Generation of intra- and interspecific *Saccharomyces* hybrids with improved oenological and aromatic properties

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Summary

Non-wine yeasts could enhance the aroma and organoleptic profile of wines. However, compared to wine strains, they have specific intolerances to winemaking conditions. To solve this problem, we generated intra- and interspecific hybrids using a non-GMO technique (rare-mating) in which non-wine strains of *S. uvarum*, *S. kudriavzevii* and *S. cerevisiae species* were crossed with a wine *S. cerevisiae* yeast. The hybrid that inherited the wine yeast mitochondrial showed better fermentation capacities, whereas hybrids carrying the non-wine strain mitotype reduced ethanol levels and increased glycerol, 2,3-butanediol and organic acid production. Moreover, all the hybrids produced several fruity and floral aromas compared to the wine yeast: β-phenylethyl acetate, isobutyl acetate, γ-octalactone, ethyl cinnamate in both varietal wines. *Sc × Sk* crosses produced three- to sixfold higher polyfunctional mercaptans, 4-mercapto-4-methylpentan-2-one (4MMP) and 3-mercaptohexanol (3MH). We proposed that the exceptional 3MH release observed in an *S. cerevisiae × S. kudriavzevii* hybrid was due to the cleavage of the non-volatile glutathione precursor (Glt-3MH) to detoxify the cell from the presence of methylglyoxal, a compound related to the high glycerol yield reached by this hybrid. In conclusion, hybrid generation allows us to obtain aromatically improved yeasts concerning their wine parent. In addition, they reduced ethanol and increased organic acids yields, which counteracts climate change effect on grapes.

Introduction

Today, the role of yeasts in winemaking is no longer only focused on producing ethanol but on specific objectives such as providing aroma complexity, reducing ethanol, increasing acidity and others. As a result, the research and selection of yeasts have been extended to other species and genera different from the wine yeasts *Saccharomyces cerevisiae*. Many interesting yeasts of non-*Saccharomyces* genera have been selected, fulfilling the objectives mentioned above (Gobbi *et al.*, 2013; Varela *et al.*, 2016; Ravasio *et al.*, 2018; Oliveira and Ferreira, 2019). However, due to their low competitiveness and poor winemaking tolerance, co-inoculation or sequential fermentation with *S. cerevisiae* wine yeasts are crucial to complete the fermentation.

Within *Saccharomyces* genus, yeasts of cryophilic species and non-wine *S. cerevisiae* yeasts also have the ability to decrease alcohol yield, increase acidity and enhance fruity fermentative and varietal aromas in wines (Gamero *et al.*, 2011a; Oliveira *et al.*, 2014; Stribny *et al.*, 2015). In addition, cryotolerant species, such as *S. kudriavzevii* and *S. uvarum*, can grow and ferment at low temperatures, thus favouring wine aroma retention. Moreover, these attributes can mitigate the effects of climate change on grapes and fulfil new consumer demands (Querol *et al.*, 2018).

These wild *Saccharomyces* strains also show poor competitive ability under different fermentation environments (Arroyo-López *et al.*, 2010; Alonso-del-Real *et al.*, 2017; Origone *et al.*, 2017; Su *et al.*, 2019a). Consequently, as for non-*Saccharomyces* yeasts, their

application as pure culture starters at the industrial level is somehow restricted (Deroite *et al.*, 2018). However, despite these limitations, many studies have found natural hybrid yeasts derived from these wild strains having enhanced winemaking tolerance (Belloch *et al.*, 2008; Gangl *et al.*, 2009; Gamero *et al.*, 2011b; da Silva *et al.*, 2015; Ortiz-Tovar *et al.*, 2019). The presence of natural hybrids in fermentative and wild environments is relatively frequent and results from adapting to the prevailing environmental conditions (González *et al.*, 2006; Peris *et al.*, 2018). Thus, this process originates organisms (intra- and interspecific) with inherited physiological properties from both parents, resulting in improved oenological characteristics (González *et al.*, 2007).

Therefore, the use of hybrid yeasts that overcome some wild strain limitations is more practical for the wine industry than the mixed culture strategies required with non-*Saccharomyces* yeasts. In addition, the resulting hybrids can cover a wide range of oenological objectives. For example, *S. cerevisiae* × *S. uvarum* hybrids have been generated with increased ethanol tolerance, high glycerol synthesis and increased capacity to release volatile thiols (Masneuf *et al.*, 2002; Lairón-Peris *et al.*, 2020). Likewise, *S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. eubayanus* hybrids with improved fermentative capacity at low temperatures and under low nitrogen conditions have been developed (Su *et al.*, 2019b). Commercial wine yeasts have also been improved by generating *S. cerevisiae* × *S. cerevisiae* hybrids (Pérez-Través *et al.*, 2015).

Hybrid yeasts can be generated by different techniques, including the so-called rare-mating. Besides being a non-genetic modified organism (GMO) and easy-to-use method, rare-mating results in the formation of organisms with sufficient genetic information from both parental yeasts (Schillberg *et al.*, 1991; Pérez-Través *et al.*, 2012).

In our previous studies, wild yeasts having attractive oenological traits for Tempranillo and Albariño wines have been characterized. In this regard, a wild *S. uvarum* strain producing a significant number of high-impact aromas in Tempranillo wines compared to a wine *S. cerevisiae* strain was found. This *S. uvarum* strain also developed Albariño wines of great oenological interest, releasing typical varietal aromas. Besides the acidity provided by its highest succinic acid production, it synthesized the highest concentration of fruity linear and branched ethyl esters and showed a significant ability to release varietal polyfunctional mercaptans (Pérez *et al.*, 2022b).

Moreover, we found two *S. kudriavzevii* strains, isolated from natural niches, that revealed a remarkable capacity to release high amounts of polyfunctional mercaptans (PMFs), mainly 4-methyl-4-mercaptopentan-2-one (4MMP) and 3-mercaptohexanol (3MH) in Albariño wines (Pérez *et al.*, 2022b). This aromatic potential, together with the cryophilic aptitude of these *S. kudriavzevii* yeasts, suggests their suitability for fermentation of white grape varieties such as Sauvignon Blanc. Additionally, among young Tempranillo wines, one of these *S. kudriavzevii* strains showed the highest yields of fruity ethyl esters. Futhermore, a cachaça *S. cerevisiae* strain developed, in Tempranillo wines, significant C_{13}-norisoprenoid and vanillin derivative levels (Pérez *et al.*, 2022a).

These wild yeasts described above would be ideal for obtaining wines following the current demands of wine consumers. However, their application under wine conditions and as a pure culture also requires certain aptitudes specific to wine-producing strains. The hybridization between *Saccharomyces* species by non-transgenic techniques can provide strains suitable for their industrial application. Therefore, the present work aims to generate non-GMO hybrid yeasts that inherit an optimal fermentative activity from wine yeasts and produce wines with improved oenological and aromatic characteristics inherited from wild strains.

Results

Hybrid obtention and molecular characterization of the stable hybrids

Four crosses were made using the wine yeast LALL as a common parental strain and four wild strains (Table 1), resulting in five different original hybrids (Table 2), one for each cross and two for the cross with KR strain.

According to the parental ploidy (Table 1) and DNA content of the resulting hybrids (Table 2), the rare-mating crosses were indeed made between two spores (n × n crosses) or between a spore and a diploid cell (n × $2n$ crosses), indicating the ability of all the parental strains to sporulate in these conditions.

