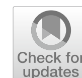
Food and Bioprocess Technology
https://doi.org/10.1007/s11947-023-03108-y

RESEARCH



Approaching Study on the Relationship Between *Saccharomyces cerevisiae* Production of Tyrosol, Hydroxytyrosol, and Melatonin with Volatile Compounds in Fermented Must

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Received: 30 January 2023 / Accepted: 19 April 2023
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Abstract

Yeasts are feasible and effective bioreactors and, therefore, there is a great interest in their industrial employment for the production of a wide range of molecules. In this study, the production by *Saccharomyces cerevisiae* of bioactive compounds such as hydroxytyrosol (HT), tyrosol (TYR) and melatonin (MEL) vs. volatile compounds in fermented must was studied. The concentration of the bioactive compounds HT and MEL in fermented must employing different yeast strains revealed that the higher the concentrations, the lower the amount of volatile compounds determined. This inverse correlation was especially remarkable with respect to the production of higher alcohols, especially 2-phenylethanol (2-PE) and esters. Furthermore, the employment of a modified Aro4p^{K229L} *S. cerevisiae* QA23 yeast strain which overproduces HT, gave rise to fermented must also higher in 2-PE and their corresponding esters but with an outstanding less presence of other important esters such as ethyl hexanoate and ethyl octanoate. Both premises could point out that *S. cerevisiae* might have different approaches to handling cell stress/toxicity due to their nitrogen metabolism. One detoxifying pathway could be through the production of higher alcohols and these in turn to esters and the other be more related to synthesizing antioxidant molecules such as MEL and HT.

Keywords *S. cerevisiae* · Fermentation · Tyrosol · Hydroxytyrosol · Melatonin · Volatile compounds

Introduction

Currently, the industrial employment of yeast to produce a wide range of molecules is booming because they are feasible and effective bioreactors, leading to efficient production performance (Pretorius, 2020). Some of the applications include the production of biodiesel, perfume ingredients, pharmaceuticals, enzymes, pigments, plastic, lubricants, and other bio-based chemicals, etc. (Bettencourt et al., 2020; Diamantopoulou et al., 2020; Franco-Duarte et al., 2017; Huang & Demirci, 2009; Mantzouridou & Paraskevopoulou, 2013; Mata-Gómez et al., 2014; Perpetuini et al., 2020; Vandermies & Fickers, 2019; Zhang et al., 2017). The main primary metabolite from the alcoholic fermentation of a sugar substrate with *Saccharomyces cerevisiae* is ethanol; however, the molecules derived from the secondary metabolism of the yeast, specifically from nitrogen metabolisms such as aroma and bioactive compounds are of special interest to the industry (Cordente et al., 2019).

Regarding volatile compounds, depending on the yeast employed, the volatile composition may vary in a huge extent, being one of the main factors responsible for the aroma of a wine (Garde-Cerdán & Ancín-Azpilicueta, 2006; Varela et al., 2009). Moreover, the interaction of some yeasts with other types has been described to influence on the volatile compounds production (Kosel et al., 2017). In addition, it has been demonstrated that the volatile compounds produced by the yeast are strongly linked to the amino acids

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Published online: 25 May 2023

composition of the substrate and to the amount of yeast available nitrogen (YAN) in the media (Rapp & Versini, 1995; Hernández-Orte et al., 2002, 2005; Garde-Cerdán & Ancín-Azpilicueta, 2008), or even the temperature of fermentation (Morakul et al., 2013) among other factors. Therefore, the elucidation of the clues to modulate yeast aromatic amino acid metabolism is the subject of several research works (Dickinson et al., 2003; Hazelwood et al., 2008). Moreover, a useful system for monitoring fermentative aromas has established a strong relationship between nitrogen sources and the higher alcohols and esters production along winemaking fermentation (Mouret et al., 2014). Apart from these volatile compounds, among the higher alcohols with importance from an organoleptic perspective is tyrosol (TYR), also produced by the yeast, being published evidence of the in-mouth sensory properties related to wine bitterness (Sáenz-Navajas et al., 2012). The origin of higher alcohols by *S. cerevisiae* derives from α-ketoacids generated either through the anabolic (de novo synthesis from sugar) (Fig. 1a) or the catabolic (from amino acids present in the media-via Ehrlich) pathways (Fig. 1b) (Mas et al., 2014). Some studies have observed that the addition of the corresponding precursor amino acid may yield the specific higher alcohol in increased concentrations (Hernández-Orte et al., 2002, 2005; Garde-Cerdán & Ancín-Azpilicueta, 2008; Bordiga et al., 2016).

Conversely, by using isotopically ^{13}C-labeled amino acid precursors (Crépin et al., 2017; Rollero et al., 2017) and glucose (Nisbet et al., 2014), it has been shown that the vast majority of the higher alcohols are formed through the anabolic pathway. On the other hand, *S. cerevisiae* prefers ammonium and glutamine as nitrogen sources but in conditions of depletion of these nitrogen sources, the yeast can use amino acids through the Ehrlich pathway (Hazelwood et al., 2008) yielding acids, aldehydes, and higher alcohols which subsequently can give rise to esters, mainly with acetate (Cordente et al., 2021). At the same time, it has been suggested that the production of higher alcohols and their corresponding esters could be a result of a detoxifying mechanism used by the yeast to diminish aldehydes and medium-chain fatty acids (MCFAs) (Borrull et al., 2015; Boulton et al., 1995; Saerens et al., 2010).

In addition to the synthesis of volatile compounds, other molecules with bioactive potential are generated by the metabolism of *S. cerevisiae,* among them are melatonin (MEL) and hydroxytyrosol (HT) (Álvarez-Fernández et al., 2019a). MEL has been proposed as being a signaling molecule (Fernandez-Cruz et al., 2019; Morcillo-Parra et al., 2019) and also has been shown to possess remarkable antioxidant, neuroprotective, anti-inflammatory and cardiovascular protection properties in humans (Reiter et al., 2007; Sanchez-Barcelo et al., 2012). The secondary metabolism may also yield HT, which is produced from TYR. Hence, HT may be produced by yeast following the Ehrlich pathway from the tyrosine present in the fermentation media or from glucose (anabolic pathway) (Gallardo-Fernández et al., 2022) being therefore closely related to the synthesis of higher alcohols. Certain health benefits, such as cardioprotection and neuroprotection, have been reported for its precursor TYR (Gris et al., 2011; Samuel et al., 2008), but the positive health effects of HT are much more remarkable (Gallardo-Fernández et al., 2022).

The employment of yeast, both as cell factories for the production of valuable volatile and bioactive compounds and as fermentative microorganisms to produce foods with enhanced organoleptic properties as well as a convenient bioactive profile, is feasible and profitable. For this purpose, genetic engineering techniques have been employed for

