microorganisms

MDPI

Article

Characterizing the Potential of the Non-Conventional Yeast *Saccharomycodes ludwigii* UTAD17 in Winemaking

Marcos Esteves [1,2], Catarina Barbosa [1,2,3], Isabel Vasconcelos [4], Maria João Tavares [5], Arlete Mendes-Faia [1,2], Nuno Pereira Mira [5] and Ana Mendes-Ferreira [1,2,*]

1 WM&B—Laboratory of Wine Microbiology & Biotechnology, Department of Biology and Environment, Universidade de Trás-os-Montes e Alto Douro, 5000-801 Vila Real, Portugal; mjcesteves@utad.pt (M.E.); crbarbosa@utad.pt (C.B.); afaia@utad.pt (A.M.-F.)
2 BioISI—Biosystems & Integrative Sciences Institute, Campo Grande, 1749-016 Lisboa, Portugal
3 CoLAB Vines&Wines - National Collaborative Laboratory for the Portuguese Wine Sector, Associação para o Desenvolvimento da Viticultura Duriense (ADVID), Edifício Centro de Excelência da Vinha e do Vinho, Régia Douro Park, 5000-033 Vila Real, Portugal
4 CBQF/Centro de Biotecnologia e Química Fina, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, 4169-005 Porto, Portugal; ivasconcelos@porto.ucp.pt
5 iBB - Institute of Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal; tavares.mja@gmail.com (M.J.T.); nuno.mira@tecnico.ulisboa.pt (N.P.M.)
* Correspondence: anamf@utad.pt; Tel.: +351-259-350-975

Received: 30 September 2019; Accepted: 18 October 2019; Published: 23 October 2019

Abstract: Non-*Saccharomyces* yeasts have received increased attention by researchers and winemakers, due to their particular contributions to the characteristics of wine. In this group, *Saccharomycodes ludwigii* is one of the less studied species. In the present study, a native *S. ludwigii* strain, UTAD17 isolated from the Douro wine region was characterized for relevant oenological traits. The genome of UTAD17 was recently sequenced. Its potential use in winemaking was further evaluated by conducting grape-juice fermentations, either in single or in mixed-cultures, with *Saccharomyces cerevisiae*, following two inoculation strategies (simultaneous and sequential). In a pure culture, *S. ludwigii* UTAD17 was able to ferment all sugars in a reasonable time without impairing the wine quality, producing low levels of acetic acid and ethyl acetate. The overall effects of *S. ludwigii* UTAD17 in a mixed-culture fermentation were highly dependent on the inoculation strategy which dictated the dominance of each yeast strain. Wines whose fermentation was governed by *S. ludwigii* UTAD17 presented low levels of secondary aroma compounds and were chemically distinct from those fermented by *S. cerevisiae*. Based on these results, a future use of this non-*Saccharomyces* yeast either in monoculture fermentations or as a co-starter culture with *S. cerevisiae* for the production of wines with greater expression of the grape varietal character and with flavor diversity could be foreseen.

Keywords: *Saccharomycodes ludwigii*; non-*Saccharomyces*; wine fermentation; mixed-culture; wine aroma

1. Introduction

Inoculation with active dry yeasts of *Saccharomyces cerevisiae* is a common practice in most wine-producing regions since the middle of the 20th century, to assure prompt and reliable fermentations and wines with a consistent and predictable quality. The main drawback of this practice is the loss of the typical distinctive characteristics found in wines obtained by spontaneous fermentation,

carried out by winery- and grape-resident yeasts [1–5]. Grape-must microbiota is dominated by non-*Saccharomyces* yeast strains which have for long been considered spoilage agents, not only due to their low fermentative ability but also because of the assumption that they overproduce off-flavor compounds, such as acetic acid, acetaldehyde, acetoin, or ethyl acetate [1,6]. However, the demonstration that these negative traits are species and strain-dependent [7] and that some non-*Saccharomyces* yeasts even exhibit beneficial traits, not found in *S. cerevisiae* [8–14] have led winemakers to take a fresh look at these formerly disregarded species. In this line, over the last years, a massive number of studies searching for autochthonous non-*Saccharomyces* strains that might impart a unique aroma complexity or mouthfeel to wines, while expressing terroir-associated characteristics have been published [15–23]. Among the most studied non-*Saccharomyces* yeasts are the members of the genus *Hanseniaspora* (*H. uvarum* [24–28], *H. guillermondii* [24–26,29,30], and *H. vineae* [25,31–34]), *Metschnikowia pulcherrima* [21,33,35–37], *Torulaspora delbrueckii* [38–43], *Kluyveromyces/Lachancea thermotolerans* [44–48], and *Starmerella bacillaris* (formerly *Candida stellata/Candida zemplinina*) [27,49–53]. In addition to their contribution to the enhancement and diversification of wine aroma, it was found that these yeasts might display other oenological relevant traits, such as increased glycerol, mannoprotein, and total acidity contents [16,46,52,54,55], contributing to color stability [56,57] and reducing volatile acidity or ethanol levels [21,33,36,38,58]. In this context, the potential of developing starter cultures based on non-*Saccharomyces* yeast species has flourished in the wine world and several non-*Saccharomyces* strains that can be used as starter cultures are now commercially available [10,59]. Nevertheless, there are still a number of species whose potential in winemaking remains to be discovered. One such species is *Saccharomycodes ludwigii* (*S. ludwigii*), a bipolar budding yeast, first isolated from deciduous trees in Europe [60], which has a long history as a spoilage agent in winemaking. This yeast is rarely found in grapes but appears to be a usual contaminant of sulfite-preserved musts [6]. It has also been found in wines, at the end of the alcoholic fermentation or during storage [61], where it contributes for sedimentation or cloudiness formation [62]. The persistence of *S. ludwigii* in wineries is largely explained by its high tolerance to sulfur dioxide [63] and ethanol [7]. Regardless of its association with spoilage, *S. ludwigii* has been proposed as a starter-culture for the production of feijoa fermented beverages [61], cider [64], and low-alcohol or non-alcoholic beers [65,66]. In winemaking, the few studies undertaken with this yeast have shown that it could also be promising, since, depending on the strain, *S. ludwigii* possesses a good fermentative capacity [7] and is able to shape the aroma profile [8,18] and mouthfeel perception of wines [52]. Thus, the aim of this work was to examine the oenological potential of *S. ludwigii* UTAD17, an indigenous Douro Wine Region strain whose genome sequence has been recently released [67]. For this purpose, besides phenotyping the strain for relevant oenological traits, fermentations of a natural grape-must were performed, either in pure or in co-culture with *S. cerevisiae*. The growth and fermentation behavior, as well as the analytical profiles of the final wines, were also evaluated, revealing that this strain could be useful for tailoring wines with enhanced varietal characters.

2. Materials and Methods

2.1. Yeast Strains and Maintenance Conditions

The yeast *S. ludwigii* UTAD17, an autochthonous Douro Wine Region strain isolated in our laboratory [67], and the *S. cerevisiae* Lalvin QA23 (Lallemand-Proenol 4410-308 Canelas, Portugal), obtained from the market as an active dried yeast, were used in this study. Yeasts were routinely maintained at 4 °C on Yeast Extract Peptone Dextrose agar plates (YPD) containing per liter: 20 g of glucose, 10 g of peptone, 5 g of yeast extract, and 20 g of agar from stocks stored at −80 °C. Prior to use, the yeasts were transferred to a new slant of YPD and incubated for 24–48 h at 28 °C, unless otherwise stated.

2.2. Phenotypic Characterization

S. ludwigii UTAD17 was screened for relevant enological features [68]. The evaluation of stress resistance and the activity of the enzymes of enological interest was performed as described in [21]. Briefly, for all assays, after growth in YPD medium until the mid-exponential growth phase, the yeast strain was inoculated in the appropriate culture media for stress tolerance and enzymatic activities evaluation. YPD agar plates without a stress agent, was used as the control. Accordingly, the following concentrations were used—6%, 9%, or 12% (v:v^{-1}) of ethanol; 1, 2, or 4 mM of sulfur dioxide (SO_2); 0.5, 1, or 2 mM of copper, supplied as copper sulfate; and 0.25, 0.5, or 1 mM of H_2O_2. Yeast growth in the presence of cerulenin or 5,5′,5′′-trifluoro-D,L-leucine (TFL) was screened to evaluate the potential to produce particular flavor compounds, using agar plates and a minimal medium (YNB) supplemented with glucose (2%) and TFL (0.6 mM) or cerulenin (6 µM).