Initial hybrids from KR (*S. kudriavzevii, Sk*) and CS (*S. cerevisiae, Sc*) parental yeasts inherited LALL mitochondrial DNA (mtDNA), whereas crosses with KA (*S. kudriavzevii*) and UE (*S. uvarum, Su*) parental yeasts presented a mixture of mtDNA profiles. Each original HKA and HUE hybrid derived in two different stable profiles that differed in their mtDNA (HKA4, HKA5, HUE2 and HUE5), which were again subjected to stabilization. In contrast, HKRI, HKRII and HCS original hybrids maintained the same stable profile after the stabilization process naming them HKR1, HKR8 and HCS3. For this reason, seven stable hybrids were finally recovered from the four crosses made.

More precisely, mtDNA analysis revealed that *Sc* × *Sk* and *Sc* × *Sc* crosses mainly retained *Sc* LALL mtDNA except for the recombinant mitochondrial profile

Table 1. Genotype and phenotype characteristics of parental strains used.

Parental	Species	Code	Source	DNA content[a]	Aromas in Tempranillo[b]	Aromas in Albariño[b]	Oenological properties[b]
CR89D1	*S. kudriavzevii*	**KR**	oak (*Q. faginea*)	2.20 ± 0.002	neutral	3MHA, 4MMP,3MH, R-limonene	low ethanol, high glycerol
CA111F1	*S. kudriavzevii*	**KA**	oak (*Q. faginea*)	2.10 ± 0.006	branched ethyl esters	3MHA, 4MMP,3MH, R-limonene	low ethanol, high glycerol
CECT12600	*S. uvarum*	**UE**	Liquor	2.01 ± 0.053	geraniol, β-citronellol, α-ionone vanillin derivatives, PEA	3MHA, 4MMP,3MH, geraniol, PEA, branched ethyl esters	low ethanol, high glycerol, high succinic acid, low acetic acid
CSC1	*S. cerevisiae*	**CS**	Cachaça	0.99 ± 0.021	vanillin derivatives, ethyl leucate, monoterpenes, isobutyl acetate	neutral	neutral
LALL	*S. cerevisiae*	**LALL**	Wine commercial	1.98 ± 0.01	–	–	–

3MHA, 3-mercaptohexyl acetate; 4MMP, 4-mercapto-4-methylpentan- 2-one; 3MH, 3-mercaptohexanol, PEA, β-phenylethyl acetate.
a. DNA content values measured from two replicates (mean ± SD).
b. Data obtained in previous studies (Pérez *et al*., 2022a, 2022b).

Table 2. Molecular characterization of original and stable hybrids showing differential molecular profiles.

Crosses	Original hybrid	*DNA content (cross type)	mtDNA	δ-PCR	Stable hybrid	*DNA content	mtDNA	δ-PCR
LALL x KR (*Sc* × *Sk*)	**HKRI**	2.13 ± 0.022[a] (nxn)	LALL	1	**HKR1**	2.01 ± 0.002[b]	LALL	1
	HKRII	3.08 ± 0.026[a] (nx2n)	LALL	2	**HKR8**	2.95 ± 0.012[b]	LALL	2
LALL x KA (*Sc* × *Sk*)	**HKA**	2.03 ± 0.038 (nxn)	mixture	1	**HKA4**	1.81 ± 0.105	r1	1
					HKA5	2.07 ± 0.011	LALL	1
LALL x UE (*Sc* × *Su*)	**HUE**	2.86 ± 0.014(nx2n)	mixture	1	**HUE2**	2.87 ± 0.025	r1	1
					HUE5	2.83 ± 0.011	UE	1
LALL x CS (*Sc* × *Sc*)	**HCS**	3.02 ± 0.021[a] (nx2n)	LALL	1	**HCS3**	2.48 ± 0.008[b]	LALL	1

Sc, Saccharomyces cerevisiae; Sk, Saccharomyces kudriavzevii; Su, Saccharomyces uvarum, r1, recombinant mitochondrial genome. *DNA content values measured from two replicates (mean ± SD). According to ANOVA ($p < 0.05$) and Tukey's HSD test, superscript letters denote DNA content statistically different from the original hybrid. Molecular profiles were determined by mtDNA-RFLP analysis (Querol *et al*., 1992) and inter-δ sequence analysis (Legras and Karst, 2003).

(r1) found in the HKA4 stable hybrid. While *Sc* × *Su* crosses had the UE (*Su*) mtDNA and a recombinant mtDNA in each stable yeast. In addition, the delta pattern (δ-PCR) remained unchanged compared with their original hybrids. On the contrary, a DNA loss was observed, showing a statistically significant reduction ($P < 0.05$) concerning ploidy after stabilization in HCS3 (*Sc* × *Sc*) by 0.5 points, and to a lesser extent in the hybrids HKR1 and HKR8 (Table 2).

Characterization of fermentation capacity and metabolite production of hybrids

Albariño fermentations were carried out at 16°C by the two *Sc* × *Su* hybrids and the four *Sc* × *Sk* hybrids. Tempranillo fermentations were performed at 25°C with three hybrids: HSC3 (*Sc* × *Sc*), HUE5 (*Sc* × *Su),* and HKA5 (*Sc* × *Sk*). Additionally, the shared parental strain among all hybrids (wine *Sc* strain, LALL) was included as a control in both fermentation sets (Fig. 1). Fermentations were completed in 14 days (< 1 g l^{-1} sugar).

In Albariño fermentations, hybrids HUE2 and HUE5 (*Sc* × *Su*) and HKR8 (*Sc* × *Sk*) showed shorter lag phases than LALL. Despite starting fermentations earlier, their specific maximum rates (Vmax) were lower to LALL and HKR1. The latter hybrid, HKR1, showed the best performance, equal to LALL, while HKA4 was the slowest, with a more extended lag phase and the lowest maximum rate. However, HKA4 finished fermentation simultaneously as LLAL, while it took longer for HUE2 and HUE5 strains.

The Vmax was considerably higher in Tempranillo fermentations than Albariño, probably because of the higher fermentation temperature, resulting in a greater volume of CO_2 released in less time (Fig. 1). HCS3 and HKA4 hybrids displayed the best fermentation performances in these conditions. The latter strain, despite showing a longer lag phase at the beginning of fermentation, later reached the highest maximum fermentation rate, even higher than LALL, finishing the process without difficulty. HUE5 strain showed the slowest fermentation rates as in Albariño fermentations.
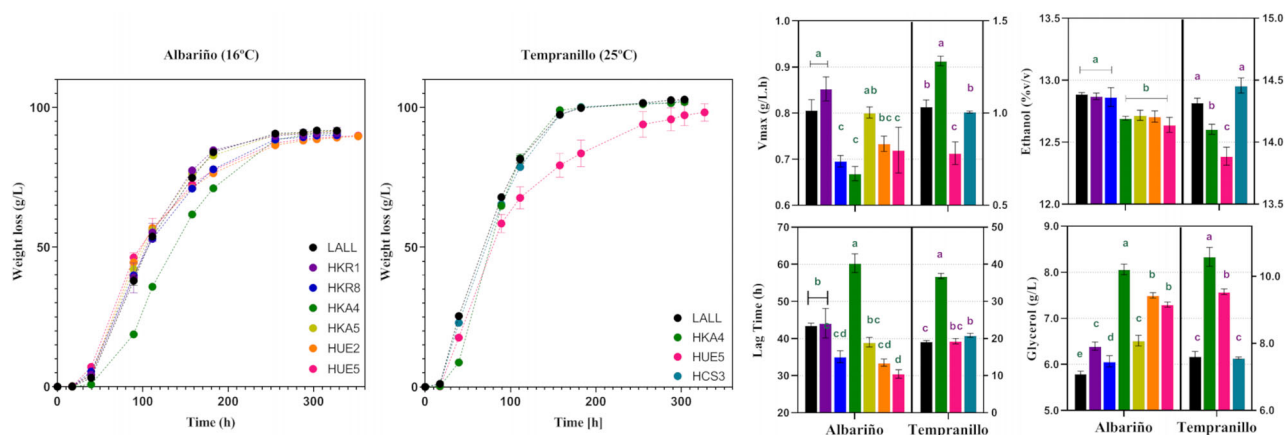
Fig. 1. Weight loss curves, kinetic fermentation parameters and primary metabolites (mean ± SD; *n* = 3) produced by hybrid yeasts and parental wine strain LALL during fermentation in semi-synthetic Albariño and Tempranillo musts at 16°C and 25°C respectively. Different letters in the same varietal grape indicate significant differences according to Tukey's HSD test (*P* < 0.05). The resulting weight loss curves were fitted to a non-linear regression mathematical Gompertz model (Zwietering *et al.*, 1990) adapted for fermentation parameters (maximum specific fermentation rate and Lag time) as previously described in Pérez *et al.* (2021).