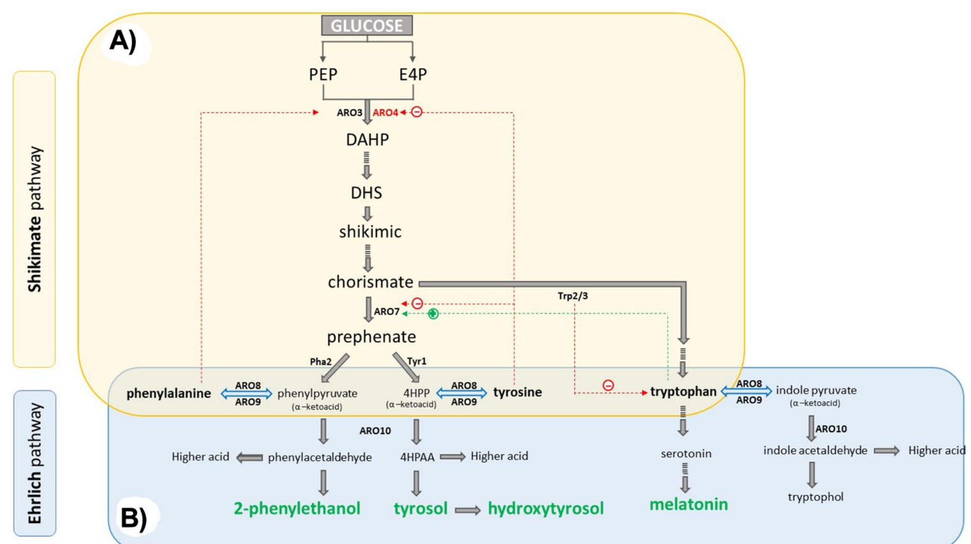

Fig. 1 Shikimate (**A**) and Ehrlich (**B**) pathways for the production of tyrosol, hydroxytyrosol, melatonin and 2-phenylethanol. This figure is adapted from Cordente et al. (2019) and Bisquert et al. (2022)

example for the sobreexpresion of those genes responsible for the synthesis of HT (Bisquert et al., 2022). However, the potential for the production of these compounds from the secondary metabolism of a very common yeast such as *S. cerevisiae* has been scarcely explored. Several research addressing the characterization of the volatile compounds produced by different yeast strains have been published (Albanese et al., 2013; Ivanova et al., 2013; Morakul et al., 2013; Mouret et al., 2014); however, these studies do not approach simultaneous production of bioactive compounds and the influence and relationship with aromatic metabolites as the present work analyzes. Very recently, Cordente et al. (2021) have pointed out the relation between 2-PE and the production of TYR in synthetic and natural grape must, showing that those genetically modified yeast strains able to overproduce 2-PE, are also capable to generate increased concentrations of TYR.

Therefore, the aims of this study were to evaluate the simultaneous production of volatile compounds and the bioactive compounds melatonin, tyrosol, and hydroxytyrosol of some commonly used *S. cerevisiae* yeast strains trying to ascertain the relationship between aroma and bioactive composition and also the influence of the initial must composition. At the same time, the production of these compounds by a modified *S. cerevisiae* HT overproductive yeast strain was tested with the purpose of ascertaining if an increased production of the alcohols TYR and HT has an impact on the aroma composition of the fermented must.

Materials and Methods

Chemical Standards

Standards of tyrosol, hydroxytyrosol, and melatonin were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade methanol was supplied from Merck (Darmstadt, Germany) and formic acid was provided by Prolabo (Obregon, Mexico). 4-Methyl-2-pentanol was used as the internal standard for the gas chromatography determinations and was provided by Merck (Darmstadt Germany). An alkane standard mixture C_{10}–C_{40} purchased by Fluka (Madrid, Spain) was used for calculating the Linear Retention Index (LRI) for GC–MS analysis.

Fermentation Trials

All the samples analyzed in this study are samples at the end of the fermentation. The first set of fermentations was performed employing different commercial *S. cerevisiae* Uvaferm VRB (UVR), Enoferm M2 (EM2), P23 Lallemand (P23), and a yeast from Murviedro winery (MUW). The fermentations were carried out in glass bottles and they took place as described in Table 1. Each strain was inoculated from a culture grown overnight in YPD in their stationary phase. Fermentations were performed in triplicate. To control the development of the fermentations, 1.5 mL of sample was extracted at 24 h, 48 h and 120 h to measure the density using a Densito 30PX densimeter and the OD600nm with a UVmini-12 spectrophotometer. At the end of the fermentation, the amount of residual sugars and the percentage of ethanol were measured (Pérez et al., 2021). A total of 12 samples (4 yeast × 3 biological replicates) were centrifuged 5 min at 4000 rpm to separate the cells, and the supernatant was stored at −20 °C prior to the analysis.

The second trial of fermentations employing a commercial wine yeast strain of *S. cerevisiae* Lalvin YSEO QA23 (Lallemand) and its genetically modified yeast was performed. The genetic modifications carried out on QA23 were multiple integration of HpaBC complex under the control of strong constitutive promoters TEF1p and PGK1p into the genome using Ty1Cons2 sequences as homologous recombination targets, and the single integration of allelic variant ARO4K229L under the control of GPD promoter in chromosome X-3. Such modifications were performed according to previous works (Bisquert et al., 2022; Muñiz-Calvo et al., 2020). Briefly, genes hpaB and hpaC and bidirectional promoter TEF1-PGK1 were PCR-amplified from plasmids p426GPD-hpaB, p425GPD-hpaC and pCfB2628 respectively (Germann et al., 2016), while ARO4K229L with GPD promoter was amplified from plasmid p423GPD-ARO4*. In parallel, vectors bearing the Ty1Cons2 and X-3 sequence from the EasyCloneMulti and EasyClone vector set pCfB2988 and pCfB257 (Jensen et al., 2014; Maury et al., 2016), were prepared by sequential treatment with enzymes AsiSI (SfaAI) (Thermo Fisher Scientific, Waltham, MA, USA) and BsmI (New England Biolabs, Ipswich, MA, USA). After purification, PCR products were cloned into pre-treated vectors by USERTM method (New England Biolabs). The ligation product was transformed into *E. coli* and successful cloning of both vectors was verified by Sanger sequencing (EUROFINS genomics, Ebersberg, Germany).

Table 1 Conditions of the two different fermentation trials

	Non-modified/commercial strains trial	Modified QA23 strain trial
Temperature	28	25
Agitation	150 rpm	
Volume	80 mL	750 mL
YAN	300 mg/L	200 mg/L
Cells/mL	2×10^6 cells/mL	10^6 cells/mL
SM employed	Riou et al. (1997)	

YAN yeast assimilable nitrogen, *SM* synthetic must

Prior to yeast transformation, the resulting integrative vectors pCfB2988, HpaBC and pCfB257 were linearized by FastDigest NotI (Thermo scientific, Vilnius, Lithuania) and the fragment containing the desired sequences to integrate were purified from agarose gel. Yeast cells were transformed with 1–1.5 μg of the linear fragment from the integrative vectors by the PEG/LiAc method according to Gietz (2014) and selected on selective agar medium according to strain auxotrophic markers.

The fermentations were also carried out following Riou et al. (1997) specifications for synthetic must with some modifications as described: 10 L of SM was prepared for experiment with slight differences following Rebollo-Romero et al. (2020). Sugars: fructose (100 g/L) and glucose (100 g/L); NH_4Cl (0.46 g/L); acids: malic acid (5 g/L), citric acid (0.5 g/L) and tartaric acid (3 g/L); the minerals added were: K_2SO_4 (0.5 g/L), KH_2PO_4 (0.75 g/L), $MgSO_4$ $7H_2O$ (0.25 g/L), $CaCl_2$ (0.155 g/L) and NaCl (0.2 g/L). Also it was added to the SM 1 mL of trace elements was added including 13.09 mL of an amino acids solution composed by isoleucine (2.5 g/L), tyrosine (1.5 g/L), tryptophan (13.4 g/L), threonine (5.8 g/L), aspartic acid (3.4 g/L), glutamic acid (9.2 g/L), leucine (3.7 g/L), glycine (1.4 g/L), histidine (2.6 g/L), glutamine (38.4 g/L), alanine (11.2 g/L), valine (3.4 g/L), methionine (2.4 g/L), cysteine (1.6 g/L), phenylalanine (2.9 g/L), arginine (28.3 g/L), serine (6 g/L), lysine (1.3 g/L), and proline (46.1 g/L). Finally to complete this SM 10 mL of a vitamins solution containing myoinositol (2 g/L), thiamine hydrochloride (0.025 g/L), calcium pantothenate (0.15 g/L), pyridoxine (0.025 g/L), nicotinic acid (0.2 g/L) and biotin (3 mL). The pH was adjusted to 3.31 with NaOH. The final YAN was 210 mg/L.