Enzymatic activities were evaluated using qualitative assays. The activity of β-lyase was screened using a medium containing 0.1% *S*-methyl-L-cysteine, 0.01% pyridoxal-50-phosphate, 1.2% Yeast Carbon Base, and 2% agar, with pH adjusted to 3.5. β-glycosidase activity was tested using a medium containing 0.5% cellobiose (4-*O*-β-D-glucopyranosyl-D-glucose), 0.67% yeast nitrogen base, and 2% agar. Proteolytic activity was evaluated by spotting yeast strain on skim milk agar medium. Qualitative detection of biogenic amines (histamine, tyramine and putrescine) was performed using differential culture media containing yeast extract (3%), glucose (1%), the amino acid precursor (2%), histidine, tyrosine or ornithine, and bromocresol purple (0.015 g.L^{-1}), at a final pH adjusted to 5.2. The potential ability to produce hydrogen sulfide (H_2S) associated to sulfite reductase activity was evaluated by growing yeast cells on BiGGY agar.

2.3. Grape Juice and Inocula Preparation

Natural grape-juice was obtained by crushing grapes of the *Vitis vinifera* L. cv. Touriga Nacional; after homogenization, the juice was clarified by centrifugation at 12,734× *g* for 10 min (Sorvall centrifuge GSA 6-Place Rotor, Marshall Scientific, Hampton, NH 03842, USA) and was carefully separated from the solid fraction. A sample of the grape-juice was collected at this point for routine analysis (Table 1). After pasteurization at 70 °C for 10 min, the grape-juice was immediately cooled on ice. For each strain, the inoculum was prepared by separately pre-growing the yeast cells in 50 mL-flasks, containing 25 mL of synthetic grape-juice medium (GJM), original recipe of [69] with minor modifications in the nitrogen composition. Nitrogen was added up to 267 mg YAN/L, supplied as di-ammonium phosphate (DAP). The flasks were incubated overnight at 25 °C in an orbital shaker (IKA KS 4000 ic Control, VWR International, Radnor, PA 19087-8660, USA) set at 150 rpm.min^{-1}. Both strains were inoculated in grape-juice with an initial cellular concentration of 10^6 cfu·mL^{-1}.

2.4. Fermentation Trials

Fermentations trials were conducted by inoculating (1) a single culture of *S. ludwigii* UTAD17 (Sl), (2) a single culture of *S. cerevisiae* (Sc), (3) a mixed culture of *S. ludwigii* UTAD17 and *S. cerevisiae* (Sl+Sc) inoculated simultaneously, prior to fermentation, or (4) a mixed culture in which *S. cerevisiae* was inoculated sequentially, 72 h after *S. ludwigii* UTAD17 (Sl_Sc). Single and mixed culture fermentations were carried out in duplicates and triplicates, respectively, using a previously described system [70] consisting of 100 mL flasks filled to 2/3 of their volume (80 mL) and fitted with a side-arm port sealed with a rubber septum for anaerobic sampling. Two flasks containing uninoculated grape-must were used as control. The flasks were maintained at 25 °C under static conditions. Fermentations were monitored daily by weight loss as an estimation of CO_2 production and were allowed to proceed until no further weight loss was observed. For the assessment of growth parameters and analytical determinations, aseptic sampling was performed using a syringe-type system. After fermentation, the wines were centrifuged (10 min at 5500 rpm, Sigma 3-18K refrigerated Centrifuge, 37520 Osterode

am Harz, DE) to remove yeast cells and were kept at −20 °C until the analytical determinations were performed.

2.5. Determination of Growth and Fermentation Parameters

Growth kinetics were monitored by viable cell plate counting (cfu·mL^{-1}) on YPD agar or Lysine agar medium plates incubated at 28 °C for 48–72 h. The lysine agar medium was used to directly assess *S. ludwigii* UTAD17 viability in mixed-culture fermentations, since *S. cerevisiae* is unable to grow in a culture medium in which lysine is the sole nitrogen source [71]. The maximum fermentation rate ($M_{ax}FR$) was determined from the slope of the linear dependence of the steepest incline in weight (g) at the corresponding time points (h), and fermentation purity (FP) was determined as acetic acid (g L^{-1})/ethanol (%*v*:*v*$^{-1}$).

2.6. Analytical Determinations

The amount of glucose and fructose, acetic acid, as well as Yeast Assimilable Nitrogen (YAN), comprising primary amino nitrogen (PAN) and ammonium, were enzymatically determined using a Y15 autoanalyzer (Biosystems S.A, Barcelona, Spain). Total SO_2, pH, and titratable acidity were determined according to the standard methods compiled in the Compendium of International Methods of Analysis of Musts and Wines [72].

Ethanol and glycerol concentrations were determined in a high-performance liquid chromatography system (HPLC Flexar, PerkinElmer, Shelton, Connecticut, EE. UU) equipped with the ion exclusion cation exchange column Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA, USA) and refractive index detector. The column was eluted using sulfuric acid (0.005 N) at 60 °C and a 0.6 mL/min flow rate. Samples were previously filtered through a membrane (Millipore, 0.22 µm pore size) before an injection of 6 µL. The components were identified through their relative retention times, compared to the respective standards, using the Perkin Elmer Chromera Software.

Aliphatic higher alcohols (1-propanol, 1-butanol, 2-methyl-1-butanol and 3-methyl-1-butanol), acetaldehyde, and ethyl acetate were analyzed as described Moreira et al. [73] by using a Hewlett-Packard 5890 (Hewlett-Packard, Palo Alto, CA 94304, USA) gas chromatograph equipped with a flame ionization detector (GC-FID) and connected to a H.P. 3396 Integrator. Fifty microliters of 4-methyl-2- pentanol at 10 g L^{-1} was added to 5 mL of wine as the internal standard. The sample (1 µL) was injected (split, 1: 30) into a CP-WAX 57 CB column (Chrompack) of 50 m × 0.25 mm and 0.2 µm phase thickness. The program temperature varied from 40 °C (10 min) to 80 °C (10 min) at 3 °C min^{-1} and from 80 °C to 200 °C (4 min) at 15 °C min^{-1}. Injector and detector temperatures were set at 220 °C. Carrier gas was H_2 at 1–2 mL min^{-1}.

The determination of 2-phenylethanol, acetates of higher alcohols (isoamyl acetate, 2-phenylethyl acetate) and ethyl esters of fatty acids (ethyl butanoate, ethyl hexanoate and ethyl octanoate), volatile fatty acids (butyric, isobutyric, isovaleric acids) and free fatty acids (hexanoic, octanoic and decanoic acids) was performed in a Hewlett Packard 5890 gas chromatograph, equipped with a flame ionization detector. For this purpose, 50 mL of wine, with 4-decanol at 1.5 mg/L as the internal standard, was extracted successively with 4, 2, and 2 mL of ether–hexane (1:1 *v*:*v*$^{-1}$) for 5 min. The organic phase (1 µL) was injected (splitless) into a BP21 (SGE) column of 50 m × 0.22 mm and 0.25 µm phase thickness. The temperature program was 40 °C (1 min) to 220 °C (15 min), at 2 °C·min^{-1}. Injector and detector temperatures were set at 220 °C. The carrier gas used was H_2 at 1–2 mL min^{-1}.

2.7. Statistical Analysis

The data are presented as mean values with their standard deviation. One-way analysis of variance (ANOVA) of the inoculation strategy on yeast growth, fermentation activity, and volatile and non-volatile compounds was performed using the JMP 7.0 software (SAS Inc., 2007). If significant differences were found with ANOVA ($p < 0.05$), then Student's *t*-test was used for the paired comparisons. Partial least squares linear discriminant analysis (PLS–DA) was performed to

discriminate the wines, based on volatile and non-volatile compounds, using the MATLAB R2018b environment (The MathWorks Inc.; version 9.5.; Natick, MA, USA) state abbreviation). All data were previously standardized.

2.8. Comparison of S. ludwigii UTAD17 'ORFeome' with S. cerevisiae s288c

Recently *S. ludwigii* UTAD17 has been sequenced and annotated with the predicted set of open reading frames (ORFeome) estimated to be about 4015 protein-coding genes [67]. This whole-genome shotgun is available in the European Nucleotide Archive (ENA) under the accession number UFAJ01000000 (contigs UFAJ01000001 through UFAJ01001360; study accession number PRJEB27462; read accession number SAMEA4945973). Herein, a supervised analysis was performed by BLASTp using the proteomes of *S. ludwigii* UTAD17 and *S. cerevisiae* S288c, looking for the presence or absence of protein-coding genes that could underlie the observed physiological traits. *S. ludwigii* UTAD17 proteins were considered similar to those present in *S. cerevisiae* S288c when the resulting alignment had an associated e-value below e^{-20} and a minimum identity of 30% (Table S1).

