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Genetic basis of oenological traits in *Saccharomyces* interspecific hybrids

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Sous la direction de Carole CAMARASA,
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A Rafael, Amalia, Regino y Dolores.

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Communications related to this work

Poster presentations at conferences

- Álvarez, R., Louis, E.J., Dequin, S., and Camarasa, C., 'Screening of aroma metabolites within a set of 90 *Saccharomyces* strains', EFB2021, 10th – 14th May 2021, organised by the European Federation of Biotechnology (online).

Oral presentations at conferences

- Álvarez, R., Louis, E.J., Dequin, S., and Camarasa, C., 'Screening of aroma metabolites within a set of 90 *Saccharomyces* strains', MACROWINE 2021, 23rd – 30th June 2021, organised by Università di Verona, Università di Torino and Fondazione Edmund Mach, Italy (online).

- Álvarez, R., Louis, E.J., Dequin, S., and Camarasa, C., 'Screening of aroma metabolites within a set of 90 *Saccharomyces* strains', ICY15 meets ICYGMB30, 23th – 27th August 2021, organised by the University of Vienna, Austria (online).

- Álvarez, R., Garces, F., Louis, E.J., Dequin, S., and Camarasa, C., 'Phenotypic diversity in the genus *Saccharomyces* for winemaking', ISSY36, organised by UBC (University of British Columbia), Vancouver, Canada.

- Álvarez, R., Maçna, F., Hu, Y., Dequin, S., Camarasa, C., and Louis, E.J., 'Genetic basis of oenological traits in an interspecific *Saccharomyces uvarum* x *S. mikatae* hybrid', PYFF8, 5th – 8th June 2023, organised by UCC (University College Cork), Cork, Ireland.

Peer-reviewed publications

- Álvarez, R., Garces, F., Louis, E.J., Dequin, S., Camarasa, C. (2023) "Beyond *S. cerevisiae* for winemaking: Fermentation-related trait diversity in the genus *Saccharomyces*", *Food Microbiology*, 113(August), https://doi.org/10.1016/j.fm.2023.104270.

Abbreviations

ADH: alcohol dehydrogenase

ADY: active dry yeast

Agr: arginine (amino acid)

Ala: alanine (amino acid)

ALD: aldehyde dehydrogenase

ANOVA: analysis of variance

Asn: asparagine (amino acid)

Asp: aspartic acid (amino acid)

ATP: adenosine triphosphate

BLAST: Basic Local Alignment Search Tool (by NCBI)

Bp: base pairs

BSA: bulk segregant analysis

CCM: central carbon metabolism

CIM: composite interval mapping

CNV: copy number variation

CoA: coenzyme A

Cys: cysteine (amino acid)

DNA: deoxyribonucleic acid

EDTA: etheylenediaminetetraacetic acid

EtBr: ethidium bromide

FAS: fatty acid synthase

FID: flame ionization detector

FOT: fungal oligopeptide transporter

GC: gas chromatography

GCR: gross chromosomal rearrangement

Gln: glutamine (amino acid)

Glu: glutamate (amino acid)

Gly: glycine (amino acid)

GMO: genetically modified organism

HGT: horizontal gene transfer

His: histidine (amino acid)

HPLC: high performance liquid chromatography

Ile: isoleucine (amino acid)

ISA: individual segregant analysis

ISTD: internal standard

Kbp: kilo-base pairs (i.e. x 1000 bp)

Leu: leucine (amino acid)

LOH: loss-of-heterozygosity

Lys: lysine (amino acid)

MAS: marker-assisted selection

MAT: mating type locus (genotypes: MATa or MATalpha)

MCFA: medium-chain fatty acid

Met: methionine (amino acid)

MMR: mismatch repair system

MPP: multi-parent population

MS: mass spectrometry

mtDNA: mitochondrial DNA

NAD^+/NADH: Nicotinamide adenine dinucleotide (oxidized / reduced form, respectively)

$NADP^+$/NADPH: Nicotinamide adenine dinucleotide phosphate (oxidized / reduced form, respectively)

NGS: next generation sequencing

OAA: oxaloacetate

OAC: odour-active compound

OIV: International Organisation of Vine and Wine

ORF: open reading frame

PCA: principal component analysis

PDC: pyruvate decarboxylase

Phe: phenylalanine (amino acid)

PPP: pentose phosphate pathway

Pro: proline (amino acid)

QTG: quantitative trait gene

QTL: quantitative trait locus

QTN: quantitative trait nucleotide

R_{60} / R_{80}: CO_2 production rate when 60 or 80 g/L of CO_2 have been released

R_{max}: maximum CO_2 production rate

RTG: return to (mitotic) growth

SCFA: short-chain fatty acid

Ser: serine (amino acid)

SFA: saturated fatty acid

SGD: *Saccharomyces* Genome Database

SIM: simple interval mapping

SNP: single nucleotide polymorphism

TAE: tris-acetate-EDTA (buffer)

TCA: tricarboxylic acid cycle, or Krebs cycle

Thr: threonine (amino acid)

Trp: tryptophan (amino acid)

Tyr: tyrosine (amino acid)

UFA: unsaturated fatty acid

Val: valine (amino acid)

WGD: whole-genome duplication

YAN: yeast assimilable nitrogen

YPD: yeast extract peptone dextrose

αKG: α-ketoglutarate

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Chapter 1

Résumé en français

La vinification est très certainement le processus biotechnologique le plus ancien maîtrisé par l'humanité. Les premières preuves de vinification datent du néolithique, autour de 6000 à 5800 av. J.C. (McGovern et al., 2017). De ces jours, l'homme a essayé de maîtriser et d'améliorer ce procédé, étudiant les mécanismes sous-jacents. Progressivement, le vin est devenu un élément essentiel de la culture, tout d'abord européenne, puis au niveau mondial. Cependant, ces dix dernières années la production de vin des pays européens a diminué. Cela est à mettre en relation avec un surplus de production, qui est le principal problème rencontré actuellement par le secteur viticole : 260 mhL de vin ont été produits en 2021, mais seulement 236 mhL ont été consommés (OIV, 2022). Cela s'explique, au moins partiellement, par des changements profonds des pratiques de consommation du vin dans nos sociétés. En raison des campagnes de sensibilisation du grand public sur les effets de l'alcool sur la santé, la consommation régulière de grands volumes de vin, observée jusqu'à la moitié du XXe siècle, a évolué vers une consommation plus occasionnelle, recherchant la qualité plutôt que la quantité. Par conséquent, le secteur du vin est progressivement devenu un marché axé sur la qualité, où les consommateurs recherchent des vins à la fois avec moins d'alcool et des profils organoleptiques marqués, caractéristiques d'un terroir ou d'une appellation (Swiegers et al., 2005).

D'un autre côté, le changement climatique exerce une influence de plus en plus prégnante sur la composition des moûts de raisin. Pendant la maturation, les raisins subissent une augmentation progressive de la teneur en sucre et une diminution progressive des acides organiques. Le réchauffement climatique accélère ce déséquilibre en conduisant à une teneur en sucre excessivement élevée – entraînant une augmentation des concentrations d'alcool – et des quantités d'acide trop faibles dans les moûts (Mira de Orduña, 2010). Pour rester compétitifs, les vignerons cherchent des méthodes pour diversifier les propriétés organoleptiques de leurs produits, visant à répondre aux attentes des consommateurs tout en faisant face aux effets du changement climatique sur la composition du vin.