Six replicate fermentation experiments were used for each strain with the fermentation conditions showed in Table 1. The flasks were weighed daily before and after sampling, in order to monitor the fermentation. The optical density was also measured daily by (Cytoflex S, Beckman Coulter, California, EEUU). The samples of final fermented musts resulting from the fermentations were stored at −80 °C, before the analysis in UHPLC-HRMS and GC–MS.

Tyrosol, Hydroxytyrosol, and Melatonin Extraction

Before the analysis, samples were cleaned up using SPE C18 cartridges (Variant, Agilent). The first step was the conditioning of the cartridge using 2 mL of methanol and sequentially 2 mL of Milli-Q water. Once the cartridge is conditioned, 2 mL of the sample was passed through the cartridge. Then 2 mL of a 10% v/v methanol/water solution was used to wash it.

The compounds of interest were eluted with 1 mL of methanol. A vacuum concentrator (HyperVAC-LITE, Gyrozen, Korea) was used at 2000 rpm at 30 °C for 8 h, to dry samples as previously described (Álvarez-Fernández et al., 2019b). Once the samples were dried, they were reconstituted with 200 μL of 10% v/v acetonitrile/water in the case of hydroxytyrosol and tyrosol and 10% v/v methanol/water with 0.1% formic acid for melatonin, and they were stored at −80 °C until analysis.

UHPLC-HRMS Analyses

A Thermo Scientific Liquid Chromatography system consisting of a binary UHPLC Dionex Ultimate 3000 RS connected to a quadrupole-orbitrap Qexactive hybrid mass spectrometer (ThermoFisher Scientific, USA) with HESI ionization probe (HESI-II) was used for the determination of the bioactive compounds tyrosol, hydroxytyrosol and melatonin. A Zorbax SB-C18 (2.1 × 100 mm, 1.8 μm particle size) column (Agilent) with a flow of 0.4 mL/min for hydroxytyrosol and tyrosol and 0.5 mL/min for melatonin. The chromatographic conditions for melatonin consisted of two mobile phases, water (A) and methanol (B), both with 0.1% formic acid with a gradient elution programmed as follows: 95% A, 5% B (0–2 min); 0% A, 100% B (2–13 min); and 95% A, 5% B (13.1–15 min). The flow selected was 0.5 mL min^{-1}. The gradient for hydroxytyrosol and tyrosol consists of (A) water with 0.2% of acetic acid and (B) acetonitrile. The binary gradient was used with the following profile: 0–1 min 5% B, 1–7 min from 5 to 100% B, 7–8.5 min 100% B, and 8.5–10 min 5% B. 5 μL was the volume elected for the injection. The compounds were dissolved in 10% v/v acetonitrile/water.

A method of parallel reaction monitoring (PRM) was used in a negative mode with a resolution of 35,000 at m/z 200 FWHM and an isolation window of 1 m/z and the normalized collision energy was set at 40 eV. The masses were optimized as follows: tyrosol (137.06080) and hydroxytyrosol (153.05572). HESI source parameters were spray voltage, 3.0 kV; S lens level, 50; capillary temperature, 320 °C; sheath, auxiliary, and sweep gas flow, 50, 15, and 2 respectively (arbitrary units); and probe heater temperature, 425 °C.

For melatonin, the positive mode was used. The following parameters were optimized to carry out the analysis: HESI source parameters were as follows: source voltage 3.5 kV; tube lens voltage 50 V; capillary temperature 230 °C; source heater temperature 440 °C; sheath, auxiliary and sweep gas flow rate (N2) 53, 14 and 3. The masses were optimized as follows for melatonin (233.12845). The data were treated with the TraceFinder 5.1 software.

SPME/GC/MS Analyses

Volatile compounds' extraction was performed using Headspace Solid Phase Microextraction (HS-SPME), employing

a triple fiber of 1 cm DVB (divinylbenzene)/C-WR (carbon wide range)/PDMS (polydimethylsiloxane) (Agilent Technologies, Switzerland). For this purpose, 4 mL of each sample was transferred into 20 mL glass vials with 0.8 g of sodium chloride and 10 µL of 4-methyl-2-pentanol (0.75 mg/L) (used as an internal standard), which were then placed in the autosampler.

For the static headspace extraction, incubation lasted 40 min at 45 °C and 250 rpm agitation speed, and the fiber was then exposed to the headspace for 40 min, with a penetration into the vial of 22 mm. Once the adsorption finished, the fiber was desorbed for 180 s in the injector using the splitless mode.

For the analyses of the samples, a Bruker 450 Gas Chromatograph was employed coupled to a Mass Spectrometer Bruker 300-MS. For the chomatographic analysis, the conditions followed are described in Ubeda et al. (2019).

For identification purposes, linear retention indices (LRIs) were calculated after the injection of C_{10}-C_{40} alkanes solution by applying the same conditions of sample analysis. The identification was made by matching the LRIs of each compound from the standards NIST library (2.0 version)

Table 2 Volatile compounds determined after alcoholic fermentation of ***Uvaferm® VRB***, ***Enoferm® M2***, ***P23 Lallemand*** and ***Murviedro*** winery yeast of the synthetic must

