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,
the consumer trends have changed, looking for rich and ripe fruit-flavoured 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).

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 (Ebele, 2001), making a positive aromatic contribution at concentrations below 300 mg/L (Swiegler & 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 & Duszkieiwicz-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; Luttik 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 & Gonalves, 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 & Duszkieiwicz-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 ARO4 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 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, 0.024 g/L Lysine, 0.5 g/L Phenylalanine, 1 g/L Glutamic acid, 1 g/L Arginine, 0.3 g/L Tyrosine, 0.6 g/L Leucine, 0.24 g/L Lysine, 0.6 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.
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.
(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 (Stovichek 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 μl 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 alleles. Finally, the inserted cassette was treated with DpnI (Thermo Fisher Scientific). A total of 30 μl of PCR product was amplified, and one of the corresponding to 72 YAN) and 5.54 ml of a mixture of 19 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 NH₄Cl (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 μm filters.

**Fermentation conditions**

Fermentations were carried out in 100 ml bottles coupled with an airlock containing 80 ml of SWM with a 30 mm × 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 OD₆₀₀ of 0.2 at 28°C with an agitation of 120 rpm. The fermentation kinetics was monitored through the weight loss, which represents the CO₂ 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.

**TABLE 1** Strains used in this study.

| Strain      | Specie                      | Genotype                          | Reference    |
|-------------|-----------------------------|-----------------------------------|--------------|
| AQ2775      | Saccharomyces cerevisiae    | T73 MATalpha                      | This study   |
| AQ4013      | Saccharomyces kudriavzevii  | CR85 MATalpha ho::MX4dsdA         | This study   |
| ST41        | Saccharomyces cerevisiae    | MATalpha, aro4::kanMX             | This study   |
| ST41-Sc     | Saccharomyces cerevisiae    | MATalpha, aro4::kanmx::ARO4(T73)  | This study   |
| ST41-Sk     | Saccharomyces cerevisiae    | MATalpha, aro4::kanmx::ARO4(CR85) | This study   |
| ST41-Sc-3Δ  | Saccharomyces cerevisiae    | MATalpha, aro4::kanmx::ARO4(T73), aro3::KanMX | This study   |
| ST41-Sk-3Δ  | Saccharomyces cerevisiae    | MATalpha, aro4::kanmx::ARO4(CR85), aro3::KanMX | This study   |

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 KH₂PO₄, 0.5 g/L K₂SO₄, 0.25 g/L MgSO₄·7H₂O, 0.155 g/L CaCl₂·2H₂O 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 MnSO₄·H₂O, 4 g/L ZnSO₄·7H₂O, 1 g/L CuSO₄·5H₂O, 1 g/L KI, 0.4 g/L CoCl₂·5H₂O, 1 g/L H₃BO₃ and 1 g/L (NH₄)₆Mo₇O₂₄·4H₂O) 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). Once the fermentation ended, samples were collected by centrifugation, and the supernatant was recovered and frozen until the analytical procedures.

**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,
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-μm nylon filter. The run conditions were 1.5 mM of H₂SO₄ 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 detector.
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-μm nylon filters. The separation was performed in a 4.6 mm × 150 mm × 2.6 μm 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 μl 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 μl of SC-aro media containing different concentrations of tyrosine (0.1, 0.5, 1, 2 and 5 mM) with an initial OD 600 of 0.2 on 96 well-microtiter plates at 25°C. Growth curves were monitored through OD measurement at 600nm wavelength in a SPECTROstar Nano® microplate reader (BMG LABTECH GmbH). Maximum specific growth rates (μmax) 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
(S4S2, P5E3, P13G11, Q37E15, 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.

**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. 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 (***)..

![Figure 3](https://www.graphpad.com)
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 total impaired function 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.

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-arox media with different concentrations of the inhibitor tyrosine (Figure 4). We did not observe appreciable 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 show 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 Macias 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 (S4S2, P5E3, P13G11, Q17E15 and L26Q24), being the change L26Q24 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 β0 of the first monomer involved in the interaction with the beta-sheet β6b of the second monomer (Figure 5B). The beta-sheet β6b belongs to one of the two-stranded β-sheets (denominated internal extension) inserted between helix α5 and strand β6 of the eightfold β/α barrel, which is essential for the regulation of Aro4p by tyrosine (Hartmann et al., 2003). The change L26Q24 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 A332S330 which is in the loop that connects the beta-sheet β8 with the alpha-helix α8, 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.
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 S4S2 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 Macias 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 α3, α4, the loop between α4 and β5, β6a and β6b (Hartmann et al., 2003). Based on single random (S195G, S196G) and directed mutations (T162L, G193K, S195A, G226A, K229L, T236R), 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 G226S 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 (D22G, T44I, E49G, R55G) and the deletion of the first 20 N-terminal residues also generate an unregulatable DAHP synthase (Hartmann et al., 2003). The N-terminal extension is composed of j0 (residues 24–29), α0 (residues 33–41) and α00 (residues 44–63) structures, where the j0 is involved in the interaction between the two Aro4p monomers (Hartmann et al., 2003). Therefore, the L26Q24 substitution, which is in the beta-sheet j0, generates a new hydrogen bond between the glutamline and the threonine located in the β6b that could confer a more stable interaction between the monomers, and hence, a differential downregulation pattern by tyrosine. Moreover, the changes in P5E3, P13G11 and Q17E15, which were not resolved in the crystal structure, could play a role in protein regulation as well. The replacement S322A330 with the second-highest score, is located in the loop that connects theβ8α8. 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 β8α8 of the Aro4p, but the mutation Q322R that is in the alpha-helix α7 has been reported as partially recovering the sensitiveness to tyrosine of an insensitive tyrosine mutant P165G (Helmstaedt et al., 2005). Thus, the mutation A332S330 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 0.42 tyrosine μM/μM asparagine and 0.172 tyrosine μM/μM 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 100 g 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 × 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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CONFICT OF INTEREST
None declared.

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