

RESEARCH ARTICLE

Adaptive evolution in the *Saccharomyces kudriavzevii* Aro4p promoted a reduced production of higher alcohols

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Abstract

The use of unconventional yeast species in human-driven fermentations has attracted a lot of attention in the last few years. This tool allows the alcoholic beverage industries to solve problems related to climate change or the consumer demand for newer high-quality products. In this sense, one of the most attractive species is *Saccharomyces kudriavzevii*, which shows interesting fermentative traits such as the increased and diverse aroma compound production in wines. Specifically, it has been observed that different isolates of this species can produce higher amounts of higher alcohols such as phenylethanol compared with *Saccharomyces cerevisiae*. In this work, we have shed light on this feature relating it to the *S. kudriavzevii* aromatic amino acid anabolic pathway in which the enzyme Aro4p plays an essential role. Unexpectedly, we observed that the presence of the *S. kudriavzevii* ARO4 variant reduces phenylethanol production compared with the *S. cerevisiae* ARO4 allele. Our experiments suggest that this can be explained by increased feedback inhibition, which might be a consequence of the changes detected in the Aro4p amino end such as L₂₆Q₂₄ that have been under positive selection in the *S. kudriavzevii* specie.

INTRODUCTION

Saccharomyces cerevisiae is the most preferred yeast starter in winemaking since it can face the variety of stresses encountered throughout the fermentation and because of its robust fermentative traits (Pretorius, 2000). Nevertheless, in recent years the consumer trends have changed, looking for rich and ripe fruit flavour wines (Bucher et al., 2018; Varela et al., 2015). Moreover, the wine's ethanol concentration has increased due to the increment of sugar content in grapes (van Leeuwen & Darriet, 2016), as a consequence of Viticultural practices and climate

change (Nicholas, 2014; Varela et al., 2008, 2015). The increment of ethanol impairs the wine's organoleptic properties masking the perception of its fruity and floral aromas (King et al., 2013). Therefore, this has led to the search for alternatives to reduce it (Varela et al., 2015). Among the existing strategies, the usage of other *Saccharomyces* species such as *S. kudriavzevii* and *S. uvarum* as starters has been explored since they exhibit desired traits including lower ethanol production, higher glycerol production and the increment of aromatic compounds such as higher alcohols and esters (Gamero et al., 2013; Pérez-Torrado et al., 2015; Peris et al., 2016; Querol et al., 2018); in recent years,

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Higher (or fusel) alcohols are considered the most important contributors to wine's flavour (Cordente et al., 2012; Holt et al., 2019; Ugliano & Henschke, 2009), whose concentration can vary between 140 and 420 mg/L (Ebeler, 2001), making a positive aromatic contribution at concentrations below 300 mg/L (Swiegers & Pretorius, 2005). They are synthesized through the Ehrlich pathway, which is used by yeasts for the catabolism of the aromatic, branched-chain and sulfur-containing amino acids (Hazelwood et al., 2008). They are first transaminated to form keto-acids, which are decarboxylated to produce their corresponding aldehydes, and then, reduced and transformed into their alcohols. Among them, the presence of phenylethanol in wine is desired since it confers a rose-like aroma (Ugliano & Henschke, 2009). However, in winemaking conditions, the production of phenylethanol relies preferably on the catabolism of sugars (Rollero et al., 2019; Ugliano & Henschke, 2009) since the keto acid intermediate phenylpyruvate can be synthesized de novo from sugars (Figure 1). It begins with the Shikimate pathway, whose final product chorismate is used for the biosynthesis of aromatic amino acids (Gientka & Duszkiwicz-Reinhard, 2009). Nevertheless, during the biosynthesis of phenylalanine and tyrosine, the intermediate prephenate can be transformed into either 4-hydroxyphenylpyruvate (4HPP) or phenylpyruvate, which enters directly into the Ehrlich pathway to produce tyrosol and phenylethanol, respectively (Figure 1; Luttk et al., 2008).

It has been reported that phenylethanol concentration is increased in fermentations carried out using wild European *S. kudriavzevii* isolates (Lopes et al., 2010; Sampaio & Gonçalves, 2008) in Tempranillo and Macabeo musts (Peris et al., 2016). The *Saccharomyces* genus is composed of eight species and several natural interspecific hybrids, which have been isolated from different natural and man-made niches (Alsammar &

Delneri, 2020). Given the vast availability of genome sequences of different *Saccharomyces* isolates, comparative genomics is a suitable approach to understanding the molecular mechanism behind the oenological traits exhibited by *S. kudriavzevii* (Macías et al., 2019). A previous study searched for genes under positive selection in *S. kudriavzevii* compared with *S. cerevisiae*, which allowed the identification of the gene *ARO4* among the candidates (Macías et al., 2019). *ARO4* encodes 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase involved in the first step of the Shikimate pathway, which carries out the condensation between the glycolysis intermediate phosphoenolpyruvate (PEP) and the ribose pentose pathway intermediate erythrose-4-phosphate (E4P) to generate DAHP (Gientka & Duszkiwicz-Reinhard, 2009; Künzler et al., 1992).

Therefore, since *ARO4* contributes to the biosynthesis of higher alcohols and the presence of adaptive selection, it has been proposed that this gene might be contributing to the ability of *S. kudriavzevii* to increase phenylethanol production (Macías et al., 2019). Therefore, in the present study, we analysed the impact of the *S. kudriavzevii* *ARO4* allele on the production of phenylethanol and tyrosol in winemaking conditions. Besides, we have got insights into the regulation of the *S. kudriavzevii* Aro4p that might explain the observed differences in phenylethanol production.

EXPERIMENTAL PROCEDURES

Yeast strains and growth conditions

The parental and engineered strains used in this study are listed in Table 1. The haploid strains AQ2775 and AQ4013 are derived from the wine *S. cerevisiae* T73 (Querol et al., 1992) and wild *S. kudriavzevii* CR85 (Lopes et al., 2010), respectively. YPD solid media (1% yeast extract, 2% peptone, 2% glucose and 2% agar) plus either 200 µg/ml G418 (YPD-KAN) or 100 µg/ml nourseothricin (YPD-NAT) were used for the selection of transformants. The synthetic complete medium (SC) was prepared according to Yeast Nitrogen Base without amino acids 6.7 g/L, 10% v/v Amino acid mix 10× and 2% glucose (Ear et al., 2016). The L-amino acids and nucleic acids mix were prepared 10× (0.29 g/L Adenine, 0.2 g/L Uracil, 0.4 g/L Tryptophan, 0.16 g/L Histidine, 0.17 g/L Arginine, 0.3 g/L Tyrosine, 0.6 g/L Leucine, 0.24 g/L Lysine, 0.5 g/L Phenylalanine, 1 g/L Glutamic acid, 1 g/L Asparagine, 1.5 g/L Valine, 2 g/L Threonine, 3.75 g/L Serine and 0.2 g/L Methionine). The synthetic medium without aromatic amino acids (SC-aro) was prepared as SC but added an amino acid mix 10× without the L-amino acids phenylalanine, tryptophan and tyrosine.

