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Effect of *Saccharomyces*, Non-*Saccharomyces* Yeasts and Malolactic Fermentation Strategies on Fermentation Kinetics and Flavor of Shiraz Wines

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Abstract: The use of non-*Saccharomyces* yeasts to improve complexity and diversify wine style is increasing; however, the interactions between non-*Saccharomyces* yeasts and lactic acid bacteria (LAB) have not received much attention. This study investigated the interactions of seven non-*Saccharomyces* yeast strains of the genera *Candida*, *Hanseniaspora*, *Lachancea*, *Metschnikowia* and *Torulaspora* in combination with *S. cerevisiae* and three malolactic fermentation (MLF) strategies in a Shiraz winemaking trial. Standard oenological parameters, volatile composition and sensory profiles of wines were investigated. Wines produced with non-*Saccharomyces* yeasts had lower alcohol and glycerol levels than wines produced with *S. cerevisiae* only. Malolactic fermentation also completed faster in these wines. Wines produced with non-*Saccharomyces* yeasts differed chemically and sensorially from wines produced with *S. cerevisiae* only. The *Candida zemplinina* and the one *L. thermotolerans* isolate slightly inhibited LAB growth in wines that underwent simultaneous MLF. Malolactic fermentation strategy had a greater impact on sensory profiles than yeast treatment. Both yeast selection and MLF strategy had a significant effect on berry aroma, but MLF strategy also had a significant effect on acid balance and astringency of wines. Winemakers should apply the optimal yeast combination and MLF strategy to ensure fast completion of MLF and improve wine complexity.

Keywords: yeast selection; lactic acid bacteria; inoculation; volatile compounds; chemical profile; sensory evaluation; aroma

1. Introduction

Shiraz, also known as Syrah (*Vitis vinifera* L.), is a red cultivar used internationally to produce dark-coloured and full-bodied wines that are suitable for ageing. Shiraz is cultivated in all wine producing regions of the world, including Australia, South Africa and South American countries [1]. Shiraz is renowned for "spicy", "dark fruit"- and "berry"-like flavors and different wine styles can be produced, depending on the region of origin, viticultural and winemaking practices [2]. Wine flavor contributes to the final quality of wine and is the product of the combined effects of several volatile compounds, such as alcohols, aldehydes, esters, acids, monoterpenes and other minor components already present in the grapes, or that are formed during fermentation or maturation [1].

Wine production includes two important fermentation processes, i.e., alcoholic fermentation conducted by yeast, and malolactic fermentation (MLF) conducted by lactic acid bacteria (LAB) [3,4]. The yeasts drive alcoholic fermentation by converting grape sugar to alcohol, carbon dioxide and volatile compounds that affect the aroma and taste of wine [3,5]. At the onset of alcoholic fermentation, a large number of non-*Saccharomyces* species may be present, but the final stage is dominated by alcohol-tolerant *Saccharomyces cerevisiae* strains [3,5–7].

Recent studies have shown that non-*Saccharomyces* yeasts have different oenological properties to those of *S. cerevisiae*, and can be used to modulate and improve the aroma and complexity of wines [8–11]. Most non-*Saccharomyces* yeasts are poor fermenters and therefore are used in combination with *S. cerevisiae* in sequential inoculations, to complete the fermentation [9].

In studies carried out with Shiraz, using *Candida zemplinina*, *Kazachstania aerobia*, *K. gamospora*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Torulaspora delbrueckii* and *Zygosaccharomyces kombuchaensis*, the wines produced with these non-*Saccharomyces* yeasts had distinct volatile chemical profiles that were different to the *S. cerevisiae* reference [10,11]. These non-*Saccharomyces* wines had lower concentrations of esters, alcohols and terpenes than the *S. cerevisiae* wines. In a study carried out on Sauvignon blanc, using some of the aforementioned non-*Saccharomyces* yeasts, the wines also showed distinct chemical and sensory profiles [12]. Sauvignon blanc wines produced with *S. cerevisiae* had guava, grapefruit, banana, and pineapple aromas, while *C. zemplinina* wines were driven by fermented apple, dried peach/apricot, and stewed fruit aromas, as well as a sour flavor.

Non-*Saccharomyces* yeast can also be used to reduce ethanol content and a reduction from 0.64% *v*/*v* at pilot scale in grape juice to 1.60% *v*/*v* in laboratory scale trials using synthetic grape juice was reported [13]. Sequential fermentation trials using *L. thermotolerans* (formerly *Kluyveromyces thermotolerans*) under industry conditions with a two day delay of the second inoculum (*S. cerevisiae*), resulted in an ethanol reduction of 0.7% *v*/*v* [8]. A sequential inoculation of *M. pulcherrima* AWRI 1149 followed by a *S. cerevisiae* wine strain lowered ethanol concentration to 0.9% and 1.6% *v*/*v* for Chardonnay and Shiraz wines, respectively [14].

Malolactic fermentation is an enzymatic decarboxylation of L-malic acid to L-lactic acid and CO_2 and is required for the production of some red wines, full-bodied white and sparkling wines [4,15]. Malolactic fermentation increases microbiological stability and can affect wine flavor through the modification of compounds such as diacetyl, esters, higher alcohols and volatile acids by LAB [16–19]. *Oenococcus oeni* is the preferred LAB species for MLF due to its resistance to harsh conditions found in wine [17–19]. Various MLF strategies have been investigated with simultaneous (at the start of alcoholic fermentation) and sequential inoculation (after alcoholic fermentation) receiving the most attention [15]. Selecting compatible yeast and LAB strains are essential for successful alcoholic and malolactic fermentation, as certain yeast strains have been shown to have a negative effect on LAB growth and MLF [20,21]. However, some LAB strains can also cause slow or stuck fermentations [22]. Yeast and LAB interactions differ for the various MLF inoculation strategies, so the optimal yeast/LAB combinations may not be the same for simultaneous and sequential MLF [15,23]. Wine sensory profiles following simultaneous inoculation of LAB, can differ from those of sequential MLF inoculation [24,25]. The interactions between *S. cerevisiae*, non-*Saccharomyces* yeasts and LAB are not as well researched as the interactions between *S. cerevisiae* and LAB.

There is still a lack of understanding of how specific non-*Saccharomyces* yeasts alter the sensory properties of wine, as well as the interactions of these non-*Saccharomyces* with *S. cerevisiae* yeasts in wines from various grape cultivars [11]. Little is known about the interactions of *Saccharomyces*, non-*Saccharomyces* yeasts and lactic acid bacteria, and how their interactions affect wine aroma and flavor. In a previous study [26], 37 non-*Saccharomyces* yeast strains were evaluated for use in wine production. The current study narrowed the non-*Saccharomyces* yeasts down to seven strains from five species, i.e., *C. zemplinina*, *Hanseniaspora uvarum*, *M. pulcherrima*, *L. thermotolerans* and *T. delbrueckii*. These non-*Saccharomyces* strains were used in combination with *S. cerevisiae* and three MLF strategies in a small-scale Shiraz wine production trial. The aims were to investigate the interactions between

Saccharomyces, non-*Saccharomyces* yeast and *Oenococcus oeni*, as well as the resulting effect of these interactions on duration of MLF and Shiraz wine flavor.

2. Materials and Methods

2.1. Cultivation and Enumeration of Microorganisms

The yeasts and LAB used in this study are listed in Table 1. The two commercial non-*Saccharomyces* yeast strains, i.e., *T. delbrueckii* (Level^2 TD™, Lallemand Inc. Montreal, QC, Canada) and *L. thermotolerans* (Viniflora® Rhythm™, Chr Hansen, Hørsholm, Denmark), were isolated from active dried yeast blends [26] and used as wet cultures. All non-*Saccharomyces* yeasts were stored under cryo-preservation at −80 °C. The non-*Saccharomyces* yeasts were propagated in a four step protocol: (i) on yeast peptone dextrose agar (YPDA, Merck, Modderfontein, South Africa) at 28 °C for 48 h or until sufficient growth was observed, (ii) then single colonies were inoculated into 10 mL YPD broth and grown for 24 h at 28 °C, (iii) transferred to 100 mL YPD broth and incubated for 24 h at 28 °C, and (iv) finally transferred to containers holding 3–4 L YPD broth and incubated at 28 °C for 24 h. The containers were shaken during propagation to ensure aerobic conditions. Non-*Saccharomyces* yeasts were inoculated into the Shiraz grape juice at a concentration of ~1×10^6 cells/mL. *S. cerevisiae* was used as an active dried yeast culture and rehydrated according to the supplier's recommendations and inoculated at 0.3 g/L. A commercial *O. oeni* culture was used to induce MLF (Table 1). This MLF culture was used at the dosage prescribed by the supplier for the simultaneous MLF treatment, but a higher dosage (15 mg/L) was used to induce sequential MLF due to higher alcohol concentrations of the wines.

Table 1. Yeasts and lactic acid bacterium used in this study.

Reference Code	Species Name	Source
Sc	*Saccharomyces cerevisiae*	VIN 13, commercial yeast strain from Anchor Wine Yeast, South Africa
C7	*Candida zemplinina* (synonym: *Starmerella bacillaris*)	Yeast isolate from ARC Infruitec-Nietvoorbij culture collection
H4	*Hanseniaspora uvarum*	Yeast isolate from ARC Infruitec-Nietvoorbij culture collection, South Africa
L1	*Lachancea thermotolerans*	Viniflora® Rhythm™, commercial yeast strain from Chr. Hansen A/S, Denmark
L2	*Lachancea thermotolerans*	Yeast isolate from ARC Infruitec-Nietvoorbij culture collection
M2	*Metschnikowia pulcherrima*	Yeast isolate from ARC Infruitec-Nietvoorbij culture collection
T3	*Torulaspora delbrueckii*	Level^2 TD™, commercial yeast strain from Lallemand Inc.
T6	*Torulaspora delbrueckii*	Yeast isolate from ARC Infruitec-Nietvoorbij culture collection
O. oeni	*Oenococcus oeni*	Commercial malolactic bacteria culture Viniflora® oenos from Chr. Hansen A/S

Yeast counts of Shiraz juice and wines were obtained by plating on Wallerstein Laboratory (WL) Nutrient medium (Biolab, Merck, Modderfontein, South Africa) and bacterial counts by plating out on De Man Rogosa, Sharpe (MRS) agar (Biolab, Merck) supplemented with 25% (*v*/*v*) grape juice and 100 mg/L cycloheximide (Sigma-Aldrich, Munich, Germany). Yeast growth media were incubated aerobically and the LAB growth media were incubated under facultative anaerobic conditions at 28 °C for 2–7 days, after which the colonies were counted. The naturally occurring non-*Saccharomyces* yeast populations were determined by counting the non-*Saccharomyces* yeast colonies present in the reference treatment, which only received a *S. cerevisiae* inoculum. The naturally occurring *Saccharomyces* yeast

populations were determined by counting the *Saccharomyces* yeast colonies in the treatments that did not receive any *S. cerevisiae* inoculum. The development of the naturally occurring LAB during fermentation was monitored by sampling the wines that did not undergo MLF and the sequential MLF treatments until day 19, when the commercial *O. oeni* starter culture was added to the sequential MLF wines.

2.2. Wine Production

Shiraz grapes, obtained from the Nietvoorbij research farm (Stellenbosch, South Africa), were crushed, the juice separated from skins and the volume measured. The skins were weighed and each 70 L fermentation container received the same volume and ratio of juice and skins. The method of grape must preparation ensured a homogenous matrix so that treatments could be compared. Fermentations were carried out at ca. 24 °C using a standardized winemaking protocol as described by Minnaar et al. [27]. Eight yeast strains in combination with three MLF strategies, i.e., (1) yeast treatment without MLF, (2) yeast treatment and LAB inoculated simultaneously (sim MLF) and (3) yeast treatment with sequential MLF (seq MLF), were investigated (Table 2). In total 72 wines were produced, which included 24 different treatments and each treatment had three replicates. *S. cerevisiae* (Sc) on its own served as the reference treatment. The non-*Saccharomyces* yeasts and the *S. cerevisiae* only treatment were inoculated on day 0. In the sequential yeast fermentations, the *S. cerevisiae* yeast was only inoculated 24 h after the non-*Saccharomyces* monocultures (day 1). For the wines that underwent the simultaneous MLF treatment, *O. oeni* was also added on day 1, but an hour after the addition of *S. cerevisiae*. For the sequential MLF treatments, *O. oeni* was added to the wines after alcoholic fermentation was completed. All treatments were racked, fined, cold stabilized and bottled as described by Minnaar et al. [27]. All wines were stored at 15 °C until needed.

2.3. Juice and Wine Analyses

The following were measured on the grape must: sugar in °Brix (Refractometer), free and total SO_2 (Ripper method), pH and titratable acidity analyses as described in the South African Wine Laboratories Association Manual (SALWA) [28]. Standard chemical parameters (glucose and fructose concentrations, pH, malic and lactic acid, total acidity (TA), alcohol, volatile acidity (VA) and glycerol) were determined for the bottled wine using a WineScan^{TM} FT120 instrument (FOSS Analytical A/S, Hillerød, Denmark) at the Institute for Wine Biotechnology (Stellenbosch University, Stellenbosch, South Africa). Data were predicted from infrared spectra using in-house calibration models as described by Louw et al. [29]. The concentrations of major volatile compounds in bottled wines were determined by the Chemical Analytical Laboratory (Institute for Wine Biotechnology and Department of Viticulture and Oenology, Stellenbosch University), using a gas chromatograph coupled to a flame ionization detector (GC-FID) as described by Louw et al. [29].

Table 2. Standard chemical parameters and duration of malolactic fermentation (MLF) of Shiraz juice [1] and wines produced with *Saccharomyces* and non-*Saccharomyces* yeasts in combination with MLF strategies (none, simultaneous and sequential). Values are averages of three replicates.

