applied sciences

MDPI

Article

Improved *Saccharomyces cerevisiae* Strain in Pure and Sequential Fermentation with *Torulaspora delbrueckii* for the Production of Verdicchio Wine with Reduced Sulfites

Alice Agarbati, Laura Canonico *, Francesca Comitini and Maurizio Ciani

Department of Life and Environmental Sciences, Polytechnic University of Marche, Via Brecce Bianche, 60131 Ancona, Italy; a.agarbati@pm.univpm.it (A.A.); f.comitini@univpm.it (F.C.); m.ciani@univpm.it (M.C.)
* Correspondence: l.canonico@univpm.it; Tel.: +39-071-2204987

Received: 3 September 2020; Accepted: 24 September 2020; Published: 25 September 2020

Featured Application: The present research is finalized to propose a potential application of improved native *Saccharomyces cerevisiae* strain in pure and/or mixed fermentation with Torulaspora delbrueckii to obtain wine with a reduced sulfites content and a peculiar aromatic taste at winery condition.

Abstract: The application of yeast strains that are low producers of sulfur compounds is actually required by winemakers for the production of organic wine. This purpose could be satisfied using a native *Saccharomyces cerevisiae* strain improved for oenological aptitudes. Moreover, to improve the aromatic complexity of wines, sequential fermentations carried out with *S. cerevisiae*/non-*Saccharomyces* yeast is widely used. For these reasons, in the present work an improved native *S. cerevisiae* low producer of sulfite and sulfide compounds was evaluated in pure and in sequential fermentation with a selected *Torulaspora delbrueckii*. Additionally, the influence of grape juices coming from three different vintages under winery conditions was evaluated. In pure fermentation, improved native *S. cerevisiae* strain exhibited a behavior related to vintage, highlighting that the composition of grape juice affects the fermentation process. In particular, an increase in ethyl octanoate (vintage 2017) and phenyl ethyl acetate (vintage 2018) was detected. Moreover, isoamyl acetate was highly consistent and could be a distinctive aroma of the strain. The sequential fermentation *T. delbrueckii*/*S. cerevisiae* determined an increase in aroma compounds such as phenyl ethyl acetate and ethyl hexanoate. In this way, it was possible to produce Verdicchio wine with reduced sulfites and characterized by a peculiar aromatic taste.

Keywords: *Torulaspora delbrueckii*; *Saccharomyces cerevisiae*; sulfites; organic wine; Verdicchio; aroma profile

1. Introduction

The use of *Saccharomyces cerevisiae* as a starter culture significantly improves the control fermentation process, avoiding negative repercussions in winemaking. On the other hand, this biotechnological practice may determine a standardization of wine flavor [1]. Different approaches and alternative strategies to improve the analytical and aroma profile, enhancing the complexity of wines, were proposed. In this regard, several research studies evaluated the positive influence of non-*Saccharomyces* yeasts in mixed fermentation on the flavor complexity [2–10]. In addition, non-*Saccharomyces* yeasts exhibited other features such as bio-protective action, antimicrobial activity, and variations on the production of some compounds such as ethanol, glycerol, lactic acid in mixed

fermentation that could be of interest in winemaking. In particular, *Torulaspora delbrueckii* showed the capacity to reduce the final ethanol content of wine and to act against spoilage yeasts [11–13].

The use of selected *S. cerevisiae* strains as starter cultures of fermentation could represent another possibility to confer a specific aromatic imprinting to the wine. This aspect could be further influenced by the metabolic interaction between *S. cerevisiae*/non-*Saccharomyces* strains in mixed starter cultures [14,15]. Indeed, *S. cerevisiae* strains can metabolically interact through the modification of fermentation products when they grow in mixed cultures. For this reason, in recent decades, researchers proposed the use of tailored strains, often isolated from grapes (native yeast strains) that possibly reflect a specific geographical area, as a valid alternative to the use of selected starter cultures, with the purpose of conferring uniqueness to the wines [16–21]. On the other hand, the native *S. cerevisiae* strains often do not show all the desired oenological aptitudes. About this, several studies described different approaches and strategies to improve *S. cerevisiae* strains obtaining yeasts with desired characteristics suitable for winemaking [21–23]. For these reasons, in the present study, we evaluated the use of the improved native *S. cerevisiae* for the low production of sulfite and sulfide compounds coming from Verdicchio wine area [21]. This *S. cerevisiae* strain was evaluated in pure and in sequential fermentation with a selected *T. delbrueckii* strain for the production of low sulfites wine under winery conditions. The fermentation performance and the aroma profile of the resulted wines were assessed.

2. Materials and Methods

2.1. Yeast Strains

The improved *S. cerevisiae* native strain, DiSVA 708, belonging to the Yeast Collection of the Department of Life and Environmental Sciences (DiSVA) of the Polytechnic University of Marche (Italy) [21] and the commercial *S. cerevisiae* starter strain, Lalvin ICV OKAY® (Lallemand Inc., Toulouse, France) were used. The commercial strain is characterized by the absence of H_2S production and reduced production of SO_2.

The non-*Saccharomyces* strain, *T. delbrueckii* DiSVA 130, was used in the sequential fermentations with the improved native *S. cerevisiae* DiSVA 708 over the two vintages (2017–2018). It was originally isolated from grapes and used in mixed fermentation to enhance the analytical and aromatic profile of the wine [24].

All of the yeast strains were maintained on yeast extract–peptone–dextrose (YPD) agar medium (Oxoid, Basingstoke, U.K.) at 4 °C for short-term storage and in YPD broth supplemented with 80% (*w*/*v*) glycerol at −80 °C for long-term storage.

2.2. Fermentation Trials

Fermentation trials were performed over three consecutive years: 2016–2018, using organic Verdicchio grape juice. For each vintage, the grapes were treated following the same winemaking process (soft hydraulic press pressing and cold clarification at 8 °C for 5 days). The analytical characters of the grape musts (initial sugars, pH, total acidity, malic acid, total SO_2 and nitrogen content) are shown in Table 1. The diammonium phosphate and yeast derivative (Genesis Lift® Oenofrance, Bordeaux, France) used were adjusted to 250 mg N/L as yeast assimilable nitrogen. The fermentation trials were carried out in a steel vessel containing 40 L of grape juice in duplicate under static conditions at 18 °C.