Compound	LRI	Qion	ID	Uvaferm® VRB	Enoferm® M2	P23 Lallemand	Murviedro winery
Esters							
Ethyl acetate	**890**	43	A	70.843 ± 14.826[a]	96.937 ± 25.443[ab]	120.828 ± 35.555[b]	84.381 ± 16.648[ab]
Isoamyl acetate	**1100**	43	A	3.706 ± 0.439[a]	10.067 ± 1.305[ab]	13.324 ± 5.763[b]	27.525 ± 7.412[c]
Ethyl octanoate	**1433**	88	A	17.909 ± 2.494[b]	6.947 ± 4.656[a]	18.939 ± 3.939[b]	18.298 ± 9.077[b]
Ethyl nonanoate	**1535**	88	A	0.112 ± 0.019	0.073 ± 0.025	0.115 ± 0.046	0.139 ± 0.102
Ethyl decanoate	**1638**	88	A	13.151 ± 5.605[a]	12.968 ± 3.623[a]	14.931 ± 4.186[a]	24.817 ± 5.844[b]
Gamma-butyrolactone	**1667**	42	A	0.112 ± 0.023[a]	0.562 ± 0.109[b]	0.815 ± 0.279[b]	0.859 ± 0.221[b]
Ethy-9-decenoate	**1687**	55	A	0.559 ± 0.117[b]	0.258 ± 0.202[a]	0.606 ± 0.154[b]	0.763 ± 0.048[b]
2-Phenylethyl acetate	**1831**	104	A	4.211 ± 1.253[a]	13.716 ± 0.694[bc]	12.687 ± 1.449[b]	15.413 ± 1.526[c]
2,2-Dimethyl-1-(2-hydroxy-1-methylethyl)propyl 2-Methylpropanoate	**1884**	71	A	1.057 ± 0.479	1.203 ± 0.101	1.679 ± 0.526	1.454 ± 0.129
Ethyl 3-hydroxytridecanoate	**1903**	117	A	0.051 ± 0.020[a]	0.118 ± 0.014[b]	0.191 ± 0.024[c]	0.082 ± 0.004[a]
Octyl octanoate	**2018**	55	A	0.036 ± 0.015	0.055 ± 0.004	0.062 ± 0.030	0.061 ± 0.011
Alcohols							
Isobutanol	**1071**	43	A	6.794 ± 2.672[a]	9.513 ± 3.247[a]	22.399 ± 4.524[b]	18.177 ± 1.039[b]
3-Methyl-1-butanol	**1247**	55	A	76.103 ± 11.585[a]	157.988 ± 23.123[b]	141.778 ± 15.416[b]	170.228 ± 30.359[b]
2-Nonanol	**1520**	45	A	0.324 ± 0.176[a]	0.460 ± 0.104[a]	0.488 ± 0.053[a]	1.121 ± 0.377[b]
2,3-Butanediol	**1568**	45	A	18.966 ± 6.935[a]	16.479 ± 7.082[a]	37.934 ± 11.796[b]	8.133 ± 1.591[a]
Methionol	**1743**	106	A	0.082 ± 0.035[a]	0.194 ± 0.031[ab]	0.321 ± 0.105[c]	0.235 ± 0.067[bc]
Decanol	**1776**	70	A	0.187 ± 0.072[ab]	0.129 ± 0.035[a]	0.263 ± 0.026[ab]	0.324 ± 0.171[b]
2-Phenylethanol	**1935**	91	A	43.676 ± 8.136[a]	143.284 ± 15.285[b]	129.773 ± 18.784[b]	118.421 ± 14.324[b]
Dodecanol	**1983**	55	A	0.849 ± 0.220[ab]	0.764 ± 0.238[a]	1.306 ± 0.226[b]	1.315 ± 0.374[b]
Acids							
Acetic acid	**1458**	43	A	49.808 ± 26.266[a]	50.581 ± 7.305[a]	82.482 ± 15.164[b]	69.293 ± 3.815[ab]
2-Methyl propanoic acid	**1589**	43	A	0.514 ± 0.190[a]	0.923 ± 0.139[b]	0.523 ± 0.233[a]	0.705 ± 0.100[ab]
4-Methyl pentanoic acid	**1681**	57	B	0.119 ± 0.039[a]	0.435 ± 0.059[b]	0.201 ± 0.064[a]	0.403 ± 0.101[b]
Hexanoic acid	**1862**	60	A	1.863 ± 0.226[a]	4.442 ± 0.479[c]	3.024 ± 0.301[b]	3.762 ± 0.885[bc]
Octanoic acid	**2063**	60	A	7.463 ± 1.514[ab]	6.162 ± 0.118[a]	11.267 ± 0.973[b]	10.020 ± 4.223[ab]
Decanoic acid	**2228**	60	A	3.675 ± 1.183[ab]	2.863 ± 0.738[a]	4.364 ± 0.851[ab]	5.398 ± 1.858[b]
Aldehydes							
2,4-Decadienal	**1826**	81	A	0.013 ± 0.002[a]	0.015 ± 0.004[ab]	0.020 ± 0.001[b]	0.019 ± 0.004[ab]
2,5-Dimethylbenzaldehyde	**1837**	134	B	0.095 ± 0.018[a]	0.097 ± 0.022[ab]	0.153 ± 0.027[b]	0.154 ± 0.047[b]

Values are expressed in relative areas. Values with different superscript letters indicate statistically significant differences ($p < 0.05$) LSDFisher test

LRI linear retention index, *Qion* major ion employed for quantitation, *ID* reliability of identification: A, mass spectrum agreed with NIST mass spectral database and LRI agreed with the literature data (Pherobase: www.pherobase.com; NIST Mass Spectrometry Data Center: https://webbook.nist.gov/; Pubchem: https://pubchem.ncbi.nlm.nih.gov/); B, mass spectrum agreed with NIST mass spectral database

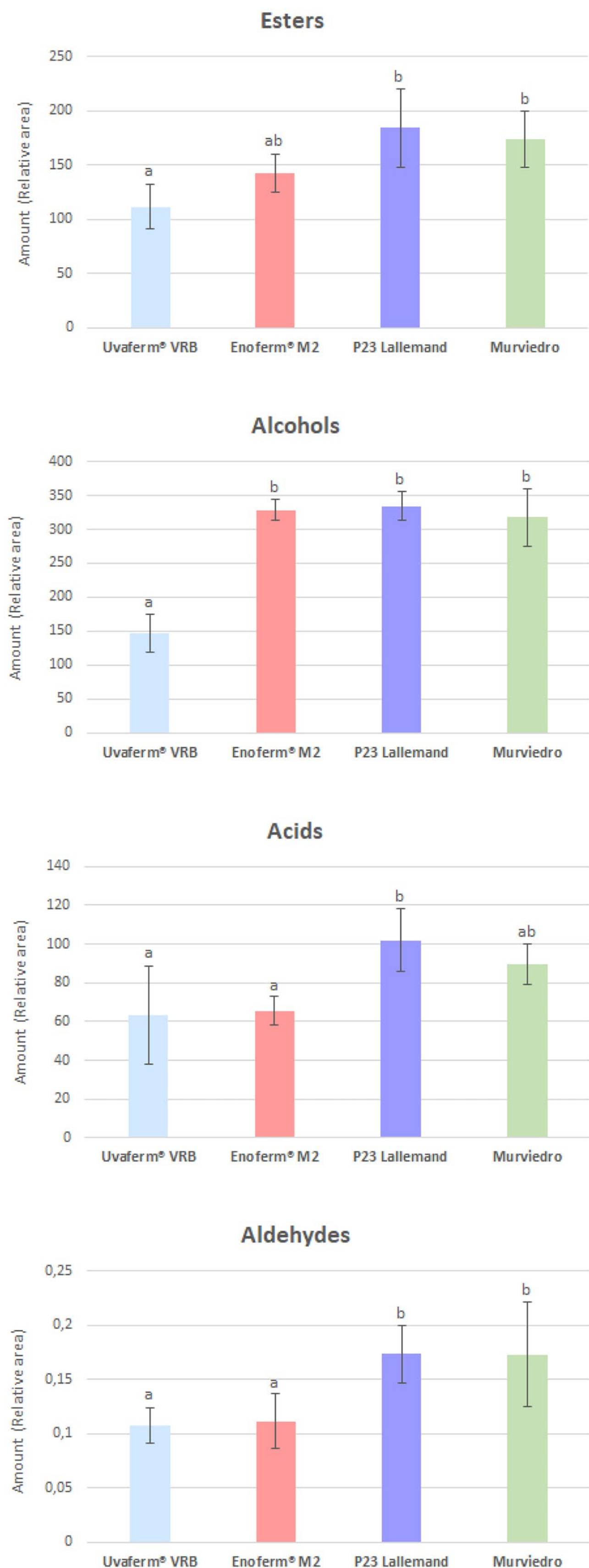

Fig. 2 Total amounts of esters, alcohols, acids and aldehydes present in the fermented must produced with every *Uvaferm*® *VRB*, *Enoferm*® *M2*, *P23 Lallemand* and *Murviedro* winery yeast. Bars with different superscript letters indicate statistically significant differences ($p < 0.05$) by Fisher Least Significant Difference (LSD) among the samples