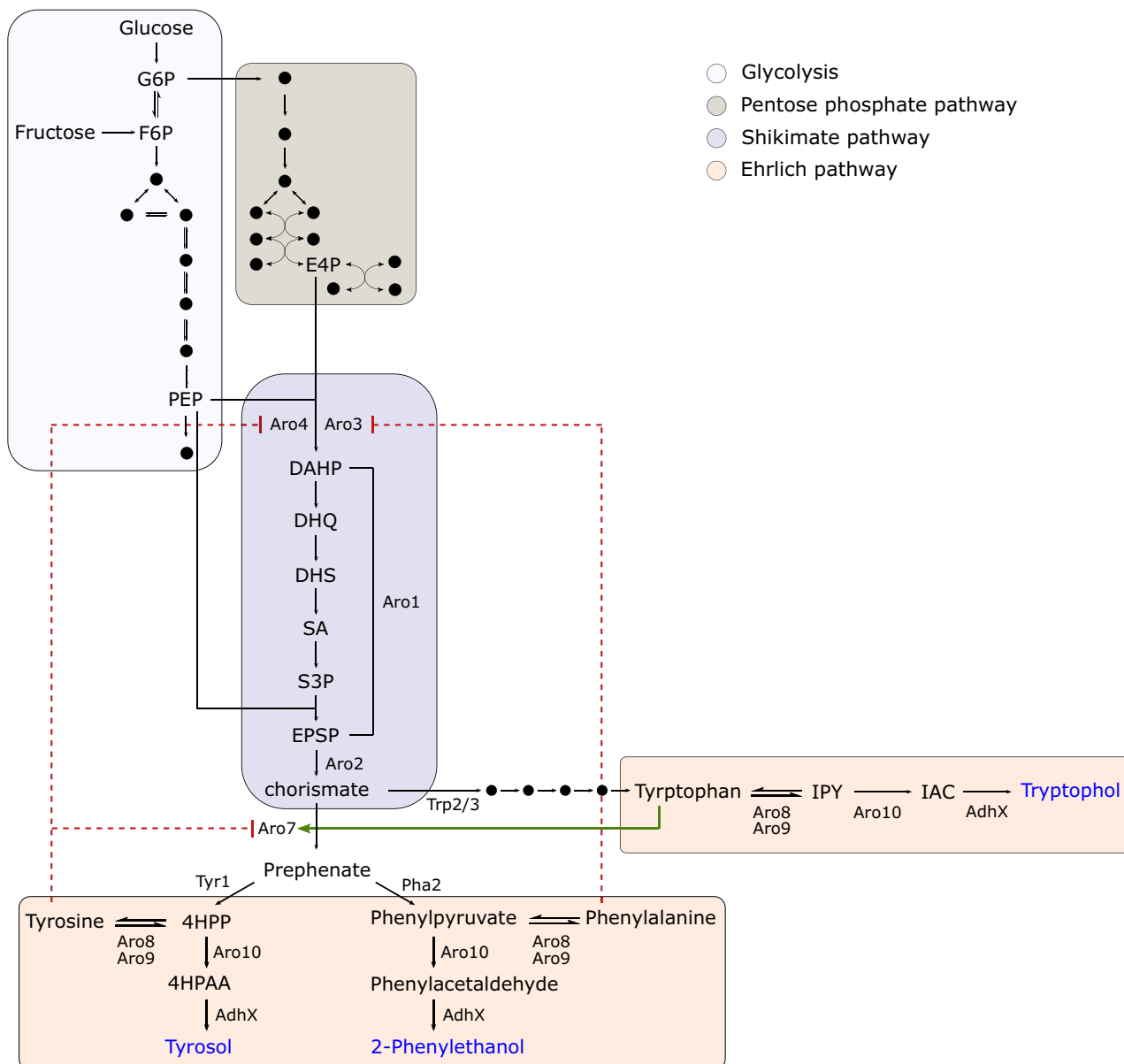


FIGURE 1 Metabolic pathways are involved in the biosynthesis de novo of aromatic higher alcohols from sugars. The enzymes involved in both Ehrlich (Hazelwood et al., 2008) and Shikimate (Gientka & Duszkiwicz-Reinhard, 2009) pathways are shown. The biosynthesis begins with the condensation of phosphoenolpyruvate (PEP) with erythrose-4-phosphate (E4P) carried out by the DAHP synthases Aro4 and Aro3, which are inhibited by the tyrosine and phenylalanine (dotted red lines), respectively. Then, it follows a series of reactions to finally produce chorismate. The chorismate can be converted into prephenate by the chorismite mutase Aro7, which is also downregulated by tyrosine but activated by threonine (green line). During the tyrosine/phenylalanine biosynthetic branch, the prephenate can be converted into tyrosine or phenylalanine or pass through the Ehrlich pathway to produce the higher alcohols tyrosol or phenylethanol. The higher alcohols are highlighted in blue. 4HPAA, 4-hydroxyphenylacetaldehyde; 4HPP, 4-hydroxyphenylpyruvate; AdhX, alcohol dehydrogenases Adh1 to Adh7; DAHP, 3-deoxy-*arabino*-heptulosonate-7-phosphate; DHQ, 3-dehydroquinone; DHS, 3-dehydroshikimate; EPSP, 5-enolpyruvateshikimate-3-phosphate; F6P, fructose-6-phosphate; G6P, glycerol-6-phosphate; IAC, lidole acetaldehyde; IPY, indole pyruvate; S3P, 3-phosphoshikimate; SA, Shikimic acid. Figure adapted from Luttkik et al. (2008), Gientka and Duszkiwicz-Reinhard (2009) and Bisquert et al. (2022).

Strain construction

To generate strains, carrying the different *ARO4* alleles was used the same approach as has been described by Tapia et al. (2022) and summarized in Figure 2. Briefly, the deletion of the *ARO4* open reading frame (ORF) in strain AQ2775 was performed through

PCR-mediated gene disruption (Baudin et al., 1993) using the amplified *KanMX* cassette from the plasmid pUG6 (Güldener et al., 1996) as the selection marker. The PCR was carried out using NZYtaq II DNA polymerase (NZYTech) with the primers listed in Table 2 and following the provided instructions. The strains were transformed through the lithium acetate method

TABLE 1 Strains used in this study.