All of the yeast strains were pre-cultured in modified YPD medium (0.5% yeast extract, 0.1% peptone and 2% glucose) for 72 h at 25 °C in an orbital shaker (150 rpm). The biomass was collected by centrifugation, washed three times with sterile distilled water and inoculated into the grape juice to obtain an initial concentration of approximately 1×10^6 cell mL^{-1}. For vintages 2017 and 2018, the sequential fermentations were carried out by inoculation of *T. delbrueckii* DiSVA 130 followed by inoculation of *S. cerevisiae* DiSVA 708 after two days from the start of fermentation. The growth kinetics of the yeast strains were monitored during the fermentation at established

intervals using WL nutrient Agar medium (Oxoid, Hampshire, U.K.) and Lysine Agar medium (Oxoid, Hampshire, U.K.) [25]. The sugar consumption during the fermentation process was measured by Baumé (°Bé) densimeter.

Table 1. Main analytical parameters of the organic Verdicchio grape juice used across the vintages 2016–2018.

Grape Juice Parameters	Vintage 2016	Vintage 2017	Vintage 2018
Initial sugars (g L^{-1})	236.00	212.00	203.00
pH	3.22	3.22	3.26
Total acidity (g L^{-1})	4.42	4.58	4.83
Malic acid (g L^{-1})	2.70	2.50	2.40
Total SO_2 (mg L^{-1})	35.00	27.00	26.00
Nitrogen content YAN * (mg N L^{-1})	89.00	60.00	62.00

* YAN = yeast assimilable nitrogen.

2.3. Analytical Procedures

Total acidity, volatile acidity, pH, ethanol content and total SO_2 were determined according to the Official European Union Methods (EC Regulation No. 2870/00) [26]. Enzymatic kits (Megazyme International Ireland) were used to measure the amounts of glucose and fructose (K-FRUGL), glycerol (K-GCROL), and malic acid (K-DMAL) according to the manufacturer instructions. Acetate strips (CARLO ERBA Reagents S.r.l., Milan, Italy) were used to evaluate the H_2S production during the fermentation process. The production of H_2S was detected by the increase in color of acetate strips from white to black in function of H_2S during the fermentation. The semi-quantitative evaluation was expressed using an arbitrary scoring scale from zero (no H_2S production, white strip) to 5 (maximum level of H_2S production, black strip).

A specific enzymatic kit (kit no. 112732; Roche Diagnostics, Germany) was used to determine the ammonium content. The free α-amino acids were evaluated following Dukes and Butzke protocol [27].

Ethyl acetate, acetaldehyde and alcohols (n-propanol, isobutanol, amyl and isoamyl alcohols) were quantified using a gas chromatograph system (GC-2014; Shimadzu, Kjoto, Japan) by direct injection. The final wines were prepared as described by Canonico et al. [24]. The solid-phase microextraction (HS-SPME) method was used to quantify the main volatile compounds as described by Canonico et al. [28]. The compounds were desorbed by inserting the fiber Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Sigma-Aldrich, St. Louis, MO, USA) into a gas chromatograph (GC) injector.

2.4. Sensory Analysis

At the end of the fermentation, the wines obtained were transferred into completely filled 750 mL bottles, closed with the crown cap and maintained at 4 °C until sensory analysis. After storage for 3 months, wines were subjected to sensory analysis on the basis of smell and taste. A group of ten testers (80% expert and 20% non-expert), using a score scale of 1 to 10, expressed their opinion regarding smell and taste of each wine tested. The data obtained were used to compare the wines and provide information regarding the organoleptic quality and probable consumers' acceptability of the wines obtained.

2.5. Statistical Analysis

Analysis of variance (ANOVA) was used to elaborate the analytical character data of wine. The means were analyzed using the statistical software package JMP® 11. Duncan tests were used to detect the significant differences. The experimental data were significant with associated p-values < 0.05.

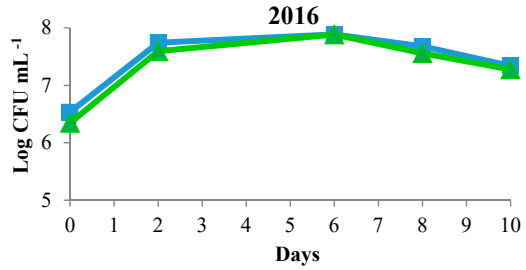
Principal Component Analysis (PCA) carried out using JMP 11® statistical software (Statistical discovery from SAS, New York, NY, USA) was used to analyze the mean values of each volatile compound and the main by-products of fermentation. The mean data were normalized to eliminate the influence of hidden factors.

3. Results

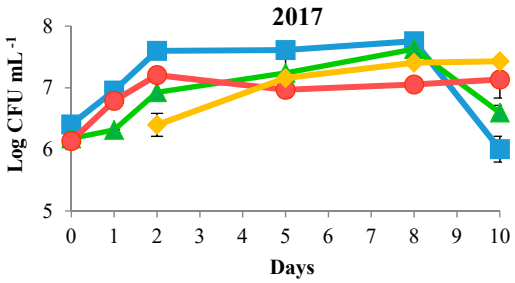
3.1. Biomass Evolution and Sugar Consumption

Growth kinetics of the pure and sequential fermentations carried out during the vintages 2016–2018 are reported in Figure 1. The results showed that improved *S. cerevisiae* DiSVA 708 exhibited the same growth kinetics of OKAY® starter strain in vintage 2016 (Figure 1a), while in vintages 2017–2018, DiSVA 708 showed lower growth kinetics. In particular, DiSVA 708 achieved a similar biomass concentration (about 5×10^7 CFU/mL) exhibited by OKAY® on the 8th day and 5th day of fermentation and in vintage 2017 and 2018, respectively (Figure 1b,c). In sequential fermentations, *T. delbrueckii* on the 2nd day of fermentation (2017–2018) achieved the maximum cell concentration (about 10^7 cell/mL). In vintage 2017 (Figure 1b), this value was constant until the end of fermentation, while in vintage 2018 the cell concentration decreased until 10^6 cell/mL (Figure 1c). In both vintages (2017–2018), the DiSVA 708 strain, inoculated after 48 h of fermentation, reached on the 5th day of fermentation the maximum cell concentration and remained approximatively constant until the end of fermentation (about 5×10^7 cell/mL) (Figure 1b,c).

The sugar consumption (Figure 2) exhibited a similar trend independently by fermentation trials and vintages, completing the fermentation process on the 10th day. Only *S. cerevisiae* OKAY® showed a faster rate of sugar consumption in vintage 2016 (Figure 2).