Treatment [2]	Residual Sugar (g/L)	pH	Volatile Acidity (g/L)	Total Acidity (g/L)	Malic Acid (g/L)	Lactic Acid (g/L)	Alcohol (% *v*/*v*)	Glycerol (g/L)	Duration of MLF (Days)
Sc	2.23 ± 0.13 ef [3]	3.66 ± 0.01 jkl	0.25 ± 0.01 k	6.19 ± 0.04 a	1.26 ± 0.06 c	<0.20 i	15.99 ± 0.03 abcd	11.43 ± 0.05 fgh	No MLF
Sc + sim MLF	2.16 ± 0.16 ef	3.74 ± 0.04 defg	0.39 ± 0.02 gh	5.89 ± 0.12 cde	<0.20 f	1.01 ± 0.04 b	16.09 ± 0.12 a	11.84 ± 0.09 ab	54
Sc + seq MLF	2.18 ± 0.27 ef	3.76 ± 0.01 cdef	0.39 ± 0.02 gh	5.52 ± 0.03 ij	<0.20 f	0.86 ± 0.02 d	16.01 ± 0.07 abc	11.75 ± 0.09 bc	53
C7 + Sc	2.23 ± 0.13 ef	3.59 ± 0.01 m	0.33 ± 0.01 i	6.21 ± 0.03 a	1.21 ± 0.05 c	<0.20 i	15.49 ± 0.01 k	11.08 ± 0.13 k	No MLF
C7 + Sc + sim MLF	2.20 ± 0.25 ef	3.67 ± 0 ijkl	0.40 ± 0.01 fg	6.03 ± 0.13 bc	<0.20 f	0.95 ± 0.04 c	15.93 ± 0.04 bcdef	11.85 ± 0.14 ab	63
C7 + Sc + seq MLF	2.32 ± 0.22 bcdef	3.70 ± 0.01 ghij	0.47 ± 0.02 c	5.65 ± 0.04 ghi	<0.20 f	0.77 ± 0.02 g	15.54 ± 0.03 k	11.24 ± 0.04 ij	40
H4 + Sc	2.77 ± 0.16 a	3.76 ± 0.04 cdef	0.37 ± 0.01 h	5.69 ± 0.03 gh	0.77 ± 0.09 e	<0.20 i	15.94 ± 0.03 bcde	11.26 ± 0.04 ij	No MLF
H4 + Sc + sim MLF	2.42 ± 0.19 bcde	3.73 ± 0.04 efgh	0.42 ± 0.02 ef	5.76 ± 0.05 efg	<0.20 f	1.06 ± 0.06 a	15.82 ± 0.09 fghij	11.76 ± 0.16 bc	48
H4 + Sc + seq MLF	2.78 ± 0.18 a	3.85 ± 0.01 b	0.52 ± 0.01 b	5.19 ± 0.04 m	<0.20 f	0.81 ± 0.02 efg	15.96 ± 0.04 bcd	11.59 ± 0.05 de	38
L1 + Sc	2.32 ± 0.26 bcdef	3.72 ± 0.01 efghi	0.30 ± 0.02 j	5.88 ± 0.01 def	1.12 ± 0.02 d	<0.20 i	15.77 ± 0.02 ij	11.33 ± 0.05 hi	No MLF
L1 + Sc + sim MLF	2.60 ± 0.09 ab	3.76 ± 0.25 def	0.43 ± 0.01 ef	5.58 ± 0.06 hij	<0.20 f	1.00 ± 0.02 b	15.93 ± 0.08 bcdef	11.61 ± 0.08 cde	48
L1 + Sc + seq MLF	2.22 ± 0.27 ef	3.80 ± 0.01 bcd	0.44 ± 0.01 de	5.35 ± 0.02 kl	<0.20 f	0.86 ± 0.02 d	15.80 ± 0.04 hij	11.71 ± 0.03 bcd	48
L2 + Sc	2.55 ± 0.13 abcd	3.83 ± 0 b	0.42 ± 0.01 ef	5.48 ± 0.05 jk	1.68 ± 0.01 a	<0.20 i	16.04 ± 0.04 ab	11.06 ± 0.05 k	No MLF
L2 + Sc + sim MLF	2.18 ± 0.30 ef	3.62 ± 0.13 lm	0.39 ± 0.05 gh	6.04 ± 0.03 b	<0.20 f	0.82 ± 0.07 def	15.89 ± 0.02 defgh	11.62 ± 0.26 cde	68
L2 + Sc + seq MLF	2.59 ± 0.18 abc	3.92 ± 0.02 a	0.56 ± 0.01 a	4.95 ± 0.03 n	<0.20 f	0.70 ± 0.04 h	16.08 ± 0.02 a	11.37 ± 0.06 ghi	40
M2 + Sc	2.20 ± 0.28 ef	3.67 ± 0.02 hijk	0.31 ± 0.10 ij	6.01 ± 0.02 bcd	1.21 ± 0.03 c	<0.20 i	15.81 ± 0.13 ghij	11.32 ± 0.03 hi	No MLF
M2 + Sc + sim MLF	2.08 ± 0.22 f	3.74 ± 0.04 defg	0.43 ± 0.02 de	5.76 ± 0.12 efg	<0.20 f	0.96 ± 0.012 bc	15.81 ± 0.09 ghij	11.71 ± 0.02 bcd	52
M2 + Sc + seq MLF	2.28 ± 0.19 cdef	3.77 ± 0.02 cde	0.48 ± 0.01 c	5.44 ± 0.03 jk	<0.20 f	0.78 ± 0.03 fg	15.92 ± 0.02 cdefg	11.57 ± 0.04 def	48
T3 + Sc	2.45 ± 0.20 bcde	3.63 ± 0.01 klm	0.31 ± 0.01 ij	6.08 ± 0.07 ab	1.25 ± 0.08 c	<0.20 i	15.80 ± 0.15 hij	10.98 ± 0.11 k	No MLF
T3 + Sc + sim MLF	2.26 ± 0.11 def	3.74 ± 0.02 defg	0.41 ± 0.01 fg	5.76 ± 0.10 efg	<0.20 f	0.99 ± 0.04 bc	15.91 ± 0.01 cdefgh	11.98 ± 0.07 a	51
T3 + Sc + seq MLF	2.29 ± 0.21 bcdef	3.73 ± 0.01 efg	0.46 ± 0.01 cd	5.48 ± 0.03 jk	<0.20 f	0.85 ± 0.04 de	15.88 ± 0.02 defghi	11.25 ± 0.78 ij	48
T6 + Sc	2.45 ± 0.06 bcde	3.72 ± 0.02 fghi	0.40 ± 0.01 fg	5.74 ± 0.05 fg	1.59 ± 0.04 b	<0.20 i	15.84 ± 0.14 efghij	11.14 ± 0.04 jk	No MLF
T6 + Sc + sim MLF	2.22 ± 0.10 ef	3.67 ± 0.01 ijkl	0.41 ± 0.02 fg	6.00 ± 0.06 bcd	<0.20 f	0.95 ± 0.04 c	15.75 ± 0.09 j	11.75 ± 0.09 bc	53
T6 + Sc + seq MLF	2.58 ± 0.16 abcd	3.82 ± 0.01 bc	0.54 ± 0.01 a	5.27 ± 0.02 lm	<0.20 f	0.65 ± 0.02 h	15.94 ± 0.02 bcde	11.49 ± 0.11 efg	40

[1] Juice analysis: Balling = 26.9°Brix, pH = 3.41, total acidity = 6.5 g/L, malic acid = 1.80 g/L, free SO_2 = 16 mg/L and total SO_2 = 29 mg/L. [2] *Saccharomyces cerevisiae* (Sc), *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, simultaneous (sim) MLF and sequential (seq) MLF induced with *Oenococcus oeni*. [3] Values in the same column followed by the same letters did not differ significantly ($p \leq 0.5$).

2.4. Sensory Evaluation

A panel consisting of 15 experienced wine judges (3 women and 11 men, aged 22 to 50 years) evaluated the wines after 24 months of bottle ageing. The panellists were commercial winemakers or staff of ARC Infruitec-Nietvoorbij (The Fruit, Vine and Wine Institute of the Agricultural Research Council, Stellenbosch, South Africa). Panel members were experienced in wine evaluation (from 2 to 20 years of experience) and did not receive collective training. Wines were evaluated during three sessions (24 wines per session) over three consecutive days in a temperature-controlled room at ±20 °C. During each session, panel members had to take a compulsory break after tasting 12 wines. Each replicate was evaluated on a separate day. The descriptors were chosen from a predefined lexicon and the wines were subjected to classical profiling [30]. The panel members were asked to evaluate the wines orthonasally and to score the intensity of each descriptor individually on a 100 mm unstructured line scale. The descriptors were berry, fruity, fresh vegetative, cooked vegetative, floral, spicy, acid balance, body, astringency, bitterness and overall quality. Each panellist had a separate tasting booth and ca. 30 mL of the wine was presented, in a randomized order, in a standard international wine tasting glass, labeled with a three digit code. Research Randomizer (Version 4.0, Lancaster, PA, USA, http://randomizer.org) was used to generate the three digit code and to randomize the order in which the wines were presented to each panellist.

2.5. Data and Statistical Analysis

The chemical and sensory data were tested for normality using the method of [31] and then subjected to analysis of variance (ANOVA) using the general linear means procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Student's *t* least significant difference (LSD) values were calculated at the 5% probability level ($p = 0.05$) to facilitate comparison between treatment means [32]. Additionally, the sensory data were subjected to mixed model ANOVA using Statistica 13.0 (Quest software Inc., Aliso Viejo, CA, USA). Means within data sets that differed at the 5% probability level were considered significantly different. Principal component analysis (PCA) was performed, using XLSTAT software (Version 18.07.39157, Addinsoft, New York, NY, USA), to examine the correlation between wine samples and the chemical compounds determined with GC-FID.

3. Results and Discussion

3.1. Fermentation Kinetics and Progress of MLF

3.1.1. Yeast Growth in Wines without MLF

Counts of the naturally occurring *Saccharomyces* and non-*Saccharomyces* yeast populations in the Shiraz juice were ca. 2.1×10^4 and 1.9×10^5 colony forming units (CFU)/mL, respectively (Figure 1). Monitoring the naturally occurring non-*Saccharomyces* yeasts population in the *S. cerevisiae* reference fermentation showed an increase over the first 24 h, before decreasing to ~3.8×10^5 CFU/mL after 48 h. This is a normal occurrence for natural non-*Saccharomyces* populations during fermentation [33]. The non-*Saccharomyces* yeast count decreased during the remainder of alcoholic fermentation and at the end of fermentation (18 days) the count was lower than 1×10^4 CFU/mL (data not shown). After 48 h, the expected dominance by the inoculated *S. cerevisiae* was observed in all wines (Figure 1).

In the non-*Saccharomyces* inoculated wines, these yeasts were present at higher levels (10^6–10^7 CFU/mL) during the first two days of alcoholic fermentation than the naturally occurring non-*Saccharomyces* population in the *S. cerevisiae* reference wine. For the first 24 h, the inoculated non-*Saccharomyces* yeasts were also present at higher levels than the naturally occurring *Saccharomyces* yeasts. It is expected that these yeasts could have made a notable contribution to the flavor profiles of the various wines [34].

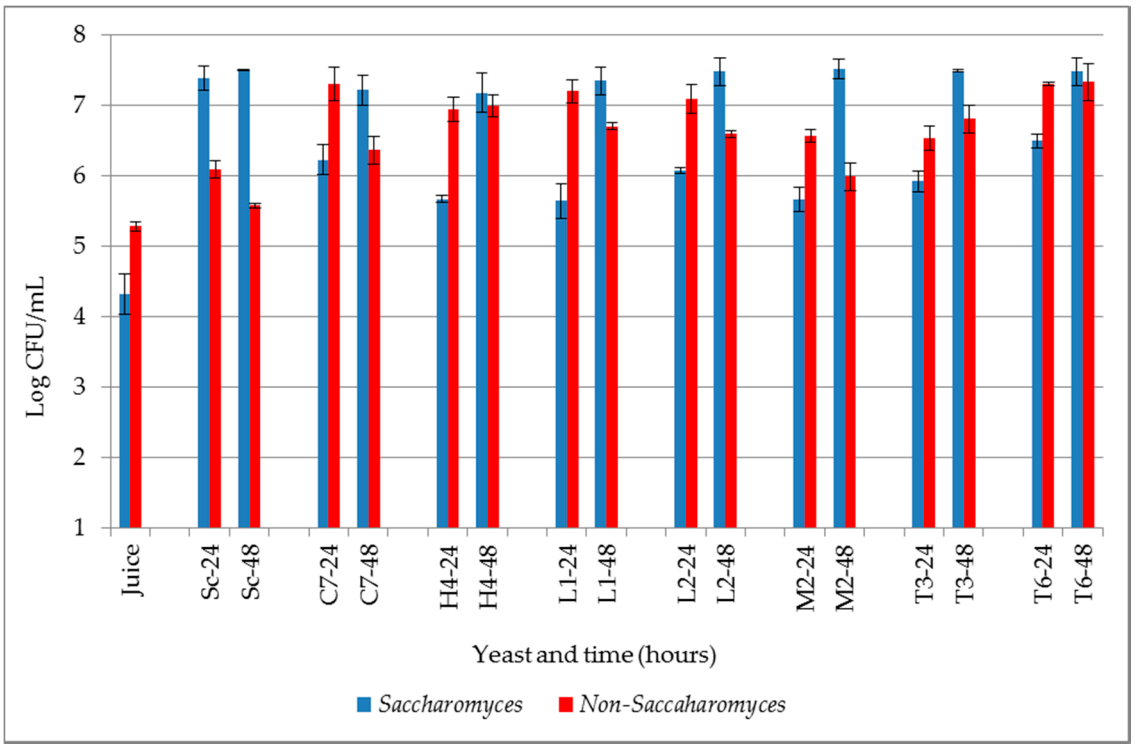

Figure 1. *Saccharomyces* and non-*Saccharomyces* yeast counts in colony forming units/millilitres (CFU/mL) of Shiraz juice, wines inoculated with a commercial *Saccharomyces cerevisiae* (Sc) strain on its own and wines with *S. cerevisiae* in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6 were evaluated. The yeast counts were performed after 24 and 48 h of the alcoholic fermentation. Values are averages of three replicates and the error bars indicate the standard deviation.