Strain	Specie	Genotype	Reference
AQ2775	<i>Saccharomyces cerevisiae</i>	T73 MAT α pha	This study
AQ4013	<i>Saccharomyces kudriavzevii</i>	CR85 MAT α pha ho::MX4dsdA	This study
ST41	<i>Saccharomyces cerevisiae</i>	MAT α pha, aro4::KanMX	This study
ST41-Sc	<i>Saccharomyces cerevisiae</i>	MAT α pha, aro4::kanmx::ARO4(T73)	This study
ST41-Sk	<i>Saccharomyces cerevisiae</i>	MAT α pha, aro4::kanmx::ARO4(CR85)	This study
ST41-Sc-3 Δ	<i>Saccharomyces cerevisiae</i>	MAT α pha, aro4::kanmx::ARO4(T73), aro3::KanMX	This study
ST41-Sk-3 Δ	<i>Saccharomyces cerevisiae</i>	MAT α pha, aro4::kanmx::ARO4(CR85), aro3::KanMX	This study

(Gietz & Schiestl, 2007) and cultivated into YPD-KAN plates at 28°C for 4 days. The deletions were confirmed by PCR using the diagnostic primers. We used the CRISPR-Cas9 system (Stovicek et al., 2017) to swap the *KanMX* at the T73 *ARO4* locus with *ARO4* alleles. A spacer that targets the *KanMX* coding region was designed according to Doench et al. (2014), in which, the T73 genome was used as the reference to avoid gRNA showing off-target activity. Then, the whole plasmid pRCC-N (Generoso et al., 2016) was amplified with the primers carrying the protospacer sequence at their 5' ends using the Phusion™ High-Fidelity Polymerase (Thermo Fisher Scientific). A total of 30 μ l of PCR product was treated with DpnI (Thermo Fisher Scientific) for 3 h to digest the pRCC-N template. Simultaneously, we amplified both CR85 and T73 *ARO4* alleles as the donor DNA from the AQ4013 and AQ2775 total DNA using Phusion™ High-Fidelity Polymerase, where the primers carried homologous regions to the T73 *ARO4* locus at their 5' ends, respectively. We transformed the *aro4* mutant ST41, with a transformation mix containing both protospacer-carrying pRCC-N PCR product and one of the *ARO4* alleles, and the cells were cultivated on YPD-NAT plates for 4 days. The swapping was confirmed by PCR from nourseothricin-resistant transformant strains whose resistance against G418 was lost and whose ability to grow in a minimal medium containing phenylalanine (Phe) as the sole nitrogen source was recovered (Künzler et al., 1992). Finally, the inserted *ARO4* alleles were amplified, and their sequences were checked through Sanger sequencing from Eurofins Genomics Mix2Seq service. To specifically study the activity of the *ARO4* alleles, we disrupted the *ARO3* genes of the recombinant strains ST41-Sc and ST41-Sk using again the *KanMX* cassette as we mentioned above.

Fermentation medium

The fermentations were performed in synthetic wine must (SWM) that mimics the grape juice (Bely et al., 1990) and was prepared based on Rollero et al. (2015) but with some modifications. Briefly, the must is composed of 100 g/L glucose, 100 g/L fructose,

5 g/L malic acid, 3 g/L tartaric acid, 0.5 g/L citric acid 1-hydrate, 0.75 g/L KH_2PO_4 , 0.5 g/L K_2SO_4 , 0.25 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.155 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.2 g/L NaCl. Different concentrated stocks were prepared and added to the must: 10 ml/L of vitamins stock (2 g/L myo-inositol, 0.15 g/L calcium pantothenate, 0.025 g/L thiamine hydrochloride, 0.2 g/L nicotinic acid, 0.025 g/L pyridoxine and 0.0003 g/L biotin), 1 ml/L of trace salts stock (4 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 4 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 g/L KI, 0.4 g/L $\text{CoCl}_2 \cdot 5\text{H}_2\text{O}$, 1 g/L H_3BO_3 and 1 g/L $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$) and 1 ml of anaerobiosis factors mixture (15 g/L ergosterol and 5 g/L oleic acids mixed with 1/1 volume of Ethanol/Tween 80). A total of 180 mg/L of yeast assimilable nitrogen (YAN) was provided through 0.195 g/L NH_4Cl (corresponding to 72 YAN) and 5.54 ml of a mixture of 19L-amino acids (corresponding to 108 YAN) composed of 11.2 g/L alanine, 28.3 g/L arginine, 3.4 g/L aspartic acid, 1.5 g/L cysteine, 9.2 g/L glutamic acid, 38.4 g/L glutamine, 1.4 g/L glycine, 2.6 g/L histidine, 2.5 g/L isoleucine, 3.7 g/L leucine, 1.3 g/L lysine, 2.4 g/L methionine, 2.9 g/L phenylalanine, 46.1 g/L proline, 6.0 g/L serine, 5.8 g/L threonine, 13.4 g/L tryptophan, 1.5 g/L tyrosine and 3.4 g/L valine. Finally, the must is buffered to pH 3.3 with 1 M NaOH and sterilized with 0.22 μ m filters.

Fermentation conditions

Fermentations were carried out in 100 ml bottles coupled with an airlock containing 80 ml of SWM with a 30 mm \times 5 mm magnetic stirrer on a 15 spot-multistirrer (VELP Scientifica). Overnight precultures were incubated in a 5 ml YPD medium at 28°C. Then, they were washed in SWM once and the biomass was inoculated into the 80 ml of SWM with an initial OD_{600} of 0.2 at 28°C with an agitation of 120 rpm. The fermentation kinetics was monitored through the weight loss, which represents the CO_2 produced by yeast during alcoholic fermentation, until the end of fermentation when no more weight loss is measured (Bely et al., 1990). Once the fermentation ended, samples were collected by centrifugation, and the supernatant was recovered and frozen until the analytical procedures.