3. Results and Discussion

3.1. Phenotypic Characterization of S. ludwigii UTAD17

In order to assess the potential of *S. ludwigii* UTAD17 to be used in winemaking, a phenotypic profiling was performed for a number of oenological traits, as determined by the International Organisation of Vine and Wine (OIV) [68] (Figure S1). Ethanol is the main metabolite produced during wine fermentation while SO_2 and copper are applied by winemakers as antimicrobial agents to control spoilage in wineries and vineyards, respectively. These compounds have recognized negative effects on yeast growth and fermentative activity which could lead to stuck and sluggish fermentations [74]. The results obtained showed that *S. ludwigii* UTAD17 displayed high resistance to SO_2 (4 mM), ethanol (12% $v{:}v^{-1}$), and copper (2 mM) (Figure S1). The ability to produce biogenic animes which are toxic to humans, [21] was also evaluated. Interestingly, *S. ludwigii* UTAD17 did not present decarboxylase activities responsible for the production of histamine, tyramine and putrescine. On the other hand, *S. ludwigii* UTAD17 exhibited β-glucosidase and β-lyase activities involved in the liberation of terpenes from glycosylated precursors [12] and volatile thiols from cysteinylated precursors [19]. In line with the results obtained, *S. ludwigii* UTAD17 presents important features for a wine yeast starter, since it is able to adjust to winemaking stress, can contribute to the improvement of wine aromatic profile and does not compromise consumers' health.

3.2. Yeast Growth Kinetics and Fermentation Profiles

To evaluate the performance of *S. ludwigii* UTAD17 in winemaking conditions, fermentations were conducted either in single culture or in consortium with the commercial wine strain *Saccharomyces cerevisiae* QA23, inoculated simultaneously or sequentially, at 72 h. In parallel, a control fermentation was carried out using *Saccharomyces cerevisiae* QA23 in single culture, for comparison.

The growth dynamics and fermentation profiles for each single and mixed culture trials are presented in Figure 1. All fermentations were completed, although differences in the total time of fermentation were observed. The *S. ludwigii* UTAD17 showed a sugar uptake preference similar to *S. cerevisiae* strains, consuming glucose more rapidly than fructose. While in pure culture, *S. ludwigii* UTAD17 displayed a significantly lower fermentation rate than *S. cerevisiae*, although it was able to ferment the grape must sugar to dryness (below 4 g L^{-1}) within 11 days, six more days than the time required for the high fermenter strain *S. cerevisiae* Lalvin QA23 (Table 1 and Figure 1B). This lower fermentative activity of *S. ludwigii* UTAD17 was not attributable to the differences in the biomass, which is known to have a great influence in determining the fermentation activity [69,75]. Indeed, both species, inoculated at the same amount (1×10^6 cfu·mL^{-1}), resumed growth almost immediately after inoculation and, albeit with differences in the growth rate (Figure 1A), achieved similar

maximum cell populations—1.2 × 10^8 cfu·mL^{-1} for *S. cerevisiae* (after 48 h) and 1.1 × 10^8 cfu·mL^{-1} for *S. ludwigii* UTAD17 (after 72 h). A low fermentative capacity together with a high susceptibility to ethanol is believed to underlie the reduced competitiveness of non-*Saccharomyces* species along wine fermentation [76]. The phenotypic screening performed showed that *S. ludwigii* UTAD17 is able to tolerate up to 12% (*v*:*v*$^{-1}$) of ethanol (Figure S1), which is above the 10.2–10.4% (*v*:*v*$^{-1}$) achieved in the final wines, indicating that the decline in cell viability registered in the later stage of the fermentation should result from other factors. The predicted ORFeome of *S. ludwigii* UTAD17 showed that this species is equipped with enzymes required for ethanol production from glucose, including hexoses transporters, glycolytic enzymes, and alcohol dehydrogenases (Table S1). Recently, genomic sequencing of an *H. guilliermondii* wine strain revealed that one of the key factors contributing to the reduced fermentation ability of this species is the lack of genes for the biosynthesis of thiamine [77], a cofactor of the pyruvate decarboxylase enzyme that is known to play an essential role in determining the regulation of the glycolytic flux [78,79]. This is not apparently the case in *S. ludwigii* UTAD17, since the thiamine-biosynthesis genes could be predicted from the genomic sequence of the UTAD17 strain (Table S1) [67]. Further studies are required to understand the lower fermentation rate exhibited by the *S. ludwigii* UTAD17, in comparison with *S. cerevisae*, one of the possibilities being a low activity of critical glycolytic enzymes, as recently shown to be the case in *H. uvarum* pyruvate kinase [80]. Contrary to that observed for *S. cerevisiae* which almost entirely consumed the nitrogen available in the medium, *S. ludwigii* UTAD17 displayed a preferential consumption of amino acids (PAN) over ammonium and left about 60 mg/L of YAN in the final wine (Table 1). It has been proposed that differences in the efficiency of nitrogen consumption, in general, and in the ability to uptake specific nitrogen sources from the grape-must account for variations in the fermentative activity of *S. cerevisiae* strains [81,82]. In this context, it would be interesting to determine whether the differences observed in the fermentation performances of *S. cerevisiae* and *S. ludwigii* UTAD17 (Figure 1) are the result of differences in their nitrogen uptake capability (Table 1).

Table 1. Physicochemical composition of initial grape-must and wines obtained by single-cultures of *S. ludwigii* UTAD17 (Sl) and *S. cerevisiae* QA23 (Sc) or in consortium—mixed simultaneously (Sl+Sc) and sequentially (Sl_Sc) in natural grape-juice of *Vitis vinifera* L. cv. Touriga Nacional at 25 °C under static conditions.

Compound	Grape-Must	Sl	Sc	Sl+Sc	Sl_Sc
Sugars (g L^{-1})	182.140 ± 3.62	2.273 ± 0.733 ^{a}	0.328 ± 0.284 ^{b}	0.190 ± 0.242 ^{b}	0.018 ± 0.009 ^{b}
Ethanol (% *v*:*v*$^{-1}$)	-	10.195 ± 0.194 ^{a}	10.391 ± 0.025 ^{a}	10.201 ± 0.077 ^{a}	10.355 ± 0.271 ^{a}
Glycerol (g L^{-1})	-	6.279 ± 0.024 ^{c}	7.671 ± 0.060 ^{ab}	7.659 ± 0.302 ^{a}	6.800 ± 0.658 ^{bc}
Acetic Acid (g L^{-1})	-	0.138 ± 0.004 ^{bc}	0.170 ± 0.018 ^{a}	0.149 ± 0.004 ^{ab}	0.120 ± 0.011 ^{c}
Titratable Acidity (g L^{-1})	8.010 ± 0.350	8.100 ± 0.120 ^{ab}	7.980 ± 0.000 ^{a}	8.390 ± 0.010 ^{a}	7.690 ± 0.130 ^{b}
Total SO_2 (mg L^{-1})	-	14.830 ± 0.430 ^{a}	15.300 ± 0.820 ^{a}	15.360 ± 0.000 ^{a}	16.380 ± 3.020 ^{a}
pH	2.990 ± 0.011	2.957 ± 0.003 ^{a}	2.926 ± 0.031 ^{b}	2.961 ± 0.000 ^{a}	2.956 ± 0.004 ^{a}
YAN (mg L^{-1})	196.869 ± 2.339	61.214 ± 5.029 ^{a}	5.250 ± 0.354 ^{c}	4.000 ± 0.000 ^{c}	22.594 ± 6.731 ^{b}
$YAN_{(72\,h)}$ (mg L^{-1})	-	79.584 ± 1.996 ^{a}	12.250 ± 3.889 ^{b}	13.168 ± 0.289 ^{b}	68.418 ± 19.340 ^{a}
PAN (mg L^{-1})	92.000 ± 9.899	20.500 ± 2.121 ^{a}	5.250 ± 0.354 ^{b}	4.000 ± 0.000 ^{b}	19.167 ± 4.537 ^{a}
$PAN_{(72\,h)}$ (mg L^{-1})	-	29.000 ± 1.414 ^{a}	12.250 ± 3.889 ^{b}	13.168 ± 0.289 ^{b}	26.333 ± 4.646 ^{a}
NH_4 (mg L^{-1})	127.500 ± 9.192	49.500 ± 3.536 ^{a}	nd ^{c}	nd ^{c}	4.170 ± 2.843 ^{b}
$NH_{4(72\,h)}$ (mg L^{-1})	-	61.500 ± 0.707 ^{a}	nd ^{b}	nd ^{b}	51.167 ± 17.905 ^{a}
Sugars 72 h (g L^{-1})	-	109.252 ± 1.555 ^{a}	41.174 ± 0.246 ^{b}	42.883 ± 2.115 ^{b}	101.200 ± 11.232 ^{a}
M_{ax}FR (g of CO_2 V h^{-1}).	-	0.051 ± 0.004 ^{b}	0.100 ± 0.005 ^{a}	0.098 ± 0.002 ^{a}	0.058 ± 0.006 ^{b}
FP	-	0.014 ± 0.001 ^{a,b}	0.017 ± 0.002 ^{a}	0.015 ± 0.001 ^{a}	0.011 ± 0.002 ^{b}

Data are expressed as triplicate means for mixed trials and duplicate means for single culture trials ± standard deviations. Values in the same row with different superscript letters are significantly different ($p < 0.05$). YAN—yeast assimilable nitrogen. PAN—primary amino nitrogen. MaxFR—maximum fermentation rate. FP—fermentation purity (acetic acid (g L^{-1})/ ethanol (% *v*:*v*$^{-1}$)). nd—not detected. -: not measured.