3.1.2. LAB Growth

The naturally occurring LAB populations in the grape must were initially present at moderate numbers (6×10^3 CFU/mL) (Figure 2). Thereafter, the population size was either maintained at 10^2–10^4 CFU/mL or decreased during fermentation, before increasing at the end of alcoholic fermentation. The decrease of LAB numbers during alcoholic fermentation, with the subsequent increase after fermentation [3,35], as well as the occurrence at low to moderate numbers and increasimg during alcoholic fermentation [36,37], are both typical winemaking scenarios. Factors such as pH, SO_2 concentration, ethanol levels, temperature, yeast strain, etc. are important and can affect the growth of LAB during wine production [3,4,36].

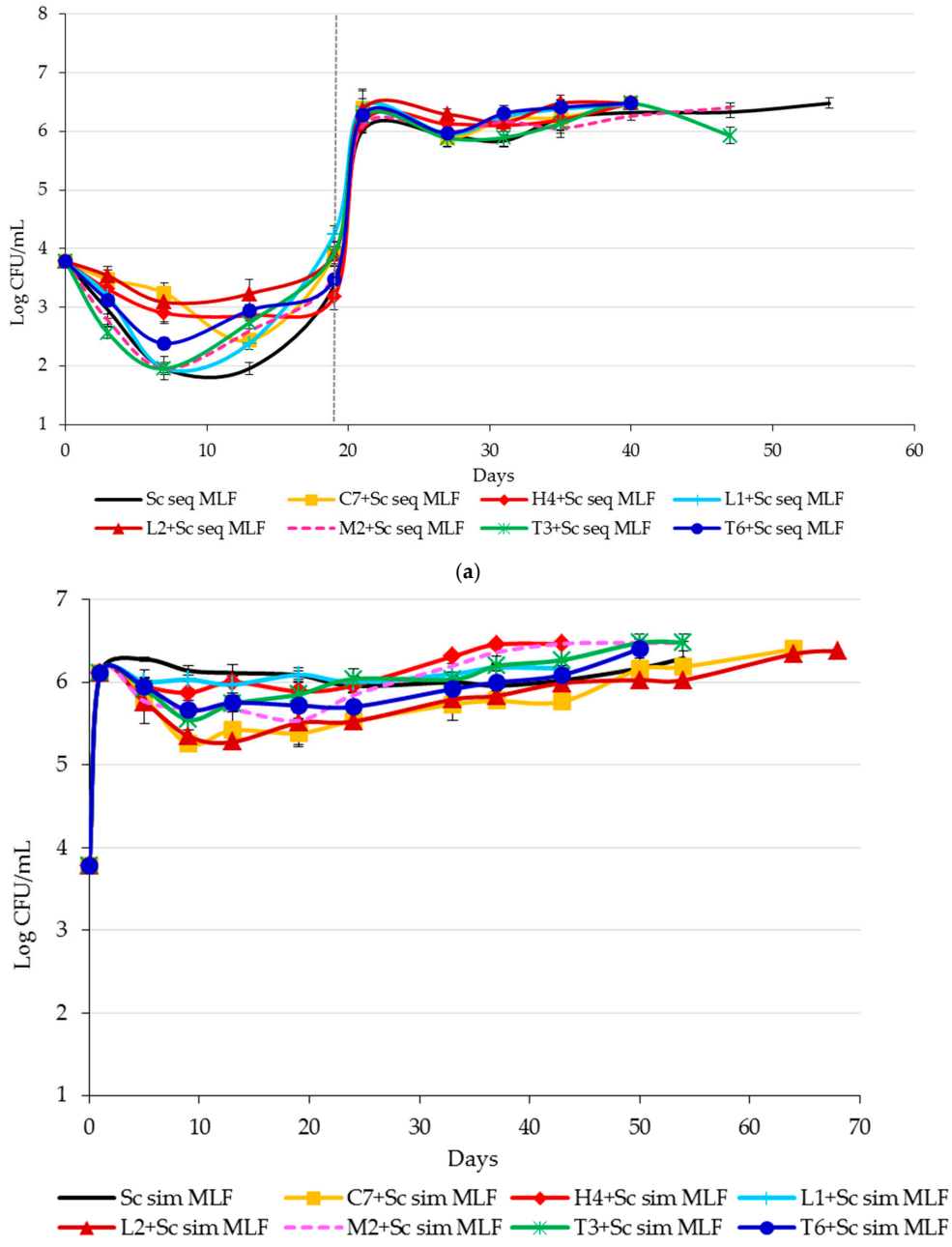

Figure 2. Cell counts (colony forming units per millilitres) of the naturally occurring lactic acid bacteria and inoculated *Oenococcus oeni* in Shiraz wines produced with *Saccharomyces cerevisiae* (Sc) on its own or in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, as well as three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). (**a**) Wines that underwent sequential malolactic fermentation (seq MLF) and the dashed vertical line at day 19 indicates when the commercial *O. oeni* was inoculated. (**b**) Wines where the commercial *O. oeni* was inoculated after 24 h (day 1) to induce MLF as a simultaneous inoculation (sim MLF). Values are averages of three replicates and the error bars indicate the standard deviation.

Individually, the numbers of naturally occurring LAB varied notably in wines, fermented with the selected non-*Saccharomyces* yeast combinations, which underwent sequential MLF (Figure 2a). The variation in LAB numbers can be ascribed to the effect of the different yeasts that conducted the

primary fermentation and support the findings of Muñoz et al. [22]. Based on the LAB counts from day 1 to 12, it was observed that yeast strains *S. cerevisiae* (Sc), *T. delbrueckii* T3 and T6, *M. pulcherrima* M2 and *L. thermotolerans* L1 had a larger inhibitory effect on the levels of the naturally occurring LAB (decreased from 6×10^3 to 90 CFU/mL) than *C. zemplinina* C7, *H. uvarum* H4 and *L. thermotolerans* L2 (decreased from 6×10^3 to 2.7×10^2 CFU/mL) (Figure 2a). However, as previously mentioned, all the LAB populations started to recover at the end of alcoholic fermentation (days 18–19). Inoculation with the commercial *O. oeni* strain on day 19 resulted in the dramatic and expected increase of LAB from ~ 1×10^3 to $\geq 1 \times 10^6$ CFU/mL. During the subsequent sequential MLF, wines produced with *S. cerevisiae*, *M. pulcherrima* and *T. delbrueckii* T3 had the lowest *O. oeni* counts, indicating that these yeast strains had a negative effect on the viability of *O. oeni*, which also explains why MLF took longer to complete. Wines produced with *C. zemplinina* also had low *O. oeni* counts (7.9×10^5 CFU/mL on day 27), but this did not result in delays in MLF. In wines produced with *H. uvarum*, *O. oeni* counts remained high (>1.2×10^6 CFU/mL) throughout MLF, which explains why this treatment completed MLF the fastest (38 days) (Table 2).

The naturally occurring LAB numbers (Figure 2a) in the simultaneous MLF treatments were notably lower than the inoculated *O. oeni* numbers (Figure 2b). This indicates that the inoculated *O. oeni* was probably responsible for completion of MLF. Non-*Saccharomyces* treatments, C7 + Sc sim MLF and L2 + Sc sim MLF had a negative (inhibitory) effect on *O. oeni*, resulting in lower counts for these wines (Figure 2b). Simultaneous MLF also took longer to complete than in wines produced with the other yeast strains (Table 2). The inhibitory effect of C7 was already noted by Du Plessis et al. [26] and inhibition could be alleviated by nutrient supplementation. Therefore, it can be concluded that inhibition of *O. oeni* growth by C7 was due to competition for essential nutrients. These wines did not contain notably higher alcohol concentrations (Table 2) or SO_2 levels (supplementary Table S1) than the other yeast treatments that could also lead to inhibition.

The inhibitory effect of *L. thermotolerans* strain L2 was not noted when it was evaluated in synthetic wine [26], but delays in MLF were observed for Chardonnay wines that underwent simultaneous MLF when the same *S. cerevisiae*/LAB combination was used (unpublished data). In the current study, the inhibition by L2 might be due to the combination of L2 with this specific *S. cerevisiae* strain, which resulted in the production of toxic metabolites or depletion of essential nutrients necessary for LAB growth. However, without further investigation it is difficult to draw a conclusion.

3.1.3. Progression of MLF

In most cases, wines produced with non-*Saccharomyces* yeasts completed MLF in a shorter period than wines produced with *S. cerevisiae* only. Duration of MLF varied amongst the wines produced with the different non-*Saccharomyces* yeast strains, with sequential MLF taking less time to complete than simultaneous MLF (Table 2). However, the success of sequential MLF is mainly due to the higher *O. oeni* dosage applied, which resulted in higher concentrations of viable cells. Due to circumstances outside the control of the researcher, the Shiraz grapes were harvested at a different ripeness level than initially planned, resulting in higher sugar concentration (26.9°Brix) and wines with high alcohol levels (>15% *v*/*v*) (Table 2). As the supplier does not recommend the use of Viniflora® oenos in high alcohol wines, a higher dosage was used for the sequential MLF treatments to ensure the successful completion of MLF and to compensate for cell death due to alcohol toxicity.

The H4 + Sc combination was most compatible with inoculated *O. oeni* and progress of simultaneous and sequential MLF. Results clearly show that there were differences between the non-*Saccharomyces* strains with regard to their effect on LAB growth and progress of MLF. The use of a different *S. cerevisiae* or LAB strain might have generated different results. These findings agree with reports of Bartowsky et al. [15] and Muñoz et al. [22] that optimal yeast LAB combinations may indeed differ for simultaneous and sequential MLF.

3.2. Standard Oenological Parameters

3.2.1. Wines without MLF

The alcoholic fermentation was completed after 18 days and all treatments fermented to dryness (residual sugar <4 g/L) (Table 2). In most cases, wines produced with non-*Saccharomyces* yeast had lower alcohol levels (15.49 to 15.94% *v*/*v*) than wines produced with *S. cerevisiae* only (~16% *v*/*v*), except L2 + Sc wines (16.04% *v*/*v*). A similar trend was observed by various authors [38,39]. Wines produced with *C. zemplinina* in combination with *S. cerevisiae* (C7 + Sc) contained the lowest alcohol levels (15.49% *v*/*v*). In most of the wines produced with non-*Saccharomyces* yeasts, glycerol levels were significantly lower than wines produced with *S. cerevisiae* only. Mendoza and Farías [40] reported similar results, but Comitini et al. [38] and Benito et al. [39] reported the contrary. The differences in reports might be due to the fact that different yeast strains and different grape varieties were used.

Acetic acid is the main contributor to volatile acidity (VA) and above the sensory threshold of 0.7–1.1 g/L can impart a vinegar aroma [41]. Although the wines produced with non-*Saccharomyces* yeasts had significantly higher VA levels than wines produced with *S. cerevisiae* only, the levels were well below the sensory threshold and legal limit of 1.2 g/L [42]. This is similar to the findings of Mendoza et al. [43]. *T. delbrueckii* has been described as producing low to high VA levels [44,45]. In this study, *T. delbrueckii* wines contained higher VA levels than *S. cerevisiae* only wines (0.25 vs. 0.4 g/L). The *H. uvarum* strain used in this study produced relatively low VA levels, confirming reports about the high strain variability of this species, and that some strains are comparable to *S. cerevisiae* with regard to levels of VA produced [46,47].

Malic acid levels varied significantly among the different yeast treatments and wines produced with *L. thermotolerans* L2 in combination with *S. cerevisiae* (L2 + Sc) had the highest concentration (1.68 g/L), while wines produced with *H. uvarum* in combination with *S. cerevisiae* (H4 + Sc) contained the lowest concentration (0.77 g/L) (Table 2). Significantly lower malic acid concentrations for the H4 + Sc and L1 + Sc treatments indicate possible malic acid degradation by these strains. The low lactic acid concentrations (0.2 g/L) and naturally occurring LAB levels (~2×10^3 and 2×10^4 CFU/mL, respectively) at the end of alcoholic fermentation, excludes the occurrence of spontaneous MLF in these wines. Du Plessis et al. [26] showed that strains H4 and L1 had limited malic acid degradation ability in MLF broth and synthetic media, but the ability of these strains to degrade malic acid was not tested in grape juice or must.

3.2.2. Wines that Underwent MLF

Wines that underwent MLF had significantly higher VA values (0.39 to 0.56 g/L) than the wines that did not undergo MLF (Table 2). Acetic acid, together with carbon dioxide, ethanol and lactic acid are produced by heterofermentative bacteria such as *O. oeni* during MLF [3], which impact on VA levels. In general, the sequential MLF wines contained higher VA levels than wines that underwent simultaneous MLF. This is similar to results reported by other researchers [48,49].

For most treatments, wines that did not undergo MLF had lower alcohol levels than wines that underwent MLF. Similar results have been reported by Benito et al. [48] and Izquierdo-Cañas et al. [50]. The *S. cerevisiae* simultaneous MLF treatment had the highest alcohol level (16.09% *v*/*v*), but no clear trend with regard to alcohol levels was observed in wines produced with non-*Saccharomyces* yeasts that underwent simultaneous or sequential MLF. However, there appeared to be an increase or decrease in the alcohol levels in wines that underwent MLF that was dependent on the yeast strain used. These results contradict those of Izquierdo-Cañas et al. [51], who found that sequential MLF wines had lower alcohol levels than simultaneous MLF wines.