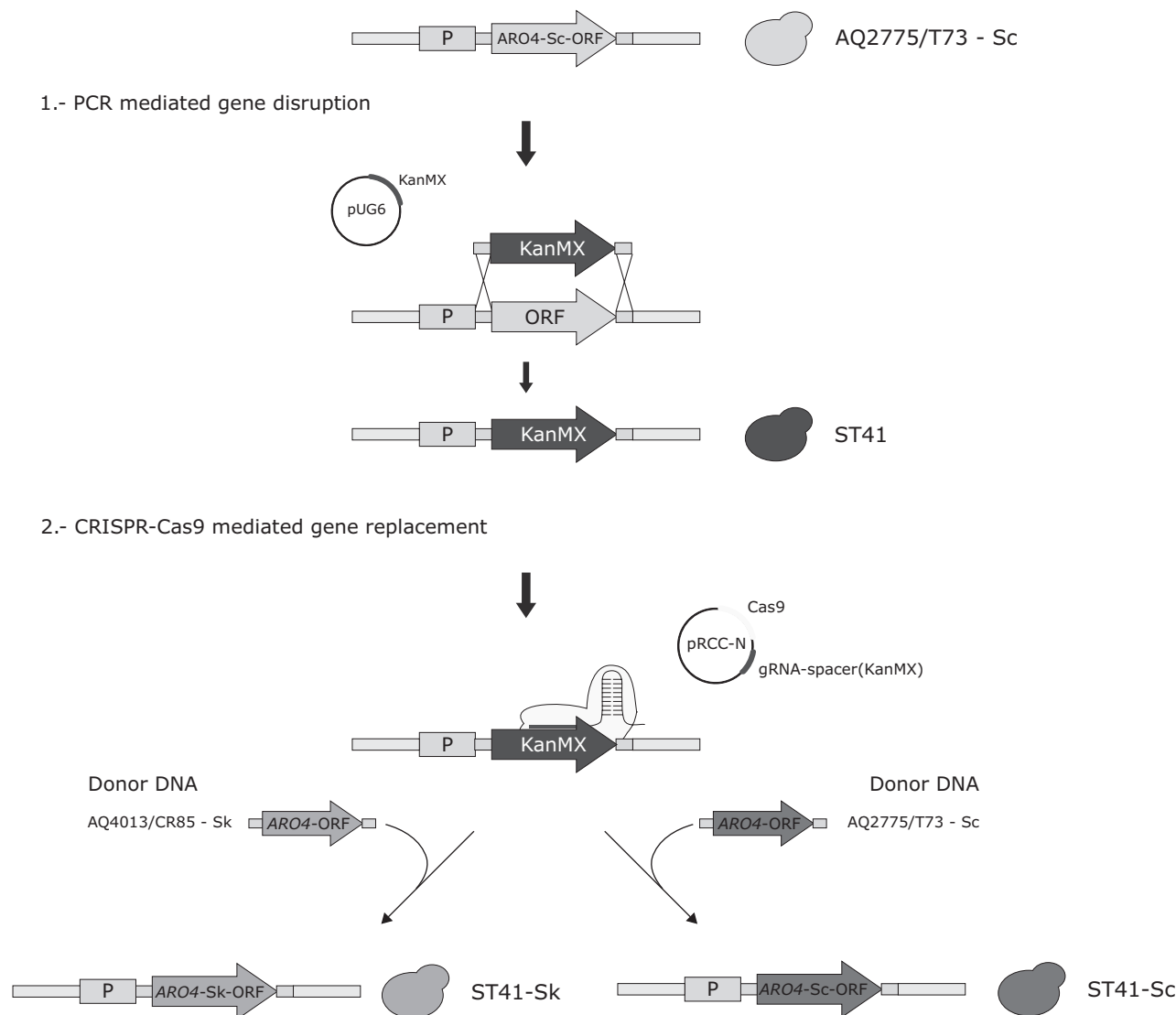


FIGURE 2 Two-step *ARO4* allele swapping approach is used for recombinant strain generation. (1) PCR-mediated gene disruption: The *KanMX* cassette was amplified from the plasmid pUG6 (Güldener et al., 1996) using the primers pair *aro4* Δ -Fw/*aro4* Δ -Rv to disrupt the *S. cerevisiae* (AQ2775/T73) *ARO4* open reading frame by the PCR-mediated gene disruption method generating the strain ST41. (2) CRISPR-Cas9 mediated gene replacement: the whole pRCC-N plasmid (Generoso et al., 2016) was amplified using the primers pair gRNA-*KanMX*-Fw/gRNA-*KanMX*-Rv to add the spacer sequence to target the *KanMX* cassette located in the *ARO4* locus (strain ST41). Once the Cas9 performed the double-stranded break in the *KanMX* cassette, the DNA reparation was promoted by homologous recombination adding to the transformation mix as donor DNA either the *S. cerevisiae* (AQ2775/T73) or *S. kudriavzevii* (AQ4013/CR85) *ARO4* ORF alleles, which were amplified using the pair primers *ARO4*-Sc-Fw/*ARO4*-Sc-Rv and *ARO4*-Sk-Fw/*ARO4*-Sk-Rv, respectively, generating the recombinant strain ST41-Sc and ST41-Sk. P: *S. cerevisiae* *ARO4* promoter.

Determination of sugars and alcohols

The quantification of alcohols and sugars was carried out by HPLC (Thermo Fisher Scientific) using a refraction index detector equipped with a HyperREZ™ XP Carbohydrate H+ 8 column (Thermo Fisher Scientific) coupled with a HyperRETZ™ XP Carbohydrate Guard (Thermo Fisher Scientific). The chromatography conditions were the same as described by Minebois et al. (2020b). Briefly, 1 ml of samples was diluted with Milli-Q water depending on the remaining sugar amount and filtered through a 0.22- μ m nylon filter. The run conditions were 1.5 mM

of H_2SO_4 at 0.6 ml/min flux with 35 bars of pressure, and the oven temperature was maintained at 50°C. The concentration of the different compounds was determined through their corresponding standard calibration curve.

Quantification of higher alcohols by HPLC-PDA

The higher alcohols tyrosol and phenylethanol were quantified by HPLC on an Acquity ARC system core (Waters) equipped with a photodiode array

TABLE 2 Primers used in this study.

Primer	Sequence (5'–3')
Primers for gene disruption	
aro3Δ-Fw ^a	TACCCCTATTACGTTACAAGAACAACACTTTATAGCATATGTAGAGATCTGTTTAGCTTGCCT
aro3Δ-Rv ^a	CATTCAAGATTATTTGCATTTTTCCCTCATTTACAGGCTAGTTTTTCGACACTGGATGGC
aro4Δ-Fw ^a	TAAATTTAGTAAACAAAAGAATCTATCAGAAATGAGTGATAGAGATCTGTTTAGCTTGCCT
aro4Δ-Rv ^a	GAGGAAAGAATGTACGTTACATATATCATTAAAAAACATGTTTTTCGACACTGGATGGC
Primers for ARO4 allele swapping	
Protospacer incorporation in pRRC-N plasmid	
gRNA-KanMX-Fw ^b	<u>TGTTTTGCCGGGGATCGCAG</u> TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG
gRNA-KanMX-Rv ^b	CTGCGATCCCCGGCAAACAGATCATTATCTTTCACTGCGGAG
Primer for ARO4 alleles amplification as donor DNA	
ARO4-Sc-Fw ^c	<u>ACC</u> GCTAAATTTAGTAAACAAAAGA
ARO4-Sc-Rv ^c	AATACCGAATTGGCAGTGGTAG
ARO4-Sk-Fw ^c	TAAATTTAGTAAACAAAAGAATCTATCAGAAATGAGTGAAATGTTCCGGTG
ARO4-Sk-Rv ^c	GAGGAAAGAATGTACGTTACATATATCATTAAAAAACATTTATTTCTTGTAACTTCTCTTC
Diagnostic primers	
ARO3-Sc-test-Fw	GCAGCTGCGTATCTTCTCAA
ARO3-Sc-test-Rv	CCTTTGGCTGAAATTGGAAA
ARO4-Sc-test-Fw	GGGACGCATTGTTAGCTCAT
ARO4-Sc/Sk-test-Rv	CTGGAGAAGCTAATGGGTCG
K2	GGGACAATTCAACGCGTCTG
Sequencing primers	
ARO4-Sc/Sk-Seq_1	TCTTTCGTCATTTTTAGCATCG
ARO4-Sc-Seq_1	AATGCTTGATACCATTCTCTCT
ARO4-Sk-Seq_1	TAAAGGGTGATTTGTCTGTCAT
ARO4-Sk-Seq_2	GGACTTCAGAAATCAACCAAAG

^aHomologous sequences to *KanMX* from the pUG6 plasmid are underlined.