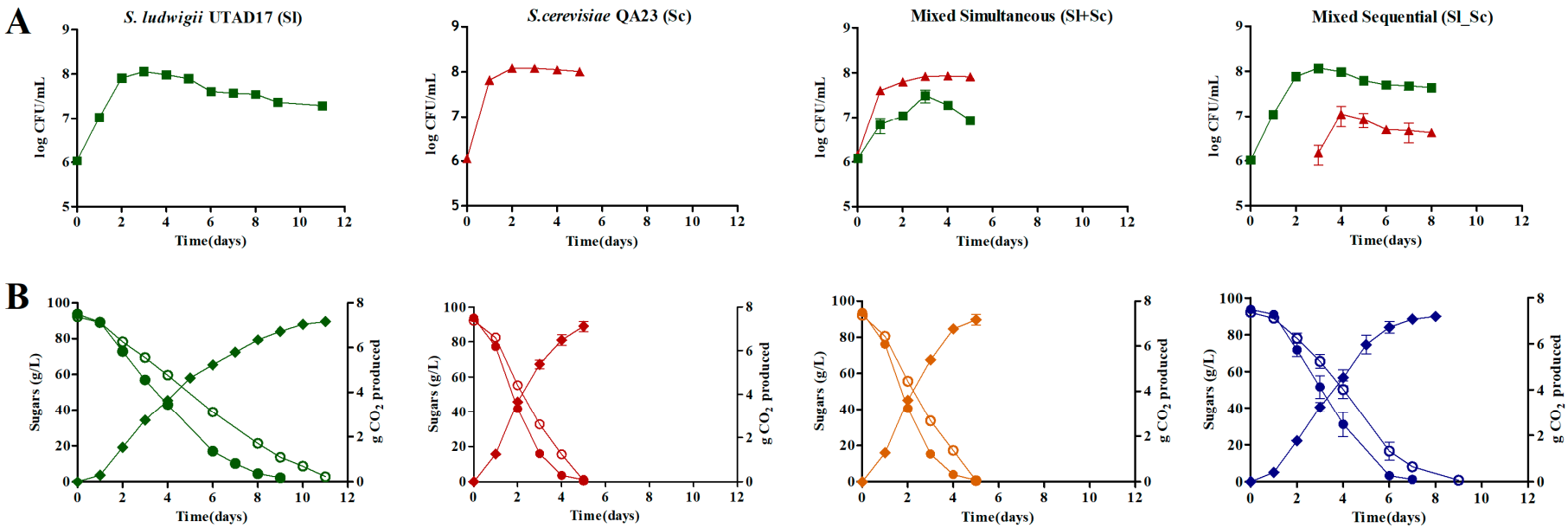

Figure 1. Means ± standard deviations of (**A**) yeast cell counts of *S. ludwigii* UTAD17 (green squares) and *S. cerevisiae* QA23 (red triangles) in single and mixed cultures; and (**B**) Fermentation profiles (diamonds), glucose (filled circles), and fructose (clear circles) concentrations during single and mixed culture trials.

When the two strains were co-inoculated simultaneously (Sc+Sl), a decrease in both strain populations was noted to likely reflect the more competitive environment in terms of space [83] and nutrients, two factors that were previously found to determine yeast–yeast interactions [32,74]. Yet, *S. cerevisiae* dominated over *S. ludwigii* UTAD17, in line with the notion that *S. cerevisiae* is better adapted to grape-juice per se than non-*Saccharomyces* yeasts [84]. Notably, although *S. ludwigii* UTAD17 was able to maintain a substantial viable population throughout these Sc+Sl fermentations, the rate and total time of fermentation was mostly similar, compared to *S. cerevisiae* in a single culture. In the sequential mixed-culture trials (Sl_Sc), *S. ludwigii* UTAD17 initiated fermentation, and *S. cerevisiae* QA23 was later inoculated at 72 h. At this stage, about 40% of the initial sugars were fermented by *S. ludwigii* UTAD17 and the assimilable nitrogen concentration was significantly lowered (Table 1). Contrary to that observed in simultaneous fermentations, in this case, *S. ludwigii* UTAD17 dominated over *S. cerevisiae*. Indeed, growth of the non-*Saccharomyces* strain proceeded as in a single culture, exhibiting a similar growth rate and loss of viability. On the other hand, in these experiments *S. cerevisiae* QA23 growth was limited to a maximum population of about 1.1×10^7 cfu mL^{-1}, attained 24 h after its inoculation, most probably due to the low assimilable nitrogen available in the medium. Nevertheless, there was an increase in the fermentation rate and sequential fermentations were successfully completed in eight days, taking 72 h more than *S. cerevisiae* in single culture. Adjusting the YAN levels of the fermenting must at this stage, could be an option to improve the growth of *S. cerevisiae* and the fermentation performance.

3.3. Effect of S. ludwigii UTAD17 on Wine Composition and Aroma Profile

The primary physiochemical parameters of the wines obtained are presented in Table 1. The production of ethanol is an essential attribute to define the use of yeasts in the production of fermented beverages. *S. ludwigii* UTAD17 showed a similar efficiency of sugar-to-ethanol conversion to that of *S. cerevisiae* QA23, as the ethanol levels of the final wines, which ranged from 10.2 to 10.4% ($v{:}v^{-1}$), were not significantly different. Likewise, no significant differences were found on the amount of SO_2 formed in each fermentation. Both strains produced up to 20 mg mg L^{-1} and thus they were considered low-sulfite-forming yeasts [85]. Overall, all fermentations resulted in lower levels of acetic acid. Slightly lower levels of acetic acid were found in wines where *S. ludwigii* UTAD17 was involved, as compared to the wines only fermented by *S. cerevisiae* and significantly lower levels of this compound were detected on the sequentially inoculated wines (Table 1). These are promising features since the amount of both metabolites is tightly limited by regulations, might depreciate wine aroma (especially acetic acid) or raise concerns about consumers' safety (SO_2) and, thus, they should be kept at the lowest possible levels. The lower acetic acid produced in the fermentations dominated by the non-*Saccharomyces* yeast was accompanied by significantly lower levels of glycerol in all fermentations, suggesting that *S. ludwigii* UTAD17 management strategy for NADH/NAD^+ recycling and maintenance of redox balance is similar to that of *S. cerevisiae* [86]. This connection between acetic acid and glycerol production is not as clear in other non-*Saccharomyces*. For instance, in single culture fermentations, *Starmerella bacillaris* appears to be a high glycerol and low acetic acid producer [53], while *H. uvarum* seems to be a low glycerol and high acetic acid producer of yeast [87].

In order to evaluate how *S. ludwigii* UTAD17 affected the final aroma composition, the different wines were analyzed by gas chromatography. Eighteen yeast-derived aroma compounds were quantified, five alcohols, six acids, four ethyl-esters, two acetates, and one aldehyde (Table 2). Those compounds that were found to be significantly different ($p < 0.05$), along with glycerol and acetic acid (Table 1), were used for Partial Least Squares–Discriminant Analysis (PLS–DA), in order to distinguish the wines obtained with the different inoculation strategies (Figure 2). The first component accounted for 69.99%, while component 2 explained 18.83% of the total variation. Replicate experiments were well grouped on the PLS. A clear separation was observed between the wines whose fermentation were dominated by *S. cerevisiae*, and those governed by *S. ludwigii* UTAD17. Although *S. ludwigii* UTAD17 produced overall significantly lower levels of volatile compounds (Table 2), it should be highlighted

that the wines obtained by simultaneous co-inoculation of both strains (Sl+Sc), located in the upper-left quadrant (Figure 2), were characterized by a greater diversity of flavors and complexity, as compared with those fermented by *S. cerevisiae* alone. Overall, fermentations conducted by *S. ludwigii* UTAD17 resulted in wines characterized by higher levels of 1-butanol and butyric and isobutyric acids, which were found to be 2 to 3 times higher than that in Sc and Sc+Sl wines (Table 2). 1-butanol and the short-chain fatty acid, butyric acid, are synthetized from 2-ketovalerate following decarboxylation to the aldehyde precursor, butyraldehyde, which is either reduced or oxidized, respectively [88]. In this study we could only speculate that the high levels of both compounds produced by *S. ludwigii* UTAD17 could result from either an excess of the intermediate α-keto acid or the use of this redox duality of the last steps of the Ehrlich pathway to help maintain the redox balance of the cell [89].