Glycerol levels were significantly higher in wines that underwent MLF than in wines that did not and this is in agreement with the findings of Tristezza et al. [49] and Benito et al. [48]. In most cases, glycerol levels were also higher in wines that underwent simultaneous MLF than in wines that

underwent sequential MLF, with the highest being 11.98 g/L for T3 + Sc. These results confirm the findings of Mendoza and Farías [40] and Mendoza et al. [43], but contradict those of Tristezza et al. [49].

3.3. Flavor Compounds

ANOVA of volatile compounds showed that there was a significant interaction for all volatile compounds between wines produced with the three MLF strategies (none, simultaneous and sequential MLF) and eight yeast combinations (Table 3 and supplementary Table S2). This resulted in all 24 treatments delivering wines with significantly different volatile chemical profiles. These variations will have an impact on the perceived flavor profiles of the wines. The aforementioned results are in agreement with the findings of Whitener et al. [10–12], who reported that wines produced with different non-*Saccharomyces* and *Saccharomyces* yeast combinations had distinctive flavor profiles. However, unlike this investigation, those studies did not address yeast-LAB interactions.

To determine the potential contribution of the various volatile compounds to wine flavor, the odor activity values (OAVs) were determined. The OAV values were calculated by dividing the mean concentration of a compound by its odor threshold value (OTH, Table 4) as described by Guth [52]. Volatile compounds with OAV > 1 could potentially make an active contribution to wine aroma [52]. However, compounds with high OAVs do not always have an effect on wine aroma and the OAV is only an indication of the potential aroma contribution of individual compounds [53]. In a similar manner, the contribution by volatile compounds that are present at sub-threshold concentrations (i.e., OAVs < 1) should also not be excluded, as these aroma-active compounds can have additive, interactive effects, masking or suppressing effects [54].

Sixteen of the 31 quantified volatile compounds had OAVs > 1 (Table 3). They were ethyl acetate, ethyl hexanoate, ethyl butanoate, ethyl-3-hydroxybutanoate, isoamyl acetate, 2-phenylethyl acetate, ethyl phenylacetate, diethyl succinate, 2-phenylethanol, isoamyl alcohol, 3-ethoxy-1-propanol, hexanol, isobutanol, acetic acid, isovaleric acid and valeric acid.

Wines produced with *S. cerevisiae* only that did not undergo MLF contained higher diethyl succinate (fruity, melon, berry aroma) and 2-phenylethanol (floral, rose, honey, spice, lilac aroma) concentrations than wines produced with non-*Saccharomyces* yeasts that did not undergo MLF (Table 3). Whitener et al. [10] reported similar results.

The concentrations of MLF marker compounds such as diethyl succinate, ethyl lactate and ethyl acetate were higher in wines that underwent MLF, which is in agreement with literature [23,25]. In most cases, ethyl acetate concentrations were lower in wines that underwent simultaneous MLF than wines that underwent sequential MLF. This finding is in agreement with those of Abrahamse and Bartowsky [25] and Izquierdo-Cañas et al. [51], but contrary to findings of Antalick et al. [23]. Ethyl lactate and diethyl succinate concentrations were higher in wines that underwent simultaneous MLF than in wines that underwent sequential MLF. Izquierdo-Cañas et al. [51] reported similar results. The other ethyl and acetate esters are known as odorant esters because of their impact on wine aroma, despite being present at low concentrations (g/L) [23]. The concentrations of these esters varied and some (ethyl-3-hydroxybutanoate, ethyl decanoate and ethyl phenylacetate) were higher in wines that underwent simultaneous MLF, while others (ethyl butanoate, isoamyl acetate, ethyl hexanoate, ethyl octanoate and 2-phenylethyl aceate) were higher in wines that underwent sequential MLF.

Diacetyl is one of the most important compounds associated with MLF and contributes to buttery, nutty and butterscotch characters in wine [3,4,16]. However, diacetyl is chemically unstable and can be reduced to acetoin, which in turn can be reduced to 2,3-butanediol. Reduction of diacetyl to acetoin and 2,3-butanediol is advantageous because these products are less toxic to yeasts. Acetoin does not contribute to wine flavor due to its high aroma threshold of 150 mg/L [4]. In this study, only the concentration of acetoin was analyzed and, as expected, was significantly higher in wines that underwent MLF (Table 3).

Table 3. Concentrations of volatile compounds (mg/L) and their calculated odor activity values (OAV) of bottled Shiraz wines produced with different yeast [1] strains in combination with three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Values are averages of three replicates.

Treatment [1]	Ethyl Acetate	OAV	Ethyl Butanoate	OAV	Isoamyl Acetate	OAV	Ethyl Lactate	OAV	Ethyl-3-hydroxy Butanoate	OAV	Diethyl Succinate	OAV	Ethyl Hexanoate	OAV	Ethyl Octanoate	OAV
Sc	40.20 p [2]	3.3	0.49 kl	1.2	1.33 ijk	8.3	1.59 l	0.1	1.68 efg	1.7	2.103 f	1.8	0.77 ij	9.6	0.33 f	0.6
Sc + sim MLF	52.98 m	4.4	0.49 kl	1.2	1.05 n	6.6	9.22 b	0.7	1.68 ef	1.7	2.466 c	2.1	0.76 j	9.4	0.25 i	0.4
Sc + seq MLF	55.77 l	4.6	0.48 l	1.2	1.48 def	9.3	6.48 g	0.5	1.60 j	1.6	2.076 f	1.7	0.77 ij	9.6	0.33 f	0.6
C7 + Sc	58.55 jk	4.9	0.53 efgh	1.3	1.06 n	6.7	1.62 l	0.1	1.76 bc	1.8	1.941 h	1.6	0.80 fg	10.0	0.32 f	0.6
C7 + Sc + sim MLF	62.08 ghi	5.2	0.53 efgh	1.3	1.07 n	6.7	8.81 c	0.6	1.75 cd	1.8	2.581 b	2.2	0.80 fg	10.0	0.28 gh	0.5
C7 + Sc + seq MLF	76.02 b	6.3	0.55 ab	1.4	1.20 lm	7.5	6.77 ef	0.5	1.79 ab	1.8	2.002 g	1.7	0.83 cd	10.4	0.37 e	0.6
H4 + Sc	65.72 f	5.5	0.52 efgh	1.3	1.47 defg	9.2	2.08 k	0.1	1.67 fgh	1.7	1.544 m	1.3	0.81 ef	10.1	0.37 e	0.6
H4 + Sc + sim MLF	64.15 fg	5.3	0.50 jk	1.3	1.23 klm	7.7	7.54 d	0.5	1.70 e	1.7	2.486 c	2.1	0.78 hi	9.8	0.27 h	0.5
H4 + Sc + seq MLF	73.35 c	6.1	0.54 bcd	1.4	1.60 abc	10.0	4.80 j	0.3	1.64 hi	1.6	1.586 m	1.3	0.84 c	10.5	0.41 c	0.7
L1 + Sc	45.83 o	3.8	0.53 cdef	1.3	1.44 efgh	9.0	1.53 l	0.1	1.59 j	1.6	1.828 k	1.5	0.80 efg	10.0	0.36 e	0.6
L1 + Sc + sim MLF	63.54 fgh	5.3	0.52 ghi	1.3	1.39 fghi	8.7	7.46 d	0.5	1.67 fgh	1.7	2.459 c	2.0	0.79 gh	9.9	0.29 gh	0.5
L1 + Sc + seq MLF	60.39 ij	5.0	0.53 cde	1.3	1.53 cde	9.5	5.63 hi	0.4	1.64 i	1.6	1.854 jk	1.5	0.81 efg	10.1	0.38 de	0.7
L2 + Sc	69.35 e	5.8	0.55 ab	1.4	1.64 ab	10.2	2.01 k	0.1	1.74 cd	1.7	1.710 l	1.4	0.88 a	11.0	0.43 b	0.7
L2 + Sc + sim MLF	72.04 cd	6.0	0.52 fghi	1.3	0.95 o	5.9	9.64 a	0.7	1.80 a	1.8	2.595 b	2.2	0.81 ef	10.1	0.31 f	0.5
L2 + Sc + seq MLF	81.31 a	6.8	0.56 a	1.4	1.67 a	10.5	5.03 j	0.4	1.74 cd	1.7	1.714 l	1.4	0.88 a	11.1	0.46 a	0.8
M2 + Sc	48.61 n	4.1	0.54 bc	1.4	1.52 cde	9.5	1.39 l	0.1	1.65 ghi	1.7	1.870 ijk	1.6	0.83 c	10.4	0.40 cd	0.7
M2 + Sc + sim MLF	57.62 kl	4.8	0.51 ij	1.3	1.21 lm	7.6	6.69 fg	0.5	1.69 ef	1.7	2.283 e	1.9	0.77 ij	9.6	0.27 gh	0.5
M2 + Sc + seq MLF	63.71 fgh	5.3	0.53 cdefg	1.3	1.44 defgh	9.0	5.69 hi	0.4	1.67 fgh	1.7	1.852 jk	1.5	0.82 de	10.2	0.39 cd	0.7
T3 + Sc	51.47 m	4.3	0.54 bcd	1.3	1.32 ijk	8.3	1.50 l	0.1	1.73 d	1.7	1.885 ij	1.6	0.83 cd	10.3	0.37 e	0.6
T3 + Sc + sim MLF	60.75 ij	5.1	0.53 cdefg	1.3	1.36 hij	8.5	6.99 e	0.5	1.66 fghi	1.7	2.353 d	2.0	0.79 gh	9.9	0.29 g	0.5
T3 + Sc + seq MLF	63.41 fgh	5.3	0.54 bcd	1.4	1.29 jkl	8.0	5.88 h	0.4	1.73 d	1.7	1.912 hi	1.6	0.84 c	10.5	0.40 cd	0.7
T6 + Sc	70.27 de	5.9	0.55 ab	1.4	1.38 ghij	8.6	2.10 k	0.1	1.67 fgh	1.7	1.581 m	1.3	0.86 b	10.7	0.40 cd	0.7
T6 + Sc + sim MLF	61.55 hi	5.1	0.52 hi	1.3	1.18 m	7.4	8.65 c	0.6	1.75 cd	1.8	2.717 a	2.3	0.80 fgh	10.0	0.28 gh	0.5
T6 + Sc + seq MLF	83.26 a	6.9	0.56 a	1.4	1.55 bcd	9.7	5.59 i	0.4	1.68 efg	1.7	1.595 m	1.3	0.88 a	11.0	0.46 a	0.8

Table 3. *Cont.*

Treatment	Ethyl Decanoate	OAV	Ethyl Phenyl Acetate	OAV	2-Phenyl ethyl Acetate	OAV	Methanol	OAV	Propanol	OAV	Butanol	OAV	Isobutanol	OAV	Pentanol	OAV
Sc	0.097 ij	0.2	0.61 c	8.4	1.18 jk	0.7	156.39 de	0.3	47.65 fg	0.2	3.34 a	0.02	42.39 hi	1.1	0.710 j	0.01
Sc + sim MLF	0.123 bc	0.2	0.64 b	8.7	1.15 k	0.6	184.84 abc	0.4	53.65 bc	0.2	2.62 e	0.02	47.77 cd	1.2	0.734 b	0.01
Sc + seq MLF	0.096 j	0.2	0.67 a	9.2	1.18 jk	0.7	146.68 gh	0.3	43.27 ij	0.1	3.15 b	0.02	38.50 k	1.0	0.706 kl	0.01
C7 + Sc	0.098 hij	0.2	0.44 n	6.1	1.31 efg	0.7	178.68 c	0.4	42.25 j	0.1	2.07 m	0.01	52.69 b	1.3	0.723 gh	0.01
C7 + Sc + sim MLF	0.132 a	0.3	0.49 kl	6.8	1.27 hi	0.7	184.83 abc	0.4	51.70 cd	0.2	2.50 fg	0.02	51.95 b	1.3	0.737 ab	0.01
C7 + Sc + seq MLF	0.125 abc	0.2	0.48 klm	6.6	1.38 d	0.8	177.87 c	0.4	42.76 ij	0.1	2.15 l	0.01	54.62 a	1.4	0.733 cd	0.01
H4 + Sc	0.101 ghij	0.2	0.51 ij	7.0	1.27 hi	0.7	179.10 c	0.4	49.41 ef	0.2	2.36 i	0.02	38.02 k	1.0	0.719 h	0.01
H4 + Sc + sim MLF	0.121 bcd	0.2	0.59 d	8.0	1.19 j	0.7	182.98 bc	0.4	43.93 hij	0.1	2.35 ij	0.02	49.38 c	1.2	0.733 cd	0.01
H4 + Sc + seq MLF	0.111 ef	0.2	0.55 fg	7.6	1.37 d	0.8	161.97 d	0.3	44.95 hi	0.1	2.26 k	0.02	35.61 l	0.9	0.723 gh	0.01
L1 + Sc	0.102 ghij	0.2	0.50 kl	6.8	1.28 ghi	0.7	152.48 efg	0.3	54.69 ab	0.2	2.98 c	0.02	34.72 l	0.9	0.715 i	0.01
L1 + Sc + sim MLF	0.096 j	0.2	0.57 de	7.8	1.28 fghi	0.7	190.70 a	0.4	49.37 ef	0.2	2.24 k	0.01	52.49 b	1.3	0.727 ef	0.01
L1 + Sc + seq MLF	0.105 fghi	0.2	0.56 ef	7.7	1.29 fgh	0.7	156.00 def	0.3	56.13 a	0.2	3.03 c	0.02	35.65 l	0.9	0.721 h	0.01
L2 + Sc	0.113 def	0.2	0.57 de	7.8	1.47 b	0.8	183.45 abc	0.4	42.93 ij	0.1	2.51 fg	0.02	44.82 efg	1.1	0.726 efg	0.01
L2 + Sc + sim MLF	0.117 cde	0.2	0.48 lm	6.6	1.29 fgh	0.7	190.62 a	0.4	50.31 de	0.2	2.70 d	0.02	56.45 a	1.4	0.739 a	0.01
L2 + Sc + seq MLF	0.122 bcd	0.2	0.67 a	9.2	1.80 a	1.0	186.66 ab	0.4	43.49 ij	0.1	2.51 fg	0.02	45.19 ef	1.1	0.732 cd	0.01
M2 + Sc	0.107 fg	0.2	0.50 jk	6.8	1.34 de	0.7	143.03 h	0.3	38.41 k	0.1	2.52 fg	0.02	39.81 jk	1.0	0.704 kl	0.01
M2 + Sc + sim MLF	0.126 ab	0.3	0.57 ef	7.8	1.21 j	0.7	183.91 abc	0.4	43.72 ij	0.1	2.40 hi	0.02	49.32 c	1.2	0.720 h	0.01
M2 + Sc + seq MLF	0.105 fghi	0.2	0.54 gh	7.4	1.32 ef	0.7	148.78 fgh	0.3	39.55 k	0.1	2.56 ef	0.02	40.80 ij	1.0	0.706 kl	0.01
T3 + Sc	0.106 fgh	0.2	0.47 m	6.5	1.35 e	0.7	149.18 efgh	0.3	35.70 l	0.1	2.22 lk	0.01	42.94 gh	1.1	0.703 l	0.01
T3 + Sc + sim MLF	0.100 ghij	0.2	0.53 gh	7.3	1.25 i	0.7	178.55 c	0.4	49.52 def	0.2	2.46 gh	0.02	46.60 de	1.2	0.729 de	0.01
T3 + Sc + seq MLF	0.103 ghij	0.2	0.54 gh	7.4	1.38 d	0.8	150.39 efgh	0.3	35.75 l	0.1	2.28 jk	0.02	43.44 fgh	1.1	0.707 jk	0.01
T6 + Sc	0.107 fg	0.2	0.47 m	6.4	1.43 c	0.8	179.67 bc	0.4	46.10 gh	0.2	2.35 ij	0.02	38.90 jk	1.0	0.721 h	0.01
T6 + Sc + sim MLF	0.126 abc	0.3	0.53 hi	7.2	1.28 fghi	0.7	179.36 bc	0.4	54.32 ab	0.2	2.38 hi	0.02	52.08 b	1.3	0.740 a	0.01
T6 + Sc + seq MLF	0.120 bcd	0.2	0.51 ij	7.1	1.47 b	0.8	181.11 bc	0.4	46.09 gh	0.2	2.40 hi	0.02	39.14 jk	1.0	0.725 fg	0.01