Les levures sont les acteurs principaux de la fermentation alcoolique. Elles transforment les hexoses en éthanol et en CO_2 et produisent des milliers de métabolites primaires et secondaires qui façonnent le goût, l'odeur et la perception en bouche des vins. Par conséquent, la composition du vin peut être partiellement modulée en sélectionnant les levures qui mènent la fermentation. Ainsi, différentes souches de levure peuvent produire quantités différentes d'éthanol et d'acides organiques à partir d'un même moût de départ. De plus, elles contribuent différemment à l'arôme et goût du vin en libérant des composés volatiles depuis leurs précurseurs inodores (arômes variétaux) et par la synthèse *de novo*

d'autres molécules volatiles telles que des alcools supérieurs et des esters (arômes fermentaires).

De ce fait, les approches fondées sur la sélection et l'amélioration des souches de levure semblent prometteuses pour relever les défis auxquels l'industrie vinicole est actuellement confrontée. Dans ce contexte, les microbiologistes cherchent à élargir la gamme des levures commerciales, en proposant des souches avec des phénotypes spécifiques. Pour cela, principalement, deux approches complémentaires peuvent être suivies : d'une part, l'étude de la biodiversité de levures en conditions œnologiques permet de découvrir des nouveaux phénotypes d'intérêt ; d'autre part, il est possible d'étendre cette diversité phénotypique par les approches d'amélioration génétique de souches, actuellement disponibles en laboratoire. Pour cette dernière approche, les connaissances approfondies sur les bases génétiques des nouveaux phénotypes sont nécessaires pour mettre en place et cibler les stratégies d'amélioration.

L'histoire de la vinification a été focalisée sur un acteur majeur : *Saccharomyces cerevisiae*. Cette espèce intervient dans l'élaboration de la plupart des boissons fermentées, soit en souche pure, soit sous forme d'hybride interspécifique avec d'autres espèces de *Saccharomyces* comme *S. eubayanus* ou *S. kudriavzevii*. Cet organisme modèle est connu pour ses excellentes capacités fermentaires, et transforme efficacement le sucre des moûts de raisin en alcool même en conditions aérobies (effet Crabtree). Malgré leur intérêt, les souches de *S. cerevisiae* et ses hybrides disponibles commercialement présentent une variabilité limitée, démontrée insuffisante pour relever efficacement les défis actuels de l'industrie des boissons mentionnés ci-dessus, surtout d'un point de vue métabolique (Molinet et Cubillos 2020).

De ce fait, les microbiologistes se sont intéressés de plus en plus sur des espèces de levure alternatives, principalement des non-*Saccharomyces*. Ces genres de levure, longtemps considérés comme des contaminants, ont révélé leur potentiel de produire une gamme diversifiée de métabolites fermentaires, y compris des composés volatils contribuant positivement à la qualité sensorielle des vins (Jolly *et al.,* 2014). Certaines de ces espèces permettent aussi d'obtenir un rendement en éthanol considérablement réduit, réorientant les flux de carbone vers d'autres composés du métabolisme carboné central ou de la biomasse (Padilla *et al.,* 2016). Malgré leurs propriétés intéressantes, la plupart des non-*Saccharomyces* ont un inconvénient majeur : du fait de leur sensibilité à l'éthanol, elles ne sont pas capables de mener à terme la fermentation (consommation totale des sucres). Par conséquent, les souches de *S. cerevisiae* naturellement présentes dans les fermentations spontanées surpassent généralement les souches non-*Saccharomyces*, minimisant ainsi leur contribution à la composition du vin (Jolly et al. 2014). Les co-cultures et les inoculations séquentielles de *S. cerevisiae* et de non-*Saccharomyces* constituent une approche pertinente, mais présentent toutefois des inconvénients dus à des

interactions négatives entre espèces, principalement liées à des phénomènes de compétition vis-à-vis des nutriments (Zilelidou et Nisiotou 2021). Par conséquent, l'inoculation en culture pure est l'approche préférée par les vignerons, permettant plus de contrôle sur le processus et la composition du produit final. Ce contexte souligne le besoin de l'industrie pour de nouvelles souches capables d'effectuer une fermentation efficace tout en produisant une gamme variée de métabolites à faible concentration d'éthanol.

En plus de *S. cerevisiae*, le genre *Saccharomyces* comporte sept autres espèces. Parmi elles, *S. cerevisiae* et *S. uvarum* ont été trouvées dans des fermentations industrielles en tant qu'espèces pures et *S. eubayanus* et *S. kudriavzevii* ont été isolées dans des environnements industriels, mais seulement en tant que composant d'hybrides interspécifiques avec *S. cerevisiae*. A l'inverse, à ce jour, les espèces *S. mikatae, S. jurei, S. arboricola* et *S. paradoxus* n'ont été isolées que dans les écosystèmes naturels (Alsammar et Delneri 2020). Fait intéressant, certaines levures *Saccharomyces* non *cerevisiae* peuvent fermenter le moût de raisin jusqu'à épuisement du sucre. Par exemple, il a été établi que les populations de *S. uvarum* prédominent dans certaines fermentations spontanées même lorsque *S. cerevisiae* est présent (Demuyter et al. 2004). D'autres études ont permis d'identifier des souches de *S. paradoxus* (Orlić et al. 2007), *S. eubayanus* (Parpinello *et al.* 2020) et *S. kudriavzevii* (Pérez *et al.* 2021) montrant de bonnes performances fermentaires. D'un point de vue métabolique, il a été démontré que *S. kudriavzevii, S. eubayanus* et *S. uvarum* produisent des vins avec moins d'ethanol et des quantités plus élevées en glycérol et certains esters et alcools supérieurs (Minebois et al. 2020; Pérez et al. 2021; 2022), tandis que *S. paradoxus* peut également réduire l'acidité volatile, comparativement à *S. cerevisiae* (Orlić *et al.* 2007). Toutefois, les connaissances sur la diversité phénotypique des souches de *Saccharomyces* non-*cerevisiae* en œnologie restent très empiriques et limitées à un petit nombre de souches, ce qui limite l'exploitation des potentialités de ces espèces dans l'industrie.

Dans ce contexte, l'objectif général de ce projet de thèse a été d'étudier la diversité phénotypique dans le genre *Saccharomyces* en fermentation alcoolique ainsi que les bases génétiques sous-jacentes à leurs phénotypes d'intérêt.

Dans un premier temps, nous nous sommes intéressés à la diversité phénotypique chez les huit espèces de *Saccharomyces* actuelles – dont certaines (*S. mikatae, S. jurei* et *S. arboricola*) n'ont jamais été caractérisées dans des conditions œnologiques – visant à déterminer ses propriétés cinétiques et métaboliques en fermentation alcoolique. Pour cela, nous avons sélectionné 92 souches de *Saccharomyces*, y compris des souches commerciales actuellement utilisées par l'industrie. Nous avons conduit des fermentations à échelle de laboratoire (250 mL) en moût synthétique (200 g/L de sucres et 200 mg/L d'azote) à 16 et 22 ºC et réalisé un suivi détaillé de la cinétique fermentaire. Nous avons également quantifié les principaux métabolites contribuant à la qualité sensorielle des vins et dont la

production doit être contrôlée : l'éthanol, les acides organiques, les arômes fermentaires, et d'autres composés jouant un rôle dans les propriétés organoleptiques des vins comme le glycérol.