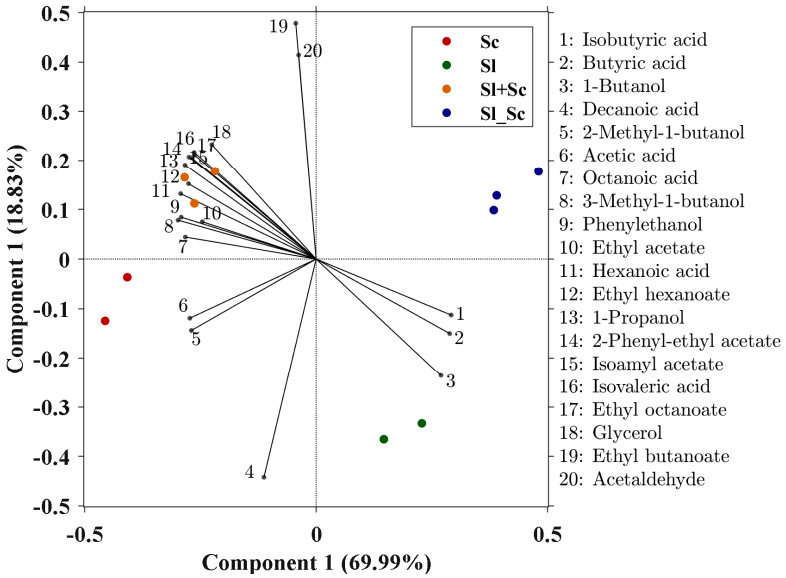

Figure 2. Partial least squares-discriminant analysis (PLS–DA) plot of wines obtained with the different inoculation strategies using volatile and non-volatile compounds that were significantly different among treatments—single-culture of *S. ludwigii* UTAD17(Sl) and *S. cerevisiae* (Sc) or in consortium—mixed simultaneously (Sl+Sc) and sequentially (Sl_Sc).

Previous studies have put aside the use of *S. ludwigii* in the winemaking industry, given the large amounts of ethyl acetate and acetaldehyde produced by these strains [7,8,61]. Notably, the *S. ludwigii* UTAD17 strain is, apparently, a low producer of both compounds as the levels detected were significantly inferior to those obtained in both Sc and Sl+Sc fermentations. Additionally, none-to-low levels of acetate and ethyl esters, which are responsible for the pleasant fruity and floral bouquet of wines, were found to be produced by *S. ludwigii* UTAD17. The predicted UTAD17 ORFeome did not include proteins similar to the *S. cerevisiae* acetyl transferases ScATF1, ScATF2, and ScAYT1, required for synthesis of acetate esters (Table S1). As such, the minor amounts of these compounds found on Sl_Sc fermentations was likely attributable to *S. cerevisiae* QA23 activity. Contrarily, we found that *S. ludwigii* UTAD17 harbors proteins similar to *S. cerevisiae* ethanol acyl-coA transferases ScEHT1 and ScEEB1, responsible for ethyl ester synthesis through the condensation between ethanol and fatty acyl-CoA [90], and ScIAH1 involved in esters hydrolysis [91]. In this case, the limited production of ethyl esters by *S. ludwigii* UTAD17 might result from differences in the activity levels of these enzymes. A more in-depth analysis of this genomic information is being undertaken that will shed light on the molecular foundations underlying some of the intriguing physiological traits of this yeast such as high SO_2 resistance, lower fermentative power, and wine aroma. From a practical point of view, this information will be very useful for a rational application of this strain, depending on the winemaking conditions

Table 2. Concentration of volatile compounds detected and quantified by Gas Chromatography equipped with Flame ionization Detector (GC–FID) in wine obtained by a single-culture of *S. ludwigii* UTAD17(Sl) and *S. cerevisiae* (Sc) or in consortium—mixed simultaneous (Sl+Sc) and sequential (Sl_Sc), in natural grape-juice of *Vitis vinifera* L. cv. Touriga Nacional at 25 °C, under static conditions.

Compound (mg L^{-1})	Sl	Sc	Sl+Sc	Sl_Sc	OT (mg/L)	OD
Alcohols						
1-propanol	14.315 ± 0.402 ^{c}	44.383 ± 1.298 ^{a}	43.985 ± 0.516 ^{a}	17.934 ± 1.557 ^{b}	306.000	Alcohol, ripe fruit
1-butanol	35.428 ± 1.045 ^{a}	15.040 ± 0.220 ^{c}	15.335 ± 1.428 ^{c}	30.792 ± 0.316 ^{b}	150.000	Medicinal
2-Methyl-1-butanol	17.882 ± 1.033 ^{ab}	19.040 ± 0.721 ^{a}	18.966 ± 1.087 ^{a}	15.123 ± 0.436 ^{b}	30.000	Alcohol, nail polish
3-Methyl-1-butanol	74.963 ± 6.858 ^{b}	102.984 ± 3.465 ^{a}	101.176 ± 1.794 ^{a}	69.184 ± 1.217 ^{b}	30.000	Whiskey, nail polish
2-Phenylethanol	18.945 ± 0.728 ^{b}	27.040 ± 1.881 ^{a}	26.997 ± 1.398 ^{a}	16.869 ± 1.015 ^{b}	14.000	Rose, honey
$\sum$	161.534 ± 8.609 ^{b}	208.486 ± 3.824 ^{a}	206.459 ± 6.122 ^{a}	149.962 ± 4.092 ^{b}		
Acetate Esters						
Phenylethyl Acetate	nd^{b}	0.181 ± 0.011 ^{a}	0.177 ± 0.024 ^{a}	nd^{b}	0.250	Flowery
Isoamyl Acetate	nd^{c}	0.807 ± 0.106 ^{a}	0.725 ± 0.155 ^{a}	0.171 ± 0.120 ^{b}	0.030	Banana
$\sum$	nd^{c}	0.988 ± 0.117 ^{a}	0.902 ± 0.163 ^{a}	0.171 ± 0.120 ^{b}		
Ethyl Esters						
Ethyl Acetate	32.679 ± 6.895 ^{b}	40.995 ± 0.393 ^{ab}	47.392 ± 7.071 ^{a}	28.387 ± 5.742 ^{b}	7.500	Fruity, vinegar, nail polish, acetic
Ethyl Butanoate	nd^{b}	0.604 ± 0.182 ^{a}	0.630 ± 0.066 ^{a}	0.669 ± 0.118 ^{a}	0.020	Apple, strawberry, fruity
Ethyl Hexanoate	nd^{d}	0.301 ± 0.012 ^{a}	0.231 ± 0.024 ^{b}	0.082 ± 0.020 ^{c}	0.005	Green apple, fruity
Ethyl Octanoate	nd^{b}	0.312 ± 0.046 ^{a}	0.410 ± 0.080 ^{a}	nd^{b}	0.002	Pear, fruity
$\sum$ **(except ethyl acetate)**	nd^{c}	1.216 ± 0.149 ^{a}	1.271 ± 0.160 ^{a}	0.752 ± 0.132 ^{b}		
Fatty Acids						
Isobutyric Acid	2.430 ± 0.072 ^{a}	1.077 ± 0.194 ^{b}	1.242 ± 0.205 ^{b}	2.494 ± 0.172 ^{a}	2.300	Fatty
Butyric Acid	1.892 ± 0.074 ^{a}	0.631 ± 0.089 ^{b}	0.726 ± 0.056 ^{b}	1.880 ± 0.074 ^{a}	10.000	Fatty, rancid
Isovaleric Acid	nd^{b}	0.231 ± 0.040 ^{a}	0.251 ± 0.045 ^{a}	nd^{b}	0.033	Fatty, rancid
Hexanoic Acid	0.591 ± 0.023 ^{c}	1.435 ± 0.064 ^{a}	1.350 ± 0.027 ^{b}	0.558 ± 0.034 ^{c}	0.420	Cheese, fatty
Octanoic Acid	0.727 ± 0.175 ^{c}	2.383 ± 0.060 ^{a}	1.559 ± 0.127 ^{b}	0.710 ± 0.145 ^{c}	0.500	Fatty, unpleasant
Decanoic Acid	0.222 ± 0.005 ^{a}	0.224 ± 0.031 ^{a}	nd ^{b}	nd ^{b}	1.000	Fat, rancid
$\sum$	5.861 ± 0.293 ^{a}	5.980 ±0.475 ^{a}	5.127 ± 0.145 ^{b}	5.642 ± 0.329 ^{ab}		
Acetaldehyde	20.279 ± 0.472 ^{b}	25.328 ± 1.400 ^{a}	25.661 ± 1.500 ^{a}	25.944 ± 2.672 ^{a}	10.000	Sherry, nutty, bruised apple

Data are expressed as means ± standard deviations resulting from triplicate experiments for mixed trials and duplicates for single culture trials. OT—odor threshold; OD—odor descriptions. Odor thresholds and odor descriptions can be found in the literature [92–98]. Values in the same row with different superscript letter are significantly different ($p < 0.05$). nd—not detected.