In Albariño wines, an ethanol difference of 0.2% v/v was observed in HKA4, HKA5, HUE2 and HUE5 hybrids differing statistically from LALL, HKR1 and HKR8 strains (Fig. 1). For Tempranillo wines, the differences were more significant between the strains. HKA4 and HUE5 hybrids produced wines with 0.3% v/v and 0.48% v/v less ethanol than LALL and HCS3.

In the case of glycerol, HKA4 produced the highest amounts, reaching 8 g l^{-1} in Albariño and 10.5 g l^{-1} in Tempranillo (Fig. 1). *Sc* × *Su* hybrids also produced large amounts of glycerol (7.5 g l^{-1} in Albariño and 9.5 g l^{-1} in Tempranillo). HCS3, HKR1, HKR8, HKA5 and the parental *Sc* LALL strain reached the lowest glycerol values in both musts.

Regarding wine's organic acid content (Table S1), HKA4 strain exhibited the highest citric acid contents, although differing slightly. The two *Sc* × *Su* hybrids (HUE2 and HUE5) showed the highest malic acid values in Albariño and the highest succinic acid values in both varietal wines. However, it was also interesting to note the high amount of 2,3-butanediol determined in both young wines of the HKA4 hybrid.

Interestingly, the hybrids HKR1, HKR8, HCS3 and HKA5 having *Sc* LALL mtDNA, were the most similar to the wine yeast in terms of fermentation performance and by-products production. The rest of the hybrids with *Su* mtDNA (HUE5) or recombinant mtDNA (HKA4 and HUE2) reduced ethanol and increased organic acid yields, where HKA4 was the highest glycerol producer in both varietal wines.

Production of volatile compounds by hybrid strains

Aroma composition of Albariño wines. Of the 58 aroma compounds determined above detection limits, 37

showed statistical differences between strains (*P* < 0.05; Table S2) in Albariño wines. To estimate the potential sensorial impact of yeasts on Albariño wines, concentrations of volatiles considered active aroma vectors (Ferreira *et al.*, 2021) were normalized by their odour threshold (OT) and grouped according to a shared sensory descriptor (Table S3).

Therefore, a principal component analysis was applied to the significant compounds and their sensory descriptors associated according to the literature (Fig. 2A and B). In addition, the assembly of a dendrogram allowed a more general view of the differences between hybrids (Fig. 2C). The resulting PCA plot describes 64.3% of the total variance, separating strains into three main groups. PC1 (44.5%) showed the most significant separation between wines, clustering the *Sc* × *Su* hybrids, HUE2 and HUE5, on PCA's right quadrant. Indeed HUE2 and HUE5 were also separated from the rest by the highest dissimilarity in the dendrogram plot (Fig. 2C). Their wines were composed of most fermentative compounds, including branched acids and ethyl esters, β-phenylethanol and acetate, and two lactones, γ-butyrolactone and γ-octalactone. In contrast, the lowest values of 1-butanol and 4-methyl-4-mercaptopentan-2-one (4MMP) were found in their wines. Given the estimated OAVs, these wines would be sensorially characterized by the floral, spicy/woody and lactic/acidic descriptors (Fig. 2A).

The rest of the strains were mainly opposed to HUE2 and HUE5 due to the superior contents of PFMs. HKA4 wines were located in the upper left PCA's quadrant characterized by 3-mercaptohexanol (3MH) and the floral compounds ethyl cinnamate, linalool and β-citronellol. Due to the highest content in 3MH, their wines would be
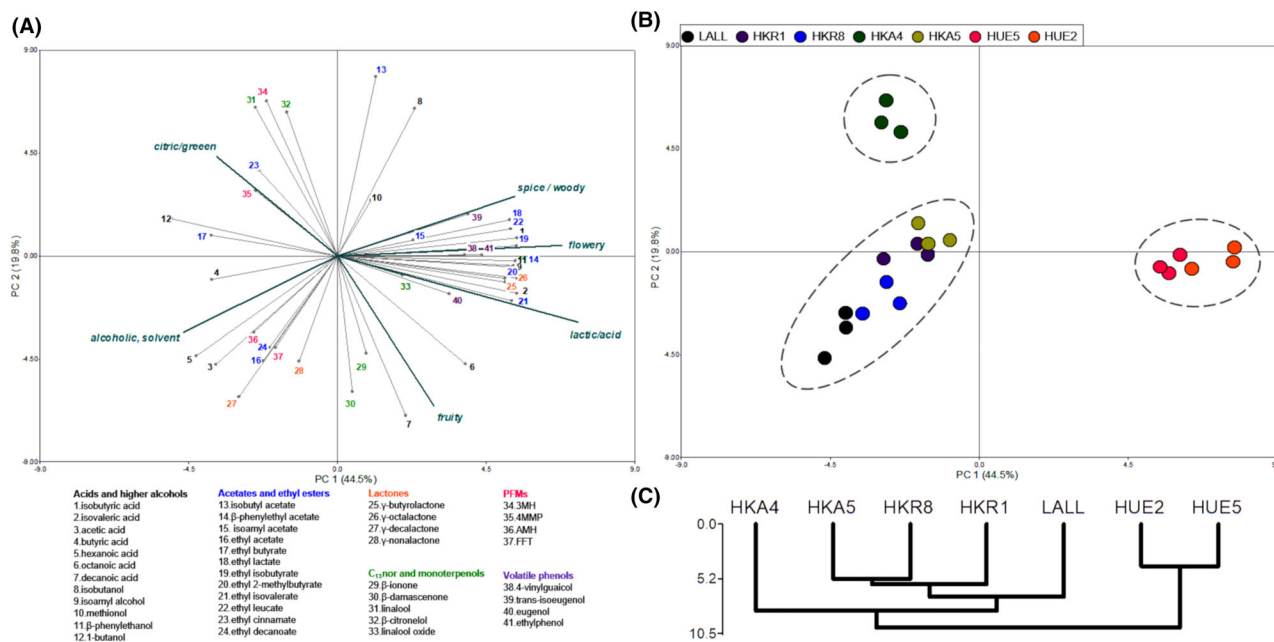
Fig. 2. Principal component analysis (A and B) and hierarchical clustering analysis (C) of the major and minor aroma compounds produced by yeast hybrids and parental wine strain LALL in Albariño wines. 3MH, 3-mercaptohexanol; 4MMP, 4-mercapto-4-methylpentan-2-one; 3MHA, 3-mercaptohexyl acetate; FFT, 2-furfurylthiol.

mainly characterized by the citric/green notes. LALL, HKR1 and HKR8 strains showed the highest acetic acid values, and their wines were identified by the most increased ethanol and ethyl acetate contents, involved in the alcoholic/solvent aroma vector. However, HKA5, positioned within this group but within the upper quadrant, shows shared aromatic characters with HKA4.