Treatment	Isoamyl Alcohol	OAV	3-Ethoxy-1-propanol	OAV	3-Methyl-1-pentanol	OAV	Hexanol	OAV	2-Phenyl Ethanol	OAV	Acetoin	OAV	Acetic Acid	OAV
Sc	338.70 ef	5.6	2.43 jk	24.3	0.65 d	0.6	37.73 kl	4.7	79.45 bc	5.7	5.26 kl	0.04	180.02 p	0.9
Sc + sim MLF	370.68 c	6.2	2.71 h	27.1	0.69 a	0.7	45.05 bc	5.6	75.78 e	5.4	13.95 de	0.09	269.45 l	1.3
Sc + seq MLF	335.27 f	5.6	2.41 k	24.1	0.65 d	0.7	36.92 klm	4.6	77.00 cde	5.5	12.59 efg	0.08	278.61 kl	1.4
C7 + Sc	302.06 h	5.0	3.86 b	38.6	0.59 i	0.6	39.42 hi	4.9	57.37 j	4.1	4.59 l	0.03	272.79l	1.4
C7 + Sc + sim MLF	381.58 b	6.4	2.51 ij	25.1	0.67 b	0.7	44.20 bcde	5.5	80.14 b	5.7	13.57 efg	0.09	298.04 ij	1.5
C7 + Sc + seq MLF	319.82 g	5.3	4.06 a	40.6	0.60 h	0.6	42.16 g	5.3	59.73 ij	4.3	13.64 def	0.09	385.22 c	1.9
H4 + Sc	228.40 m	3.8	3.23 f	32.3	0.56 k	0.6	40.21 h	5.0	39.14 l	2.8	5.44 kl	0.04	306.70 ghi	1.5
H4 + Sc + sim MLF	366.74 cd	6.1	2.44 jk	24.4	0.67 b	0.7	45.58 ab	5.7	76.33 de	5.5	8.65 ij	0.06	316.47 gh	1.6
H4 + Sc + seq MLF	228.74 m	3.8	3.38 e	33.8	0.56 jk	0.6	43.17 efg	5.4	39.77 l	2.8	15.90 b	0.11	384.48 c	1.9
L1 + Sc	285.62 j	4.8	2.56 i	25.6	0.62 e	0.6	37.94 jk	4.7	63.25 gh	4.5	5.45 kl	0.04	201.73 o	1.0
L1 + Sc + sim MLF	366.73 cd	6.1	2.79 h	27.9	0.62 e	0.6	44.76 bcd	5.6	74.72 e	5.3	15.54 bc	0.10	318.76 fg	1.6
L1 + Sc + seq MLF	290.64 ij	4.8	2.57 i	25.7	0.62 e	0.6	38.13 ijk	4.8	64.89 g	4.6	12.25 gh	0.08	313.81 gh	1.6
L2 + Sc	260.06 k	4.3	3.34 e	33.4	0.57 j	0.6	43.55 defg	5.4	44.24 k	3.2	6.02 k	0.04	350.01 d	1.8
L2 + Sc + sim MLF	400.33 a	6.7	2.57 i	25.7	0.65 d	0.7	45.52 ab	5.7	87.48 a	6.2	11.78 h	0.08	305.54 hi	1.5
L2 + Sc + seq MLF	259.57 k	4.3	3.53 d	35.3	0.57 j	0.6	44.27 bcde	5.5	43.81 k	3.1	12.53 fgh	0.08	468.89 a	2.3
M2 + Sc	290.00 ij	4.8	3.56 d	35.6	0.61 ef	0.6	36.44 lm	4.6	62.50 gh	4.5	4.19 l	0.03	219.49 n	1.1
M2 + Sc + sim MLF	346.22 e	5.8	3.58 cd	35.8	0.65 d	0.7	43.28 efg	5.4	69.30 f	4.9	9.79 i	0.07	290.89 jk	1.5
M2 + Sc + seq MLF	290.23 ij	4.8	3.61 cd	36.1	0.61 fg	0.6	36.24 m	4.5	61.54 hi	4.4	7.94 j	0.05	334.87 e	1.7
T3 + Sc	294.95 hi	4.9	3.54 d	35.4	0.60 gh	0.6	38.02 ijk	4.8	57.63 j	4.1	4.28 l	0.03	242.29 m	1.2
T3 + Sc + sim MLF	358.92 d	6.0	2.56 i	25.6	0.66 c	0.7	42.66 fg	5.3	78.99 bcd	5.6	12.16 h	0.08	298.90 ij	1.5
T3 + Sc + seq MLF	297.42 hi	5.0	3.67 c	36.7	0.61 fg	0.6	39.18 hij	4.9	57.92 j	4.1	8.40 ij	0.06	338.07 de	1.7
T6 + Sc	235.74 m	3.9	3.23 f	32.3	0.56 jk	0.6	43.08 efg	5.4	37.94 l	2.7	6.49 k	0.04	331.14 ef	1.7
T6 + Sc + sim MLF	401.63 a	6.7	2.91 g	29.1	0.70 a	0.7	46.61 a	5.8	86.07 a	6.1	14.30 cd	0.10	294.87 ij	1.5
T6 + Sc + seq MLF	237.91 l	4.0	3.36 e	33.6	0.56 jk	0.6	43.86 cdef	5.5	38.32 l	2.7	17.89 a	0.12	432.88 b	2.2

Table 3. *Cont.*

Treatment	Propionic Acid	OAV	Butyric Acid	OAV	Isobutyric Acid	OAV	Valeric Acid	OAV	Isovaleric Acid	OAV	Hexanoic Acid	OAV	Octanoic Acid	OAV	Decanoic Acid	OAV
Sc	3.89 bcde	0.2	1.13 ghi	0.5	1.524 g	0.05	0.417 ab	1.4	9.07 i	6.0	0.64 b	0.2	1.57 j	0.2	1.09 cd	0.2
Sc + sim MLF	3.92 bcd	0.2	1.10 ij	0.5	1.759 e	0.06	0.416 b	1.4	18.67 g	12.4	0.52 j	0.2	1.29 m	0.1	1.04 fgh	0.2
Sc + seq MLF	3.91 bcd	0.2	1.09 j	0.5	1.443 h	0.05	0.423 a	1.4	18.80 g	12.5	0.64 ab	0.2	1.57 j	0.2	1.06 efg	0.2
C7 + Sc	3.52 hi	0.2	1.21 bc	0.6	1.930 c	0.06	0.39 kl	1.3	9.24 i	6.2	0.55 hi	0.2	1.64 i	0.2	1.08 d	0.2
C7 + Sc + sim MLF	4.05 ab	0.2	1.21 c	0.6	2.168 a	0.07	0.398 ghij	1.3	22.58 b	15.1	0.55 i	0.2	1.43 kl	0.1	1.06 ef	0.2
C7 + Sc + seq MLF	3.49 ij	0.2	1.25 ab	0.6	1.972 c	0.07	0.395 ijkl	1.3	22.82 b	15.2	0.55 i	0.2	1.77 efg	0.2	1.09 cd	0.2
H4 + Sc	4.18 a	0.2	1.15 fgh	0.5	1.283 k	0.04	0.404 efg	1.3	8.78 i	5.9	0.57 fgh	0.2	1.67 hi	0.2	1.06 efg	0.2
H4 + Sc + sim MLF	3.66 efghi	0.2	1.12 hij	0.5	1.818d	0.06	0.405ef	1.4	18.94 g	12.6	0.58 efg	0.2	1.37 l	0.1	1.03 h	0.2
H4 + Sc + seq MLF	3.76 defgh	0.2	1.15 fgh	0.5	1.197 l	0.04	0.406 def	1.4	20.91 ef	13.9	0.59 de	0.2	1.86 bcd	0.2	1.08 d	0.2
L1 + Sc	3.54 ghi	0.2	1.17 def	0.5	1.333 ijk	0.04	0.406 def	1.4	10.19 h	6.8	0.66 ab	0.2	1.73 gh	0.2	1.08 de	0.2
L1 + Sc + sim MLF	4.09 ab	0.2	1.16 fg	0.5	2.039 b	0.07	0.412 bcd	1.4	20.31 f	13.5	0.59 de	0.2	1.45 k	0.1	1.06 ef	0.2
L1 + Sc + seq MLF	3.77 cdefg	0.2	1.20 cde	0.5	1.282 k	0.04	0.416 b	1.4	24.19 a	16.1	0.65 ab	0.2	1.78 efg	0.2	1.08 d	0.2
L2 + Sc	3.63 fghi	0.2	1.25 a	0.6	1.525 g	0.05	0.410 cde	1.4	9.07 i	6.0	0.61 c	0.2	1.92 b	0.2	1.10 bc	0.2
L2 + Sc + sim MLF	4.07 ab	0.2	1.26 a	0.6	1.940 c	0.06	0.405 ef	1.3	22.59 b	15.1	0.52 j	0.2	1.44 k	0.1	1.10 bcd	0.2
L2 + Sc + seq MLF	4.08 ab	0.2	1.26 a	0.6	1.453 h	0.05	0.415 bc	1.4	21.43 de	14.3	0.58 def	0.2	2.00 a	0.2	1.09 cd	0.2
M2 + Sc	2.95 l	0.1	1.15 fgh	0.5	1.378 i	0.05	0.394 jkl	1.3	9.09 i	6.1	0.66 a	0.2	1.74 g	0.2	1.10 bcd	0.2
M2 + Sc + sim MLF	3.55 ghi	0.2	1.09 j	0.5	1.676 f	0.06	0.398 ghij	1.3	20.78 ef	13.9	0.56 ghi	0.2	1.39 kl	0.1	1.03 h	0.2
M2 + Sc + seq MLF	3.24 jk	0.2	1.15 g	0.5	1.302 k	0.04	0.396 hijk	1.3	22.33 bcd	14.9	0.62c	0.2	1.80 def	0.2	1.08 d	0.2
T3 + Sc	3.47 ij	0.2	1.21 cd	0.5	1.482 gh	0.05	0.390 l	1.3	9.21 i	6.1	0.60 cd	0.2	1.75 fg	0.2	1.09 cd	0.2
T3 + Sc + sim MLF	3.85 cdef	0.2	1.17 ef	0.5	1.734 e	0.06	0.407 def	1.4	21.34 e	14.2	0.59 de	0.2	1.44 k	0.1	1.04 gh	0.2
T3 + Sc + seq MLF	3.03 kl	0.2	1.21 c	0.5	1.440 h	0.05	0.397 hij	1.3	22.81 b	15.2	0.58 def	0.2	1.88 bc	0.2	1.11 ab	0.2
T6 + Sc	4.01 abc	0.2	1.17 def	0.5	1.366 ij	0.05	0.396 ijkl	1.3	9.26 i	6.2	0.56 fghi	0.2	1.84 cde	0.2	1.08 cd	0.2
T6 + Sc + sim MLF	3.43 ij	0.2	1.14 fgh	0.5	2.058 b	0.07	0.401 fghi	1.3	22.40 bc	14.9	0.56 fghi	0.2	1.45 k	0.1	1.04 h	0.2
T6 + Sc + seq MLF	3.95 abcd	0.2	1.20 cde	0.5	1.321 jk	0.04	0.403 fgh	1.3	21.65 cde	14.4	0.57 fgh	0.2	2.04 a	0.2	1.13 a	0.2

[1] *Saccharomyces cerevisiae* (Sc), *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, simultaneous (sim) MLF and sequential (seq) MLF treatments induced with *Oenococcus oeni*. [2] Values in the same column followed by the same letter did not differ significantly ($p \leq 0.05$).