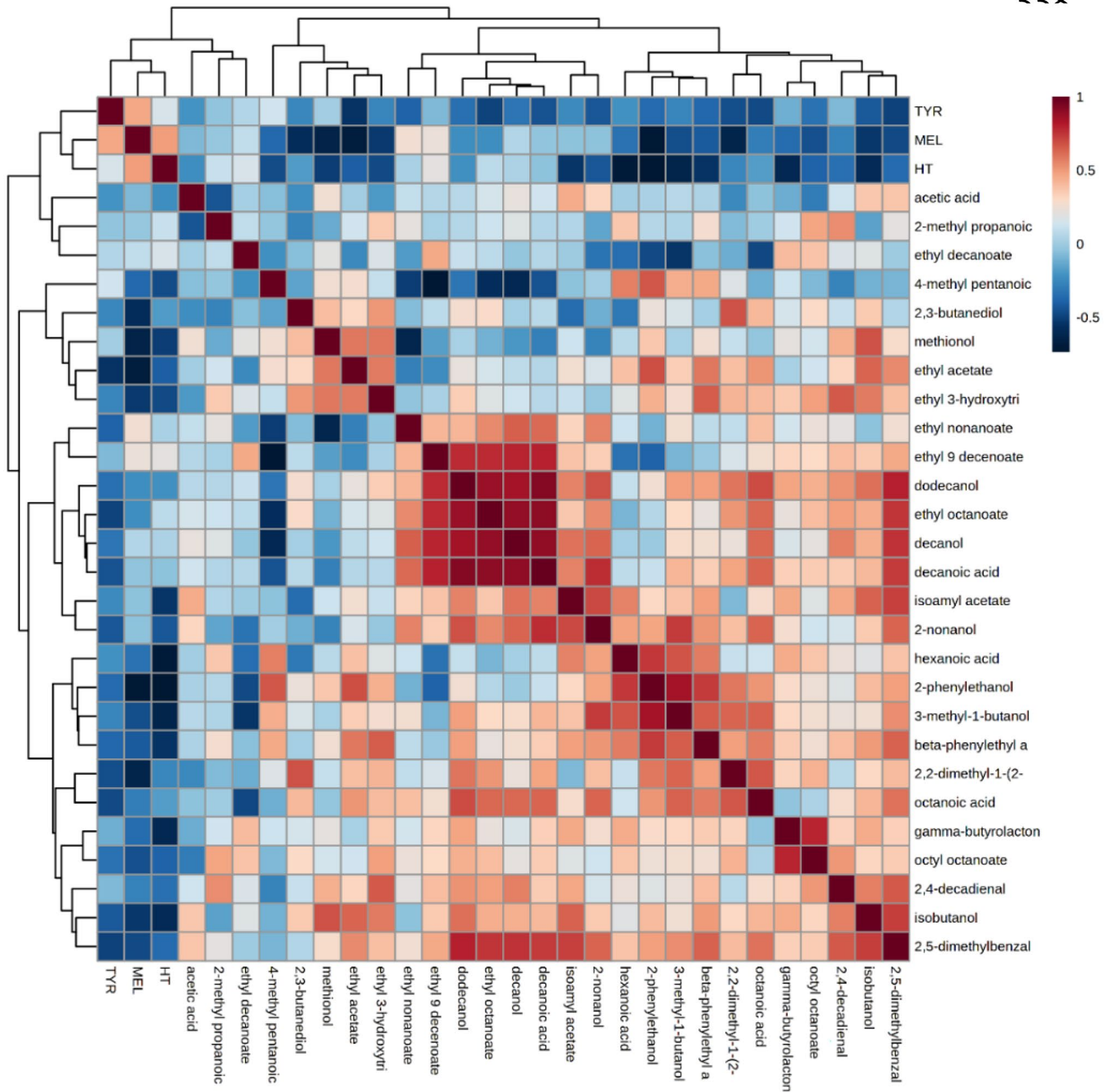

Fig. 3 Heatmap visualization based on the 27 volatile compounds (variables) in the fermented must produced with every *Uvaferm*® *VRB*, *Enoferm*® *M2*, *P23 Lallemand* and *Murviedro* winery yeast

found in the literature (Pherobase: www.pherobase.com; NIST Mass Spectrometry Data Center: (LRI Odour database: www.odour.org.uk; accessed on March 2022). The data shown in this work were expressed as the relative area with respect to 4-methyl-2-pentanol (internal standard). The relative areas were calculated by dividing the peak area of the target ion of each compound by the peak area of the target ion of the internal standard.

Statistical Analysis

Data obtained were compared using ANOVA and Fisher Least Significant Difference (LSD) Method ($p < 0.05$) employing the InfoStat software (version 2017p, FCA-Universidad Nacional de Córdoba, Argentina). Principal component analysis (PCA) was performed using IBM SPSS Statistics 26 software (IBM, Barcelona, Spain) and Partial

least squares-discriminant analysis (PLS-DA) with the 5.0 version of Metaboanalyst software (Quebec, Canada).

Results and Discussion

Volatile Compounds, Tyrosol, Hydroxytyrosol, and Melatonin Production by Yeast

Samples obtained at the end of the alcoholic fermentations performed with the yeast strains UVR, EM2, P23 and MUW were analyzed for aroma composition. A total of 27 volatile compounds were determined, 11 esters, 8 alcohols, 6 acids, and 2 aldehydes (Table 2). Figure 2 shows tendencies related to the total sum of the relative areas corresponding to each chemical group. Strains EM2, P23 and MUW produced significantly higher amounts of alcohols. Acids and aldehydes followed a similar pattern, with UVR and EM2 reaching lower quantities at the end of fermentation vs P23 and MUW. As shown in Table 2, UVR presented in general the lowest quantities of higher alcohols, especially regarding isobutanol, 3-methyl-1-butanol, and 2-PE compared to the other yeast assayed. This is in agreement with the results from Peris et al. (2016) who employed Uvaferm VRB among other yeast strains to ferment a Macabeo grape must showing a low production of higher alcohols in comparison to the quantities produced by other yeast.

Our results showed that esters (ethyl and acetate) were in fact present in significantly higher quantities in P23 and MUW than in UVR trials at the end of the fermentation (Fig. 2). While the production of ethyl esters during fermentation occurs via the enzymatic esterification reaction of ethanol and the carboxylic acids present in the media (Lambrechts & Pretorius, 2000), acetate esters are formed by enzymatic acetylation of alcohols, this last being mainly by-products of the amino acid biosynthesis such as 3-methyl-1-butanol or 2-PE (Sumby et al., 2010). Although samples EM2, MUW, and P23 reached higher amounts of ethyl acetate at the end of the fermentation as compared to UVR, with P23 reflecting significance (Table 2). Apart from this predominant ethyl ester, the acetate esters, isoamyl acetate and 2-phenylethyl acetate, stood out among all the ester family. Interestingly, they were present in the sample at levels at least three times lower (Table 2). The correlation between the formation of acetate esters and their corresponding higher alcohol has been described in wines (Cordente et al., 2012) and as can be observed, those fermented musts with a higher quantity of higher alcohols, also presented higher amounts of acetate esters. Regarding acids and aldehydes, UVR and EM2 showed similar abundance, the same happened for P23 and MUW (Fig. 2).

A heatmap was built with results on aroma composition and bioactive compounds (Fig. 3), thus allowing a global insight of data. As can be observed, there is an inverse correlation between HT and MEL and the volatile compounds as a whole. Furthermore, it was observed that TYR concentrations from the strains EM2 and MUW were higher as compared to P23 and UVR (Table 3). UVR resulted in being the lowest producer of tyrosol but the highest producer of HT, probably having a more efficient ability to hydroxylate TYR to HT.

Multivariate analysis by Principal Component Analysis (PCA) was performed employing all the secondary metabolites considered (volatile compounds, TYR, HT, and MEL). Hence, six principal components (PCs) were extracted explaining 93.3% of the total variance. Figure 4a shows the distribution of the samples (scores) in the plot of PC1 (Component 1) and PC2 (Component 2) accounting for 58.5% of the cumulative variance. The corresponding loadings distribution (Fig. 4b) clearly showed that the strain UVR gave rise to fermented musts with a lower content of volatile compounds but higher content of MEL and HT. On the other hand, samples EM2, P23 and MUW were richer in volatile compounds but their concentrations in HT and MEL were lower than those found in UVR. As mentioned above, TYR concentrations were higher in EM2 samples (Table 3); therefore, it is located in the plot closer to the fermented musts.