^bThe underlined sequences stand for the protospacers.

^cUnderlined sequences stand for the specific *ARO4* alleles sequences.

wavelength detector (Waters 2998 PDA), a quaternary pump, an autosampler and an online gasser. We used the chromatography conditions described by Bisquert et al. (2022). Briefly, the fermented SWM samples were collected in 1.5 ml-microcentrifuge tubes and centrifugated at maximum speed for 2 min. Then, the supernatants were collected and diluted 1/2nd with 100% v/v methanol. Samples were diluted 1/8th with 50% v/v methanol and filtered with 0.22- μ m nylon filters. The separation was performed in a 4.6 mm \times 150 mm \times 2.6 μ m Accucore™ C18 column (Thermo Fisher Scientific) maintained at 30°C using 0.01% TFA (Phase A) and acetonitrile (Phase B) as mobile phases at a flow rate of 1 ml/min. A total of 10 μ l of the sample was injected, and the gradient programme was as follows: (1) 0–18 min, 100% A, (2) 18–19 min 90% A, (3) 19–28 min 75% A, (4) 28–31 min A 0% and (5) 100% A. The PDA detector was set at a wavelength of 210 nm. Both tyrosol and phenylethanol were identified based on their retention times and were quantified through their corresponding standard calibration curves.

Growth inhibition of tyrosine

The approach used to test *in vivo* the Aro4p inhibition by tyrosine was based on Sousa et al. (2002). Briefly, the mutants ST41-Sk-3Δ and ST41-Sc-3Δ (Table 1) were grown in SC medium overnight at 28°C at a shaking speed of 120 rpm. Then, the cultures were washed twice in SC-aro medium and were inoculated in 200 μ l of SC-aro media containing different concentrations of tyrosine (0.1, 0.5, 1, 2 and 5 mM) with an initial OD₆₀₀ of 0.2 on 96 well-microtiter plates at 25°C. Growth curves were monitored through OD measurement at 600 nm wavelength in a SPECTROstar Nano® microplate reader (BMG LABTECH GmbH). Maximum specific growth rates (μ_{max}) were obtained by fitting the growth curves to the Gompertz model using *grofit* R package (Kahm et al., 2010).

Functional divergence analysis of Aro4p

The amino acid residues identified as being under positive selection in the *S. kudriavzevii* Aro4p

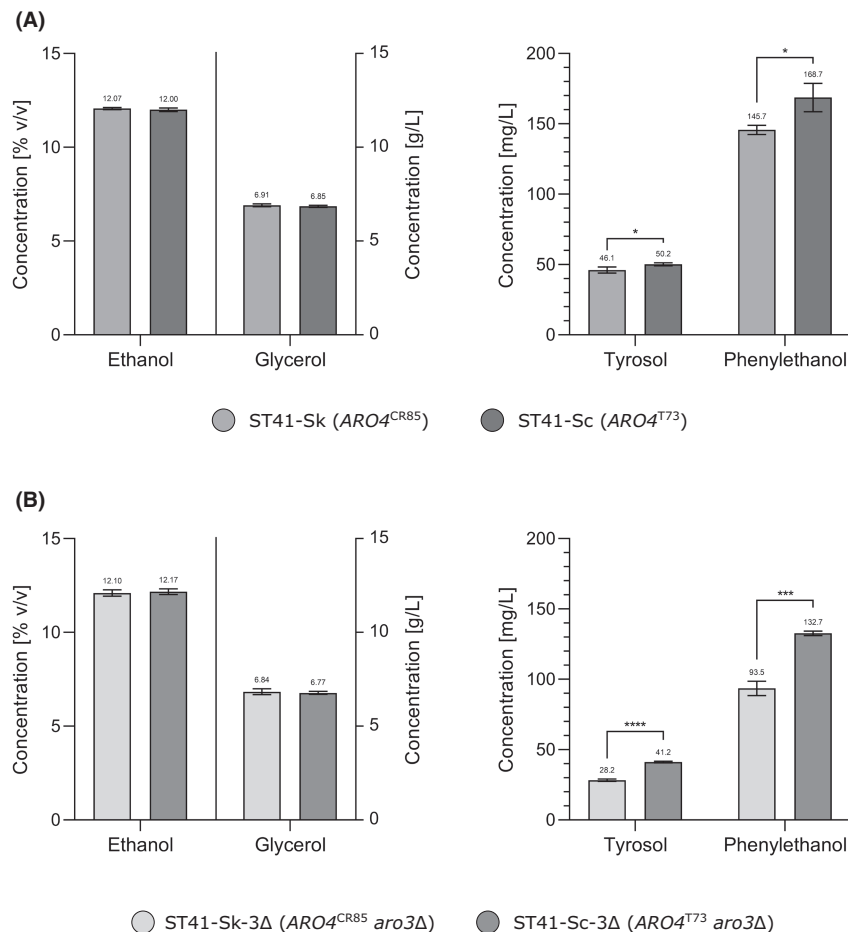


FIGURE 3 Production of ethanol, glycerol and higher alcohols by the recombinant *ARO4* strains. (A) Production was obtained by the recombinant strains ST41-Sk (*aro4::kanmx::ARO4(CR85)*) and ST41-Sc (*aro4::kanmx::ARO4(T73)*). (B) Production obtained by the *aro3* recombinant strains ST41-Sk-3Δ (*aro4::kanmx::ARO4(CR85) aro3::KanMX*) and ST41-Sc-3Δ (*aro4::kanmx::ARO4(T73) aro3::KanMX*). The fermentations were carried out in SWM at 25°C and the different compounds were detected and quantified by HPLC. Statistical differences were determined through a two-tailed unpaired *t*-test. *p*-Values style are from GraphPad Prims version 8.01: 0.0332 (*); 0.0002 (**); <0.0001 (****).

(S₄S₂, P₅E₃, P₁₃G₁₁, Q₁₇E₁₅, L₂₆Q₂₂ and A₃₃₂S₃₃₀) were extracted from the analyses performed by Macías et al. (2019). Then, Grantham's scores (Grantham, 1974) were assigned to the amino acid changes, to quantify the biochemical divergence between *S. kudriavzevii* and *S. cerevisiae* Aro4p. This method quantifies how similar or dissimilar are two amino acid residues based on their physicochemical properties such as composition, polarity and molecular volume to predict their evolutionary distance. High values between them imply that those exhibit radical differences in their properties, and the replacement could potentially generate important functional differences in the protein activity. The significance of the changes was classified according to Li et al. (1984). The software PyMOL™ Molecular Graphics System, Version 2.5.2 Schrödinger, LLC (<https://pymol.org/2/>), was used to simulate the effect of the amino acid changes on the structure and interactions of the protein Aro4p using as a template the crystal structure 1of6.4., downloaded from the SWISS-MODEL server (<https://swissmodel.expasy.org/>). The alignment of the gene products of the *S. cerevisiae* T73 and *S. kudriavzevii* CR85 *ARO4* genes was done with the Clustal Omega 1.2.2 tool using the default parameters.