En ce qui concerne les capacités fermentaires, la découverte peut-être la plus surprenante a été le fait que des souches de toutes les espèces de *Saccharomyces* ont pu fermenter le moût synthétique avec 200 g/L de glucose/fructose jusqu'à épuisement (concentration inférieure à 4 g/L de sucre) (OIV, 2021). Le profil cinétique d'une fermentation est décrit par plusieurs paramètres, incluant la phase de latence, la vitesse maximale de fermentation et le temps mis pour l'atteindre, qui traduisent l'efficacité fermentaire en début de procédé, le temps requis pour produire 60 g/L de CO2 et la vitesse à ce temps, qui reflètent l'activité des levures en fin de procédé, et enfin la durée de fermentation et la quantité finale de CO_2 produit. Pour comparer de façon globale des capacités cinétiques entre souches, nous avons développé un nouveau paramètre – le *overall kinetic score* – comme indicateur général de l'efficacité fermentaire. En utilisant ce paramètre, nous avons observé que certaines souches sauvages de *S. cerevisiae, S. paradoxus*, *S. kudriavzevii* et *S. arboricola* fermentaient plus efficacement que des souches commerciales dans les conditions utilisées. Comme la teneur initiale en sucres est l'un des facteurs les plus stressants pour la levure, nous avons ensuite conduit des fermentations avec des quantités de sucre croissantes (jusqu'a 240 g/L) avec quelques souches sélectionnées pour ses bonnes capacités fermentaires. Dans ces conditions, certaines d'entre ces souches sont incapables de terminer la fermentation avec 240 g/L de sucres, mais d'autres ont consommé la totalité des sucres plus rapidement que la souche contrôle de *S. cerevisiae* dans toutes les conditions testées. Globalement, ces résultats ont un grand intérêt pour le secteur vitivinicole, car, a priori, ils indiquent que toutes les espèces de *Saccharomyces* peuvent être utilisées en fermentation alcoolique. Par contre, ces résultats doivent être interprétés avec précaution, car les conditions que nous avons utilisées sont relativement favorables par rapport à la composition de certains moûts naturels, qui peuvent avoirdes ratios entre sucres, lipides et azote défavorables ou contenir des concentrations limitantes en vitamines et oxygène dissous, ainsi que des sources de nutriments plus complexes comme les oligosaccharides et les oligopeptides. Il est donc nécessaire d'évaluer l'effet de ces paramètres sur les capacités fermentaires des *Saccharomyces* non-*cerevisiae* avant d'envisager leur commercialisation.

En ce qui concerne la production de métabolites, nous avons établi que la diversité au sein de *Saccharomyces* non-*cerevisiae* était bien supérieure à celle de *S. cerevisiae*. Nous avons noté quelques spécificités au niveau des espèces, certaines d'entre elles différant significativement de *S. cerevisiae* dans la production d'arômes et de composés du métabolisme carboné central. Par exemple, des souches d'espèces *Saccharomyces* non-*cerevisiae* ont montré une faible production d'acide acétique (*S. mikatae* et *S. paradoxus*)

ou une forte production de glycérol (*S. arboricola, S. jurei*, *S. mikatae* et *S. eubayanus*), d'acide succinique, de 2-phényléthanol et de 2-phenylethyl acetate (*S. uvarum*), de propanol (*S. kudriavzevii*), ou encore de 2-methylbutyl acetate et d'ethyl propanoate (*S. mikatae*). Cette diversité métabolique offre de nouvelles perspectives pour résoudre certains des défis rencontrés par le secteur vinicole aujourd'hui par l'exploitation du potentiel de ces souches. L'analyse sensorielle permettrait de savoir si les différences observées peuvent avoir un impact significatif sur l'ensemble du profil aromatique du vin.

Lors de la seconde étape du projet, nous avons cherché à déterminer les bases génétiques de certains des phénotypes d'intérêt mis en évidence dans la première partie du projet. Il s'est agi d'identifier les variants alléliques supérieurs qui pourront aider à l'amélioration des *Saccharomyces* non-*cerevisiae* au laboratoire. Plus concrètement, nous avons travaillé avec des hybrides interspécifiques. De tels microorganismes ont été isolés plusieurs fois dans des fermentations industrielles, montrant souvent des propriétés supérieures à celles des espèces parentales (vigueur hybride) (Masneuf-Pomarede *et al.*, 2002; González *et al.*, 2006; Erny *et al.*, 2012; Pérez-Torrado *et al.*, 2015). Par contre, les hybrides interspécifiques sont généralement stériles ; c'est-à-dire, les spores qu'ils produisent ne sont pas viables. De ce fait, les techniques de génétique quantitative ne peuvent pas être appliquées sur des hybrides, car ces approches nécessitent une descendance viable. Au cours des deux dernières décennies, les mécanismes responsables de cette stérilité ont été décrits en détail (Louis, 2011; Bozdag and Ono, 2022) rendant possible la création d'hybrides fertiles pour des applications en génétique quantitative (Naseeb *et al.,* 2021). Ainsi, nous avons cherché à générer des hybrides interspecifiques fertiles entre plusieurs espèces de *Saccharomyces*, en contrôlant leur héritage mitochondrial, pour effectuer une étude de cartographie QTL en conditions œnologiques sur sa descendance.

Nous avons réussi à générer des hybrides tétraploïdes entre *S. mikatae* et *S. uvarum* produisant plus de 80 % spores viables, restaurant sa fertilité. Ensuite, nous avons mené sur ces hybrides 11 étapes de sporulation / intercroissement pour réduire la taille des fragments d'ADN provenant de chaque souche parentale. L'isolement, le phénotypage et le génotypage de 78 segregants F12 nous a permis d'effectuer avec succès la toute première analyse QTL chez des *Saccharomyces* non-*cerevisiae*.

Contrairement aux attentes, nous n'avons détecté aucun QTL dépendant du mitotype (c'est-à-dire un QTL présent dans les ségrégants avec un mitotype donné et absent dans les ségrégants avec l'autre). Cependant, nous avons trouvé des différences significatives entre les ségrégants ayant des mitochondries provenant de *S. mikatae* et *S. uvarum* pour certains phénotypes. Ainsi, les ségrégants avec mtDNA de *S. uvaru*m ont fermenté plus efficacement à 22 ºC que ceux ayant des mitochondries de *S. mikatae*, et l'origine de l'ADN mitochondrial a affecté significativement la production de certains

métabolites (diethyl succinate et acides propanoïque, 2-methylpropanoïque et 2-methylbutanoïque). Ceci met en évidence l'intérêt d'étudier en détail le rôle des mitochondries dans la cinétique de fermentation et d'autres traits d'intérêt œnologique.

Nous n'avons pas détecté des QTLs dans le sous-génome de *S. mikatae*, en raison de l'utilisation de deux souches très similaires de cette espèce. Cela souligne l'importance d'un screening préalable des marqueurs génétiques en plus du screening phénotypique.

Par contre, nous avons détecté plusieurs QTL dans le sous-génome de *S. uvarum*. Grâce à l'utilisation d'une population F12, ces QTL étaient généralement très courts – beaucoup d'entre eux contenant un ou deux ORFs – malgré le nombre relativement faible de ségregants utilisés. La plupart des QTLs identifiés concernent des phénotypes liés à la production de composés du métabolisme carboné central. De plus, en règle générale, les gènes candidats ne codent pas des enzymes catalysant la biosynthèse de ces métabolites mais des protéines avec des fonctions régulatrices. Ainsi, certains gènes candidats ont un rôle majeur dans la régulation de l'utilisation et le métabolisme de la glucose. Cela met en évidence le rôle clé des variations des fonctions régulatrices sur les variations des phénotypes œnologiques, ce qui a été signalé dans d'autres études (Salinas *et al.*, 2012 ; Eder *et al.*, 2018). Cependant, l'impact réel des variantes alléliques de ces gènes et d'autres gènes candidats, reste encore à déterminer. De plus, nous ne savons toujours pas si la présence du sous-génome de *S. mikatae* affecte l'impact de ces QTLs sur les phénotypes. Prochainement, l'utilisation de *reciprocal hemizygosity analysis* (RHA) (Steinmetz *et al.*, 2002) permettra de répondre à ces deux questions. Si confirmés, les gènes candidats pourront être utilisés dans des programmes de sélection assistée par marqueurs génétiques (MAS) et d'autres stratégies de croissement pour améliorer des souches de *S. uvarum* et ses hybrides dans un contexte œnologique.