Additionally, a Partial Least-Squares Discriminant Analysis (PLS-DA) was performed to bring out the variables that are more useful to classify the samples thus having discriminant potential. Variable importance in the projection (VIP) score plot shows the major metabolite features

Table 3 Tyrosol, hydroxytyrosol and melatonin determined after alcoholic fermentation of the synthetic must by ***Uvaferm® VRB***, ***Enoferm® M2***, ***P23 Lallemand, Murviedro*** winery yeast and original **Lalvin QA23** yeast and **Lalvin QA23 modified** yeast

	Non-modified/commercial strains trial				Modified QA23 strain trial	
	Uvaferm® VRB	**Enoferm® M2**	**P23 Lallemand**	**Murviedro winery**	**Lalvin QA23™**	**Lalvin QA23 modified**
Tyrosol	0.0667 ± 0.0261[c]	0.1579 ± 0.0304[ab]	0.0748 ± 0.0096[a]	0.1269 ± 0.0187[bc]	5.3641 ± 0.6722	37.3478 ± 3.9013*
Hydroxytyrosol	0.0065 ± 0.0022[b]	0.0026 ± 0.0006[a]	0.0023 ± 0.0003[a]	0.0028 ± 0.0009[a]	0.0005 ± 0.0001	0.8793 ± 0.1315*
Melatonin	0.0758 ± 0.0111	0.0598 ± 0.0067	0.0533 ± 0.0017	0.0715 ± 0.0026	-	-

Different superscript letters indicate statistically significant differences ($p < 0.05$) by LSD test among the samples of non-modified strains trial and the asterisk indicates the statistically significant difference between the original QA23 and modified yeast strain trial

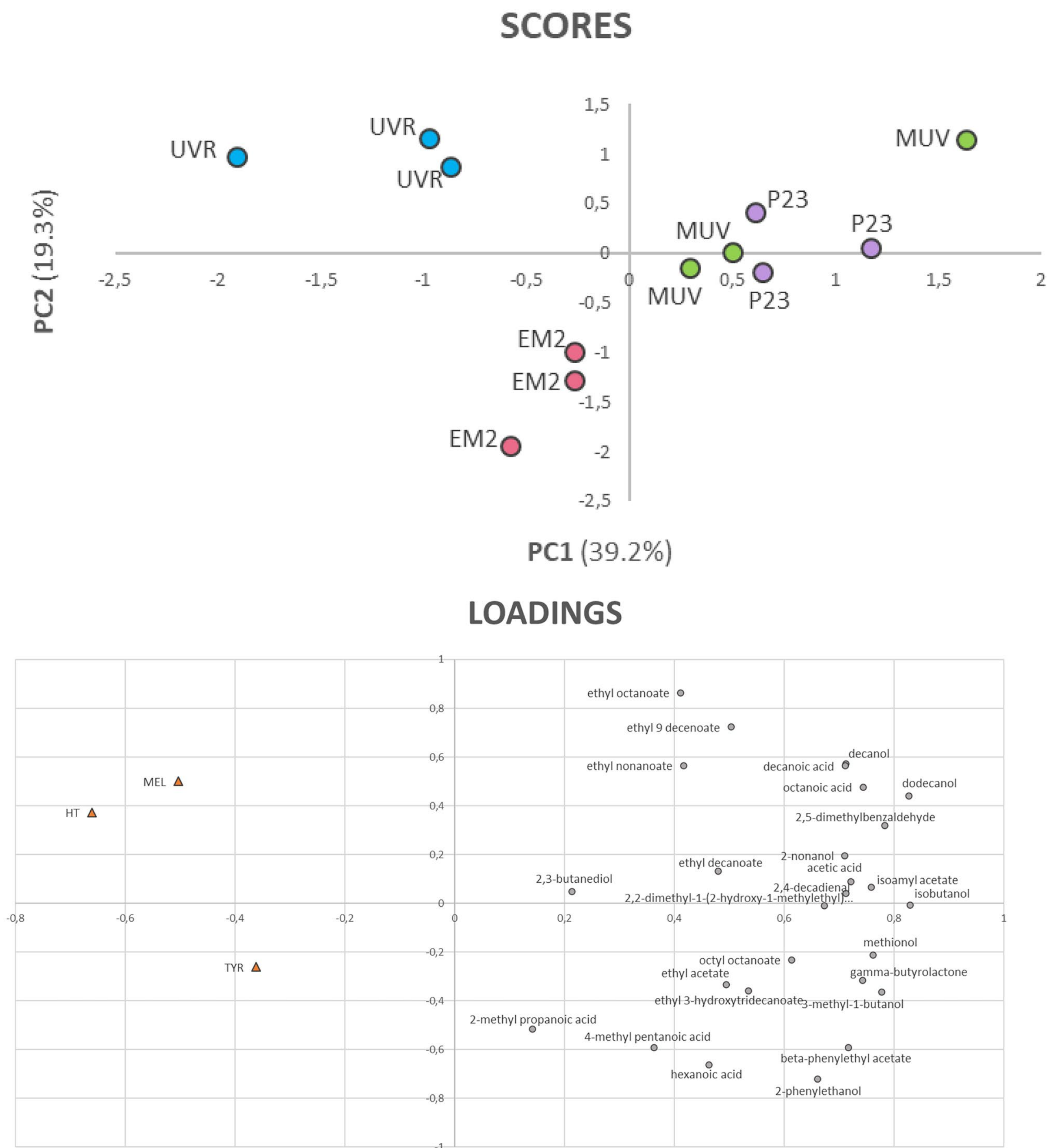

Fig. 4 Principal Component Analysis (PCA) showing the data scores (**a**) and loading (**b**) biplot on the plane of the first two principal components (PC1 against PC2) including the 27 volatile compounds, tyrosol, hydroxytyrosol, and melatonin present in *Uvaferm*® *VRB*, *Enoferm*® *M2*, *P23 Lallemand* and *Murviedro* winery yeast

(Fig. 5). Among the secondary metabolites analyzed, hexanoic acid was found the best one to classify the samples. This medium-chain fatty acid (MCFA) is produced by the yeast during alcoholic fermentation and depends on several factors such as yeast strain and temperature of fermentation (Torija et al., 2003; Tronchoni et al., 2012). In general, the overpresence of MCFAs can inhibit the growth of *S. cerevisiae* (Baroň et al., 2017). Specifically, several works have reported the strong relation of hexanoic acid with nitrogen amount in the media both in synthetic must and wine, and also a

remarkable association with the yeast strain (Barbosa et al., 2009; Bianchi et al., 2023; Carrau et al., 2008; Csutoras et al., 2022; Ugliano et al., 2010). This could indicate that given the same nitrogen in the must, the different yeast strains tested in this study have metabolized it at different rates.

Also among the most relevant VIP scores (above 1) were HT, 2-PE, 2-phenyl ethyl acetate, 3-methyl-1-butanol, and isoamyl acetate. Among them, the difference in the amounts of 2-PE found in the different fermented samples was the most remarkable (Table 2). As previously noticed, 2-PE is an exception among the higher alcohols since it presents a pleasant scent of rose/honey which is considered a positive attribute in wine, beer and cider (Hirst & Richter, 2016) partially responsible for the floral aroma of these food matrices. In addition, the esters and aldehydes synthesized from 2-PE can be important odorants of food matrices such as wine (Li et al., 2008; San-Juan et al., 2011). As mentioned above, the production of higher alcohols and their corresponding esters could be employed by the yeast to detoxify from the aldehydes and MCFAs (Saerens et al., 2010). Hereby, the difference in the production of volatile compounds and HT might partially be the result of divergent strategies of the yeast to detoxify, which would depend on the microorganism itself.