Statistical analyses

Unpaired *t*-test analyses were performed using GraphPad Prism version 8.01 for Windows 10, GraphPad Software, La Jolla California USA (<http://www.graphpad.com>).

RESULTS

The *S. kudriavzevii* *ARO4* gene decreases the phenylethanol and tyrosol production

To assay the effect of *ARO4* alleles on the production of higher alcohols, we generated the recombinant *ARO4* strains ST41-Sc and ST41-Sk (Table 1), and they were used to carry out the fermentation in SWM. At the end of fermentation, we took samples to analyse sugar content and the production of aromatic compounds by HPLC. We did not observe differences in the ethanol and glycerol production between strains ST41-Sk and ST41-Sc (Figure 3A). Strikingly, strain ST41-Sk, compared with strain ST41-Sc, showed a reduction of 8.2% and 13.3% of tyrosol and phenylethanol, respectively (Figure 3A). Since *ARO3* encodes the DAPH synthase inhibited by phenylalanine, we disrupted this gene in both recombinant

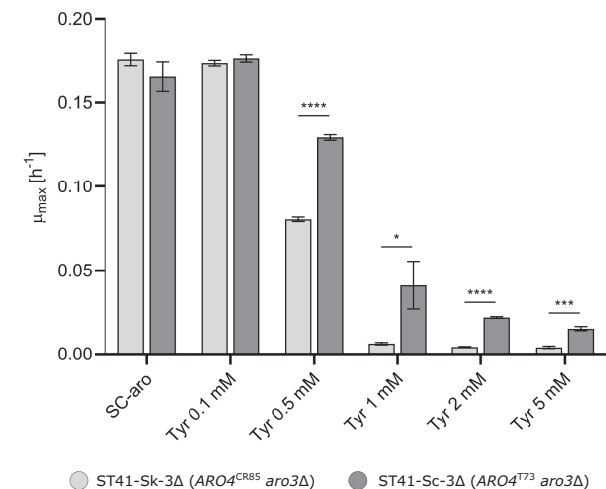


FIGURE 4 Effect of the *in vivo* Aro4p feedback inhibition on the growth rate. The strains ST41-Sk-3Δ (*aro4::kanmx::ARO4*(CR85) *aro3::KanMX*) and ST41-Sc-3Δ (*aro4::kanmx::ARO4*(T73) *aro3::KanMX*) were cultured at 28°C in 200 μl of synthetic complete medium without aromatic amino acids (SC-aro) and different tyrosine (Tyr) concentrations (0.1, 0.5, 1, 2 and 5 mM). The growth rates were determined by fitting the growth curves through the Gompertz model using the grofit R package (Kahm et al., 2010). The error bars indicate the standard deviation of three biological replicas. Statistical differences were determined through a two-tailed unpaired *t*-test. *p*-values style are from GraphPad Prims version 8.01: 0.0332 (*); 0.0002 (***); <0.0001 (****).

strains to remove DAHP synthase (DAHPs) activity interference and to analyse directly the effect of the presence of the different *ARO4* alleles. At the end of fermentation, no differences in the production of glycerol and ethanol were observed (Figure 3B). Nevertheless, we observed a reduction of 31.6% and 29.5% in the production of tyrosol and phenylethanol, respectively, in strain ST41-Sk-3Δ compared with ST41-Sc-3Δ (Figure 3B). These results suggest that the DAHPs encoded by *S. kudriavzevii* *ARO4* allele seem to reduce the metabolic flux towards the Shikimate pathway, and hence, the biosynthesis of phenylethanol and tyrosol.

The strain carrying *S. kudriavzevii* *ARO4* allele is more sensitive to tyrosine

The reduced production of phenylethanol and tyrosol exhibited by strain ST41-Sk-3Δ suggests that *S. kudriavzevii* DAHPs might exhibit less activity. Here, we conjectured this could be a consequence of higher sensitivity to the tyrosine feedback inhibition compared with the *S. cerevisiae* DAHPs. To test this hypothesis, we cultured both ST41-Sk-3Δ and ST41-Sc-3Δ strains in SC-aro media with different concentrations of the inhibitor tyrosine (Figure 4). We did not observe appreciable at 0.1 mM. However, at 0.5 mM, the μ_{max} was reduced in both strains, but the inhibition was higher in ST41-Sk-3Δ strain, which shows 37.8% less μ_{max} than strain ST41-Sc-3Δ. Moreover, we observed a total impaired

growth in strain ST41-Sk-3Δ from 1 mM to 5 mM, but strain ST41-Sc-3Δ could grow even at the highest concentration although at a low rate. These results support that the DAHPs encoded by *S. kudriavzevii* *ARO4* allele is more sensitive to tyrosine inhibition than the DAHPs encoded by *S. cerevisiae* *ARO4* allele.

Functional divergence analysis of *S. kudriavzevii* Aro4p

In the study carried out by Macías et al. (2019), the authors identified the amino acids being under positive selection in *S. kudriavzevii* Aro4p sequence. Here, we assigned Grantham's scores to those changes (Figure 5A). Five changes were N-terminal (S_4S_2 , P_5E_3 , $P_{13}G_{11}$, $Q_{17}E_{15}$ and $L_{26}Q_{24}$), being the change $L_{26}Q_{24}$ was the one showing the highest score (113) which is classified as moderately radical according to Li et al. (1984). This change is in the beta-sheet β_0 of the first monomer involved in the interaction with the beta-sheet β_6b of the second monomer (Figure 5B). The beta-sheet β_6b belongs to one of the two-stranded β -sheets (denominated internal extension) inserted between helix α_5 and strand β_6 of the eightfold β/α barrel, which is essential for the regulation of Aro4p by tyrosine (Hartmann et al., 2003). The change $L_{26}Q_{24}$ generates a new hydrogen bond of 3.3 Å between the amino group residue of the glutamine GLN-26 with the oxygen of the carboxylic group of the threonine THR-238 (Figure 5C). The change with the second-highest score is $A_{332}S_{330}$ which is in the loop that connects the beta-sheet β_8 with the alpha-helix α_8 , yet with unknown functions. Therefore, the identified changes could impact the activity regulation of the *S. kudriavzevii* Aro4p such as the feedback inhibition exerted by tyrosine.