Globalement, ce projet a contribué significativement à dévoiler le potentiel des espèces de *Saccharomyces* alternatives à *S. cerevisiae* pour relever les défis actuels de l'industrie du vin.

General introduction

Winemaking is probably the most ancient biotechnological process mastered by humankind. The earliest evidence of winemaking dates from the Neolithic, around 6000 – 5800 B.C. (McGovern et al., 2017). From those days, humans have transformed grape must into wine, certainly enjoying the outcome but ignoring the mechanisms behind the process. However, like in all other fields of science, curiosity led us to increase our knowledge of vinification, aiming for its mastery and improvement. In 1789, Lavoisier first described alcoholic fermentation as a purely chemical reaction. Later, in 1810, Gay-Lussac concluded that sugars were transformed into ethanol and CO_2. Louis Pasteur discovered the role of yeasts in alcoholic fermentation in 1861, also describing the differences between aerobic and anaerobic fermentation (Barnett, 2000; Chambers and Pretorius, 2010).

Initially, all fermentations were spontaneous processes in which naturally present yeasts from different species and genera participated. Still today, many wineries keep employing the traditional spontaneous fermentation method, as it usually yields a complex and rich beverage from an organoleptic viewpoint. However, spontaneous fermentation can present some drawbacks, such as a longer lag phase, a weaker fermentation kinetics (especially at the end of the fermentation), potential spoilage, and unmanageable output leading to heterogeneity between different batches. To overcome these obstacles, the concept of inoculation with a pure culture was introduced, and in 1965, the first active dry yeasts (ADY) were produced. The actual rise of 'starter' yeast market took place in the 1980s, and from those days, the improvement of such starters has become a key element of wine research worldwide (Chambers and Pretorius, 2010). Currently, there are about 200 commercial yeast strains available (Schmidt *et al.*, 2017), most of which are *S. cerevisiae*.

As the knowledge in the field increased, the winemaking process improved and became more popular. Wine consumption slowly developed into a habit deeply rooted in the European society, becoming a key element of its culture. According to the last OIV report (OIV, 2022), France, Italy and Spain account for almost half of the world's wine production, emphasizing the key role of the wine sector in our continent's economy. However, wine production in the countries belonging to the so-called Old World (i.e. European countries) has decreased in the last decade. The key problem that becomes evident when analysing the world wine sector is a clear surplus production: 260 mhL of wine were produced in 2021, but only 236 mhL were consumed, only marginally more than in 2020, after a constant decrease from 2017 (OIV, 2022). This surplus production is due, at least in part, to a shift in the way that society consumes wine. Mainly due to an increase in the available information on the effects of alcohol in health, there has been an evolution from a regular consumption of large volumes to a more occasional consumption, looking for quality rather than quantity. For this reason, the wine sector has progressively become a

quality-focused market, where consumers look for pleasant and novel sensory experiences (Swiegers *et al.*, 2005).

On another note, climate change exerts an increasingly profound influence on grapevine composition. During ripening, grapevines experience a gradual increase in sugar content and a gradual decrease in organic acids. Global warming accelerates this process, leading to excessively high sugar – causing increased alcohol concentrations – and overly low acid amounts (Mira de Orduña, 2010). To stay competitive, wineries seek methods to diversify their products' organoleptic properties, aiming to meet consumers' expectations while dealing with the effects of climate change on wine composition.

Yeasts are the main player in alcoholic fermentation. They transform hexose sugars into ethanol and CO_2 and produce thousands of primary and secondary metabolites, shaping wine's taste, smell, and mouthfeel properties. Consequently, wine composition can be modulated to some extent by selecting the yeasts(s) conducting the fermentation process. On the one hand, different yeasts can produce different amounts of ethanol and organic acids from an identical grape must. On the other hand, different strains contribute differently to wine aroma and taste by releasing odour-active compounds from their odourless precursors (varietal aroma) and by producing *de novo* many other volatile molecules such as higher alcohols and esters (fermentative aroma). Therefore, approaches based on the selection and improvement of yeasts seem promising to tackle the challenges currently faced by the wine industry.

The biosynthetic pathways leading to industrially relevant metabolite production are strongly modulated at a genetic level (Rossouw, Jacobson and Bauer, 2012). Additionally, those traits show continuous distributions within a population (Marullo *et al.,* 2004; Salinas *et al.,* 2012; Steyer *et al.,* 2012), as their manifestation depends on several genetic determinants acting simultaneously. Those genetic determinants can be mapped in yeast genomes by means of quantitative genetics, using strategies such as QTL (quantitative trait locus) mapping. The application of such strategies generates a knowledge that can be directly applied to the improvement of wine yeast strains.

Saccharomyces cerevisiae is the main workhorse of wine fermentation. The reasons behind its success are its ubiquity, generally robust fermentative capacities, and the ability to resist the numerous stresses encountered during the fermentation process. Despite the extensive use of *S. cerevisiae* for wine production, there is a growing interest in using alternative species, mainly beyond the *Saccharomyces* genus. Many non-*Saccharomyces* species are promising alternatives to diversify the organoleptic profile and modulate both the ethanol and the acid content of wine by producing considerably different amounts of several metabolites. However, their application is hindered because of their generally weak fermentative capacities, needing a *S. cerevisiae* strain to achieve sugar depletion.

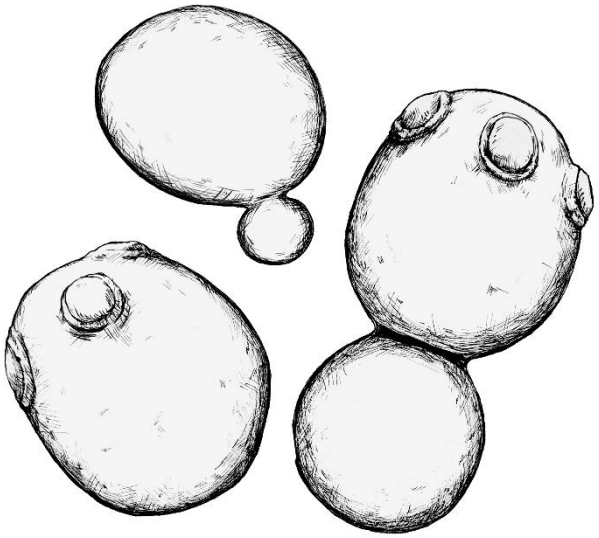
Besides non-*Saccharomyces*, previous studies have shown that non-conventional *Saccharomyces* species (i.e. alternative to *S. cerevisiae*) could be suitable candidates to overcome the challenges mentioned above. Strains of S. *paradoxus, S. kudriavzevii, S. eubayanus* and *S. uvarum* have been phenotyped in wine fermentation in the last fifteen years, producing wines with high aroma complexity, high amounts of some organic acids, and sometimes lower ethanol amounts than *S. cerevisiae*. Interestingly, unlike non-*Saccharomyces* species, alternative *Saccharomyces* species often show good fermentative capacities (Orlić *et al.* 2007; Parpinello *et al.* 2020; Pérez *et al.* 2021). Despite the promising potential of non-*cerevisiae Saccharomyces* species, most studies have focused on a handful of strains, and the oenological properties of some species is yet to be studied.