Finally, the parental yeast, LALL, had the highest values of hexanoic acid but the lowest acetate concentrations, and it was the only strain with detectable TDN and furfurylthiol amounts in the wines recently fermented.

In summary, in Albariño wines, *Sc* × *Su* hybrids were noted for their typical *S. uvarum* aromas, fruity and floral ethyl, and acetate esters, while the *Sc* × *Sk* hybrids were noted for their higher ability to release PFMs. However, HKR1 and HKR8 were the *Sc* × *Sk* hybrids most similar to LALL by the higher acetic acid content involved in the alcoholic/solvent aroma vector. It is worth noting that HKA4 (*Sc* × *Sk*) stood out for its high content in ethyl cinnamate and most varietal compounds, such as monoterpenes and PFMs.

Aroma composition of young and aged Tempranillo wines. In young Tempranillo wines, 42 out of 59 aroma compounds were found to have statistically different concentrations between strains. In contrast, in aged wines, only 16 of the 40 were statistically different (Tables S4 and S5). As in previous studies, the ageing application to wines (A) produced a significant aromatic

differentiation from the young wines (Y) as can be seen in Fig. 3A. Nevertheless, each strain's wines were still very different in each of the fermentation conditions, highlighting the great modulating effect of the yeasts. Regarding this effect, the HKA4 strain appeared to be the most different among young wines (Y:HKA4), while HUE5 yeast was for aged wines (A:HUE5). However, LALL and HCS3 yeasts remained together in both conditions, indicating that they share similar aroma profiles.

The 44 most relevant aromas determined in young Tempranillo wines showed 70.6% of the total variance among yeasts, as shown in the PCA (Fig. 3B), where PC1 (38.3%) separated the parental strain *S. cerevisiae* (LALL) and the *Sc* × *Sc* hybrid (HCS3) from the *S. cerevisiae* × non-*cerevisiae* hybrids HUE5 and HKA4. As in Albariño, isobutyl acetate was highly produced by the HKA4 strain, while HUE5 achieved the highest levels of isobutyric and isovaleric acids, the latter strain associated once more with the lactic/acidic vector. Furthermore, the highest γ-octalactone and β-phenylethyl acetate characterized these hybrids' young wines. This last compound, β-phenylethyl acetate, would be responsible for linking the flowery vector to the young wines of these hybrids.

However, LALL and HCS3 (only *Sc* genome background) were characterized by fruity compounds, such as ethyl octanoate, hexanoate, butyrate, isobutyrate and isoamyl acetate. However, the alcoholic/solvent aroma
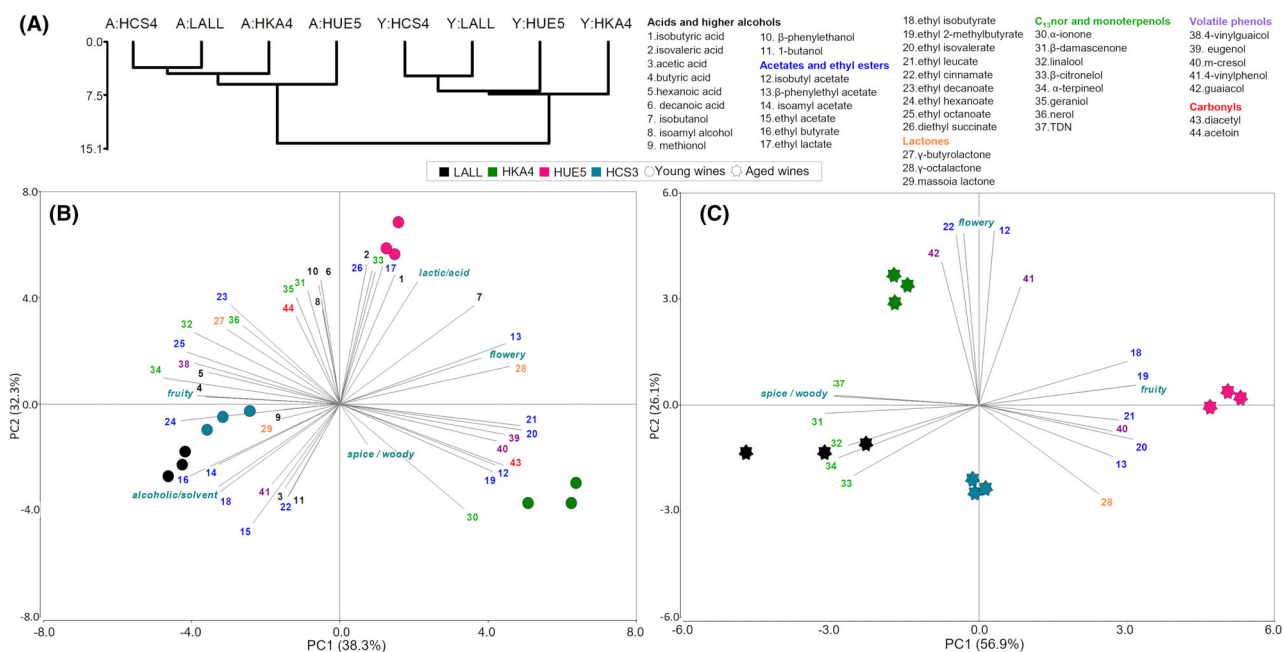
Fig. 3. Hierarchical clustering analysis (A) and principal component analysis (B and C) of major and minor aroma compounds produced by yeast hybrids and parental wine strain LALL in Tempranillo young wines ("Y") and in Tempranillo aged wines ("A").

vector would be characterizing their wines due to the highest yields in ethanol, ethyl acetate in LALL and 1-butanol and methionol in HCS3.

After accelerated ageing application, yeasts' sensory profiles changed (Fig. 3C). PC1 (56.9%) distinguished HUE5 from the rest of the yeasts; its wines were characterized by fruity volatiles. This hybrid, which in its young wines had a high content of branched fatty acids, now, due to their esterification during accelerated ageing process, the corresponding fruity ethyl esters reached the maximal concentrations. In this regard, concentrations of ethyl isobutyrate, ethyl isovalerate and ethyl 2-methylbutyrate increase 1267, 165 and 220-fold compared to its young wines (Tables S4 and S5). Likewise, ethyl cinnamate concentrations also increase after ageing, around 25-fold compared to young wines. This floral ethyl ester, highly produced by LALL in young wines, was now detected in aged HKA4 wines at the highest levels, 3.5-fold higher than the rest of the aged wines. In contrast, isobutyl and β-phenylethyl acetates decreased due to ageing.

The aged LALL wines were characterized by a higher content of some terpenes and C_{13}-norisoprenoids. Here, the most significant differences were found in β-damascenone and TDN, the latter conferring spice/woody notes was detected in higher amounts in aged LLAL wines. Finally, HCS3 and HUE5 aged wines had the highest γ-octalactone and ethyl leucate concentrations, where ethyl leucate values were close to

250 μg l^{-1} and 1.5-fold higher than LALL and HKA4 yeasts.

As a summary, in young Tempranillo wines, the *S. cerevisiae* × *S.* no-*cerevisiae* hybrids showed similar characteristics to Albariño wines, such as the highest contents of acetate esters, γ-octalactone and branched-chain fatty acids. However, when ageing was applied, these wines showed much more interesting profiles, with the highest yields of fruity esters in HUE5 and ethyl cinnamate and ethyl leucate in HKA4, having fruity and floral as general descriptors.