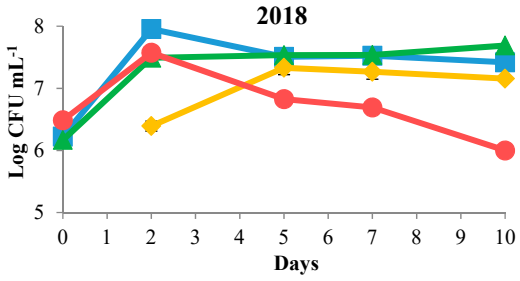


(**a**)



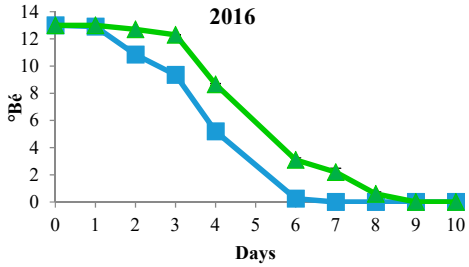
(**b**)

Figure 1. *Cont.*

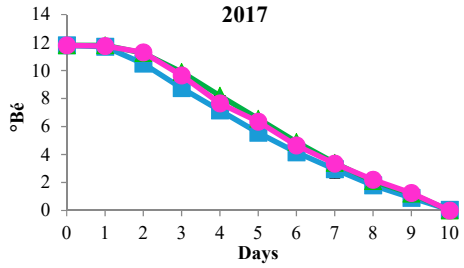


(**c**)

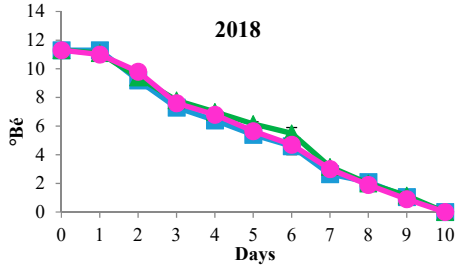
Figure 1. Growth kinetics of the pure and sequential fermentations in three consecutive years: 2016 (**a**), 2017 (**b**) and 2018 (**c**). Pure cultures of *S. cerevisiae* Lalvin ICV OKAY® (—■—) and *S. cerevisiae* DiSVA 708 (—▲—) in each vintage. Sequential fermentations of *T. delbrueckii* DiSVA 130 (—●—) and *S. cerevisiae* DiSVA 708 (—◆—) in the 2017 and 2018 vintages.



(**a**)



(**b**)



(**c**)

Figure 2. Kinetics of sugar consumption of the pure and sequential fermentations of all vintages analyzed: 2016 (**a**), 2017 (**b**), 2018 (**c**). Pure cultures of *S. cerevisiae* Lalvin ICV OKAY® (—■—) and *S. cerevisiae* DiSVA 708 (—▲—) in each vintage. Sequential fermentations of *T. delbrueckii* DiSVA 130 and *S. cerevisiae* DiSVA 708 (—●—) in the 2017 and 2018 vintages.

3.2. Main Oenological Characters and Volatile Compounds of S. cerevisiae Pure Fermentations

The main analytical characters of the fermentation trials carried out during the three consecutive vintages are reported in Table 2. With regard to the ethanol content in the resulting wines carried out by the *S. cerevisiae* pure fermentations (vintages 2017–2018), the two strains exhibited a comparable alcohol content consuming all sugars. Only in vintage 2016, DiSVA 708 showed a lower ethanol content than that exhibited by OKAY® due to a slight amount of residual sugars. On the other hand, the differences in the final ethanol content among the three vintages were due to different initial sugar content in the grape juice. OKAY® fermentation trials showed a constantly lower total acidity than the DiSVA 708 pure fermentation trials through the vintages, while the production of volatile acidity showed more variation among the vintages although it was produced in low quantities. With regard to the glycerol content, *S. cerevisiae* fermentations did not show significant differences, with the exception of *T. delbrueckii* sequential fermentation (vintage 2017). A variable trend was exhibited for malic acid content.

The starter OKAY® exhibited the lowest SO_2 production in all vintages, despite the variability found among the vintages. However, the SO_2 content of the other fermentation trials was in any case low in relation to the initial SO_2 content of grape juice. The score of H_2S production was the same for both *S. cerevisiae* pure fermentation in vintages 2017–2018 while in 2016 it was significantly different.

The results of volatile compounds are reported in Table 3. DiSVA 708 led a greater general increase in esters content than did OKAY®, with a different trend among the vintages. In particular, DiSVA 708 significantly increased ethyl acetate (2016); ethyl hexanoate (2017); phenyl ethyl acetate, ethyl octanoate and isoamyl acetate (2018). On the other hand, *S. cerevisiae* OKAY® showed a significantly high production in ethyl butyrate in all vintages, a compound characterizing the volatile profile of this strain. With regard to higher alcohols, the variations found were more dependent on the harvest than on the inoculated strains. The production of n-propanol was an exception. Indeed, OKAY® showed a production of n-propanol consistently and was significantly higher than the other trials in all vintages even when at different concentrations. Notably, an increase in acetaldehyde content in pure fermentation in vintage 2016 and a reduction in vintage 2018 by DiSVA 708 were observed.

Table 2. Main analytical parameters of the wines obtained across the vintages 2016–2018. Data are means ± standard deviations. Values displaying different superscript letters (^{a,b,c,d,e}) within each column are significantly different according to Duncan tests ($p < 0.05$). The abbreviations Td/708 indicate the sequential fermentations carried out by *T. delbrueckii*/*S. cerevisiae* DiSVA 708.