Saccharomyces species readily form interspecific hybrids. Analogous to mules arising from the breeding of a donkey stallion and a female horse, *Saccharomyces* hybrids can arise from the mating of two strains from different species. In both cases the hybrid is viable but sterile, which hinders further breeding strategies. However, the mechanisms causing sterility in *Saccharomyces* hybrids have been mostly elucidated, and fertile hybrids can be generated through genetic engineering. Recently, the overcoming of interspecific hybrid sterility by creating tetraploid intermediates has allowed the mapping of quantitative traitsin these organisms for the first time (Naseeb *et al.*, 2021). New avenues are therefore opened for the study of the genetic determinants leading to trait variations in yeast hybrids.

In this context, this thesis project aimed to uncover the potential of non-*cerevisiae Saccharomyces* species for winemaking. More specifically, we sought to provide a comprehensive picture of the phenotypic diversity within the *Saccharomyces* genus in winemaking conditions. Moreover, given the high technological interest of interspecific hybrids in fermentation, we aimed to determine the genetic basis of oenological traits in such organisms, intending to better comprehend the molecular mechanisms behind their phenotypic traits and discover allelic variants for their application in strain improvement.

The experimental results presented in this work are divided into two chapters. In the first chapter, the phenotypic diversity within the *Saccharomyces* genus in winemaking conditions was investigated. Strains belonging to all the known *Saccharomyces* species were phenotyped in synthetic grape must at two different temperatures. Their kinetic capacities and ability to produce fermentative aromas and compounds of the central carbon metabolism were examined. In the second chapter, strains of two non-conventional *Saccharomyces* species were selected for their properties described in Chapter 1, aiming to generate fertile interspecific hybrids suitable for QTL mapping. The ultimate objective was to elucidate the genomic basis underlying the observed differences in oenologically-relevant complex traits.

INTRODUCTION



1. Fermentation in winemaking

1.1. The process of alcoholic fermentation

In the context of winemaking, alcoholic fermentation is the biotransformation of grape juice or 'must' into wine by the action of yeasts. The main metabolic process taking place during this stage is the transformation of sugars (glucose and fructose) into ethanol and carbon dioxide. Because CO_2 is released in the form of gas, the weight of the remaining liquid is progressively reduced. As the activity of yeasts during alcoholic fermentation is directly correlated with the CO_2 release, fermentation progress can be followed by monitoring the weight loss of the fermenter. In this way, the CO_2 production rate (dCO_2/dt [g/L/h]) can be calculated, allowing scientists to differentiate the four phases of the microbial growth curve:

i. Lag phase. During this phase, yeast cells adapt to the new environment (Pérez-Torrado *et al.*, 2002). Grape juice is a harsh environment for most microorganisms because of the many stress factors encountered (see Section 1.2). Depending on those factors and others such as initial cell density and temperature, the lag phase can last from a few hours to a few days. Once adapted to the mentioned stresses, yeasts start fermenting and thus producing CO_2, rapidly reaching saturation (1.5 g/L CO_2) (Bely, Sablayrolles and Piere Barre, 1990). During this phase, the composition of the medium remains almost unchanged, with the exception of vitamin B1 (or thiamine) which is depleted in the first hours (Bataillon *et al.*, 1996). From this point, CO_2 is released to the gas phase, causing a noticeable mass loss.

ii. Exponential phase. After the lag phase, cells exponentially multiply until reaching the maximum population ($5 \cdot 10^7$ to $2.5 \cdot 10^8$ cells/mL), which depends mainly on the nutrient composition of the grape must (Bely, Sablayrolles and Piere Barre, 1990). Proportionally, the CO_2 production rate increases until reaching its maximum (R_{max}), then starts slowing down. In most cases, the end of the exponential phase is caused by the depletion of yeast assimilable nitrogen (YAN), but deficiencies in lipids or vitamins can also bring the exponential phase to its end. Typically, around 1/3 of the sugars initially present have been consumed at this point.

iii. Stationary phase. Once the cell population and the CO_2 production rate have reached their maximum, the culture enters in stationary phase. From this point, cell division is unnoticeable and the yeast population stays at its maximum level, while the CO_2 production rate starts slowing down. The cell viability and metabolic activity of yeasts remain high, allowing the consumption of about 2/3 of the remaining sugars.

iv. Death phase. Because of the sugar depletion and the accumulation of ethanol, acetate, acetaldehyde, short- and medium-chain fatty acids and reactive oxygen species (ROS), between other compounds, the population starts losing viability and metabolic activity. Ethanol is thought to be the main factor responsible for this decrease, as it has been shown to inhibit hexose transporters (Salmon, 1989) and increase the permeability of the cell membrane (Jones and Greenfield, 1987). However, if the yeast strains are resistant to high ethanol concentrations, they will likely deplete all sugars and complete a successful fermentation (**Fig. I-1**).

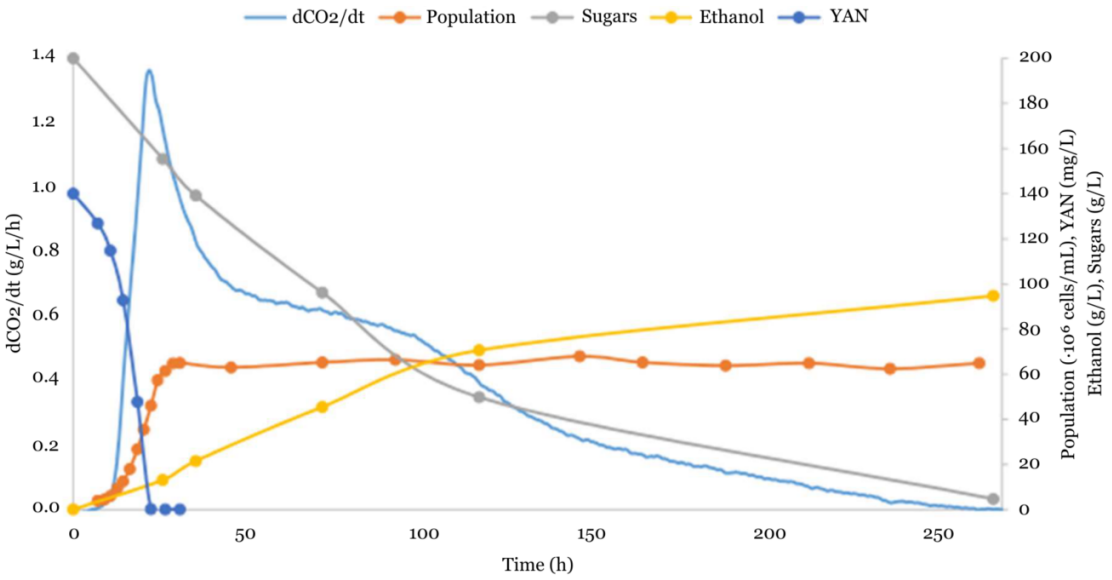


***Figure I1**. Evolution of the main parameters during alcoholic fermentation at 24 ºC using the* S. cerevisiae *Lalvin EC1118® in synthetic must.*

1.2. From must to wine: the fermentative environment

Grape must is a harsh environment for most microorganisms. It is characterized by concentrations sugar concentrations (180 – 260 g/L), a low pH (3 – 3.5), the presence of sulphites (40 – 80 mg/L) and fungicide residues from grapes, and typically limited amounts of nitrogen sources, vitamins and lipids (Marsit and Dequin, 2015; Gava *et al.*, 2021).

Fermentation progress greatly depends on grape juice composition, which in turn depends on several factors, such as the nature of the soil, the water, the climate, agronomic practices during grape development and the variety of grapevine used. Additionally, fermentation conditions differ depending on the type of vinification. For instance, red wine fermentation takes place in presence of grape seeds and skins, at a temperature around 28

ºC and a relatively high oxygen availability. On the other hand, white wines are fermented after settling (i.e. removal of suspended particles by sedimentation), at lower temperatures (above 20 ºC), and almost in total anaerobic conditions (Gonzalez and Morales, 2022).