Aroma differences with the wine strain LALL. Wines fermented by the hybrids achieved the production of desirable compounds, usually associated with attractive aromatic attributes, at concentrations significantly higher than LALL wine parental yeast ($P < 0.05$). Figure 4 represents the ratio between aroma concentrations in hybrids versus LALL.

Most hybrid yeasts differed from LALL wine strain by the highest production of fruity aromas: γ-octalactone, isobutyl acetate, branched ethyl esters and PFMs (Fig. 4). The most significant differences were found in γ-octalactone, 8–12-fold higher in hybrids than LALL, highlighting the hybrids HKA4, HCS3 in Tempranillo wines and *S. uvarum* hybrids in Albariño. As for isobutyl acetate, all hybrids in Albariño wines highly synthesized this pear-like compound, more than doubling LALL concentration. HKA4 was the higher producer of this aroma,
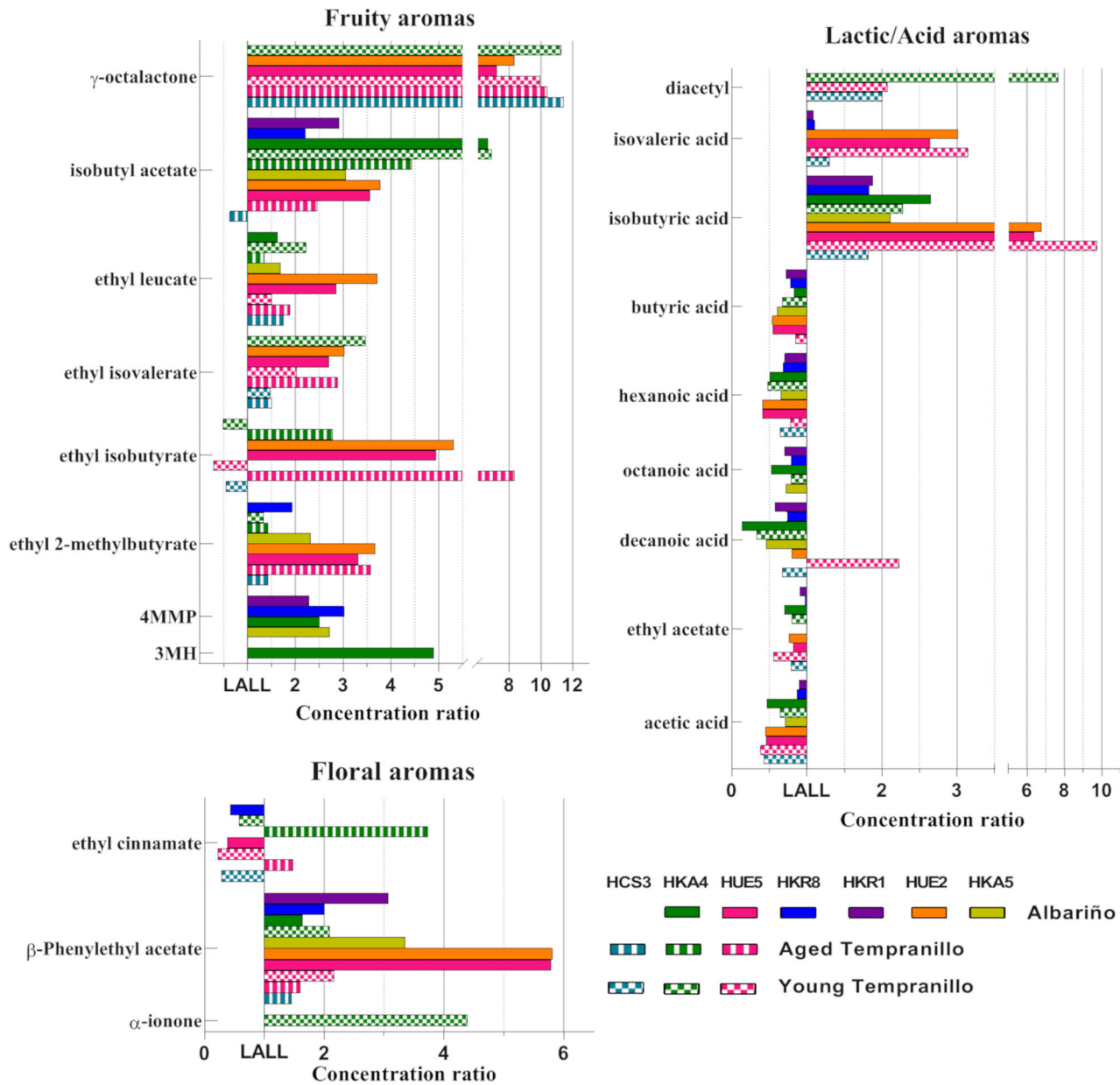
Fig. 4. Average aroma concentration of the hybrids relative to the concentrations determined in LALL wine strain, in Albariño (fulfil), young Tempranillo (square fill) and aged Tempranillo (line fill). 3MH, 3-mercaptohexanol; 4MMP, 4-mercapto-4-methylpentan-2-one.

4.5–6 times higher than the reference yeast (LALL) in all the wines analysed. Hybrids (except HKR1 and HKR8) also produced fruity esters at concentrations, over twice that of LALL, where HUE2 and HUE5 strains had the highest values, reaching fivefold and eightfold higher contents in Albariño and aged Tempranillo respectively.

Concerning polyfunctional mercaptans (PFMs) determined in Albariño wines, all *S. kudriavzevii* hybrids were the major 4MMP releasers, reaching concentrations about two- to threefold higher than those of LALL

parental strain. Interestingly, the HKA4 strain released the highest concentrations of 3MH, another important PFM, fivefold above all yeasts, including LALL.

Regarding compounds with floral aromas, hybrids involving *S. uvarum* species produced β-phenylethyl acetate at almost sixfold higher concentrations than LALL in Albariño, whereas HKA4 produced a 4.5-fold higher concentration of α-ionone in young Tempranillo; additionally, these concentrations exceeded their odour thresholds by around 1.5 OAV (Table S3) in both aroma

compounds. The HKA4 wines quadrupled the LLAL wines in ethyl cinnamate content, a compound prevalent in the other LALL wines.

Volatiles imparting lactic/acid notes (major compounds) were only determined in Albariño and Tempranillo young wines. Within this group, diacetyl was only detected in Tempranillo young wines, showing a great production by the hybrids, where HKA4 produced concentrations almost eightfold higher than LALL (Fig. 4). However, both isobutyric and isovaleric acids were overproduced by all hybrids, between 3- and 10-fold higher than LALL. On the contrary, HKR1 and HKR8 did not differ much from LALL, having the lowest amounts of these two acids and consequently the lowest values of their fruity branched esters in Albariño. In addition, all hybrids were characterized by having lower amounts than the LALL strain in the rest of fatty acids such as butyric, hexanoic, octanoic and decanoic. HUE2 and HUE5 were notable for reducing the content of these compounds, whereas HKA4 produced almost eightfold less decanoic acid in Albariño and Tempranillo young wines.

Among the undesirable flavours in the wines, all hybrids, whether Albariño or Tempranillo, produced less ethyl acetate and up to 50% less acetic acid than LALL.