		Ethanol (% *v*/*v*)	Residual Sugars (g L^{-1})	Total Acidity (Tartaric Acid g L^{-1})	Volatile Acidity (Acetic Acid g L^{-1})	Glycerol (g L^{-1})	Malic Acid (g L^{-1})	Total SO_2 (mg L^{-1})	H_2S (Score)
2016	**OKAY**	14.24 ± 0.07 ^{a}	2.13 ± 0.76 ^{c}	5.73 ± 0.03 ^{d}	0.54 ± 0.01 ^{a}	7.48 ± 0.27 ^{a}	1.35 ± 0.09 ^{b}	6.50 ± 0.71 ^{d}	1 ± 0.0 ^{c}
	DiSVA 708	13.89 ± 0.17 ^{b}	7.01 ± 2.31 ^{a}	6.17 ± 0.03 ^{b}	0.49 ± 0.01 ^{b}	6.82 ± 0.42 ^{a}	1.60 ± 0.12 ^{a}	18.00 ± 0.72 ^{c}	3 ± 0.0 ^{a}
2017	**OKAY**	13.2 ± 0.12 ^{c}	1.80 ± 0.14 ^{c}	5.79 ± 0.12 ^{d}	0.33 ± 0.05 ^{d}	7.23 ± 0.19 ^{a}	1.35 ± 0.07 ^{b}	14.00 ± 2.83 ^{c}	1 ± 0.0 ^{c}
	DiSVA 708	13.31 ± 0.04 ^{c}	2.1 ± 0.28 ^{c}	6.15 ± 0.10 ^{b}	0.42 ± 0.03 ^{c}	7.20 ± 0.24 ^{a}	1.1 ± 0.00 ^{c}	39.00 ± 2.82 ^{a}	1 ± 0.0 ^{c}
	Td/708	13.26 ± 0.07 ^{c}	3.60 ± 0.53 ^{b}	6.01 ± 0.01 ^{c}	0.30 ± 0.03 ^{d}	5.23 ± 0.18 ^{b}	1.0 ± 0.00 ^{c}	33.50 ± 0.71 ^{b}	3 ± 0.0 ^{a}
2018	**OKAY**	12.57 ± 0.01 ^{d}	1.45 ± 0.07 ^{d}	6.11 ± 0.03 ^{b,c}	0.14 ± 0.01 ^{e}	NA *	1.65 ± 0.07 ^{a}	24.00 ± 0.00 ^{c}	2 ± 0.0 ^{b}
	DiSVA 708	12.43 ± 0.01 ^{d}	1.55 ± 0.07 ^{d}	6.40 ± 0.09 ^{a}	0.15 ± 0.01 ^{e}	NA	1.55 ± 0.07 ^{a}	33.00 ± 1.41 ^{b}	2 ± 0.0 ^{b}
	Td/708	12.50 ± 0.02 ^{d}	1.77 ± 0.00 ^{c,d}	6.39 ± 0.03 ^{a}	0.15 ± 0.01 ^{e}	NA	1.65 ± 0.07 ^{a}	30.50 ± 2.12 ^{b}	3 ± 0.0 ^{a}

NA * = not available.

Table 3. The main by-products of fermentation and volatile compounds of the wines obtained for vintages 2016–2018, in pure and in sequential fermentations with the non-*Saccharomyces* strain added 2 days prior to the *S. cerevisiae* DiSVA 708 strain. Data are means ± standard deviations. Values displaying different superscript letters (^{a,b,c,d,e,f}) within each line are significantly different according to Duncan tests ($p < 0.05$). The abbreviation Td/708 indicates the sequential fermentations carried out by *T. delbrueckii*/*S. cerevisiae* DiSVA 708.

Esters (mg L^{-1})	**2016**		**2017**			**2018**		
	OKAY	**DiSVA 708**	**OKAY**	**DiSVA 708**	**Td/708**	**OKAY**	**DiSVA 708**	**Td/708**
Ethyl butyrate	3.05 ± 0.08 ^{a}	0.74 ± 0.07 ^{b,c}	1.29 ± 0.66 ^{b}	0.96 ± 0.05 ^{b,c}	0.82 ± 0.01 ^{b,c}	1.44 ± 0.54 ^{b,c}	0.59 ± 0.01 ^{c}	0.62 ± 0.00 ^{c}
Ethyl acetate	23.14 ± 0.15 ^{b}	31.14 ± 0.15 ^{a}	12.74 ± 0.23 ^{c}	13.97 ± 0.40 ^{c}	12.92 ± 0.40 ^{c}	18.18 ± 9.07 ^{b,c}	24.25 ± 1.30 ^{a,b}	16.92 ± 2.43 ^{b,c}
Phenyl ethyl acetate	0.25 ± 0.00 ^{c}	0.28 ± 0.04 ^{c}	0.21 ± 0.04 ^{c}	0.24 ± 0.01 ^{c}	0.33 ± 0.01 ^{b,c}	0.22 ± 0.07 ^{c}	0.59 ± 0.08 ^{a,b}	0.68 ± 0.06 ^{a}
Ethyl hexanoate	0.43 ± 0.07 ^{c}	0.25 ± 0.07 ^{c}	0.84 ± 0.07 ^{b}	1.69 ± 0.13 ^{a}	1.70 ± 0.02 ^{a}	0.36 ± 0.10 ^{c}	0.23 ± 0.07 ^{c}	0.71 ± 0.21 ^{b}
Ethyl octanoate	0.01 ± 0.00 ^{b}	0.01 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.01 ± 0.00 ^{b}	0.01 ± 0.00 ^{b}	0.009 ± 0.012 ^{b}	0.02 ± 0.00 ^{a}	0 ± 0 ^{b}
Isoamyl acetate	1.16 ± 0.18 ^{c}	2.14 ± 0.31 ^{b}	1.71 ± 0.35 ^{c}	2.88 ± 0.36 ^{b}	3.15 ± 0.09 ^{a,b}	2.30 ± 0.41 ^{b}	3.97 ± 1.18 ^{a}	3.83 ± 1.49 ^{a}
Alcohols (mg L^{-1})								
n-propanol	109.08 ± 0.31 ^{b}	45.75 ± 0.01 ^{e}	89.30 ± 0.47 ^{c}	33.25 ± 0.11 ^{f}	31.27 ± 0.60 ^{f}	125.18 ± 4.98 ^{a}	28.31 ± 0.74 ^{d}	45.22 ± 1.18 ^{e}
Isobutanol	13.19 ± 0.15 ^{d,e}	23.93 ± 0.22 ^{b}	12.76 ± 0.40 ^{d,e}	11.74 ± 0.43 ^{e}	20.43 ± 0.06 ^{c}	14.79 ± 0.73 ^{d}	28.31 ± 0.01 ^{a}	27.78 ± 3.18 ^{a}
Amyl alcohol	12.68 ± 0.17 ^{c,d}	18.10 ± 0.44 ^{a}	11.21 ± 0.30 ^{e}	9.88 ± 0.09 ^{f}	12.17 ± 0.95 ^{d,e}	13.89 ± 0.01 ^{b}	13.82 ± 0.35 ^{b,c}	11.68 ± 0.89 ^{d,e}
Isoamyl alcohol	116.46 ± 0.51 ^{b,c}	151.91 ± 0.81 ^{a}	120.78 ± 0.67 ^{b}	64.53 ± 0.64 ^{e}	110.11 ± 5.91 ^{c}	114.23 ± 0.99 ^{c}	60.36 ± 0.08 ^{e}	82.72 ± 4.83 ^{d}
β-Phenyl ethanol	12.03 ± 0.74 ^{b,c}	16.61 ± 0.23 ^{b}	14.22 ± 2.24 ^{b,c}	8.32 ± 0.60 ^{c}	12.97 ± 0.25 ^{b,c}	29.38 ± 0.26 ^{a}	27.86 ± 0.43 ^{a}	29.86 ± 0.56 ^{a}