Temperature influences the pace of every biochemical process, and alcoholic fermentation is not an exception. In the stainless steel fermentation tanks typically used in industrial white wine production, heat losses are minimal. As fermentation is an exothermic process, temperature needs to be controlled to avoid an excessive increase, which can cause early cell death and/or have fatal consequences for wine quality. As mentioned above, the fermentation temperature for red wine production is higher than for white wine, as a high temperature favours the extraction of polyphenolic compounds from solid particles (Setford *et al.*, 2017). Temperature also has a major effect in fermentation kinetics. It has been shown that, in isothermal fermentation, the rate can be doubled when increasing the temperature from 15 to 25 ºC (Bely, Sablayrolles and Barre, 1990). However, the temperature effect on yeasts' fermentative activity is species- and even strain-dependent.

Contrary to other fermentation processes such as beer or sake, sugars in wine production are almost solely glucose and fructose, in equimolar concentrations. They are typically found in very high amounts and therefore are always present in large excess in comparison to other nutrients. However, this fact itself constitutes an inhibitory factor for most microorganisms, due to the consequently high osmotic pressure.

Nitrogen is one of the main determinants of fermentation performance and volatile aroma production, and it is typically found in limited concentrations in grape must. Nitrogen deficiencies are in fact one the most frequent causes of stuck or sluggish fermentations in the industry (Marsit and Dequin, 2015). The main sources of yeast assimilable nitrogen (YAN) are free ammonium and amino acids, although other nitrogen sources such as oligopeptides, polypeptides, proteins, amides, biogenic amines and nucleic acids can also be incorporated and metabolized by yeasts. In practice, the YAN content of grape must ranges from 60 to 400 mg/L (Henschke and Jiranek, 1993). Typically, 140 mg/L of YAN is the minimum amount required by yeasts to complete a fermentation of a grape juice containing 200 g/L sugars, and increasing YAN concentrations up to 300 mg/L has a positive impact in fermentation kinetics (Sablayrolles, 2008).

Lipids are another crucial factor influencing wine fermentation. In principle, yeast cells are capable of producing their own sterols and unsaturated fatty acids, necessary for biomass generation and maintenance. However, this process requires oxygen (Andreasen and Stier, 1953). Therefore, under anaerobic conditions, yeasts need to incorporate lipids from the medium in order to maintain a high cell viability and metabolic activity during wine fermentation. Lipids also contribute to face the toxic effects of ethanol once this

product reaches concentrations higher than 10 % (v/v) (Alexandre *et al.*, 1994). More specifically, ethanol increases cell membrane permeability, and a sufficient lipid content in the medium minimizes its impact by maintaining an optimal membrane thickness. Almost all lipids present in grape must are in the solid fraction, and an excessive settling prior to fermentation leads to lipid deficiencies (Casalta *et al.*, 2016).

Although they are present in much lower concentrations, the amount and bioavailability of minerals and vitamins can also impact fermentation progress. For example, magnesium ions (Mg^{2+}) positively affect cell growth and sugar consumption, while an excess of calcium (Ca^{2+}) has the opposite effect (Birch, Ciani and Walker, 2003). Vitamins act as cofactors for many enzymatic transformations inside the cell, being necessary to keep a high cellular activity. Even though they are present in variable amounts in grape juices, they rarely are a limiting factor. To name a few examples, inositol (B7) participates in membrane synthesis and therefore contributes to ethanol resistance (Furukawa *et al.*, 2004), while thiamine (B1) acts as a cofactor in many enzymatic reactions, pyruvate decarboxylation being the most relevant.

Oxygen is indispensable for yeast cells to produce sterols and unsaturated fatty acids that influence the fluidity and activity of membrane-associated enzymes which, as mentioned above, influence ethanol tolerance, cell viability and fermentative capacity. On average, the amount of oxygen dissolved in grape must prior to fermentation is around 8 mg/L. However, anaerobiosis is achieved after some hours of fermentative activity. Oxygen additions during fermentation have shown to increase fermentation rates, mainly if performed at the end of the stationary phase (Julien *et al.*, 2000). Oxygen additions (often called microoxygenation) make it possible to avoid stuck fermentations, and greatly reduce fermentation times in otherwise sluggish fermentations (Blateyron and Sablayrolles, 2001). However, they have little effect on fermentations that do not present a risk of premature termination. In practice, oxygen deficiencies generally cause more problems in white wine production, as the amount of solid particles providing lipids is much lower (Alexandre *et al.*, 1994), and cells need to synthesize their own.

2. Diversity of yeasts for winemaking

2.1. Microbiology as a tool to face the wine sector's challenges

Today, the wine sector faces two major challenges. The first one is caused by climate change, mainly affecting countries with warm climates, such as the Mediterranean, many of which are important wine producers. In such countries, global warming leads to early industrial maturity coupled with suboptimal phenolic maturity (Mira de Orduña, 2010). The main consequences of these phenomena in grape must composition are increased sugar

content and decreased amounts of organic acids, mainly malic acid (Dequin *et al.*, 2017). Those changes directly affect the final product composition, yielding wines more alcohol at the end of the fermentation. High ethanol levels intensify the 'hotness' of wine and consequently reduce the perception of fruity and floral notes. Additionally, highly alcoholic beverages are subjected to high tax penalties in some countries and are now less demanded by health-conscious consumers (Schelezki *et al.*, 2018). Several viticultural and oenological practices have been proposed to reduce ethanol content in wine (Varela *et al.*, 2015), but most are expensive, difficult to implement, or negatively affect wine aroma. In that context, microbiological solutions become the most attractive from an industrial point of view.

Secondly, besides the demand for low-alcohol wines, today's consumer looks for high-quality products and novel sensory experiences, including fresh, fruity and floral notes. Besides transforming sugars into alcohol, yeast plays a significant role in wine aroma formation and modulation, generating wines with different organoleptic properties from the same grape must. Therefore, it is common to inoculate a known yeast strain with specific characteristics to provide particular aromas, while achieving product homogeneity and reducing fermentation times. Although several yeast strains are commercialized as starter cultures, their genetic diversity is limited (Borneman *et al.*, 2016) and insufficient to address the abovementioned challenges (Molinet and Cubillos, 2020). Additionally, this low diversity may negatively affect the producer's perception of yeast's impact on the final product composition (Molinet and Cubillos, 2020). In this context, the study of yeast diversity draws increased attention among wine microbiologists, aiming to discover and develop novel yeast strains with adequate fermentative power and unique metabolic properties.

2.2. Diversity within S. cerevisiae

The budding yeast *Saccharomyces cerevisiae* is certainly one of the most relevant microorganisms in human history. Humans have exploited this species for millennia to produce fermented foods and beverages such as wine, beer, cider, sake or bread. Besides its traditional importance in the food sector, although in part thanks to it, *S. cerevisiae* has become one of the most widely used eukaryotic model systems in modern biology. This is because of several characteristics that make *S. cerevisiae* especially useful and easy to work with. Some examples are its rapid growth in inexpensive media, haploid and diploid cell cycles, ease to switch between mitotic and meiotic division, and the availability of effective molecular biology methods and information in public databases (Petranovic and Nielsen, 2008; Karathia *et al.*, 2011). Its relative similarity to multicellular organisms such as humans or plants in terms of cellular and biochemical processes have made of *S. cerevisiae* an indispensable model organism in health and plant biotechnology. Moreover, it

constitutes one of the most versatile tools in synthetic biology and white biotechnology, allowing the production of valuable chemicals for application in a wide variety of sectors.