Discussion

Wild and non-wine yeasts have been extensively characterized for producing fermentative compounds that can favour the organoleptic quality of wine and counteract the effects of climate change on grapes. Despite the recent interest in non-*Saccharomyces* yeasts to reduce the ethanol content of wines and to improve their aromas, most of these species are easily replaced by *S. cerevisiae* during wine fermentations. A similar situation has been also described for other *Saccharomyces* species (*S. uvarum* and *S. kudriavzevii*), they can be replaced by *S. cerevisiae* in wine fermentation conditions (Arroyo-López *et al.*, 2010; Alonso-del-Real *et al.*, 2017). Since several studies have found natural hybrid yeasts possessing enhanced fermentative properties acquired from wine and cryotolerant wild strains (González *et al.*, 2007; Gangl *et al.*, 2009; Ortiz-Tovar *et al.*, 2019), hybrid-induced generation would be a solution. In previous studies (Pérez *et al.*, 2022a, 2022b), we selected non-wine yeasts of the species *S. uvarum*, *S. kudriavzevii* and *S. cerevisiae* with attractive oenological attributes, and we generated hybrids with a wine *S. cerevisiae* strain by rare-mating.

Hybrid strains can inherit the nuclear genome of both parentals. This statement suggests that they may acquire different capabilities, such as tolerance to low temperatures from their cryophilic parental or resistance

to high ethanol concentrations or high fermentation temperatures from their wine parental *S. cerevisiae* strain (Masneuf *et al.*, 2002; González *et al.*, 2006; Belloch *et al.*, 2008; Arroyo-López *et al.*, 2010; Lairón-Peris *et al.*, 2020). In our study, we observed that hybrids HKR1, HKR8, HKA5 and HCS3 that inherited the mitochondrial DNA of the wine strain, were the most similar to LALL in terms of fermentation pattern, ethanol and glycerol production in both, Albariño (16°C) and Tempranillo (25°C) fermentations. However, hybrids having recombinant or *S. uvarum* mtDNA such as HKA4, HUE2 and HUE5 strains, showed a higher lag phase affecting the fermentation beginning, lower maximum fermentation rates (Vmax), and longer fermentations indicating a low tolerance to high-temperature fermentation, and low tolerance to ethanol presence resembling those characteristics of the cryotolerant *S. uvarum* and *S. kudriavzevii* parental species (Alonso-del-Real *et al.*, 2017; Lairón-Peris *et al.*, 2020).

But the most interesting is that these hybrids showed differences in the ethanol, glycerol and organic acid yields depending on the mtDNA inherited. The hybrids with *S. cerevisiae* mitochondria have more evident similarities with *S. cerevisiae* parental strain (LALL) showing lower glycerol production and high ethanol yields. On the contrary, HKA4 (*Sc × Sk*) produced the highest glycerol and 2,3-butanediol yields, while *Sc × Su* hybrids having the most significant amounts of succinic acid and the lowest of acetic acid reinforces the fact that they mainly inherited typical features extensively studied in previous works on their cryotolerant parental species (Querol *et al.*, 2018). It has been suggested that hybrid yeast carrying the mitochondrial genome of one parental yeast confers the optimal growth temperature of the donor parent (Baker *et al.*, 2019), however, according to our results, the mitotype inhered could be contributing to fermentation capability as well as with the main fermentative by-products involved in redox homeostasis, namely glycerol, succinic acid, 2,3-butanediol.

Aroma profiles inherited

We also observed a good correlation between the mitochondria and the aroma in Albariño wines, where yeast sharing the same mitochondrial DNA of *S. cerevisiae* (LALL) aromatically resembled the wine strain by the low fruity ethyl ester content. At the same time, the ability to produce certain aroma compounds typical of non-*cerevisiae* strains seems to have been inherited, such as the high production of PFMs in Albariño wines and acetate esters in both varietal wines. However, they shared with LALL the highest levels of ethanol, belonging to the alcoholic/solvent aroma vector and that tend to decrease the fruity flavours (Ferreira *et al.*, 2021). Therefore HKA4,

HUE2 and HUE5 hybrids possessing mitochondria of non-*cerevisiae* species were the most different on the aroma production level, noted by the floral and fruity descriptors. In future works, we need to confirm if this is only associated with inherited mitochondria, or there may also be a larger fraction of genomic DNA from *S. cerevisiae* or non-*cerevisiae* parentals.

The differences observed in HUE2 and HUE5 compared to LALL were mainly on the production of fermentative aromas typically found in *S. uvarum* species, such as fruity ethyl esters (Pérez *et al.*, 2022a,b), phenylethyl acetate and its alcohol (Stribny *et al.*, 2015). Minebois *et al.* (2020) have recently reported that this species highly produced these fermentative compounds to compensate for the fermentation redox balance, and in addition, they are high acetyl-CoA producers by consuming acetate during the first stages of fermentation. For this reason, the two *Sc* × *Su* hybrids liberated the lowest values of acetic acid. As observed previously in their parental *S. uvarum* strain (Pérez *et al.*, 2022b, Table 1), these hybrids produced large amounts of fruity ethyl esters, corresponding to the large production of isobutyric and isovaleric acid. During accelerated ageing, these acids continued to be esterified, which resulted in the significantly highest concentrations of ethyl isobutyrate and ethyl isovalerate observed in HUE5 aged Tempranillo wines. In addition, during the ageing process, acetates, such as the rose-like aroma β-phenylethyl acetate, suffered a decrease by hydrolysis (Díaz-Maroto *et al.*, 2005). As a result of this chemical process, we could expect an evolution from a floral profile in young wines to a fruity profile in aged wines. At the same time, the opposite happened with the HKA4 wines, where the floral-like ethyl cinnamate compound increased considerably during ageing, evolving from fruity to floral aged wines.

Polyfunctional mercaptans are desired high-impact aromas that, despite being at very low concentrations in wines, are generally above their perception thresholds (Mateo-Vivaracho *et al.*, 2010), providing tropical-citric notes to white wines such as Sauvignon Blanc (Tominaga *et al.*, 1998; Roland *et al.*, 2011). These aromas, mainly 4MMP, were previously determined to be highly released by the parental strains KR (CR89D1), followed by KA (CA111F1) (Pérez *et al.*, 2022b). Fortunately, one interesting finding was that all the *Sc* × *Sk* hybrids generated using these latter wild yeasts showed a solid ability to free 4MMP (2-3 fold higher than LALL) and 3MH. Natural yeast hybrids of *Sc* × *Sk* cross have already been reported to release PFMs in high amounts, but they are also shown to produce high acetic acid yields under certain winemaking conditions (Murat *et al.*, 2001; Gangl *et al.*, 2009; Deroite *et al.*, 2018). Among this interspecific cross, we found that the HKA4 hybrid

produced the lowest acetic acid levels than the wine strain LALL and the other *Sc* × *Sk* hybrids while yielding the maximal 3MH levels.

This latter compound (3MH) is one of the most appreciated thiols in Sauvignon blanc wines (Tominaga *et al.*, 1998), providing tropical notes such as grapefruit (Swiegers and Pretorius, 2007). Recently, several studies have determined that Gluthatione-3MH is the 3MH major precursor (Subileau *et al.*, 2008; Alegre *et al.*, 2017). PFMs are released from their precursors by the action of a β-lyase enzyme. Protein encoded by gene *GLO1* is not only known to participate in the condensation reaction between methylglyoxal and glutathione as a detoxification mechanism (Inoue and Kimura, 1996) but also was reported to have C-S lyase activity on thiol precursors (Howell *et al.*, 2005). Since methylglyoxal and glycerol shares the same precursor, namely dihydroxyacetone phosphate, and given the very high level of glycerol produced by HKA4 strain, we inferred that this strain could take advantage Glut-3MH to utilize glutathione for the methylglyoxal detoxification, ultimately releasing 3MH.

Finally, since these precursors enter through specific amino acid transporters whose efficiency depends on several regulatory mechanisms, this implies differences in the uptake of the two precursors depending on the yeast strain (Thibon *et al.*, 2008; Winter *et al.*, 2011; Pinu, 2018). According to this, overexpression of genes related to amino acid transporters or β-lyase enzymes could explain the substantial 4MMP release by *S. cerevisiae* × *S. kudriavzevii* strains from Cys-4MMP precursor. In contrast, overexpression of the gene *GLO1* involved in the methylglyoxal detoxification mechanism could explain the 3MH massive release from Glut-3MH by HKA4 yeast.