DISCUSSION

In this study, we focused on the role of *ARO4* *S. kudriavzevii* allele in the production of the desired higher alcohol phenylethanol. Macías et al. (2019) reported the gene *ARO4* from *S. kudriavzevii* to be under positive selection compared with *S. cerevisiae* gene, and they suggested that the observed amino acidic changes could have a role in the ability of *S. kudriavzevii* to produce more phenylethanol than *S. cerevisiae* (Peris et al., 2016). The results obtained in this study suggest that DAHP synthase encoded by *S. kudriavzevii* *ARO4* allele is less efficient than the protein encoded by *S. cerevisiae* counterpart during winemaking fermentation. This result agrees with the lower intracellular flux throughout the shikimate pathway exhibited by the *S. kudriavzevii* strain CR85 compared with *S. cerevisiae* strain T73 during fermentation in grape must at low temperatures (12°C; Minebois et al., 2020b). Moreover, Rollero et al. (2019) demonstrated in *S. cerevisiae* that the

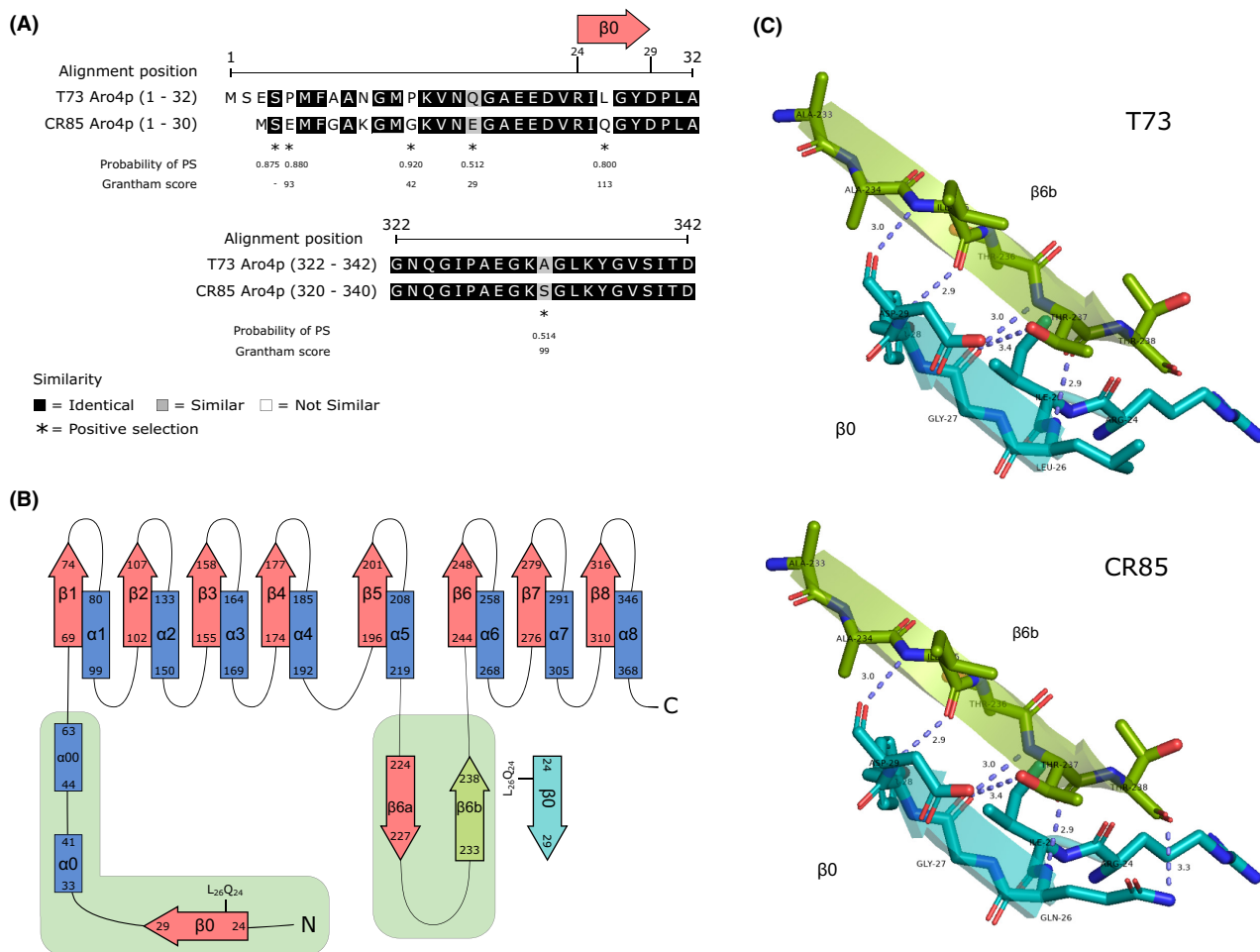


FIGURE 5 Amino acids are under positive selection. (A) The amino acid residues were identified by Macías et al. (2019). The probability of the positive selection (PS) is shown. Grantham's scores were assigned to the amino changes based on Grantham (1974). According to Li et al. (1984), amino acid changes are considered: Conservative (score ≤ 50), moderately conservative ($50 < \text{score} \leq 100$), moderately radical ($100 < \text{score} \leq 150$) and radical (score > 150). (B) Topology of the Aro4p, adapted from Hartmann et al. (2003). The beta-sheet and alpha-helix structures are coloured in red and blue, respectively. The additional structures of the Aro4p β/α barrel, the N-terminal extension and the two-stranded β -sheet internal extension, are highlighted in green. The beta-sheets β_0 and β_{6b} , involved in the interaction between the two monomers of Aro4p, are coloured in cyan and green, respectively. The location of the change $L_{26}Q_{24}$ is indicated. (C) Tridimensional model of the interaction between the beta-sheets β_0 and β_{6b} of the *S. cerevisiae* (up) and *S. kudriavzevii* (down) Aro4p. The PyMOL™ 2.5.2 software was used to carry out the *in-silico* mutation using as a template the Aro4p crystal structure 1 of 6.4 downloaded from the SWISS-MODEL server. The conformation of GLN-26 with the highest mutation probability (23.2%) is shown. The blue dotted lines indicated polar interactions.

phenylethanol is generated mainly from the catabolism of the phenylalanine rather than through a *de novo* synthesis from the sugar catabolism. Moreover, *S. kudriavzevii* produces higher phenylethanol amounts from the amino acid precursor phenylalanine compared with *S. cerevisiae* (Stribny et al., 2015). It has been demonstrated that the expression of *S. kudriavzevii* *ARO10* allele in the wine *S. cerevisiae* strain T73 increases the production of phenylethanol during fermentation in synthetic wine must (Stribny et al., 2016). Therefore, the ability of *S. kudriavzevii* to produce more phenylethanol (Peris et al., 2016) seems to rely on the Ehrlich Pathway. However, it remained unclear why *S. kudriavzevii* Aro4p is less efficient than *S. cerevisiae* Aro4p. The DAHP encoded by *ARO4* is feedback regulated by tyrosine (Helmstaedt et al., 2005; Künzler et al., 1992), and yeasts with the genotype *aro3 ARO4* are sensitive to the presence of tyrosine if it is used as

the sole aromatic amino acid source (Sousa et al., 2002). Therefore, we propose that the lower efficiency of the *S. kudriavzevii* Aro4p could be a consequence of a higher sensitivity to the feedback regulation exerted by tyrosine. Indeed, we demonstrated that the strain carrying the *S. kudriavzevii* *ARO4* gene is more sensitive to tyrosine than the recombinant strain carrying the *S. cerevisiae* one.