Table 4. Odor threshold (OTH) values (mg/L) and descriptions of aroma and flavor compounds found in wine. Superscript values denote the appropriate reference.

Compounds	OTH Values (mg/L)	Aroma/Flavor Descriptors
Esters		
Ethyl acetate	12 [55]	Fruit, nail polish [41,56]
Ethyl butanoate (butyrate)	0.4 [57]	Strawberry [57], apple [56], fruity [21]
Isoamyl acetate	0.16 [57]	Banana, pear [16,41]
Ethyl lactate	14 [58]	Butter, cream, fruit [56]
Ethyl-3-hydroxy butanoate	1 [55]	Fruity, grape [55], strawberry [59]
Diethyl succinate	1.2 [57]	Fruity, melon [57], berry [56]
Ethyl hexanoate (ethyl caproate)	0.08 [57]	Apple [56], fruity, anise [53], strawberry [58]
Ethyl octanoate (ethyl caprylate)	0.58 [57]	Fruit [56], pear, pineapple [41]
Ethyl decanoate (ethyl caprate)	0.5 [57]	Floral [41,56], grape, soap [16,56]
Ethyl phenylacetate	0.073 [60]	Honey-like [60]
2-Phenylethyl acetate	0.25 [52]	Flowery, fruity, rose [16,41]
Alcohols		
Methanol	500 [57]	Alcohol [57]
N-Propanol	306 [57]	Alcohol, ripe fruit [57], pungent, harsh [16,56]
N-Butanol	150 [57]	Fusel, spirituous [16,56]
Isobutanol	40 [52]	Wine, solvent, fusel [16]
Pentanol	64 [61]	Fusel, alcoholic, fermented, pungent, bready, yeasty [11]
Isoamyl alcohol	60 [57]	Herbaceous [59], whiskey, malt, burnt [56]
3-Ethoxy-1-propanol	0.1 [55]	Fruity [57]
3-Methyl-1-pentanol	1 [55]	Green, pungent, solvent [55]
Hexanol	8 [52]	Herbaceous [55], grass [16,53], resin [53]
2-Phenylethanol	14 [62]	Floral, rose [16,41], honey, spice, lilac [56]
Ketones		
Acetoin	150 [57]	Buttery, cream [57]
Acids		
Acetic acid	200 [52]	Vinegar [41,62]
Propionic acid	0.42 [41]	Pungent, rancid [41,56], sweat [56]
N-Butyric acid	2.2 [55]	Cheese [53], pungent [41]
Isobutyric acid	30 [55]	Rancid, butter, cheese [56], pungent [41]
N-Valeric acid	3 [63]	unpleasant [41]
Isovaleric acid	1.5 [55]	Cheese [41,52], rancid, sweaty [41]
Hexanoic acid	3 [55]	Sweat [41,56], sour, vinegar, cheese, rancid, fatty, pungent [41]
Octanoic acid	10 [55]	Sweat, cheese [56], oily, fatty, rancid, soapy, sweet, faint fruity, butter [41]
Decanoic acid	6 [57]	Rancid, fat [41,56], unpleasant, citrus, phenolic [41]

3.4. Multivariate Data Analysis of Wines

To investigate the correlation between the chemical composition of the Shiraz wines and the various yeast combinations and MLF strategies, a PCA was performed, using the data of the 31 volatile compounds (GC-FID analysis) and nine standard chemical parameters (glucose, fructose, pH, volatile acidity, total acidity, malic acid, lactic acid, ethanol and glycerol). The first two principal components explained 58.37% of the variance in the data set (Figure 3). Subsequently, 11 variables (pH, glucose, ethanol, propanol, butanol, 3-ethoxy-1-propanol, ethyl-3-hydroxybutanoate, ethyl phenylacetate, propionic acid, butyric acid and valeric acid) that did not contribute to the separation on PC1 and PC2 were removed. The PCA biplot of the 29 variables explained 72.69% (PC1 = 44.96% and PC2 = 27.73%) of the variance in the data set. Three groups were observed, i.e., the wines that underwent simultaneous MLF (top right quadrant of PC1), the wines that underwent sequential MLF (Top left quadrant of PC1) and those that did not undergo MLF (bottom left quadrant PC2). However, there was some overlapping between wines that did not undergo MLF and wines that underwent sequential MLF. The clustering of the wines indicates that MLF strategy had a greater effect on chemical composition of the wines than yeast treatment, but that yeast treatment also played a role with regard to the clustering. The effects of the yeast combinations can be seen in the variations within the three clusters. The association of the wines within the clusters indicates that there are similarities, but also some differences among the wines. Results also show that the chemical profiles of wines that underwent sequential MLF and wines that did not undergo MLF were similar and were notably different from wines that underwent simultaneous MLF. The association of *S. cerevisiae* only wines that did not

undergo MLF and *S. cerevisiae* only wines that underwent sequential MLF is a good example of the aforementioned observation.

Based on the contribution and the squared cosines of the variables, the most important compounds for differentiating between wines produced with the selected yeast combinations and three MLF strategies were volatile acidity, acetic acid, ethyl acetate, isoamyl alcohol, 3-methyl-1-pentanol, ethyl octanoate, diethyl succinate, 2-phenyl ethanol and octanoic acid.

Most of the wines that did not undergo MLF were positively correlated with malic acid, hexanoic acid and total acidity. These wines were also negatively correlated with most of the other compounds. Wines that did not undergo MLF had higher total acidity than wines that underwent MLF and consequently, lower pH levels as shown in Table 2. The wines produced with *L. thermotolerans* L2 (ARC culture collection isolate) differed the most from the *S. cerevisiae* reference wines with regard to chemical composition. This is in agreement with the finding of Whitener et al. [10], who reported that Shiraz wines produced with *L. thermotolerans* in combination with *S. cerevisiae* were significantly different from wines produced with *S. cerevisiae* only. However, the finding of the aforementioned authors was for different *S. cerevisiae* and *L. thermotolerans* strains than reported in this study. Wines produced with the two *L. thermotolerans* strains (L1 and L2) were not closely associated, indicating that wines produced with L1 (commercial strain) is significantly different from wines produced with L2 (ARC culture collection isolate) with regard to chemical composition (Figure 3 and Table 3). Wines produced with L1 + Sc contain significantly higher levels of diethyl succinate, isoamyl alcohol, 2-phenylethanol, propanol, butanol, 3-methyl-1-pentanol and hexanoic acid than L2 + Sc wines, while wines produced with L2 + Sc contained significantly higher levels of most of the other volatile compounds. A similar trend with regard to differences in chemical composition was observed for wines produced with *T. delbrueckii* T3 (commercial strain) and T6 (ARC culture collection isolate) (Figure 3 and Table 3).

Most of the wines that underwent sequential MLF were positively correlated with volatile acidity, ethyl acetate, acetic acid, 2-phenylethyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, octanoic acid, isoamyl acetate, decanoic acid and fructose (Figure 3). Clear variation was observed with regard to the clustering of wines produced with the different yeast combinations that underwent sequential MLF, indicating that their chemical compositions differed from each other and the other MLF treatments. Similar to what was observed for the wines that did not undergo MLF, wines produced with M2 + Sc and L1 + Sc that underwent sequential MLF were similar.

Wines that underwent simultaneous MLF were closely associated and positively correlated with ethyl decanoate, hexanol, acetoin, methanol, pentanol, isovaleric acid, lactic acid, ethyl lactate, isobutanol, isobutyric acid, glycerol, diethyl succinate, isoamyl alcohol, 3-methyl-1-pentanol and 2-phenyl ethanol. Despite the close association of wines that underwent simultaneous MLF, differences were observed for wines produced with the selected yeast combinations. Wines produced with M2 + Sc and L1 + Sc that underwent simultaneous MLF did not cluster together as observed for the no MLF and sequential MLF strategies. For wines that underwent simultaneous MLF, M2 + Sc and T3 + Sc wines clustered together.

Results also showed that the variation in chemical composition of wines produced by strains from the same non-*Saccharomyces* species can be as significant as the variation between different non-*Saccharomyces* species, or as significant as the differences between non-*Saccharomyces* and *Saccharomyces* yeasts.

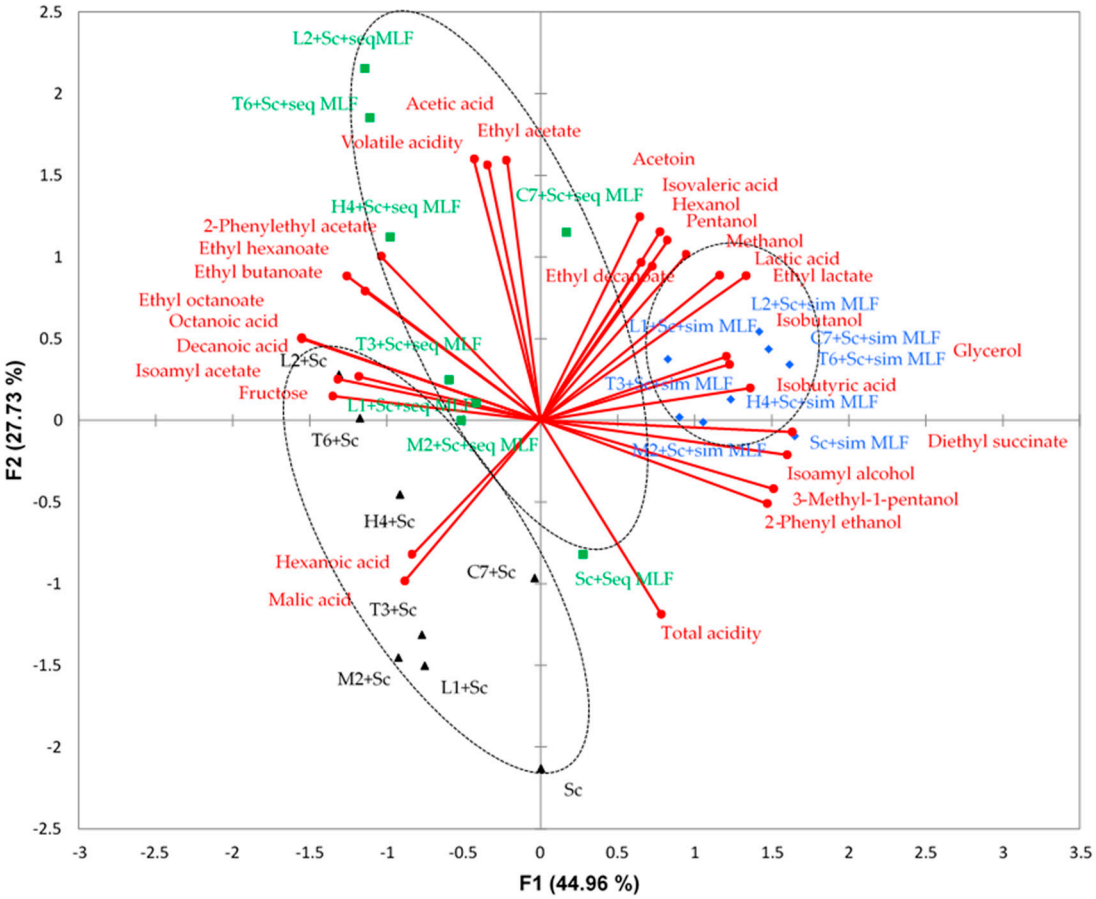

Figure 3. Principal component analysis (PCA) bi-plot derived from volatile compounds and standard chemical parameters of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc) on its own or in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, as well as three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Circles are for illustrative purpose only.

3.5. Sensory Evaluation

The sensory data show that the different yeast combinations had a significant effect on berry aroma (p = 0.0036), while MLF strategy (none, simultaneous and sequential MLF) had a significant effect on berry aroma (p = 0.0053), acid balance (p = 0.0447) and astringency (p = 0.0271) (Table 5). At the 90% confidence level ($p \leq 0.1$) yeast treatment had a significant effect on fresh vegetative aroma and MLF strategy had a significant effect on fruity aroma. Overall, there was no significant interaction effect between yeast treatment and MLF strategy (Table 5), but for certain wines significant differences were observed (Table S3). Only the treatment effects for berry, acid balance and astringency are discussed, but the additional sensory data for all descriptors and treatment interactions are listed in the supplementary information (Table S3). Although the interactive effect of yeast treatment and MLF strategy was not significant, the effects of all the treatment combinations on the aforementioned descriptors are shown for illustrative purposes (Figures 4–6).

Table 5. Probability (p) values [1] of Shiraz wines produced with the different yeast treatments and malolactic fermentation (MLF) strategies.

Descriptor	Treatments		
	Yeast	MLF Strategy	Yeast × MLF Strategy
Berry	0.0036	0.0053	0.1643
Fruity	0.1696	0.0857	0.4701
Fresh vegetative	0.0989	0.8366	0.9774
Cooked vegetative	0.6539	0.1068	0.9403
Spicy	0.1848	0.5088	0.2219
Floral	0.3241	0.6223	0.8284
Acid balance	0.2679	0.0447	0.5892
Body	0.4319	0.2718	0.1424
Astringency	0.1749	0.0271	0.2493
Bitterness	0.1547	0.3787	0.6995
Overall quality	0.2355	0.8938	0.2737

[1] Differences between treatments are significant if $p \leq 0.05$.