However, HUE2 and HUE5 hybrids, whose parental yeast UE was described as good PFM producer (Pérez *et al.*, 2022b), produced the lowest amounts and did not differ from LALL among these compounds, which also contrasts with previous works that have characterized *Sc* × *Su* hybrids as high PFMs releasing (Masneuf *et al.*, 2002; Dubourdieu *et al.*, 2006).

Another interesting finding was that in both varietal wines, γ-octalactone and isobutyl acetate appear to be distinctive aromatic compounds present in the wines fermented by the hybrids, where the acetate was another compound that described the HKA4 aroma profile. Despite having a very high perception threshold, γ–octalactone, coconut-like lactone, could be a key compound in non-wine yeasts since all these non-wine yeast-derived hybrids highly produced it. In addition, its presence in wild strains has been reported in our previous work (Pérez *et al.*, 2022b). However, to explore this regard, there is still little information on its synthesis

pathway in *Saccharomyces spp.* Theoretically, γ–octa-lactone could be derived from the octanoic acid lac-tonization or isoamyl alcohol and acrylic acid conjugation, an acid not yet identified in wine (Berger and Zorn, 2004; Romero-Guido *et al.*, 2011).

Conclusion

In summary, in this work, we generated non-GMO hybrid yeasts that were shown to have inherited the best aro-matic and chemical properties of their wild parents in Albariño and Tempranillo wines. The wine *S. cerevisiae* parent gave to some hybrids a better fermentation capacity, which we related to having inherited its mito-chondria. The hybrids that inherited non-*cerevisiae* mito-chondria reduced ethanol levels by around 0.3–0.45%. In addition, hybrids showed typical traits inhered from its cryotolerant parental species, such as in HKA4 hybrid, which showed the highest levels of citric acid, 2,3-butanediol and glycerol, or the *Sc* × *Su* hybrids, which produced the highest succinic acid and the lowest acetic acid contents.

Regarding the aromatic profiles, several appreciated aroma compounds were significantly produced by all the hybrids compared to *S. cerevisiae* strain, such as isobu-tyl acetate, β-phenylethyl acetate and γ-octalactone. The *Sc* × *Su* crosses were characterized by higher fruity esters in both wines, mainly in the aged Tempranillo, where a significant increase was observed. On the other hand, the *Sc* × *Sk* crosses were mainly characterized by higher production of PFMs in Albariño, where the highest levels of 3MH noted HKA4 hybrid. We also pos-tulated that HKA4 releases 3MH from glutathione non-volatile precursor to detoxify the cell from the presence of methylglyoxal, a compound related to its highest glyc-erol synthesis.

Experimental procedures

Parental yeast strains used for hybridization

Hybrid yeasts used in this work were generated from 4 strains of *S. kudriavzevii* (SKR and SKA), *S. uvarum* (UE) and *S. cerevisiae* (CS) isolated from natural envi-ronments. These four strains were previously selected for their significant capacity to produce high-impact aro-mas of Albariño and Tempranillo wines (Table 1). Each of these selected strains was crossed with a wine strain (*S. cerevisiae,* coded as LALL) provided by Lallemand Bio S.L.

Hybrid generation by rare-mating methodology

The hybridization process was carried out following the steps described in Pérez-Través *et al.* (2012) with some

modifications. The first step consisted in the isolation of auxotrophic organisms from each candidate parental strain. Therefore, strains were grown overnight in 15 ml of GPY liquid medium (2% glucose, 0.5% peptone, 0.5% yeast extract) at 28°C in an agitated incubator.

Then, the recovered cells were spread on multiple agar plates containing the following selective media. α-Aminoadipic acid media (α-AA) prepared as described Zarett and Sherman (1985): 0.17% YNB [-(NH$_4$)$_2$SO$_4$] (Yeast Nitrogen Base free of amino acids and ammonia sulfate, Difco), 2% glucose, 2% agar, 30 mg l^{-1} Lysine and 2 g l^{-1} DL-α-aminodipate. 5-Fluorotic acid media (5-FOA) prepared as described Boeke *et al.* (1987): 0.7% YNB [+(NH$_4$)$_2$SO$_4$] (Difco), 2% glucose, 2% agar, 0.1% proline, 0.001% uracil and 1 g l^{-1} 5-fluoorotic acid. 5-Fluoroanthranilic acid media (5-FAA) prepared as described Toyn *et al.* (2000): 0.17% YNB [-(NH$_4$)$_2$SO$_4$] (Difco), 5% glucose, 2% agar and amino acids pool solu-tion (0.01% L-tryptophan, 0.025% adenine, 0.025% ura-cil, 0.025% L-methionine, 0.025% L-arginine, 0.025% L-histidine, 0.03% L-tyrosine, 0.08% L-leucine, 0.08% L-isoleucine, L-lysine, 0.04% L-phenylalanine, 0.15% L-valine, 0.05% L-aspartic acid, 0.2% L-threonine, 0.08% L-glutamic acid, 0.375% L-serine). In such media, yeasts containing active *URA3*, *LYS2* and *TRP1* genes convert these substances into cell-toxic compounds, inhibiting their growth; thus, only yeasts whose genes are inactive grow. Once mutants were obtained, their auxotrophic trait was again corroborated by growing them on a mini-mal medium (MM: 0.17% Yeast Nitrogen Base without amino acids, 2% glucose and 2% agar) supplemented only with the necessary specific amino acid: lysine, uracil and tryptophan respectively (Lairón-Peris *et al.*, 2020).

Secondly, these auxotrophic yeasts were grown over-night in 50 ml of GPY. The recovered cells were mixed in pairs according to the hybrids to be obtained (Table 2) and had opposing auxotrophies. Next, 100 µl of each yeast pair was incubated at 28°C without shaking in 15-ml slant tubes containing 2 ml of GPY. Every 1, 3, 5 days, 500 µl of each cross was seeded in MM plates and incubated for a maximum of 5 days at 25°C (*S. cerevisiae* × *S.* non-*cerevisiae*) or 28°C (*S. cerevisiae* × *S. cerevisiae*). As colonies appeared, they were individu-ally purified for further molecular analysis to confirm the hybrid profiles. For intraspecific hybrids (*S. cerevisiae* × *S. cerevisiae*), PCR-microsatellite loci analysis was applied following the methodology described by Pérez-Través *et al.* (2012). In this case, the hybrid nature was confirmed by finding combinations of microsatellite pat-terns (named O and P and located in chromosomes XV and XVI of *S. cerevisiae*) of both parental *S. cerevisiae* strains (LALL and CS). For interspecific hybrids (*S. cere-visiae* × *S.* non-*cerevisiae*), the combination of restric-tion patterns was verified by PCR-RFLP (restriction

fragment length polymorphism) analysis of the nuclear genes *MAG2* and *GSY1* as has been described by Pérez-Través *et al.* (2012). DNA content was then measured in hybrids and their parent strains by flow cytometry (Lopes *et al.*, 2010). At the same time, their molecular profiles were determined by mtDNA-RFLP analysis (Querol *et al.*, 1992) and inter-δ sequence analysis (Legras and Karst, 2003).