Table 3. *Cont.*

Esters (mg L^{-1})	2016		2017			2018		
	OKAY	**DiSVA 708**	**OKAY**	**DiSVA 708**	**Td/708**	**OKAY**	**DiSVA 708**	**Td/708**
Carbonyl Compounds (mg L^{-1})								
Acetaldehyde	6.01 ± 0.1 ^{d}	53.42 ± 0.27 ^{a}	8.99 ± 0.23 ^{c,d}	12.05 ± 0.01 ^{c,d}	15.77 ± 0.45 ^{c}	46.62 ± 10.47 ^{a}	29.55 ± 0.91 ^{b}	16.51 ± 1.87 ^{c}
Monoterpenes (mg L^{-1})								
Linalool	0.04 ± 0.04 ^{a,b}	0.00 ± 0.00 ^{c}	0.06 ± 0.01 ^{a,b}	0.03 ± 0.01 ^{b,c}	0.04 ± 0.00 ^{b,c}	0.046 ± 0.015 ^{a,b}	0.02 ± 0.001 ^{b,c}	0.08 ± 0.02 ^{a}
Geraniol	0.01 ± 0.01 ^{b}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}	0.01 ± 0.01 ^{b}	0.09 ± 0.02 ^{a}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}

3.3. Main Oenological Characters and Volatile Compounds of T. delbrueckii/S. cerevisiae DiSVA 708 Sequential Fermentations

The results of the main oenological characters (Table 2) of *T. delbrueckii/S. cerevisiae* DiSVA 708 sequential fermentation highlighted a comparable trend with regard to ethanol, residual sugar, total and volatile acidity and malic acid in comparison to DiSVA 708 pure fermentation. Meanwhile, significant differences among the vintages were found to highlight the differences in grape juice composition between one vintage and the other. The use of *T. delbrueckii* in sequential fermentation allowed a greater reduction in SO_2 content than in DiSVA 708 pure fermentation in both vintages (significant only vintage 2017) (Table 2). Moreover, *T. delbrueckii* sequential fermentations exhibited the same H_2S production in vintages 2017–2018, but a higher score in comparison with *S. cerevisiae* pure cultures. *T. delbrueckii* sequential fermentations showed a significant reduction in glycerol content in comparison with the other trials while a variable trend of malic acid content was observed among the fermentation trials independently by strains and vintages.

The main by-products and the main volatile compounds are reported in Table 3. Sequential fermentation *T. delbrueckii/S. cerevisiae* DiSVA 708 showed a number of esters during vintage 2017 comparable with DiSVA 708 pure fermentation. In vintage 2018, the presence of *T. delbrueckii* led to an increase in phenyl ethyl acetate and ethyl hexanoate when compared with DiSVA 708 pure fermentation. Regarding the higher alcohol content, the use of *T. delbrueckii* led to wines with an increase in isoamyl alcohol and to a lesser extent of β-phenyl ethanol (vintage 2017). Monoterpenes compounds were generally improved by the presence and fermentation activity of *T. delbrueckii*, even if the geraniol in 2018 vintage was not detected. A significant reduction in acetaldehyde content was exhibited in vintage 2018.

3.4. Principal Component Analysis of the By-Products and Main Volatile Compounds

The PCA of the main volatile compounds and all by-products was used to assess the effect of vintage and the inoculated pure and sequential fermentations (Figure 3). The graphical representation indicated that the fermentations were grouped according to both vintage and inoculated *S. cerevisiae*. Indeed, it is possible to group for the vintages (bottom-right 2016; bottom-left 2017; upper 2018). Strain effect was also detected. *S. cerevisiae* OKAY® trials were grouped together in bottom quadrants. The sequential fermentations carried out with *T. delbrueckii* were grouped together in the upper-left quadrant and closed to DiSVA 708 pure fermentations of the same vintage that showed some variations. DiSVA 708 2016 trials were different from the other trials (2017–2018), probably caused by an incomplete fermentation (residual sugar). The results obtained by PCA analysis showed that the final composition of wine was affected at different levels by the vintage, inoculated *S. cerevisiae* and non-*Saccharomyces* used in sequential fermentation.

3.5. Sensory Analysis

The wines obtained by pure (OKAY® and *S. cerevisiae* DiSVA 708) and sequential fermentations (*T. delbruekii* DiSVA 130/*S. cerevisiae* DiSVA 708) underwent sensory analysis, and the results are reported in Table 4. All the wines obtained did not show significant differences regarding smell and taste. However, the testers expressed a positive judgement regarding each wine, characterized by specific aromatic notes and without defects.