S. cerevisiae was the first eukaryotic organism to be completely sequenced, 27 years ago (Goffeau *et al.*, 1996). Since those days, sequencing technologies have kept growing in quality and velocity while reducing prices. Those advances, together with the massive isolation of *S. cerevisiae* strains from different habitats around the globe, allowed scientists to shed light on the origin and evolutionary history of the species. Although *S. cerevisiae* was frequently isolated from forest materials, including soil, decayed leaves and tree bark (Banno and Mikata, 1981), it was thought to be a fully domesticated species, strictly associated with human-made environments and with almost no presence in wild (Vaughan-Martini and Martini, 1995). From the first phylogenetic distinction between wild and domesticated populations of *S. cerevisiae* (Fay and Benavides, 2005), different clades have been identified within the species, and the classification has evolved as more isolates were included. Fay and Benavides (2005) and Legras *et al.* (2007) proposed an African and Mesopotamian origin of the wine *S. cerevisiae* strains, respectively. Liti *et al.* (2009) classified the *S. cerevisiae* populations into five distinct lineages: Malaysian, North American, Sake, West African and Wine/European. However, at that time, most available strains were still of human-related origin, restraining our knowledge on the distribution of this species in the wild. Wang *et al.* (2012) showed that *S. cerevisiae* distributes ubiquitously in nature, being present in human-associated niches and habitats remote to human activity. This study identified eight new lineages of Chinese origin (CHN-I – CHN-VIII) and suggested that *S. cerevisiae* originated in Far East Asia. Wang *et al.* (2012) also showed that the genetic diversity in industrial strains is limited compared to the full spectrum of natural biodiversity.

Duan *et al.* (2018) identified the new CHN-IX clade as the current basal lineage of the *S. cerevisiae* phylogenetic tree (**Fig. I-2**), to date isolated only in China. This study also resolved the phylogenetic separation between wild and domesticated populations. They found domesticated *S. cerevisiae* strains to fall into two monophyletic lineages, associated with liquid-state (LSF) and solid-state (SSF) fermentation processes. All lineages primarily isolated from Europe fell into the LSF group, many of which were associated with wine and beer fermentation. This study pointed out Asia as the origin of *S. cerevisiae*, including all domesticated lineages. The largest genome sequencing project to date was performed by Peter *et al.*, (2018). They revealed the presence of 10 wild and 11 domesticated clades and supported a single 'out-of-China' origin of the species.

The wide variety of environments colonised by *S. cerevisiae* has shaped the genomes of the different populations in diverse ways. Brewing strains show remarkable differentiation and are polyphyletic, while wine strains constitute a monophyletic group with much less genetic diversity. Beer strains also show a high variation in their genomic

content, with generally higher ploidies and frequent aneuploidies, while most wine isolates are pure diploids (Peter *et al.*, 2018). Those differences between wine and beer *S. cerevisiae* strains probably relate to how each industry handles its yeast cultures. While brewing yeasts grow uninterruptedly in a rich medium, being reused after each fermentation batch (Gallone *et al.*, 2016), wine strains only ferment during a short period of the year and are then returned to natural habitats (Molinet and Cubillos, 2020). This limits the number of generations and, therefore, their domestication signatures at the sequence level.

Still, wine populations of *S. cerevisiae* have undergone genetic changes that shaped their phenotypes, sometimes conferring competitive advantages in wine fermentation. For example, horizontal gene transfer (HGT) events from other species have been reported. Some originate from donor species closely related to *S. cerevisiae*, such as *S. paradoxus*. Most wine strains carry 25 to 50 ORFs from this species (Peter *et al.*, 2018). However, phylogenetically distant species have also transferred genetic material to *S. cerevisiae*. Novo *et al.* (2009) reported the presence of genes laterally acquired from *Zygosaccharomyces bailii*, a contaminant commonly found in wineries. Another example is the transfer of two *FOT* genes from *Torulaspora microellipsoides*, allowing more efficient incorporation of oligopeptides, an important nitrogen source in natural grape must (Marsit *et al.*, 2015). Another particular trait of some wine yeasts is their higher resistance to the sulphites employed to prevent wine spoilage. Chromosomal translocations (Pérez-Ortín *et al.*, 2002) and inversions (García-Ríos, Nuévalos, *et al.*, 2019) involving the promoter of the *SSU1* gene, coding for a sulphite efflux pump, have been shown to increase sulphite resistance in wine yeasts.

Smaller genetic variations such as single nucleotide polymorphisms (SNPs), insertions, deletions and copy number variations (CNVs) also led to adaptive responses to wine fermentation stresses. For instance, the *CUP1* gene (responsible for resistance to copper-based pesticides) was found in higher copy number in wine strains with increased resistance to $CuSO_4$ (Almeida *et al.*, 2015). Genetic variants of the *CUP1* promoter also increased its expression in an industrial yeast compared to the laboratory strain S288c (Liu *et al.*, 2015).

Although the mentioned phenotypes are thought to be a consequence of adaptive evolution, some authors argue that genetic drift (i.e. change in the frequency of an existing allele in a population due to random chance) had a higher impact on those traits than natural or human-enforced selection. Warringer *et al.* (2011) showed that trait variation in *S. cerevisiae* is mostly defined by population history rather than adaptation to a specific environment. According to this hypothesis, random genetic changes can be fixed through population bottlenecks, even if they do not improve fitness. However, this study also showed that copper resistance arose separately in two distinct lineages, i.e. Wine/European and Sake.