Five rounds of successive seven-day laboratory fermentations were carried out to achieve genetic stabilization of the obtained hybrid yeasts (Pérez-Través *et al.*, 2014). These fermentations were performed at 22°C using a synthetic must prepared according to the recipe described by Pérez *et al.* (2021), containing 200 g l^{-1} of sugars (50% glucose and 50% fructose) and 140 mg l^{-1} of assimilable nitrogen. From rounds 4 and 5, the molecular patterns (mtDNA-RFLP, inter-δ sequence analysis and DNA content) of ten colonies were compared with each original hybrid. The criteria for considering strains to be stable was when the molecular patterns were the same between the ten colonies of rounds 4 and 5. If colonies with different molecular profiles were observed they were selected for greater diversity and included in the second yeast stabilization programme. Once the stabilization of the different selected hybrids has been achieved, the fermentative capability of these stable hybrids was compared with the parental strains. The fermentations were carried out in shaken flasks with 50 ml synthetic must (220 g l^{-1} of reducing sugars) at 16°C and 25°C. Finally, the best fermenting stable hybrids were selected for the present work.

Must composition and fermentation conditions

The semi-synthetic musts of the two varieties, Albariño and Tempranillo, were prepared as we described in previous works (Pérez *et al.*, 2022a,b) with some modifications: the sugar must content was 210 g l^{-1} (105 g l^{-1} glucose and 105 g l^{-1} fructose) for Albariño must and 230 g l^{-1} (115 g l^{-1} glucose and 115 g l^{-1} fructose) for Tempranillo must and the must pH was adjusted to 3.3 and then filtered for sterilization (0.22 μm). The nitrogen composition was identical for both varietal musts 0.22 g l^{-1} $(NH_4)_2HPO_4$; 14.34 mg l^{-1} tyrosine; 44.37 mg l^{-1} γ-aminobutyric acid (GABA); 14.43 mg l^{-1} isoleucine; 13.42 mg l^{-1} leucine; 58.51 mg l^{-1} alanine and 17.73 mg l^{-1} valine; 86.52 mg l^{-1} aspartic acid; 85.24 mg l^{-1} glutamic acid; 60.08 mg l^{-1} serine; 6.47 mg l^{-1} glycine; 137.4 mg l^{-1} histidine; 72.27 mg l^{-1} threonine; 673.1 mg l^{-1} arginine; 302.3 mg l^{-1} proline, 25.2 mg l^{-1} methionine; 7.53 mg l^{-1} phenylalanine;13.69 mg l^{-1} lysine and 177.3 mg l^{-1} glutamine.

Solutions of phenolic and aromatic varietal fractions (PAFs) and polyfunctional mercaptans (PFMs) precursors were provided by Zaragoza University. The acquisition and composition of the PAF alcoholic solution are described in Alegre *et al.* (2020). PAFs were added to the musts at a concentration of 10% v/v just after removing ethanol using a rotary evaporator and replacing it with sterile water. Due to the higher contribution of PMFs in white wines, the solution of their cysteine and glutathione precursors was added only to Albariño must at the following concentrations: 0.05 mg l^{-1} of Glut-3MH, 0.1 mg l^{-1} of Glut-4MMP, 0.05 mg l^{-1} of Cys-3MH, 1 mg l^{-1} of Cys-4MMP.

Fermentations were carried out in a 100 ml sterile flask containing 80 ml of each varietal must, a stirrer magnet (100 rpm), and closed by a rubber stopper equipped airlock valve ($n = 3$). Temperature fermentation was set at 16°C for Albariño and 25°C for Tempranillo fermentations. Yeast strains were inoculated at 1×10^6 cells per ml from an overnight culture grown in GPY. Fermentations were monitored by daily weight loss, and once the reducing sugar content was less than 1 g l^{-1}, the fermentation process was considered finished. Then, 18 ml of Tempranillo wines were subjected to accelerated anoxic ageing (50°C, 5 weeks), simulating bottle ageing and following the methodology proposed by Oliveira and Ferreira (2019).

Determination of kinetic parameters and main fermentation by-products

Weight loss curves were fitted by the non-linear regression Gompertz model proposed by Zwietering *et al.* (1990), adapted for maximum fermentation rate (Vmax), as explained in other works (Pérez *et al.*, 2021). The daily weight loss in each must volume (80 ml in Albariño and 50 ml in Tempranillo) was expressed in grams lost per litre.

In the final wines and during the last days of fermentation, the content, in g l^{-1}, of glucose, fructose, erythritol, glycerol, tartaric, citric, malic and succinic acids and ethanol (%v/v) was analysed by high-performance liquid chromatography (HPLC, Thermo Fisher Scientific, Waltham, MA, USA) under the same equipment conditions and following the protocol described in Pérez *et al.* (2021).

Volatile compound analysis on the final wines

Major (> 0.2 mg l^{-1}) and minor (0.1–200 μg l^{-1}) volatile compounds were analysed in young wines of both varieties. Additionally, in Albariño wines, PFMs were analysed, and in aged Tempranillo samples, minor volatile compounds were quantified.

Major aromas were liquid–liquid micro-extracted, identified, and quantified by a gas chromatograph with flame

ionization detector as has been described by Ortega *et al.* (2001). Minor volatile compounds were solid-phase extracted (SPE) and quantified using a gas chromatograph coupled to a mass spectrometer detector (GC-MS, Shimadzu QP2010, Quioto, Japan) based on the methodology developed by López *et al.* (2002). PFMs were extracted by solid phase, followed by derivatization and analysed by thermal desorption GC-GC-MS, following the methods of Mateo-Vivaracho *et al.* (2010) with some modifications as described elsewhere (Pérez *et al.*, 2022b).

Statistical data analysis

All parameters (Vmax, Lag time, primary metabolites and aroma compounds) were treated by one-way ANOVA followed by Tukey's HSD test, considering a significance level of $P < 0.05$. The results were expressed as the average value $\pm$ standard deviation from three biological replicates.

Aroma data set was treated by hierarchical clustering using Euclidean distance and, principal component analysis (PCA) was applied to visualize the treatments to these measured multi-variables. For the statistical treatment, INFOSTAT software (2011 version, Grupo Infostat, Córdoba, Argentina) was used and, plots were generated using GRAPHPAD PRISM version 8.0 software (Graph-Pad Software, Inc., La Jolla, CA, USA).

Acknowledgements

Conflict of interest

The authors declare no competing interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Maximum fermentation rate (Vmax) and main metabolites analyzed in Albariño wines and young Tempranillo wines fermented by the different hybrids yeast strains and the wine parental strain (LALL).

Table S2. Concentrations (μg l^{-1}) of volatile compounds determined in Albariño wines fermented by the different hybrids yeast strains and the wine parental strain (LALL).

Table S3. Volatiles defined as active aroma vectors and their general aroma descriptor according to Ferreira et al., 2021. Mean ($n = 3$) of the odor activity values (OAV) calculated from the ratio between the concentrations of each yeast in Albariño wines and their corresponding odor thresholds (OT) for each aroma compound.

Table S4. Concentrations (μg l^{-1}) of volatile compounds determined in young Tempranillo wines fermented by the different hybrids yeast and the wine parental strain (LALL).

Table S5. Concentrations (μg l^{-1}) of volatile compounds determined in aged Tempranillo wines fermented by the different hybrids yeast strains and the wine parental strain (LALL).

Table S6. Volatiles defined as active aroma vectors and their general aroma descriptor according to Ferreira et al., 2021. Mean ($n = 3$) of the odor activity values (OAV) calculated from the ratio between the concentrations of each yeast in Tempranillo young wines and their corresponding odor thresholds (OT) for each aroma compound.

Table S7. Volatiles defined as active aroma vectors and their general aroma descriptor according to Ferreira et al., 2021. Mean ($n = 3$) of the odor activity values (OAV) calculated from the ratio between the concentrations of each yeast in Tempranillo aged wines and their corresponding odor thresholds (OT) for each aroma compound.