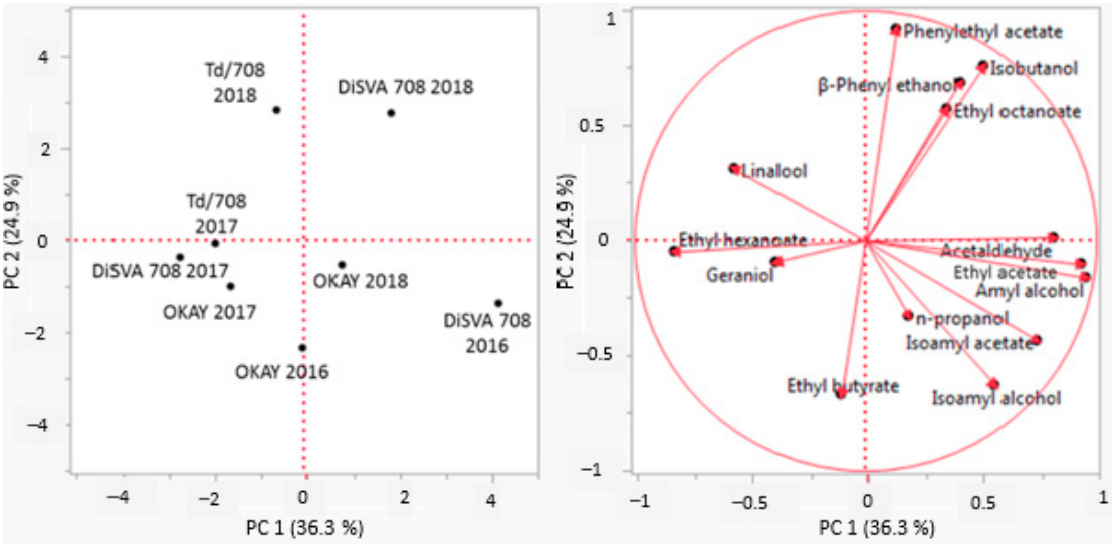


Figure 3. Principal Component Analysis based on the data regarding the main by-products of fermentation and volatile compounds of the wines obtained by pure and sequential fermentations for all vintages: 2016, 2017, 2018. The abbreviations Td/DiSVA 708 indicate the sequential fermentations carried out by *T. delbrueckii*/*S. cerevisiae* DiSVA 708.

Table 4. Sensory analysis of the wines obtained by pure and sequential fermentations in three vintages. Pure culture was carried out by *S. cerevisiae* OKAY and DiSVA 708. The abbreviations Td/708 indicate sequential fermentations carried out by *T. delbrueckii*/*S. cerevisiae* DiSVA 708, respectively. Data are means ± standard deviations. Values displaying different superscript letters (^{a,b,c}) within each column are significantly different according to Duncan tests ($p < 0.05$).

Vintages	Trials	Smell (Score)	Taste (Score)
2016	OKAY	6.78 ± 0.11 ^{a,b}	6.34 ± 0.08 ^{b}
	DiSVA 708	7.35 ± 0.12 ^{a}	7.55 ± 0.40 ^{a}
2017	OKAY	5.94 ± 0.03 ^{c}	5.89 ± 0.76 ^{c}
	DiSVA 708	6.00 ± 0.10 ^{c}	7.17 ± 0.16 ^{a}
	Td/708	5.78 ± 1.73 ^{c}	6.66 ± 0.77 ^{a,b}
2018	OKAY	7.13 ± 0.52 ^{a}	7.39 ± 1.6 ^{a}
	DiSVA 708	6.29 ± 1.78 ^{b}	6.61 ± 1.22 ^{a,b}
	Td/708	7.05 ± 0.21 ^{a}	6.82 ± 0.63 ^{a,b}

4. Discussion

The wide use of a limited number of *S. cerevisiae* strains as starter cultures of fermentation could standardize the aroma and analytical profile of wine [1,29]. To overcome this problem, the use of selected native *S. cerevisiae* strains as starter cultures of fermentation could be a suitable strategy [21,22]. Another strategy to enhance the wine aroma complexity is the use of non-*Saccharomyces* yeasts in sequential fermentations. Indeed, several studies emphasize the use of non-*Saccharomyces* yeasts in winemaking for their attitudes to produce aromatic compounds (such as esters, terpenes, thiols) and modify some structural compounds as polysaccharides, volatile acidity and ethanol content [30–36].

In this work, we evaluated the fermentation performance of a native strain of *S. cerevisiae* (DiSVA 708) improved for the absence of H_2S production and reduced production of SO_2 [21] to be used in the production of organic wines with a low SO_2 content. In pure fermentation, DiSVA 708 strain exhibited a behavior related to vintage, highlighting that the composition of grape juice can affect the fermentation performance of yeast strain. In particular, the increase in different aromatic compounds was detected among the vintages: in 2017 vintage ethyl octanoate, responsible for fruity

flavors such as apple, pear and peach [37–39] and in 2018 vintage phenyl ethyl acetate, responsible for floral and honey aroma [38]. On the other hand, isoamyl acetate (banana flavors) was consistently highly produced by DiSVA 708 strain which can be considered a distinctive aroma of the strain. On the contrary, OKAY® showed a more homogenous wine aroma across all three vintages with ethyl butyrate and n-propanol as a distinctive aroma compound [40,41].

In sequential fermentation, the results showed an influence on analytical profiles of the Verdicchio wines by *T. delbrueckii* selected strain. In particular, the sequential fermentation carried out during the 2017 vintage showed a reduced content of volatile acidity than the wine obtained by the pure fermentation of the native *S. cerevisiae* DiSVA 708. The ability to reduce the volatile acidity by *T. delbrueckii* is a feature well documented [2,42,43]. Moreover, the use of T. *delbrueckii* in sequential fermentation led to a reduction in total SO_2 content. Actually, there are no studies that directly correlate low sulfur dioxide production with the use of selected non-*Saccharomyces* yeasts. However, Bely and co-workers [42] highlighted the ability of *T. delbrueckii* to reduce SO_2-binding compounds production. We found that *T. delbrueckii* in sequential fermentation reduced acetaldehyde content.

The sequential fermentation *T. delbrueckii*/*S. cerevisiae* determined a general increase in some aroma compounds affecting the aroma complexity of the wine, such as phenyl ethyl acetate, ethyl hexanoate and β-phenyl ethanol, even if not at significant levels. Several previous works highlighted the tendency of T. *delbrueckii* to form higher levels of acetate compounds, β-phenyl ethanol and a general improvement of the quality of wine aroma increasing the perception of varietal and fruity characters [15,44,45].

On the other hand, confirming a previous work [15], the aroma profile of resulting wines was affected not only by non-*Saccharomyces*/*S. cerevisiae* used in sequential fermentation, but also by the vintage.

Overall data emphasized the use of selected non-*Saccharomyces* yeasts, such as *T. delbrueckii*, in sequential fermentation with selected native *S. cerevisiae* strains for Verdicchio wine production. Indeed, on one side, the use of native *S. cerevisiae* reinforced the distinctive aromatic characteristics of a particular enological area, usually conferred by native yeast strains. On the other side, non-*Saccharomyces* yeast produced peculiar fermentation by-products that positively influenced the analytical profile of Verdicchio wine, characterized by a well-structured style. Furthermore, a suitable strategy to obtain wine with reduced sulfites content is the use of improved native *S. cerevisiae* DiSVA 708 (low sulfite producer) in pure and mixed fermentation with a selected *T. delbrueckii* strain.