The functional divergence analysis of the amino acid, whose codons showed positive selection, might explain the observed differences in the Aro4p. Five of the six amino acids being under positive selection were located at the amino end of the Aro4p. At the S_4S_2 position, the codon TCT which appears in *S. cerevisiae* changes to the codon AGT present in the *S. kudriavzevii* *ARO4* allele. Despite both codons encoding for serine, the transition between them is produced first by a deleterious mutation followed by a compensatory substitution,

which is subject to strong purifying selection (Rogozin et al., 2016), which explains why this codon was detected in the positive selection analysis carried out by Macías et al. (2019). However, since no amino acid change was produced, no functional implications can be assigned to this change. The Aro4p is feedback regulated by tyrosine via allosteric regulation (Luttik et al., 2008), a mechanism deeply studied in the chorismate mutase encoded by *ARO7*, which is also downregulated by tyrosine (Helmstaedt et al., 2001). The Aro4p X-ray structure shows a cavity conformed of $\alpha 3$, $\alpha 4$, the loop between $\alpha 4$ and $\beta 5$, $\beta 6a$ and $\beta 6b$ (Hartmann et al., 2003). Based on single random ($S_{195}P$, $S_{196}S$) and directed mutations ($T_{162}L$, $Q_{166}E$, $G_{193}K$, $S_{195}A$, $G_{226}A$, $K_{229}L$, $T_{236}R$), it has been confirmed that the residues present in this cavity are responsible for the feedback regulation by tyrosine (Hartmann et al., 2003). Moreover, the single substitution $G_{226}S$ generated a DAHPs that showed the same regulation pattern as Aro3p, which is downregulated by phenylalanine instead of tyrosine (Hartmann et al., 2003). Interestingly, the substitutions located in the N-terminal extension ($D_{22}G$, $T_{44}I$, $E_{49}G$, $R_{55}G$) and the deletion of the first 20N-terminal residues also generate an unregulatable DAHP synthase (Hartmann et al., 2003). The N-terminal extension is composed of $\beta 0$ (residues 24–29), $\alpha 0$ (residues 33–41) and $\alpha 00$ (residues 44–63) structures, where the $\beta 0$ is involved in the interaction between the two Aro4p monomers (Hartmann et al., 2003). Therefore, the $L_{26}Q_{24}$ substitution, which is in the beta-sheet $\beta 0$, generates a new hydrogen bond between the glutamine and the threonine located in the $\beta 6b$ that could confer a more stable interaction between the monomers, and hence, a differential downregulation pattern by tyrosine. Moreover, the changes in P_5E_3 , $P_{13}G_{11}$ and $Q_{17}E_{15}$, which were not resolved in the crystal structure, could play a role in protein regulation as well. The replacement $A_{332}S_{330}$, with the second-highest score, is located in the loop that connects the $\beta 8\alpha 8$. Residues in the loops which connect the beta-strand and alpha-helix domains participate in the interaction with the substrate PEP (Hartmann et al., 2003). However, no function has been assigned to the $\beta 8\alpha 8$ of the Aro4p, but the mutation $Q_{302}R$ that is in the alpha-helix $\alpha 7$ has been reported as partially recovering the sensitiveness to tyrosine of an insensitive tyrosine mutant $P_{165}G$ (Helmstaedt et al., 2005). Thus, the mutation $A_{332}S_{330}$ could play a role either in the PEP binding or in the feedback regulation.

Despite we demonstrated that the reduced activity in *S. kudriavzevii* Aro4p is due to a higher sensitivity to the tyrosine feedback regulation, it remains unclear why the *ARO4* gene showed positive selection in *S. kudriavzevii* and which the adaptive advantages that replacements confer. The first pure *S. kudriavzevii* strains were isolated from decayed leaves in Japan (Naumov et al., 2000) and from *Quercus* tree barks (*Q. faginea*, *Q. ilex*, *Q. pyrenaica*) in the Iberian Peninsula (Lopes et al., 2010; Sampaio & Gonçalves, 2008). Sampaio and Gonçalves (2008)

analysed the sugar content in the tree barks, where the yeasts were isolated, and they presented trace amounts of sucrose in *Q. ilex* and glucose, fructose and sucrose in *Q. faginea* and *Q. pyrenaica*. Unfortunately, the amino acid content was not analysed. However, some studies have quantified the amino acid content of the xylem sap and the acorn in *Q. ilex*, which depends on the growth stage, soil composition and seasonal period (Nabais et al., 1997; Özcan, 2006). In the xylem sap, the molar concentration ratio between tyrosine and the predominant amino acid, asparagine, fluctuates from not being detected (tyrosine) up to 0.420 tyrosine $\mu M/\mu M$ asparagine and 0.172 tyrosine $\mu M/\mu M$ asparagine in *Q. ilex* trees growing in Sandy Loam and Serpentine soils (Nabais et al., 1997). However, the tyrosine amounts reach up to trace levels in ultramafic and schist-derived soils (Nabais et al., 2005). In the acorns, the molecule ratio between tyrosine and the predominant amino acid glutamate is 0.182 mmol tyrosine/mmol glutamate for each 100g dry acorn (Özcan, 2006). In contrast, the proportions between tyrosine and the predominant amino acids, arginine and glutamine, in the grape must are 5.46×10^{-2} mM tyrosine/mM arginine and 6.77×10^{-2} mM tyrosine/mM glutamine, respectively (Minebois et al., 2020a), which are similar to the proportions used in our synthetic wine must (5.10×10^{-2} mM tyrosine/mM arginine and 3.15×10^{-2} mM tyrosine/mM glutamine). Based on these observations, we propose that the gene *ARO4* in *S. kudriavzevii* evolved to reduce the aromatic amino acid biosynthetic flux according to tyrosine availability in their natural niches. In contrast, *S. cerevisiae* requires a better aromatic amino acid biosynthetic flux because of the low proportion of aromatic amino acid and higher sugar concentration present in the grape must. This agrees with the observation that, in *S. cerevisiae* \times *S. kudriavzevii* hybrids, *S. cerevisiae* *ARO4* allele is expressed more than *S. kudriavzevii* counterpart (Combina et al., 2012).

CONCLUSIONS

In the present work, we demonstrate that *S. kudriavzevii* *ARO4* allele, which shows positive selection, encodes a DAPH synthase that reduces the production of phenylethanol in wine fermentation conditions. The greater growth inhibition showed by the strain carrying *S. kudriavzevii* *ARO4* suggests that the Aro4p encoded by this allele is more sensitive to the feedback regulation by tyrosine reducing its activity. The obtained results together with previous studies support that the ability of *S. kudriavzevii* to produce larger amounts of higher alcohols relies on the aromatic amino acid catabolic pathways. Moreover, we described candidate amino acid changes that might affect the activity of *S. kudriavzevii* Aro4p and we propose that the positive selection could be related to the ecological niche of *S. kudriavzevii*.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

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

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