Overall, the above-described results gave good insights on the potential use of *S. ludwigii* UTAD17 in winemaking. Given the low fruity/estery nature of this strain, it could be a good option to obtain wines with a greater expression of grape varietal characteristics. Nowadays, winemakers make use of a blend of wines fermented with different yeast strains/species or different grape varieties to create wines tailored to meet consumer expectations. Following this line of winemaking, and considering the trend of the use of non-*Saccharomyces* yeasts to obtain wines with distinct aroma profiles, it should be underlined that the aromatic characteristics of *S. ludwigii*, UTAD17, which greatly differs from the traditional *S. cerevisiae*, might confer a peculiar imprint onto the final product. Nevertheless, our results warrant further studies to evaluate whether the observed differences in chemical composition can be perceived during sensory evaluation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/7/11/478/s1, Figure S1: Phenotypic profile of the *S. ludwigii* UTAD17; Table S1: Comparative analysis of the predicted *S. ludwigii* UTAD17 ORFeome with sets of proteins of *S. cerevisiae* S288c

Author Contributions: A.M.-F., N.P.M., and C.B. conceived and designed the experiments; M.E. and C.B. performed the experiments; I.V. performed the GC analyses; M.J.T. and M.E. performed the bioinformatics analysis. All authors analyzed the data; M.E. drafted the manuscript; All authors revised and approved the submitted version.

Funding: This work was funded by European Regional Development Fund (ERFD) through POCI-COMPETE 2020 and by FCT through the project SMARTWINE—Smarter wine fermentations: integrating OMICS tools (PTDC/AGR-TEC/3315/2014, POCI-01-0145-FEDER-016834). Support received from INTERACT project no. NORTE-01-0145-FEDER-000017 through its line of research entitled VitalityWINE, and from INNOVINE&WINE. NORTE-01-0145-FEDER-000038, co-financed by ERDF through NORTE 2020 is also acknowledged. Support from FCT to BioISI (FCT/UID/Multi/04046/2019) and iBB (through contract UID/BIO/04565/ 2013) is also acknowledged.

Acknowledgments: The authors thank Veronique Gomes for assistance in the statistical analysis and Rogério Tenreiro for the critical reading of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Fleet, G.H.; Heard, G.M. Yeast growth during fermentation. In *Wine Microbiology and Biotechnology (Fleet GH)*; Harwood Academic: Chur, Switzerland, 1993; pp. 27–54.
2. Egli, C.M.; Edinger, W.D.; Mitrakul, C.M.; Henick-Kling, T.; Henick-Kling, T. Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. *J. Appl. Microbiol.* **1998**, *85*, 779–789. [CrossRef] [PubMed]
3. Henick-Kling, T.; Edinger, W.; Daniel, P.; Monk, P. Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. *J. Appl. Microbiol.* **1998**, *84*, 865–876. [CrossRef]
4. Ciani, M.; Mannazzu, I.; Marinangeli, P.; Clementi, F.; Martini, A. Contribution of winery-resident *Saccharomyces cerevisiae* strains to spontaneous grape must fermentation. *Antonie Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **2004**, *85*, 159–164. [CrossRef] [PubMed]
5. Scholl, C.M.; Morgan, S.C.; Stone, M.L.; Tantikachornkiat, M.; Neuner, M.; Durall, D.M. Composition of *Saccharomyces cerevisiae* strains in spontaneous fermentations of Pinot Noir and Chardonnay. *Aust. J. Grape Wine Res.* **2016**, *22*, 384–390. [CrossRef]
6. Boulton, R.B.; Singleton, V.L.; Bisson, L.F.; Kunkee, R.E. Microbiological Spoilage of Wine and its Control. In *Principles and Practices of Winemaking*; Chapman & Hall: London, UK, 1996; pp. 352–381.
7. Ciani, M.; Maccarelli, F. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World J. Microbiol. Biotechnol.* **1998**, *14*, 199–203. [CrossRef]
8. Domizio, P.; Romani, C.; Comitini, F.; Gobbi, M.; Lencioni, L.; Mannazzu, I.; Ciani, M. Potential spoilage non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. *Ann. Microbiol.* **2011**, *61*, 137–144. [CrossRef]
9. Fernández, M.; Ubeda, J.; Briones, A. Typing of non-*Saccharomyces* yeasts with enzymatic activities of interest in wine-making. *Int. J. Food Microbiol.* **2000**, *59*, 29–36. [CrossRef]

10. Jolly, N.P.; Varela, C.; Pretorius, I.S. Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* **2014**, *14*, 215–237. [CrossRef]
11. Maturano, Y.P.; Assaf, L.A.R.; Toro, M.E.; Nally, M.C.; Vallejo, M.; De Figueroa, L.I.C.; Combina, M.; Vazquez, F. Multi-enzyme production by pure and mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation. *Int. J. Food Microbiol.* **2012**, *155*, 43–50. [CrossRef]
12. Mendes-Ferreira, A.; Clímaco, M.C.; Mendes-Faia, A. The role of non-*Saccharomyces* species in releasing glycosidic bound fraction of grape aroma components Ð a preliminary study. *J. Appl. Microbiol.* **2001**, *91*, 67–71. [CrossRef]
13. Rosi, I.; Vinella, M.; Domizio, P. Characterization of Beta-Glucosidase Activity in Yeasts of Enological Origin. *J. Appl. Bacteriol.* **1994**, *77*, 519–527. [CrossRef] [PubMed]
14. Strauss, M.; Jolly, N.; Lambrechts, M.; Van Rensburg, P. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. *J. Appl. Microbiol.* **2001**, *91*, 182–190. [CrossRef] [PubMed]
15. Viana, F.; Gil, J.; Genoves, S.; Valles, S.; Manzanares, P. Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiol.* **2008**, *25*, 778–785. [CrossRef] [PubMed]
16. 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]
17. De Benedictis, M.; Bleve, G.; Tristezza, M.; Tufariello, M.; Grieco, F. An optimized procedure for the enological selection of non-*Saccharomyces* starter cultures. *Antonie Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **2011**, *99*, 189–200. [CrossRef]
18. Domizio, P.; Romani, C.; Lencioni, L.; Comitini, F.; Gobbi, M.; Mannazzu, I.; Ciani, M. Outlining a future for non-*Saccharomyces* yeasts: Selection of putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation. *Int. J. Food Microbiol.* **2011**, *147*, 170–180. [CrossRef]
19. Belda, I.; Ruiz, J.; Alastruey-Izquierdo, A.; Navascués, E.; Marquina, D.; Santos, A. Unraveling the enzymatic basis of wine "Flavorome": A phylo-functional study of wine related yeast species. *Front. Microbiol.* **2016**, *7*, 1–13. [CrossRef]
20. Berbegal, C.; Spano, G.; Tristezza, M.; Grieco, F.; Capozzi, V. Microbial Resources and Innovation in the Wine Production Sector. *S. Afr. J. Enol. Vitic.* **2017**, *38*, 156–166. [CrossRef]
21. Barbosa, C.; Lage, P.; Esteves, M.; Chambel, L.; Mendes-Faia, A.; Mendes-Ferreira, A. Molecular and Phenotypic Characterization of *Metschnikowia pulcherrima* Strains from Douro Wine Region. *Fermentation* **2018**, *4*, 8. [CrossRef]
22. Nisiotou, A.; Sgouros, G.; Mallouchos, A.; Nisiotis, C.-S.; Michaelidis, C.; Tassou, C.; Banilas, G. The use of indigenous *Saccharomyces cerevisiae* and *Starmerella bacillaris* strains as a tool to create chemical complexity in local wines. *Food Res. Int.* **2018**, *111*, 498–508. [CrossRef]
23. 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] [PubMed]
24. Zironi, R.; Romano, P.; Suzzi, G.; Battistutta, F.; Comi, G. Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. *Biotechnol. Lett.* **1993**, *15*, 235–238. [CrossRef]
25. Ciani, M.; Fatichenti, F. Selective sugar consumption by apiculate yeasts. *Lett. Appl. Microbiol.* **1999**, *28*, 203–206. [CrossRef] [PubMed]
26. Moreira, N.; Mendes, F.; De Pinho, P.G.; Hogg, T.; Vasconcelos, I. Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *Int. J. Food Microbiol.* **2008**, *124*, 231–238. [CrossRef] [PubMed]
27. Andorrà, I.; Berradre, M.; Mas, A.; Esteve-Zarzoso, B.; Guillamón, J.M. Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT Food Sci. Technol.* **2012**, *49*, 8–13. [CrossRef]
28. Hu, K.; Jin, G.-J.; Mei, W.-C.; Li, T.; Tao, Y.-S. Increase of medium-chain fatty acid ethyl ester content in mixed *H. uvarum/S. cerevisiae* fermentation leads to wine fruity aroma enhancement. *Food Chem.* **2018**, *239*, 495–501. [CrossRef]