3.5.1. Berry Aroma

Wines that underwent simultaneous MLF scored slightly higher for "berry" aroma than wines that did not undergo MLF, but both treatments scored significantly higher than wines that underwent sequential MLF (Figure 4 and Table S3). Of all the treatments, wines produced with L1 + Sc that underwent simultaneous MLF scored the highest for berry aroma, and Sc and H4 + Sc wines that underwent sequential MLF scored the lowest. The *S. cerevisiae* reference wines that underwent MLF scored less for berry aroma than the *S. cerevisiae* wines that did not undergo MLF. A similar trend was observed for wines produced with L2 + Sc. Berry aroma increased in wines produced with M2 + Sc that underwent MLF.

Even though wines that underwent sequential MLF contained higher concentrations of most of the various esters than wines that underwent simultaneous MLF and wines that did not undergo MLF (Table 3), it did not contribute to more perceivable berry aroma in those wines (Figure 4). Other compounds such as volatile acids possibly masked the contribution of the esters. Wines that underwent simultaneous MLF contained higher levels of diethyl succinate (fruity, melon, berry aroma), ethyl-3-hydoxybutanoate (fruity, grape, strawberry aroma) and ethyl decanoate (floral, grape, soap aroma) than wines that underwent sequential MLF. These compounds might have contributed to the perceived berry and fruity aroma of the wines. It is also possible that the perceived berry aroma could be due to enhancement of the aforementioned compounds by other volatile compounds, such as higher alcohols, or the synergistic interactions with other compounds. Another possibility is that compounds not quantified in this study might be responsible for perceived berry aroma.

Results show that wines produced with certain non-*Saccharomyces* yeast strains in combination with simultaneous MLF had more berry aroma than wines that did not undergo MLF, while wines produced with other non-*Saccharomyces* yeast strains had more berry aroma when MLF was induced as a sequential inoculation. This indicates that the effect of MLF strategy on berry aroma is strain dependent and that yeast and LAB strain combination needs further investigation.

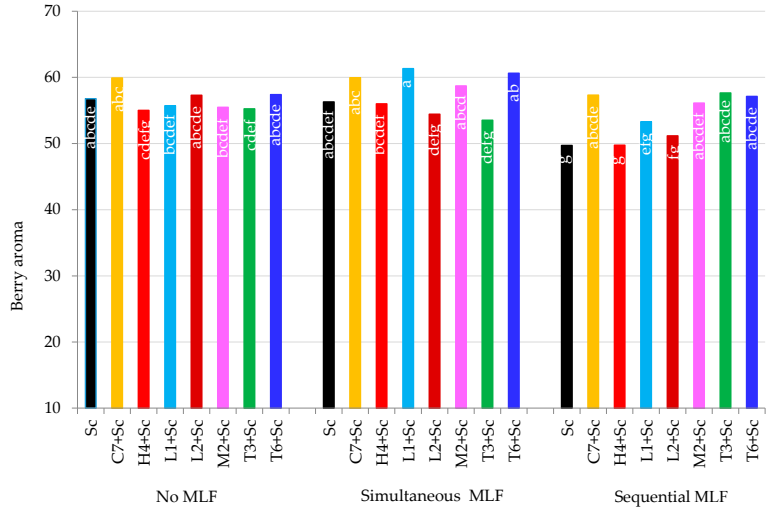

Figure 4. Percentage (%) berry aroma in Shiraz wines produced with *Saccharomyces cerevisiae* (Sc) on its own or in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, and three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Mean values followed by the same letter did not differ significantly ($p \leq 0.5$).

3.5.2. Acid Balance

In general, wines that underwent sequential MLF were less balanced and scored lower for acid balance than wines that underwent simultaneous MLF and wines that did not undergo MLF (Figure 5). The lack of acidity was confirmed by the total acidity data, which showed that wines that underwent sequential MLF had significantly lower TA levels than wines that did not undergo MLF and wines that underwent simultaneous MLF (Table 2). However, the sequential MLF wines were perceived to be less balanced and did not have a clear negative effect on the perceived quality of these wines because the wines scored similar or better for overall quality than wines that did not undergo MLF (supplementary Table S3).

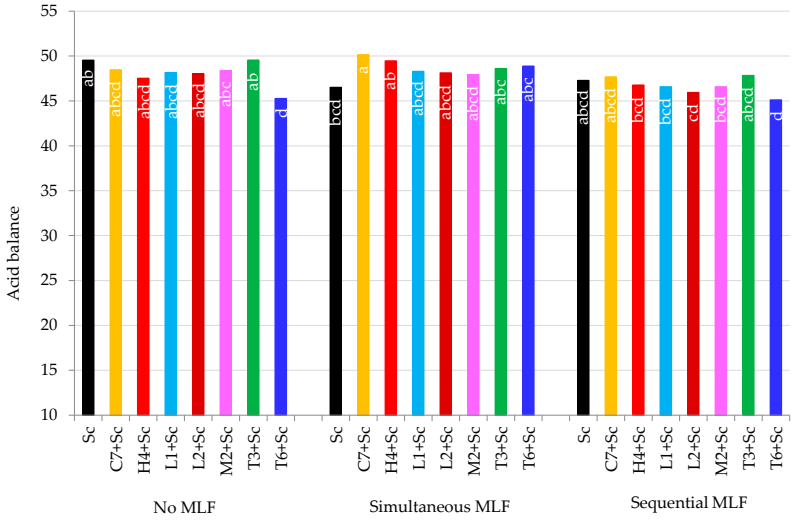

Figure 5. Acid balance (%) of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc) on its own or in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, and three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Mean values of the various treatments followed by the same letter did not differ significantly ($p \leq 0.5$).

3.5.3. Astringency

Wines that underwent simultaneous MLF were perceived to be more astringent than wines that did not undergo MLF and significantly more astringent than wines undergoing sequential MLF (Figure 6). None of the treatments produced wines that were considered unacceptable with regard to astringency. Wines that underwent sequential MLF were the least astringent, which could be beneficial to winemakers who want to get their wines on the market quickly. If a wine is too astringent, it could have a negative effect on the overall quality of wine, which was not the case for wines that underwent simultaneous MLF (supplementary Table S3). Wines that underwent simultaneous MLF scored highest for overall quality for most of the yeast combinations, even though it was not significant (Table S3). Simultaneous MLF might be beneficial for wines that are made to be aged for a long period, because astringency decreases over time and may contribute to the ageing potential of such wines.

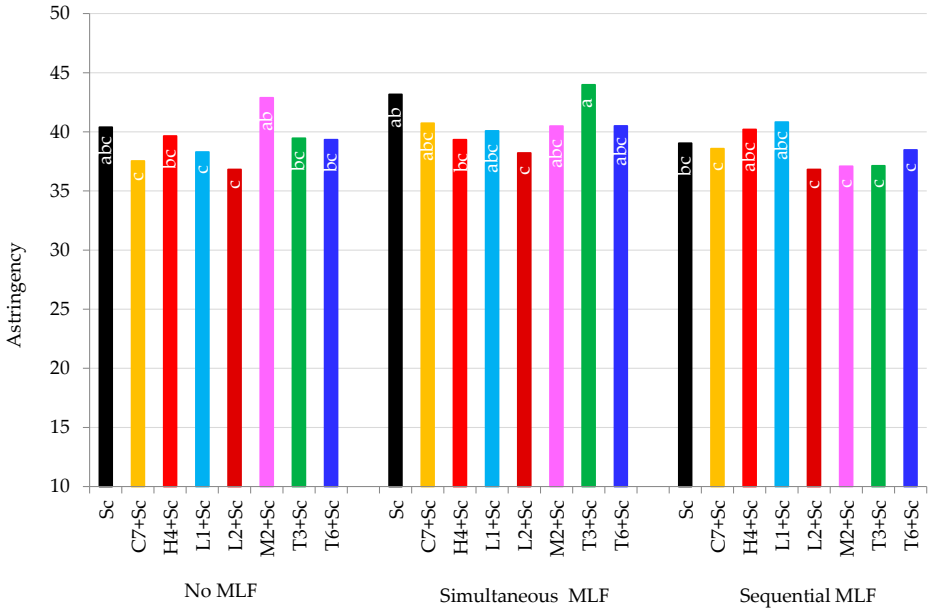

Figure 6. Percentage (%) astringency of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc) on its own or in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, as well as three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Mean values followed by the same letter did not differ significantly ($p \leq 0.5$).

3.6. Overall Effects

The selected non-*Saccharomyces* yeasts were present at high levels and long enough to contribute to wine flavor and this is supported by chemical and sensory results. The non-*Saccharomyces* isolates in combination with *S. cerevisiae* and the three MLF strategies produced wines without any off-flavors. The aforementioned wines were different to wines produced with the *S. cerevisiae* reference and also the two commercial non-*Saccharomyces* yeast strains (L1 and T3). The non-*Saccharomyces* yeast isolates showed potential for producing wines with different styles and flavor profiles, but need further evaluation in different grape cultivars/varieties and in combination with different *S. cerevisiae* yeast strains.

The yeast treatment and the stage of MLF induction had a significant effect on the standard chemical parameters and volatile composition of the wines. However, the variation in wine composition did not always translate to perceivable sensory differences and neither did the contributions of volatile compounds with OAV's above 1.

4. Conclusions

This is the first report on the use of the non-*Saccharomyces* yeast strains *C. zemplinina* C7, *H. uvarum* H4 and *L. thermotolerans* L2 in the production of Shiraz wines. Strains *C. zemplinina* C7 and *L. thermotolerans* L2 had a negative effect on LAB growth and the progress of MLF when LAB were used in a simultaneous inoculation, but the same effect was not observed for sequential MLF. Results indicated that non-*Saccharomyces* yeast strains had a beneficial effect on the progress of MLF. Therefore, if MLF is required, it is important to choose *Saccharomyces* and non-*Saccharomyces* strains that are compatible and promote MLF. On the contrary, spontaneous and inoculated MLF can be delayed if yeast strains or combinations are used that have a negative effect on LAB growth. Non-*Saccharomyces* yeasts can also be used to reduce alcohol levels. Wines that did not undergo MLF were significantly different from wines that underwent MLF in terms of chemical and sensory properties. Time of MLF induction had a significant effect on the chemical and sensory properties of the wines and had a greater effect on the sensory properties than the yeast treatment alone. However, significant variation in wine composition did not always translate to perceivable sensory differences. Wine flavor profiles can be changed by using different non-*Saccharomyces* yeast strains and MLF strategies. Differences between strains from the same non-*Saccharomyces* species can be as significant as the variation between different non-*Saccharomyces* species, or as significant as the differences between non-*Saccharomyces* and *Saccharomyces* yeasts. Induction of simultaneous or sequential MLF can also result in significant changes to wine flavor profiles. In general, wines that underwent simultaneous MLF scored higher for certain sensory descriptors than wines that underwent sequential MLF, but some yeast combinations yielded better wines with sequential MLF. The optimal MLF strategy for each yeast strain or yeast combination to improve wine flavor and quality appears to be strain dependent. The interactions between *Saccharomyces*, non-*Saccharomyces* and LAB are complex and the resulting changes to wine composition need further investigation.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/2311-5637/3/4/64/s1.

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References

1. Condurso, C.; Cincotta, F.; Tripodi, G.; Sparacio, A.; Giglio, D.M.L.; Sparla, S.; Verzera, A. Effects of cluster thinning on wine quality of Syrah cultivar (*Vitis vinifera* L.). *Eur. Food Res. Technol.* **2016**, *242*, 1719–1726. [CrossRef]
2. Mayr, C.M.; Geue, J.P.; Holt, H.E.; Pearson, W.P.; Jeffery, D.W.; Francis, I.L. Characterization of the key aroma compounds in Shiraz wine by quantitation, aroma reconstitution, and omission studies. *J. Agric. Food Chem.* **2014**, *62*, 4528–4536. [CrossRef] [PubMed]
3. Ribéreau-Gayon, P.; Dubourdieu, D.; Donéche, B.; Lonvaud, A. *Handbook of Enology*, 2nd ed.; The Microbiology of Wine and Vinifications; John Wiley & Sons Ltd.: Chichester, UK, 2006; Volume 1.
4. Du Toit, M.; Engelbrecht, L.; Lerm, E.; Krieger-Weber, S. *Lactobacillus*: The next generation of malolactic fermentation starter cultures—An overview. *Food Bioprocess Technol.* **2011**, *4*, 876–906. [CrossRef]
5. Fleet, G.H. Yeast interactions and wine flavor. *Int. J. Food Microbiol.* **2003**, *86*, 11–22. [CrossRef]
6. Granchi, L.; Bosco, M.; Messini, A.; Vincenzini, M. Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR–RFLP analysis of the rDNA ITS region. *J. Appl. Microbiol.* **1999**, *87*, 949–956. [CrossRef] [PubMed]

