-*Saccharomyces* yeasts to develop co-starter cultures for winemaking. Food Research International. 2019; 122: 432–442.
[37] Vilela A. *Lachancea thermotolerans*, the Non-*Saccharomyces* Yeast That Reduces the Volatile Acidity of Wines. Fermentation. 2018; 4: 56.
[38] Vicente J, Baran Y, Navascués E, Santos A, Calderón F, Marquina D, *et al*. Biological management of acidity in wine industry: a review. International Journal of Food Microbiology. 2022; 375: 109726.
[39] Zhang B, Tang C, Yang D, Liu H, Xue J, Duan C, *et al*. Effects of three indigenous non-*Saccharomyces* yeasts and their pairwise combinations in co-fermentation with *Saccharomyces cerevisiae* on volatile compounds of Petit Manseng wines. Food Chemistry. 2022; 368: 130807.
[40] Balikci EK, Tanguler H, Jolly NP, Erten H. Influence of *Lachancea thermotolerans* on Cv. Emir Wine Fermentation. Yeast. 2016; 33: 313–321.
[41] Escribano R, González-Arenzana L, Portu J, Garijo P, López-Alfaro I, López R, *et al*. Wine Aromatic Compound Production and Fermentative Behaviour within Different Non-*Saccharomyces* Species and Clones. Journal of Applied Microbiology. 2018; 124: 1521–1531.
[42] Zhu X, Navarro Y, Mas A, Torija M-J, Beltran G. A Rapid Method for Selecting Non-*Saccharomyces* Strains with a Low Ethanol Yield. Microorganisms. 2020; 8: 658.
[43] Hranilovic A, Gambetta JM, Jeffery DW, Grbin PR, Jiranek V. Lower-alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co-fermentations: the effect of sequential inoculation timing. International Journal of Food Microbiology. 2020; 329: 108651.
[44] Ciani, Ferraro. Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. Journal of Applied Microbiology. 1998; 85: 247–254.
[45] Englezos V, Torchio F, Cravero F, Marengo F, Giacosa S, Gerbi V, *et al*. Aroma profile and composition of Barbera wines obtained by mixed fermentations of *Starmerella bacillaris* (synonym *Candida zemplinina*) and *Saccharomyces cerevisiae*. LWT. 2016; 73: 567–575.
[46] Cordente AG, Espinase Nandorfy D, Solomon M, Schulkin A, Kolouchova R, Francis IL, *et al*. Aromatic Higher Alcohols in Wine: Implication on Aroma and Palate Attributes during Chardonnay Aging. Molecules. 2021; 26: 4979.
[47] Guth H. Quantitation and Sensory Studies of Character Impact Odorants of Different White Wine Varieties. Journal of Agricultural and Food Chemistry. 1997; 45: 3027–3032.
[48] Bagheri B, Bauer FF, Cardinali G, Setati ME. Ecological interactions are a primary driver of population dynamics in wine yeast microbiota during fermentation. Scientific Reports. 2020; 10: 4911.
[49] Renault P, Miot-Sertier C, Marullo P, Hernández-Orte P, Lagarrigue L, Lonvaud-Funel A, *et al*. Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: Potential applications in the wine industry. International Journal of Food Microbiology. 2009; 134: 201–210.
[50] Englezos V, Cravero F, Torchio F, Rantsiou K, Ortiz-Julien A, Lambri M, *et al*. Oxygen availability and strain combination modulate yeast growth dynamics in mixed culture fermentations of grape must with *Starmerella bacillaris* and *Saccharomyces cerevisiae.* Food Microbiology. 2018; 69: 179–188.
[51] Englezos V, Rantsiou K, Giacosa S, Río Segade S, Rolle L, Cocolin L. Cell-to-Cell Contact Mechanism Modulates *Starmerella bacillaris* Death in Mixed Culture Fermentations with *Saccharomyces cerevisiae*. International Journal of Food Microbiology. 2019; 289: 106–114.
[52] Harlé O, Legrand J, Tesnière C, Pradal M, Mouret JR, Nidelet T. Investigations of the mechanisms of interactions between four non-conventional species with *Saccharomyces cerevisiae* in oenological conditions. PLoS ONE. 2020; 15: e0233285.
[53] Nisiotou A, Mallouchos A, Tassou C, Banilas G. Indigenous Yeast Interactions in Dual-Starter Fermentations May Improve the Varietal Expression of Moschofilero Wine. Frontiers in Microbiology. 2019; 10: 1712.
[54] Tofalo R, Patrignani F, Lanciotti R, Perpetuini G, Schirone M, Di Gianvito P, *et al*. Aroma Profile of Montepulciano d'abruzzo Wine Fermented by Single and Co-Culture Starters of Autochthonous *Saccharomyces* and Non-*Saccharomyces* Yeasts. Frontiers in Microbiology. 2016; 7: 610.
[55] García M, Esteve-Zarzoso B, Cabellos JM, Arroyo T. Sequential Non-*Saccharomyces* and *Saccharomyces cerevisiae* Fermentations to Reduce the Alcohol Content in Wine. Fermentation. 2020; 6: 60.
[56] Blanco P, Rabuñal E, Neira N, Castrillo D. Dynamic of *Lachancea thermotolerans* Population in Monoculture and Mixed Fermentations: Impact on Wine Characteristics. Beverages. 2020; 6: 36.
[57] Blanco P, Mirás-Avalos JM, Pereira E, Orriols I. Fermentative Aroma Compounds and Sensory Profiles of Godello and Albariño Wines as Influenced by *Saccharomyces cerevisiae* Yeast Strains. Journal of the Science of Food and Agriculture. 2013; 93: 2849–2857.
[58] Englezos V, Rantsiou K, Cravero F, Torchio F, Ortiz-Julien A, Gerbi V, *et al*. *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. Applied Microbiology and Biotechnology. 2016; 100: 5515–5526.
[59] Roudil L, Russo P, Berbegal C, Albertin W, Capozzi GS and V. Non-*Saccharomyces* Commercial Starter Cultures: Scientific Trends, Recent Patents and Innovation in the Wine Sector. Recent Patents on Food, Nutrition & Agriculture. 2020; 11: 27–39.
[60] Sainz F, Pardo J, Ruiz A, Expósito D, Armero R, Querol A, *et al*. Use of Non-Conventional Yeasts to Increase Total Acidity in the Cava Base Wines. LWT. 2022; 158: 113183.