Author Contributions: A.A., L.C., F.C. and M.C. contributed equally to the manuscript. All the authors participated in the design and discussion of the research. A.A. carried out the experimental part of the work. A.A. and L.C. carried out the analysis of the data. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding

Acknowledgments: The authors wish to thank the winery Terre Cortesi Moncaro s.r.c.l. for technical assistance.

Conflicts of Interest: The authors declare to not have financial and/or personal interests that could influence the study of the present manuscript.

References

1. Vigentini, I.; Fabrizio, V.; Faccincani, M.; Picozzi, C.; Comasio, A.; Foschino, R. Dynamics of Saccharomyces cerevisiae populations in controlled and spontaneous fermentations for Franciacorta D.O.C.G. base wine production. *Ann. Microbiol.* **2013**, *64*, 639–651. [CrossRef]
2. Comitini, F.; Gobbi, M.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. Selected non-Saccharomyces wine yeasts in controlled multistarter fermentations with Saccharomyces cerevisiae. *Food Microbiol.* **2011**, *28*, 873–882. [CrossRef]

3. Jolly, N.P.; Varela, C.; Pretorius, I.S. Not your ordinary yeast: Non-Saccharomycesyeasts in wine production uncovered. *FEMS Yeast Res.* **2013**, *14*, 215–237. [CrossRef] [PubMed]
4. Azzolini, M.; Tosi, E.; Lorenzini, M.; Finato, F.; Zapparoli, G. Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with Saccharomyces cerevisiae. *World J. Microbiol. Biotechnol.* **2014**, *31*, 277–293. [CrossRef] [PubMed]
5. Liu, P.-T.; Lu, L.; Duan, C.-Q.; Yan, G.-L. The contribution of indigenous non-Saccharomyces wine yeast to improved aromatic quality of Cabernet Sauvignon wines by spontaneous fermentation. *LWT* **2016**, *71*, 356–363. [CrossRef]
6. Varela, C. The impact of non-Saccharomyces yeasts in the production of alcoholic beverages. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 9861–9874. [CrossRef]
7. Englezos, V.; Rantsiou, K.; Cravero, F.; Torchio, F.; Pollon, M.; Fracassetti, D.; Ortiz-Julien, A.; Gerbi, V.; Rolle, L.; Cocolin, L.; et al. Volatile profile of white wines fermented with sequential inoculation of Starmerella bacillaris and Saccharomyces cerevisiae. *Food Chem.* **2018**, *257*, 350–360. [CrossRef]
8. Binati, R.L.; Innocente, G.; Gatto, V.; Celebrin, A.; Polo, M.; Felis, G.E.; Torriani, S. Exploring the diversity of a collection of native non-Saccharomyces yeasts to develop co-starter cultures for winemaking. *Food Res. Int.* **2019**, *122*, 432–442. [CrossRef]
9. Ciani, M.; Comitini, F. Use of Non-*Saccharomyces* Yeasts in Red Winemaking. In *Red Wine Technology*; Morata, A., Ed.; Academic Press: Cambridge, MA, USA, 2019; pp. 51–68.
10. Binati, R.L.; Junior, W.J.L.; 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. *Int. J. Food Microbiol.* **2020**, *318*, 108470. [CrossRef]
11. Canonico, L.; Comitini, F.; Ciani, M. Metschnikowia pulcherrima Selected Strain for Ethanol Reduction in Wine: Influence of Cell Immobilization and Aeration Condition. *Foods* **2019**, *8*, 378. [CrossRef]
12. Velázquez, R.; Zamora, E.; Álvarez, M.L.; Hernández, L.M.; Ramírez, M. Effects of new Torulaspora delbrueckii killer yeasts on the must fermentation kinetics and aroma compounds of white table wine. *Front. Microbiol.* **2015**, *6*, 1222. [CrossRef] [PubMed]
13. Ramírez, M.; Velázquez, R.; Maqueda, M.; Lopez-Piñeiro, A.; Ribas, J.C. A new wine Torulaspora delbrueckii killer strain with broad antifungal activity and its toxin-encoding double-stranded RNA virus. *Front. Microbiol.* **2015**, *6*, 983. [CrossRef] [PubMed]
14. Ciani, M.; Comitini, F. Yeast interactions in multi-starter wine fermentation. *Curr. Opin. Food Sci.* **2015**, *1*, 1–6. [CrossRef]
15. Canonico, L.; Comitini, F.; Ciani, M. Influence of vintage and selected starter on Torulaspora delbrueckii/Saccharomyces cerevisiae sequential fermentation. *Eur. Food Res. Technol.* **2015**, *241*, 827–833. [CrossRef]
16. Ortiz, M.J.; Barrajón, N.; Baffi, M.A.; Arévalo-Villena, M.; Briones, A. Spontaneous must fermentation: Identification and biotechnological properties of wine yeasts. *LWT* **2013**, *50*, 371–377. [CrossRef]
17. Blanco, P.; Mirás-Avalos, J.M.; Pereira, E.; Fornos, D.; Orriols, I. Modulation of chemical and sensory characteristics of red wine from Mencía by using indigenous Saccharomyces cerevisiae yeast strains. *OENO One* **2014**, *48*, 63. [CrossRef]
18. Agarbati, A.; Canonico, L.; Ciani, M.; Comitini, F. Fitness of Selected Indigenous Saccharomyces cerevisiae Strains for White Piceno DOC Wines Production. *Fermentation* **2018**, *4*, 37. [CrossRef]
19. Lorca, G.; Uribe, S.; Martínez, C.; Godoy, L.; Ganga, M.A. Screening of native S. cerevisiae strains in the production of Pajarete wine: A tradition of Atacama Region, Chile. *J. Wine Res.* **2018**, *29*, 1–13. [CrossRef]
20. Çelik, Z.D.; Erten, H.; Cabaroglu, T. The Influence of Selected Autochthonous Saccharomyces cerevisiae Strains on the Physicochemical and Sensory Properties of Narince Wines. *Fermentation* **2019**, *5*, 70. [CrossRef]
21. Agarbati, A.; Canonico, L.; Comitini, F.; Ciani, M. Reduction of Sulfur Compounds through Genetic Improvement of Native Saccharomyces cerevisiae Useful for Organic and Sulfite-Free Wine. *Foods* **2020**, *9*, 658. [CrossRef]
22. De Vero, L.; Solieri, L.; Giudici, P. Evolution-based strategy to generate non-genetically modified organisms Saccharomyces cerevisiae strains impaired in sulfate assimilation pathway. *Lett. Appl. Microbiol.* **2011**, *53*, 572–575. [CrossRef] [PubMed]