7. Ciani, M.; Comitini, F.; Mannazzu, I.; Domizio, P. Controlled mixed culture fermentation: A new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res.* **2010**, *10*, 123–133. [CrossRef] [PubMed]
8. Gobbi, M.; Comitini, F.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.* **2013**, *33*, 271–281. [CrossRef] [PubMed]
9. Jolly, N.P.; Valera, C.; Pretorius, I.S. Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* **2014**, *14*, 215–237. [CrossRef] [PubMed]
10. Whitener, M.E.B.; Carlin, S.; Jacobson, D.; Weighill, D.; Divol, B.; Conterno, L.; du Toit, M.; Vrhovsek, U. Early fermentation volatile metabolite profile of non-*Saccharomyces* yeasts in red and white grape must: A targeted approach. *LWT Food Sci. Technol.* **2015**, *64*, 412–422. [CrossRef]
11. Whitener, M.E.B.; Stanstrup, J.; Carlin, S.; Divol, B.; Du Toit, M.; Vrhovsek, U. Effect of non-*Saccharomyces* yeasts on the volatile chemical profile of Shiraz wine. *Austr. J. Grape Wine Res.* **2017**, *23*, 179–192. [CrossRef]
12. Whitener, M.E.B.; Stanstrup, J.; Panzeri, V.; Carlin, S.; Divol, B.; Du Toit, M.; Vrhovsek, U. Untangling the wine metabolome by combining untargeted SPME–GCxGC-TOF-MS and sensory analysis to profile Sauvignon blanc co-fermented with seven different yeasts. *Metabolomics* **2016**, *12*, 1–25. [CrossRef]
13. Ciani, M.; Morales, P.; Comitini, F.; Tronchoni, J.; Canonico, L.; Curiel, J.A.; Oro, L.; Rodrigues, A.J.; Gonzalez, R. Non-conventional yeast species for lowering ethanol content of wines. *Front. Microbiol.* **2016**, *7*, 1–13. [CrossRef] [PubMed]
14. Contreras, A.; Hidalgo, C.; Henschke, P.A.; Chambers, P.J.; Curtin, C.; Varela, C. Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Appl. Environ. Microbiol.* **2014**, *80*, 1670–1678. [CrossRef] [PubMed]
15. Bartowsky, E.J.; Costello, P.J.; Chambers, P.J. Emerging trends in the application of malolactic fermentation. *Aust. J. Grape Wine Res.* **2015**, *21*, 663–669. [CrossRef]
16. Swiegers, J.H.; Bartowsky, E.J.; Henschke, P.A.; Pretorius, I.S. Yeast and bacterial modulation of wine aroma and flavor. *Aust. J. Grape Wine Res.* **2005**, *11*, 139–173. [CrossRef]
17. Lerm, E.; Engelbrecht, L.; Du Toit, M. Malolactic fermentation: The ABC's of MLF. *S. Afr. J. Enol. Vitic.* **2010**, *31*, 186–212.
18. Bartowsky, E.J. *Oenococcus oeni* and malolactic fermentation—Moving into the molecular arena. *Aust. J. Grape Wine Res.* **2005**, *11*, 174–187. [CrossRef]
19. Gammacurta, M.; Lytra, G.; Marchal, A.; Marchand, S.; Barbe, J.C.; Moine, V.; de Revel, G. Influence of lactic acid bacteria strains on ester concentrations in red wines: Specific impact on branched hydroxylated compounds. *Food Chem.* **2018**, *239*, 252–259. [CrossRef] [PubMed]
20. Alexandre, H.; Costello, P.J.; Remize, F.; Guzzo, J.; Guilloux-Benatier, M. *Saccharomyces cerevisiae-Oenococcus oeni* interactions in wine: Current knowledge and perspectives. *Int. J. Food Microbiol.* **2004**, *93*, 141–154. [CrossRef] [PubMed]
21. Arnink, K.; Henick-Kling, T. Influence of *Saccharomyces cerevisiae* and *Oenococcus oeni* strains on successful malolactic conversion in wine. *Am. J. Enol. Vitic.* **2005**, *56*, 228–237.
22. Muñoz, V.; Beccaria, B.; Abreo, E. Simultaneous and successive inoculations of yeasts and lactic acid bacteria on the fermentation of an unsulfited Tannat grape must. *Braz. J. Microbiol.* **2014**, *45*, 59–66. [CrossRef] [PubMed]
23. Antalick, G.; Perello, M.C.; de Revel, G. Co-inoculation with yeast and LAB under winery conditions: Modification of the aromatic profile of Merlot wines. *S. Afr. J. Enol. Vitic.* **2013**, *34*, 223–232. [CrossRef]
24. Massera, A.; Soria, A.; Catania, C.; Krieger, S.; Combina, M. Simultaneous inoculation of Malbec (*Vitis vinifera*) musts with yeast and bacteria: Effects on fermentation performance, sensory and sanitary attributes of wines. *Food Technol. Biotechnol.* **2009**, *47*, 192–201.
25. Abrahamse, C.; Bartowsky, E. Timing of malolactic fermentation inoculation in Shiraz grape must and wine: Influence on chemical composition. *World J. Microbiol. Biot.* **2012**, *28*, 255–265. [CrossRef]
26. Du Plessis, H.W.; du Toit, M.; Hoff, J.W.; Hart, R.S.; Ndimba, B.K.; Jolly, N.P. Characterisation of non-*Saccharomyces* yeasts using different methodologies and evaluation of their compatibility with malolactic fermentation. *S. Afr. J. Enol. Vitic.* **2017**, *38*, 46–63. [CrossRef]

27. Minnaar, P.P.; Ntushelo, N.; Ngqumba, Z.; Van Breda, V.; Jolly, N.P. Effect of *Torulaspora delbrueckii* yeast on the anthocyanin and flavanol concentrations of Cabernet franc and Pinotage wines. *S. Afr. J. Enol. Vitic.* **2015**, *36*, 50–58. [CrossRef]
28. Anonymous. Methods of Analyses for Wine Laboratories. Compiled by the South African Wine Laboratories Association. South African Society for Viticulture and Oenology, P.O. Box 2092, Dennesig, Stellenbosch 7601, South Africa. Available online: http://www.sasev.org (accessed on 30 November 2017).
29. Louw, L.; Roux, K.; Tredoux, A.; Tomic, O.; Naes, T.; Nieuwoudt, H.H.; Van Rensburg, P. Characterisation of selected South African cultivar wines using FTMIR spectroscopy, gas chromatography and multivariate data analysis. *J. Agric. Food. Chem.* **2009**, *57*, 2623–2632. [CrossRef]
30. Dehlholm, C. Descriptive Sensory Evaluations: Comparison and Applicability of Novel Rapid Methodologies. Ph.D. Thesis, Department of Food Science, University of Copenhagen, Copenhagen, Denmark, 2012.
31. Shapiro, S.S.; Wilk, M.B. An analysis of Variance Test for Normality (complete samples). *Biometrika* **1965**, *52*, 591–611. [CrossRef]
32. Ott, R.L. *An Introduction to Statistical Methods and Data Analysis*; Duxbury Press: Belmont, CA, USA, 1998; pp. 807–837.
33. Zott, K.; Miot-Sertier, C.; Claisse, O.; Lonvaud-Funel, A.; Masneuf-Pomarede, I. Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. *Int. J. Food Microbiol.* **2008**, *125*, 197–203. [CrossRef] [PubMed]
34. Lema, C.; García-Jares, C.; Orriols, I.; Angulo, L. Contribution of *Saccharomyces* and non-*Saccharomyces* populations to the production of some components of Albariño wine aroma. *Am. J. Enol. Vitic.* **1996**, *47*, 206–216.
35. Fleet, G.H.; Lafon-Lafourcade, S.; Ribéreau-Gayon, P. Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Appl. Environ. Microbiol.* **1984**, *48*, 1034–1038. [PubMed]
36. López, I.; López, R.; Santamaría, P.; Torres, C.; Ruiz-Larrea, F. Performance of malolactic fermentation by inoculation of selected *Lactobacillus plantarum* and *Oenococcus oeni* strains isolated from Rioja red wines. *VITIS—J. Grapevine Res.* **2008**, *47*, 123–129.
37. González-Arenzana, L.; López, R.; Santamaría, P.; Tenorio, C.; López-Alfaro, I. Dynamics of indigenous lactic acid bacteria populations in wine fermentations from La Rioja (Spain) during three vintages. *Microb. Ecol.* **2012**, *63*, 12–19. [CrossRef] [PubMed]
38. 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] [PubMed]
39. Benito, Á.; Calderón, F.; Palomero, F.; Benito, S. Combine use of selected *Schizosaccharomyces pombe* and *Lachancea thermotolerans* yeast strains as an alternative to the traditional malolactic fermentation in red wine production. *Molecules* **2015**, *20*, 9510–9523. [CrossRef] [PubMed]
40. Mendoza, L.; Farías, M.E. Improvement of wine organoleptic characteristics by non-*Saccharomyces* yeasts. *Curr. Res. Technol. Ed. Top. Appl. Microbiol. Microb. Biotechnol.* **2010**, *2*, 908–919.
41. Lambrechts, M.G.; Pretorius, I.S. Yeast and its Importance to Wine Aroma—A Review. *S. Afr. J. Enol. Vitic.* **2000**, *21*, 77–129.
42. Vilela-Moura, A.; Schuller, D.; Mendes-Faia, A.; Silva, R.D.; Chaves, S.R.; Sousa, M.J.; Côrte-Real, M. The impact of acetate metabolism on yeast fermentative performance and wine quality: Reduction of volatile acidity of grape musts and wines. *Appl. Microbiol. Biotechnol.* **2011**, *89*, 271–280. [CrossRef] [PubMed]
43. Mendoza, L.M.; Merín, M.G.; Morata, V.I.; Farías, M.E. Characterization of wines produced by mixed culture of autochthonous yeasts and *Oenococcus oeni* from the northwest region of Argentina. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 1777–1785. [CrossRef] [PubMed]
44. Renault, P.; Miot-Sertier, C.; Marullo, P.; Hernández-Orte, P.; Lagarrigue, L.; Lonvaud-Funel, A.; Bely, M. Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: Potential applications in the wine industry. *Int. J. Food Microbiol.* **2009**, *134*, 201–210. [CrossRef] [PubMed]
45. Loira, I.; Vejarano, R.; Bañuelos, M.A.; Morata, A.; Tesfaye, W.; Uthurry, C.; Villa, A.; Cintora, I.; Suárez-Lepe, J.A. Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality. *LWT Food Sci. Technol.* **2014**, *59*, 915–922. [CrossRef]
46. Romano, P.; Suzzi, G.; Comi, G.; Zironi, R. Higher alcohol and acetic acid production by apiculate wine yeasts. *J. Appl. Bact.* **1992**, *73*, 126–130. [CrossRef]

47. Tristezza, M.; Tufariello, M.; Capozzi, V.; Spano, G.; Mita, G.; Grieco, F. The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Front. Microbiol.* **2016**, *7*, 1–14. [CrossRef] [PubMed]
48. Benito, S.; Palomero, F.; Gálvez, L.; Morata, A.; Calderón, F.; Palmero, D.; Suárez-Lepe, J.A. Quality and composition of red wine fermented with *Schizosaccharomyces pombe* as sole fermentative yeast, and in mixed and sequential fermentations with *Saccharomyces cerevisiae*. *Food Technol. Biotechnol.* **2014**, *52*, 376–382.
49. Tristezza, M.; di Feo, L.; Tufariello, M.; Grieco, F.; Capozzi, V.; Spano, G.; Mita, G. Simultaneous inoculation of yeasts and lactic acid bacteria: Effects on fermentation dynamics and chemical composition of Negroamaro wine. *LWT Food Sci. Technol.* **2016**, *66*, 406–412. [CrossRef]
50. Izquierdo-Cañas, P.M.; Mena-Morales, A.; García-Romero, E. Malolactic fermentation before or during wine aging in barrels. *LWT Food Sci. Technol.* **2016**, *66*, 468–474. [CrossRef]
51. Izquierdo-Cañas, P.M.; Romero, E.G.; Pérez-Martín, F.; Seseña, S.; Palop, M.L. Sequential inoculation versus co-inoculation in Cabernet Franc wine fermentation. *Food Sci. Technol. Int.* **2015**, *21*, 203–212. [CrossRef]
52. Guth, H. Quantification and sensory studies of character impact odorants of different white wine varieties. *J. Agric. Food. Chem.* **1997**, *45*, 3027–3032. [CrossRef]
53. Escudero, A.; Campo, E.; Fariña, L.; Cacho, J.; Ferreira, V. Analytical characterisation of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. *J. Agric. Food Chem.* **2007**, *55*, 4501–4510. [CrossRef] [PubMed]
54. Buttery, R.G. Flavor chemistry and odor thresholds. In *Flavor Chemistry: 30 Years of Progress*; Teranishi, R., Wick, E., Hornstein, I., Eds.; Kluwer Academic: New York, NY, USA, 1999; pp. 353–365.
55. Peinado, R.A.; Mauricio, J.C.; Moreno, J. Aromatic series in sherry wines with gluconic acid subjected to different biological aging conditions by *Saccharomyces cerevisiae* var. *capensis*. *Food Chem.* **2006**, *94*, 232–239. [CrossRef]
56. Francis, I.L.; Newton, J.L. Determining wine aroma from compositional data. *Aust. J. Grape Wine Res.* **2005**, *11*, 114–126. [CrossRef]
57. Peinado, R.A.; Moreno, J.; Bueno, J.E.; Moreno, J.A.; Mauricio, J.C. Comparitive study of aromatic compounds in two young white wines subjected to pre-fermentative cryomaceration. *Food Chem.* **2004**, *84*, 589–590. [CrossRef]
58. Tao, Y.; Li, H.; Wang, H.; Zhang, L. Volatile compounds of young Cabernet Sauvignon red wine from Changli County (China). *J. Food Comp. Anal.* **2008**, *21*, 689–694. [CrossRef]
59. Ugliano, M.; Moio, L. Changes in the concentration of yeast-derived volatile compounds of red wine during malolactic fermentation with four commercial starter cultures of *Oenococcus oeni*. *J. Agric. Food Chem.* **2005**, *53*, 10134–10139. [CrossRef] [PubMed]
60. Tat, L.; Battistutta, F.; Comuzzo, P.; Zironi, R. Ethyl Phenylacetate as the probable responsible of honey-like character in Aglianico del Vulture wine. *J. Agric. Food Chem.* **2007**, *55*, 5205–5212. [CrossRef] [PubMed]
61. Etiévant, P.X. Wine. In *Volatile Compounds of Food and Beverages*; Maarse, H., Ed.; Marcel Dekker Inc.: New York, NY, USA, 1991; pp. 483–546.
62. Ferreira, V.; Lopez, R.; Cacho, J.F. Quantitative determination of the odorants of young red wines from different grape varieties. *J. Sci. Food Agric.* **2000**, *80*, 1659–1667. [CrossRef]
63. Fazzalari, F.A. *Compilation of Odor and Taste Threshold Values Data*; ASTM Data Series: West Conshohocken, PA, USA, 1978.