Thus, the capacity of conversion of TYR to HT could be more feasible for certain yeast, and for other yeast not achievable in the same extent. On the other hand, there might be yeast employing Ehrlich pathway for detoxification as quoted by Cordente et al. (2019) which in turn would explain the increased content of higher alcohols and esters in these samples.

It could be also possible that the production of any of them is being enhanced through the anabolic pathway. Also, considering that the consumption preferences of the different *S. cerevisiae* yeast are diverse (Scott et al., 2021), the strains with a preference for tyrosine, could create more 4-hydroxyphenylpyruvate (4-HPP) deriving in TYR and in a minor extent HT. Likewise, yeast with a phenylalanine preference would give rise to phenylpyruvate and therefore to increased 2-PE. Apart from HT, MEL was also located just below 1 in the VIP scores (Fig. 5), highlighting the importance of this compound in the classification of the samples under study. Similar trends were observed for HT and MEL which could be partially justified due to the activation of Aro 7. Figure 1 shows that after the condensation of two glucose-derived metabolites erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) in the anabolic pathway, some enzymatic reactions occur leading to chorismate which can form tryptophan or be converted by Aro7 into prephenate and in 4-HPP straightaway (Fig. 1). Therefore it would be plausible that the presence of tryptophan and its consumption by the yeast would generate, on the one hand, MEL, and on the other, the tryptophan in the media could activate Aro7 by tryptophan activating the pathway of HT synthesis. Despite the interesting correlation with melatonin, the most potent negative correlation was clearly stated for the case of HT.

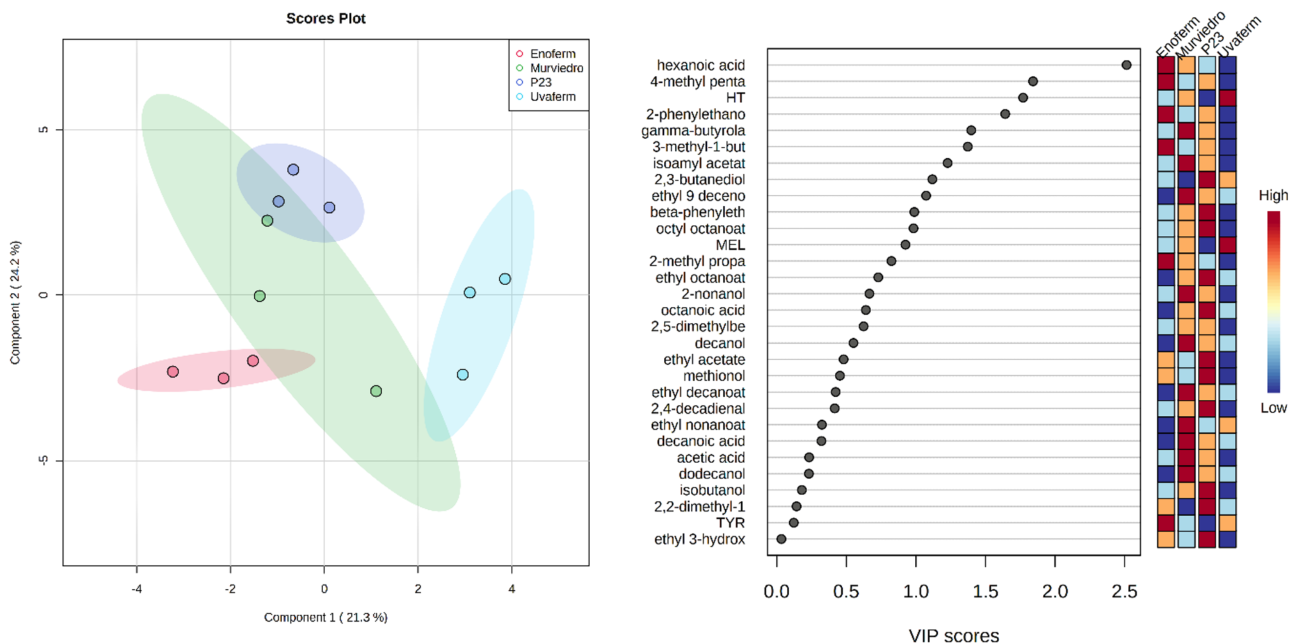

Fig. 5 Partial least squares-discriminant analysis (PLS-DA) including volatile compounds, tyrosol, hydroxytyrosol and melatonin with their corresponding variable importance in the projection (VIP) scores

Despite the lack of studies analyzing HT, TYR, and MEL together with volatile compounds produced by non-Saccharomyces yeast strains, and considering that previous reports have pointed out that these strains produce lower concentrations of HT than Saccharomyces (Rebollo-Romero et al., 2020), it would be expectable for non-Saccharomyces to contribute to the synthetic must composition with a remarkable amount of volatile compounds.

Table 4 Volatile compounds determined after alcoholic fermentation of the synthetic must employing the original **Lalvin QA23** yeast and **Lalvin QA23 modified** yeast

Compound	LRI	Qion	ID	Lalvin QA23™	Lalvin QA23 modified
Esters					
Ethyl acetate	**890**	43	A	31.297 ± 2.040	28.009 ± 1.563
Isoamyl acetate	**1100**	43	A	9.273 ± 0.924	6.705 ± 0.070*
Ethyl hexanoate	**1257**	88	A	45.872 ± 7.296	7.140 ± 1.857*
Ethyl heptanoate	**1336**	88	A	0.191 ± 0.033	0.215 ± 0.060
Hexyl formate	**1342**	56	A	0.556 ± 0.072	0.354 ± 0.034*
Ethyl octanoate	**1433**	88	A	86.674 ± 15.842	50.751 ± 1.363*
Heptyl formate	**1451**	70	B	0.617 ± 0.168	1.336 ± 0.222*
Isoamyl octanoate	**1659**	70	A	0.677 ± 0.09	2.113 ± 0.398*
Ethyl 9-decenoate	**1687**	88	A	1.088 ± 0.147	1.050 ± 0.418
2-Phenylethyl acetate	**1831**	91	A	2.019 ± 0.462	10.184 ± 0.590*
ethyl dodecanoate	**1867**	88	A	8.210 ± 0.863	14.161 ± 0.737*
2-Phenyl ethyl propionate	**1891**	104	A	1.470 ± 0.461	10.045 ± 0.678*
Isoamyl laurate	**2142**	70	B	1.824 ± 0.129	3.203 ± 0.608*
Ethyl-2-phenylethyl butylphosphonate	**2165**	104	B	11.859 ± 7.317	44.112 ± 5.713*
Ethyl hexadecanoate	**2249**	88	A	0.274 ± 0.045	0.563 ± 0.108*
Ethyl 9-hexadecenoate	**2265**	55	A	0.300 ± 0.013	0.691 ± 0.162*
Alcohols					
Isobutanol	**1071**	43	A	18.594 ± 1.305	59.000 ± 3.104*
3-Methyl-1-butanol	**1247**	55	A	106.178 ± 29.416	134.210 ± 16.791
4-Heptanol	**1395**	55	B	0.067 ± 0.003	0.126 ± 0.040
3-Ethoxy-1-propanol	**1398**	31	A	0.269 ± 0.031	0.805 ± 0.182*
2-Octanol	**1419**	45	A	1.190 ± 0.256	0.979 ± 0.062
2-Propyl-1-pentanol	**1480**	57	A	11.458 ± 4.588	21.180 ± 8.427
2-Ethyl-1-hexanol	**1482**	57	A	27.136 ± 6.617	13.943 ± 7.122
2-Nonanol	**1520**	45	A	0.514 ± 0.070	1.423 ± 0.206*
2,3-Butanediol	**1568**	45	A	2.362 ± 0.899	6.371 ± 1.736*
Undecanol	**1810**	55	A	0.158 ± 0.031	0.173 ± 0.015
2-Phenylethanol	**1935**	91	A	222.623 ± 55.713	1767.675 ± 135.003*
Hexadecanol	**2162**	55	B	0.074 ± 0.011	0.114 ± 0.018*
Acids					
Acetic acid	**1458**	43	A	12.352 ± 4.296	19.618 ± 2.568
2-Methoxy propanoic acid	**1589**	59	A	0.043 ± 0.014	0.068 ± 0.030
2-Methyl hexanoic acid	**1697**	74	B	2.458 ± 1.324	2.518 ± 0.841
Hexanoic acid	**1862**	60	A	4.933 ± 1.093	12.154 ± 0.873*
Nonanoic acid	**2216**	60	A	1.491 ± 0.125	1.866 ± 0.074*
Ketones					
2-Nonanone	**1401**	43	A	0.893 ± 0.201	2.048 ± 0.120*
2-Undecanone	**1608**	58	A	0.505 ± 0.088	0.816 ± 0.071*