29. Nevado, F.P.; Albergaria, H.; Hogg, T.; Gírio, F. Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **2006**, *108*, 336–345.
30. Lage, P.; Barbosa, C.; Mateus, B.; Vasconcelos, I.; Mendes-Faia, A.; Mendes-Ferreira, A. *H. guilliermondii* impacts growth kinetics and metabolic activity of *S. cerevisiae*: The role of initial nitrogen concentration. *Int. J. Food Microbiol.* **2014**, *172*, 62–69. [CrossRef]
31. Viana, F.; Belloch, C.; Vallés, S.; Manzanares, P. International Journal of Food Microbiology Monitoring a mixed starter of *Hanseniaspora vineae—Saccharomyces cerevisiae* in natural must: Impact on 2-phenylethyl acetate production. *Int. J. Food Microbiol.* **2011**, *151*, 235–240. [CrossRef]
32. Medina, K.; Boido, E.; Dellacassa, E.; Carrau, F. Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *Int. J. Food Microbiol.* **2012**, *157*, 245–250. [CrossRef]
33. Medina, K.; Boido, E.; Farina, L.; Gioia, O.; Gómez, M.; Barquet, M.; Gaggero, C.; Dellacassa, E.; Carrau, F. Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chem.* **2013**, *141*, 2513–2521. [CrossRef] [PubMed]
34. Lleixà, J.; Martín, V.; Portillo, M.D.C.; Carrau, F.; Beltran, G.; Mas, A. Comparison of Fermentation and Wines Produced by Inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*. *Front. Microbiol.* **2016**, *7*, 338. [CrossRef] [PubMed]
35. Cocolin, L.; Bisson, L.; Mills, D. Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiol. Lett.* **2000**, *189*, 81–87. [CrossRef] [PubMed]
36. Contreras, A.; Hidalgo, C.; Schmidt, S.; Henschke, P.; Curtin, C.; Varela, C. The application of non-*Saccharomyces yeast* in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *Int. J. Food Microbiol.* **2015**, *205*, 7–15. [CrossRef]
37. Ruiz, J.; Belda, I.; Beisert, B.; Navascués, E.; Marquina, D.; Calderón, F.; Benito, S. Analytical impact of *Metschnikowia pulcherrima* in the volatile profile of Verdejo white wines. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 8501–8509. [CrossRef]
38. Bely, M.; Stoeckle, P.; Masneuf-Pomarede, I.; Dubourdieu, D. Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. *Int. J. Food Microbiol.* **2008**, *122*, 312–320. [CrossRef]
39. Renault, P.; Coulon, J.; De Revel, G.; Barbe, J.-C.; Bely, M. Increase of fruity aroma during mixed *T. delbrueckii/S. cerevisiae* wine fermentation is linked to specific esters enhancement. *Int. J. Food Microbiol.* **2015**, *207*, 40–48. [CrossRef]
40. Azzolini, M.; Fedrizzi, B.; Tosi, E.; Finato, F.; Vagnoli, P.; Scrinzi, C.; Zapparoli, G. Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* mixed cultures on fermentation and aroma of Amarone wine. *Eur. Food Res. Technol.* **2012**, *235*, 303–313. [CrossRef]
41. Taillandier, P.; Lai, Q.P.; Julien-Ortiz, A.; Brandam, C. Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: Influence of inoculation and nitrogen content. *World J. Microbiol. Biotechnol.* **2014**, *30*, 1959–1967. [CrossRef]
42. Tondini, F.; Lang, T.; Chen, L.; Herderich, M.; Jiranek, V. Linking gene expression and oenological traits: Comparison between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* strains. *Int. J. Food Microbiol.* **2019**, *294*, 42–49. [CrossRef]
43. 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]
44. Mora, J.; Barbas, J.I.; Mulet, A. Growth of Yeast Species during the Fermentation of Musts Inoculated with *Kluyveromyces thermotolerans and Saccharomyces cerevisiae*. *Am. J. Enol. Vitic.* **1990**, *41*, 156–159.
45. Kapsopoulou, K.; Mourtzini, A.; Anthoulas, M.; Nerantzis, E. Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. *World J. Microbiol. Biotechnol.* **2007**, *23*, 735–739. [CrossRef]
46. 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]
47. Benito, Á.; Calderón, F.; Palomero, F.; Benito, S. Quality and Composition of Airén Wines Fermented by Sequential Inoculation of *Lachancea thermotolerans* and *Saccharomyces cerevisiae*. *Food Technol. Biotechnol.* **2016**, *54*, 135–144. [CrossRef]

48. Morales, M.; Fierro-Risco, J.; Ríos-Reina, R.; Ubeda, C.; Paneque, P. Influence of *Saccharomyces cerevisiae* and *Lachancea thermotolerans* co-inoculation on volatile profile in fermentations of a must with a high sugar content. *Food Chem.* **2019**, *276*, 427–435. [CrossRef]
49. Ciani, M.; Ferraro, F.R. Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. *J. Appl. Microbiol.* **1998**, *85*, 247–254. [CrossRef]
50. Soden, A.; Francis, I.; Oakey, H.; Henschke, P. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. *Aust. J. Grape Wine Res.* **2000**, *6*, 21–30. [CrossRef]
51. 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]
52. Domizio, P.; Liu, Y.; Bisson, L.; Barile, D. Use of non-*Saccharomyces* wine yeasts as novel sources of mannoproteins in wine. *Food Microbiol.* **2014**, *43*, 5–15. [CrossRef]
53. Englezos, V.; Torchio, F.; Cravero, F.; Marengo, F.; Giacosa, S.; Gerbi, V.; Cocolin, L. Aroma profile and composition of Barbera wines obtained by mixed fermentations of *Starmerella bacillaris* (*synonym Candida zemplinina*) and *Saccharomyces cerevisiae*. *LWT Food Sci. Technol.* **2016**, *73*, 567–575. [CrossRef]
54. 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] [PubMed]
55. Benito, Á.; Calderón, F.; Benito, S. Mixed alcoholic fermentation of *Schizosaccharomyces pombe* and *Lachancea thermotolerans* and its influence on mannose-containing polysaccharides wine Composition. *AMB Express* **2019**, *9*, 17. [CrossRef] [PubMed]
56. Belda, I.; Navascués, E.; Marquina, D.; Santos, A.; Calderon, F.; Benito, S. Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality. *Appl. Microbiol. Biotechnol.* **2014**, *99*, 1911–1922. [CrossRef]
57. Englezos, V.; Rantsiou, K.; Cravero, F.; Torchio, F.; Giacosa, S.; Ortiz-Julien, A.; Gerbi, V.; Rolle, L.; Cocolin, L. Volatile profiles and chromatic characteristics of red wines produced with *Starmerella bacillaris* and *Saccharomyces cerevisiae*. *Food Res. Int.* **2018**, *109*, 298–309. [CrossRef]
58. Sadoudi, M.; Tourdot-Maréchal, R.; Rousseaux, S.; Steyer, D.; Gallardo-Chacón, J.-J.; Ballester, J.; Vichi, S.; Guérin-Schneider, R.; Caixach, J.; Alexandre, H. Yeast–yeast interactions revealed by aromatic profile analysis of *Sauvignon Blanc* wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol.* **2012**, *32*, 243–253. [CrossRef]
59. Petruzzi, L.; Capozzi, V.; Berbegal, C.; Corbo, M.R.; Bevilacqua, A.; Spano, G.; Sinigaglia, M. Microbial Resources and Enological Significance: Opportunities and Benefits. *Front. Microbiol.* **2017**, *8*, 1–13. [CrossRef]
60. Phaff, H.J.; Miller, M.W.; Em, M. *The Life of Yeasts*, 2nd ed.; Harvard University Press: Cambridge, UK, 1978.
61. Romano, P.; Marchese, R.; Laurita, C.; Saleano, G.; Turbanti, L. Biotechnological suitability of *Saccharomycodes ludwigii* for fermented beverages. *World J. Microbiol. Biotechnol.* **1999**, *15*, 451–454. [CrossRef]
62. Thomas, D.S. Yeasts as Spoilage Organisms in Beverages. In *The Yeasts*, 2nd ed.; Rose, J.S., Harrison, A.H., Eds.; Academic Press: New York, NY, USA, 1993; pp. 517–561.
63. Stratford, M.; Morgan, P.; Rose, A.H. Sulphur Dioxide Resistance in *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii*. *Microbiology* **1987**, *133*, 2173–2179. [CrossRef]
64. Estela-Escalante, W.; Hatta-Sakoda, B.; Ludeña-Cervantes, Z.; Melzoch, K.; Rychtera, M.; Sarmiento-Casavilca, V.; Chaquilla-Quilca, G. Actividad Fermentativa de *Saccharomycodes ludwigii* y Evaluación de la Síntesis de Compuestos de Importancia Sensorial durante la Fermentacion de Jugo de Manzana. *Rev. Espec. Cienc. Quim. Biol.* **2011**, *14*, 12–23.
65. De Francesco, G.; Turchetti, B.; Sileoni, V.; Marconi, O.; Perretti, G. Screening of new strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* to produce low-alcohol beer. *J. Inst. Brew.* **2015**, *121*, 113–121. [CrossRef]
66. Liu, Y.; Li, H.; Du, J.-H. Non-alcoholic Beer Production by *Saccharomycodes ludwigii*. *Food Sci.* **2011**, *15*, 186–190.
67. Tavares, M.J.; Güldener, U.; Esteves, M.; Mendes-Faia, A.; Mendes-Ferreira, A.; Mira, N.P. Genome Sequence of the Wine Yeast *Saccharomycodes ludwigii* UTAD17. *Microbiol. Resour. Announc.* **2018**, *7*, e01195-18. [CrossRef]
68. Castellucci, F. *Guidelines for the Characterization of Wine Yeasts of the Genus Saccharomyces Isolated from Vitivinicultural Environments*; International Organisation of Vine and Wine: Paris, France, 2012.