23. Liu, X.; Zhang, Z. Cross Breeding and Hybrid Identification of Sulphite-tolerant Hybrids of Saccharomyces uvarum. *S. Afr. J. Enol. Vitic.* **2017**, *38*, 125–131. [CrossRef]
24. Canonico, L.; Comitini, F.; Ciani, M. Torulaspora delbrueckii for secondary fermentation in sparkling wine production. *Food Microbiol.* **2018**, *74*, 100–106. [CrossRef] [PubMed]
25. Canonico, L.; Comitini, F.; Ciani, M. Torulaspora delbrueckii contribution in mixed brewing fermentations with different Saccharomyces cerevisiae strains. *Int. J. Food Microbiol.* **2017**, *259*, 7–13. [CrossRef]
26. EEC. Council Regulation 2870/00 laying down Community reference methods for the analysis of spirit drinks. *Off. J. Eur. Comm.* **2000**, *L333*, 20–46.
27. Dukes, B.C.; Butzke, C.E. Rapid determination of primaryamino acid in grape juice using ano-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay. *Am. J. Enol. Vitic.* **1998**, *49*, 125–134.
28. Canonico, L.; Solomon, M.; Comitini, F.; Ciani, M.; Varela, C. Volatile profile of reduced alcohol wines fermented with selected non-Saccharomyces yeasts under different aeration conditions. *Food Microbiol.* **2019**, *84*, 103247. [CrossRef]
29. Guillamón, J.M.; Barrio, E. Genetic Polymorphism in Wine Yeasts: Mechanisms and Methods for Its Detection. *Front. Microbiol.* **2017**, *8*, 806. [CrossRef]
30. Anfang, N.; Brajkovich, M.; Goddard, M.R. Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Sauvignon Blanc. *Aust. J. Grape Wine Res.* **2009**, *15*, 1–8. [CrossRef]
31. Benito, S.; Hofmann, T.; Laier, M.; Lochbühler, B.; Schüttler, A.; Ebert, K.; Fritsch, S.; Röcker, J.; Rauhut, D. Effect on quality and composition of Riesling wines fermented by sequential inoculation with non-Saccharomyces and Saccharomyces cerevisiae. *Eur. Food Res. Technol.* **2015**, *241*, 707–717. [CrossRef]
32. Wang, C.; Mas, A.; Esteve-Zarzoso, B. The Interaction between Saccharomyces cerevisiae and Non-Saccharomyces Yeast during Alcoholic Fermentation Is Species and Strain Specific. *Front. Microbiol.* **2016**, *7*, 502. [CrossRef] [PubMed]
33. Benito, S. The impact of Torulaspora delbrueckii yeast in winemaking. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 3081–3094. [CrossRef] [PubMed]
34. Rollero, S.; Zietsman, J.; Buffetto, F.; Schückel, J.; Ortiz-Julien, A.; Divol, B. Kluyveromyces marxianus Secretes a Pectinase in Shiraz Grape Must That Impacts Technological Properties and Aroma Profile of Wine. *J. Agric. Food Chem.* **2018**, *66*, 11739–11747. [CrossRef] [PubMed]
35. Roudil, L.; Russo, P.; Berbegal, C.; Albertin, W.; Spano, G.; Capozzi, V. Non-Saccharomyces Commercial Starter Cultures: Scientific Trends, Recent Patents and Innovation in the Wine Sector. *Recent Patents Food Nutr. Agric.* **2020**, *11*, 27–39. [CrossRef]
36. Lin, M.M.-H.; Boss, P.K.; Walker, M.E.; Sumby, K.M.; Grbin, P.R.; Jiranek, V. Evaluation of indigenous non-Saccharomyces yeasts isolated from a South Australian vineyard for their potential as wine starter cultures. *Int. J. Food Microbiol.* **2019**, *312*, 108373. [CrossRef]
37. Rapp, A.; Mandery, H. Wine aroma. *Cell. Mol. Life Sci.* **1986**, *42*, 873–884. [CrossRef]
38. Fang, Y.; Qian, M.C. Aroma compounds in Oregon Pinot Noir wine determined by aroma extract dilution analysis (AEDA). *Flavour Fragr. J.* **2004**, *20*, 22–29. [CrossRef]
39. Gómez-Míguez, M.J.; Gómez-Míguez, M.; Vicario, I.M.; Heredia, F.J. Assessment of colour and aroma in white wines vinifications: Effects of grape maturity and soil type. *J. Food Eng.* **2007**, *79*, 758–764. [CrossRef]
40. Kutyna, D.R.; Borneman, A.R. Heterologous Production of Flavour and Aroma Compounds in Saccharomyces cerevisiae. *Genes* **2018**, *9*, 326. [CrossRef]
41. Eder, M.; Sanchez, I.; Brice, C.; Camarasa, C.; Legras, J.-L.; Dequin, S. QTL mapping of volatile compound production in Saccharomyces cerevisiae during alcoholic fermentation. *BMC Genom.* **2018**, *19*, 166. [CrossRef]
42. Bely, M.; Stoeckle, P.; Masneuf-Pomarède, I.; Dubourdieu, D. Impact of mixed Torulaspora delbrueckii–Saccharomyces cerevisiae culture on high-sugar fermentation. *Int. J. Food Microbiol.* **2008**, *122*, 312–320. [CrossRef] [PubMed]
43. Benito, S.; Calderón, F. The Influence of Non-Saccharomyces Species on Wine Fermentation Quality Parameters. *Fermentation* **2019**, *5*, 54. [CrossRef]

44. Belda, I.; Ruiz, J.; Beisert, B.; Navascués, E.; Marquina, D.; Calderón, F.; Rauhut, D.; Benito, S.; Santos, A. Influence of *Torulaspora delbrueckii* in varietal thiol (3-SHand4-MSP) release in wine sequential fermentations. *Int. J. Food Microbiol.* **2017**, *257*, 183–191. [CrossRef] [PubMed]
45. Dutraive, O.; Benito, S.; Fritsch, S.; Bei-Sert, B.; Patz, C.-D.; Rauhut, R. Patz Effect of Sequential Inoculation with Non-Saccharomyces and Saccharomyces Yeasts on Riesling Wine Chemical Composition. *Fermentation* **2019**, *5*, 79. [CrossRef]