Values are expressed in relative areas. Values with different superscript letters indicate statistically significant differences ($p < 0.05$) LSDFisher test

LRI linear retention index, *Qion* major ion employed for quantitation, *ID* reliability of identification: A, mass spectrum agreed with NIST mass spectral database and LRI agreed with the literature data (Pherobase: www.pherobase.com; NIST Mass Spectrometry Data Center: https://webbook.nist.gov/; Pubchem: https://pubchem.ncbi.nlm.nih.gov/); B, mass spectrum agreed with NIST mass spectral database

Analysis of Volatile Compounds, Tyrosol, and Hydroxytyrosol from Mutated QA23 Yeast

Considering the significant negative correlation between the production of HT by the yeasts tested and the total content in volatile compounds, fermentations were performed employing a usual yeast, QA23 strain, and an HT-overproducing QA23 yeast strain obtained with the integration of multiple copies of the gene ARO4 with point mutation K229L (Bisquert et al., 2022; Muñiz-Calvo et al., 2020). ARO4 is one of the initializing enzymes of the anabolic pathway and the sobreexpresion of modified Aro4p^{K229L} results in tyrosine-insensitive enzymes (Cordente et al., 2018) being the synthesis to TYR continuous (Fig. 1). Thus, the production of TYR and therefore HT was significantly higher and the modification resulted to be successful. This had been observed by Cordente et al. (2018) in fermented must with yeast strains with the ARO4 mutation with point mutation Q166R. They observed that the fermented must with this yeast yielded a higher concentration of TYR in comparison with the other yeast tested.

Regarding the production of volatile compounds, a total of 35 volatile compounds were determined: 16 esters, 12 alcohols, 5 acids, and 2 ketones (Table 4). The biotechnological modification caused significant changes in 23 of the 35 volatile compounds, only keeping 12 of them without significant changes as compared with QA23 strain. Noteworthy, the modified yeast QA23 produced up to 8 times more of 2-PE than the original yeast. This was expected since, as explained above, the inhibitory effect of tyrosine is annulled and thus the pathway to the intermediate prephenate was continuous. Therefore, the production of 2-PE was promoted (Fig. 1). The increased amount of 2-PE in turn resulted in higher content of its derivatives in QA23 modified yeast as compared with the original QA23. This was the case of 2-phenyl ethyl acetate, 2-phenyl ethyl propionate and ethyl-2-phenylethyl butylphosphonate which were present in the fermented must at significantly higher amounts in the modified QA23 yeast than in the original. Conversely, two ethyl esters such as ethyl hexanoate and ethyl octanoate were found in significantly higher quantities in the must fermented by the original QA23 yeast. The concentrations found for these compounds in wines usually exceed their perception thresholds and are described as impact odorants of different types of wines contributing to the fruity nuances of the beverage (Gómez-Míguez et al., 2007; Li et al., 2008; Marcq & Schieberle, 2021). Remarkably, the genetic modification did not affect the amount of ethyl acetate in the media, agreeing with the results obtained by Cordente et al. (2018). Revisiting the above-mentioned detoxification hypothesis, it may be that original QA23 and mutated QA23 yeast have different strategies to avoid oxidation. On the one hand, the original QA23 yeast could extend its life expectancy through esterification, considering that MCFAs and ethanol are toxic to yeast and compromise their survival. On the other hand, the QA23 modified, could be using the hyperproduction of HT and its antioxidant properties to reduce the oxidation and, therefore, fewer esters be produced. This hypothesis would also agree with the significantly lower amount of hexanoic acid found in the original QA23 yeast (Table 4).

Conclusions

For the first time, a detailed analysis of volatile compounds and their relation to the production of TYR, HT, and MEL in several *S. cerevisiae* strains has been performed. The final concentration of the bioactive compounds HT and MEL in fermented must employing different yeast strains studied revealed that the higher the concentrations, the lower the amount of volatile compounds determined. This inverse correlation was especially remarkable with respect to the production of higher alcohols and esters. The employment of a modified QA23 yeast strain with sobreexpresion of modified Aro4p^{K229L} which overproduces HT gave rise to a fermented must also higher in 2-PE and their corresponding esters but with an outstanding less presence of other important esters such as ethyl hexanoate and ethyl octanoate. Both premises could point out that *Saccharomyces cerevisiae* might have different approaches to handling cell stress/toxicity due to nitrogen metabolism. Some strains could be more easily detoxified through the production of higher alcohols and these in turn to esters and others have the necessary tools to convert these higher alcohols into antioxidant molecules. In any case, the nitrogenous metabolism of each strain studied has been shown to be very different between strains and to be extremely linked to the production of volatile compounds. These results could be useful with regard to the production of different food-fermented products, mainly beverages, and the use of yeasts as bioreactors for the production of bioactive and volatile compounds. Thus, the determination of the implication of certain genes in the production of antioxidant molecules and volatile compounds can have a direct application to generate improved yeast for the production of these high-value molecules or for the selection of the most proper yeasts to obtain wine with high aromatic intensity.

Acknowledgements The authors want to thank the VI PPIT-US for the pre-doctoral grant of M.Gonzalez-Ramirez and C. Ubeda's current contract (USE-18644-Z).

Author Contribution Marina Gonzalez-Ramirez performed the analyses of TYR, HT and MEL; Mar Marin Torres performed the analyses of volatile compounds; Marta Gallardo-Fernandez carried out fermentations of modified yeast; Andres Planells-Carcel executed fermentations of commercial yeast strains; Ricardo Bisquert developed and accomplished the genetic modifications of QA23 yeast; Eva Valero performed the fermentations design; Cristina Ubeda carried out data analysis, prepared figures and manuscript preparation; Ana Maria Troncoso contributed to the work conceptualization, figures and manuscript preparation; Maria Carmen Garcia-Parrilla carried out work conceptualization and manuscript preparation. All authors reviewed the manuscript.

Funding Funding for open access publishing: Universidad de Sevilla/CBUA. This work has been funded with a project from Ministerio de Ciencia, Innovación y Universidades (PID2019-108722RB-C32), a project from Junta de Andalucía-Consejería de Economía y Conocimiento (P18-RT-3098) and Universidad de Sevilla (US-1263469).

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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