69. Henschke, P.A.; Jiranek, V. *Wine Microbiology and Biotechnology (Fleet GH.)*; Harwood Academic: Chur, Switzerland, 1993.
70. Mendes-Ferreira, A.; Barbosa, C.; Falco, V.; Leão, C.; Mendes-Faia, A. The production of hydrogen sulphide and other aroma compounds by wine strains of *Saccharomyces cerevisiae* in synthetic media with different nitrogen concentrations. *J. Ind. Microbiol. Biotechnol.* **2009**, *36*, 571–583. [CrossRef] [PubMed]
71. Lin, Y. Detection of Wild Yeasts in the Brewery. *J. Inst. Brew.* **1971**, *77*, 513–516.
72. OIV. *Compendium of International Methods of Analysis of Musts and Wines*; International Organisation of Vine and Wine: Paris, France, 2016.
73. Moreira, N.; De Pinho, P.G.; Santos, C.; Vasconcelos, I. Relationship between nitrogen content in grapes and volatiles, namely heavy sulphur compounds, in wines. *Food Chem.* **2011**, *126*, 1599–1607. [CrossRef]
74. Alexandre, H.; Charpentier, C. Biochemical aspects of stuck and sluggish fermentation in grape must. *J. Ind. Microbiol. Biotechnol.* **1998**, *20*, 20–27. [CrossRef]
75. Albertin, W.; Marullo, P.; Aigle, M.; Dillmann, C.; De Vienne, D.; Bely, M.; Sicard, D. Population size drives industrial *Saccharomyces cerevisiae* alcoholic fermentation and is under genetic control. *Appl. Environ. Microbiol.* **2011**, *77*, 2772–2784. [CrossRef]
76. Heard, G.; Fleet, G. The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. *J. Appl. Bacteriol.* **1988**, *65*, 23–28. [CrossRef]
77. Seixas, I.; Barbosa, C.; Mendes-Faia, A.; Güldener, U.; Tenreiro, R.; Mendes-Ferreira, A.; Mira, N.P. Genome sequence of the non-conventional wine yeast *Hanseniaspora guilliermondii* UTAD222 unveils relevant traits of this species and of the *Hanseniaspora* genus in the context of wine fermentation. *Curr. Neuropharmacol.* **2019**, *26*, 67–83. [CrossRef]
78. Bataillon, M.; Rico, A.; Sablayrolles, J.-M.; Salmon, J.-M.; Barré, P. Early thiamin assimilation by yeasts under enological conditions: Impact on alcoholic fermentation kinetics. *J. Ferment. Bioeng.* **1996**, *82*, 145–150. [CrossRef]
79. Brion, C.; Ambroset, C.; Delobel, P.; Sanchez, I.; Blondin, B. Deciphering regulatory variation of *THI* genes in alcoholic fermentation indicate an impact of Thi3p on PDC1 expression. *BMC Genom.* **2014**, *15*, 1085. [CrossRef] [PubMed]
80. Langenberg, A.; Bink, F.J.; Wolff, L.; Walter, S.; Grossmann, M.; Schmitz, H. Glycolytic Functions Are Conserved in the Genome of the Wine Yeast. *Appl. Environ. Microbiol.* **2017**, *83*, 1–20. [CrossRef] [PubMed]
81. Barbosa, C.; Lage, P.; Vilela, A.; Mendes-Faia, A.; Mendes-Ferreira, A. Phenotypic and metabolic traits of commercial *Saccharomyces cerevisiae* yeasts. *AMB Express* **2014**, *4*, 39. [CrossRef] [PubMed]
82. Brice, C.; Cubillos, F.A.; Dequin, S.; Camarasa, C.; Martinez, C. Adaptability of the *Saccharomyces cerevisiae* yeasts to wine fermentation conditions relies on their strong ability to consume nitrogen. *PLoS ONE* **2018**, *13*, e0192383. [CrossRef] [PubMed]
83. Nissen, P.; Nielsen, D.S.; Arneborg, N. Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-Saccharomyces yeasts in mixed cultures by a cell-cell contact-mediated mechanism. *Yeast* **2003**, *20*, 331–341. [CrossRef] [PubMed]
84. Goddard, M.R. Quantifying the complexities of *Saccharomyces cerevisiae*'s ecosystem engineering via fermentation. *Ecology* **2008**, *89*, 2077–2082. [CrossRef]
85. Werner, M.; Rauhut, D.; Cottereau, P. Yeast and Natural Production of Sulphites. *Int. J. Enol. Vitic.* **2009**, *12*, 1–5.
86. Remize, F.; Andrieu, E.; Dequin, S. Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae*: Role of the cytosolic Mg2+and mitochondrial K+acetaldehyde dehydrogenases Ald6p and Ald4p in acetate formation during alcoholic fermentation. *Appl. Environ. Microbiol.* **2000**, *66*, 3151–3159. [CrossRef]
87. Andorrà, I.; Berradre, M.; Rozès, N.; Mas, A.; Guillamón, J.M.; Esteve-Zarzoso, B. Effect of pure and mixed cultures of the main wine yeast species on grape must fermentations. *Eur. Food Res. Technol.* **2010**, *231*, 215–224. [CrossRef]
88. Hazelwood, L.A.; Daran, J.-M.; Van Maris, A.J.A.; Pronk, J.T.; Dickinson, J.R. The Ehrlich Pathway for Fusel Alcohol Production: A Century of Research on *Saccharomyces cerevisiae* Metabolism. *Appl. Environ. Microbiol.* **2008**, *74*, 3920. [CrossRef]
89. Bloem, A.; Sanchez, I.; Dequin, S.; Camarasa, C. Metabolic Impact of Redox Cofactor Perturbations on the Formation of Aroma Compounds in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **2016**, *82*, 174–183. [CrossRef] [PubMed]

90. Saerens, S.M.G.; Delvaux, F.; Verstrepen, K.J.; Van Dijck, P.; Thevelein, J.M.; Delvaux, F.R. Parameters Affecting Ethyl Ester Production by *Saccharomyces cerevisiae* during Fermentation. *Appl. Environ. Microbiol.* **2008**, *74*, 454–461. [CrossRef] [PubMed]
91. Lilly, M.; Bauer, F.F.; Lambrechts, M.G.; Swiegers, J.H.; Cozzolino, D.; Pretorius, I.S. The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. *Yeast* **2006**, *23*, 641–659. [CrossRef]
92. Miyake, T.; Shibamoto, T. Quantitative analysis of acetaldehyde in foods and beverages. *J. Agric. Food Chem.* **1993**, *41*, 1968–1970. [CrossRef]
93. Guth, H. Identification of Character Impact Odorants of Different White Wine Varieties. *J. Agric. Food Chem.* **1997**, *45*, 3022–3026. [CrossRef]
94. Ferreira, V.; Ortín, N.; Escudero, A.; López, R.; Cacho, J. Chemical Characterization of the Aroma of Grenache Rosé Wines: Aroma Extract Dilution Analysis, Quantitative Determination, and Sensory Reconstitution Studies. *J. Agric. Food Chem.* **2002**, *50*, 4048–4054. [CrossRef]
95. Lopez, R.; Ortín, N.; Pérez-Trujillo, J.P.; Cacho, J.; Ferreira, V. Impact Odorants of Different Young White Wines from the Canary Islands. *J. Agric. Food Chem.* **2003**, *51*, 3419–3425. [CrossRef]
96. Peinado, R.A.; Moreno, J.; Bueno, J.E.; Moreno, J.A.; Mauricio, J.C.G. Comparative study of aromatic compounds in two young white wines subjected to pre-fermentative cryomaceration. *Food Chem.* **2004**, *84*, 585–590. [CrossRef]
97. Moreno, J.A.; Zea, L.; Moyano, L.; Medina, M. Aroma compounds as markers of the changes in sherry wines subjected to biological ageing. *Food Control* **2005**, *16*, 333–338. [CrossRef]
98. Peng, C.-T.; Wen, Y.; Tao, Y.-S.; Lan, Y.-Y. Modulating the Formation of Meili Wine Aroma by Prefermentative Freezing Process. *J. Agric. Food Chem.* **2013**, *61*, 1542–1553. [CrossRef] [PubMed]