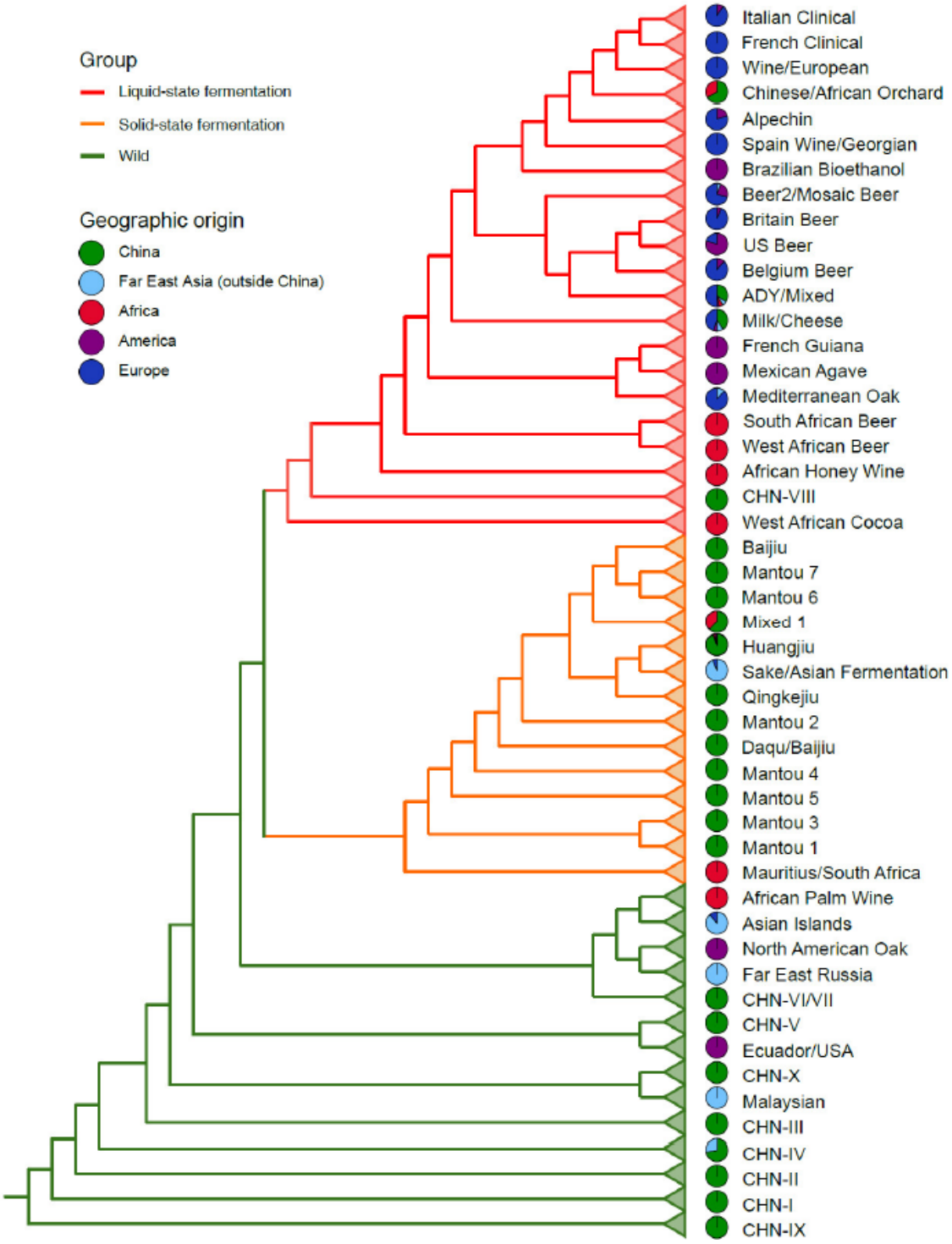


***Figure I2**. Schematic diagram showing the phylogenetic relationships between the recognised lineages of* S. cerevisiae*, from (Bai et al., 2022).*

The phenotypic diversity in *S. cerevisiae* under winemaking conditions has been widely studied in the last years. Hyma *et al.* (2011) showed that, in terms of both chemical and sensorial analysis, the aromatic profile of wild and domesticated *S. cerevisiae* strains can be differentiated, hypothesizing that aroma production is a trait selected by human. However, this study used only four wild strains. Camarasa *et al.* (2011) characterized 72 *S. cerevisiae* strains of different origins in wine fermentation and showed that strains isolated from sugar-rich environments, such as fruits, show better fermentative capacities than those isolated from low-sugar niches. Similarly, (Pérez *et al.*, 2021) found that *S. cerevisiae* strains isolated from wine, cachaça and beer fermented more efficiently than isolates from non-industrial environments. In terms of fermentation by-products, different studies have reported a high intra-species diversity within *S. cerevisiae* strains (Camarasa *et al.*, 2011; Barbosa *et al.*, 2014; Capece *et al.*, 2016; Bordet *et al.*, 2021).

Several authors have aimed to expand the existing phenotypic diversity in the laboratory. A promising approach is to combine QTL mapping to identify genetic variants leading to trait improvement and backcrossing to integrate them in a given strain. For instance, backcrossing has been used to improve *S. cerevisiae* thermo-tolerance (Marullo *et al.*, 2009) and enhance the release of volatile thiols (Dufour *et al.*, 2013). *S. cerevisiae* intraspecific hybrids have also been generated to improve fermentation-related traits such as low sulfur compound production (Agarbati *et al.*, 2020), fermentative stress tolerance (Bonciani *et al.*, 2016), low nitrogen requirements (Kessi-Pérez *et al.*, 2020), lower ethanol production (García *et al.*, 2012) or novel aroma profiles (Marullo *et al.*, 2006; Steensels *et al.*, 2014), to cite a few examples. Furthermore, adaptive laboratory evolution (ALE) strategies have been successfully used to reduce ethanol synthesis while increasing glycerol production (Tilloy, Ortiz-Julien and Dequin, 2014; Tilloy *et al.*, 2015) and to increase the flux toward the pentose-phosphate pathway, improving fermentation rates and aroma production (Cadière *et al.*, 2011).

Although significantly improving desired traits, approaches using only *S. cerevisiae* limit the potential diversity to a single species. Next two sections highlight the potential of alternative species to diversity the sensory properties of wine.

2.3. Diversity beyond the genus Saccharomyces

Non-*Saccharomyces* yeasts contribute to the sensory complexity of spontaneously fermented wines. They display an enormous diversity, and many species are much more abundant than *S. cerevisiae* in natural grape musts (**Fig. I3**).

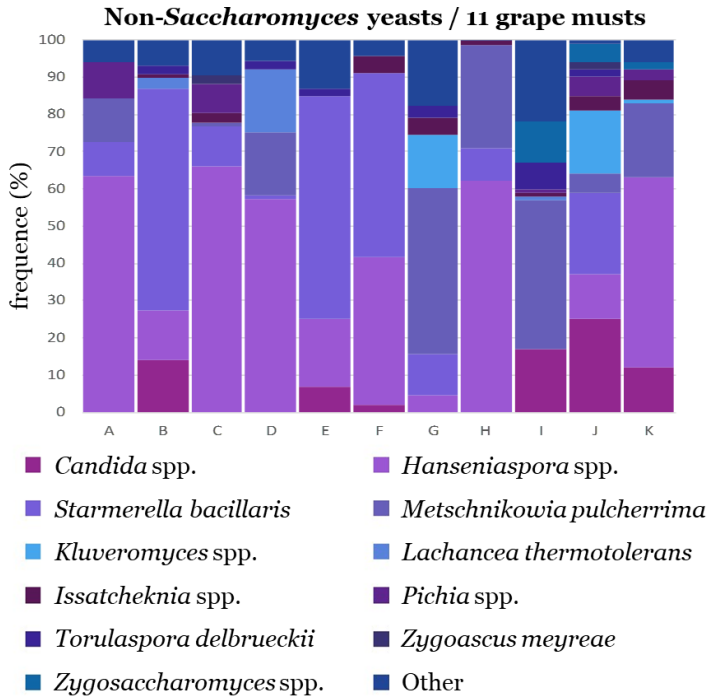


***Figure I3**. Relative abundance of yeast species naturally present in different grape musts (A – K). Data were collected from* Capozzi *et al.* (2015), Garofalo *et al.* (2016), Raymond Eder *et al.* (2017) and Mateus *et al.* (2020).

Non-*Saccharomyces* yeasts were initially seen as contaminants, potential spoilers provoking unwanted changes in wine composition. Although some of them are still mostly considered spoilage yeasts, such as *Dekkera* spp. (synonym: *Brettanomyces* spp.), others have now gained a relevant position in the wine industry. Non-*Saccharomyces* starter cultures have been available for almost two decades (Jolly, Varela and Pretorius, 2014). The reason behind this rise in attention is their vast phenotypic diversity (Rossouw and Bauer, 2016), which could help to address some of the wine sector's challenges mentioned above.

Some non-*Saccharomyces* have shown the potential to produce wines with low ethanol content. Although reductions in ethanol yields are often a result of wines with residual sugars (Jolly, Augustyn and Pretorius, 2003; Contreras *et al.*, 2014), sequential inoculation with *S. cerevisiae* allow an ethanol reduction while achieving dryness (Capece *et al.*, 2022). Additionally, this ethanol reduction is often accompanied by an increase in other interesting metabolites. This is the case of *L. thermotolerans*, a great producer of lactic acid used to acidify must (Benito, 2018b), or *Starmerella bacillaris*, which produces high amounts of glycerol (Englezos *et al.*, 2015). The latter also displays a robust fructophilic character (Ciani and Maccarelli, 1998), a phenotype mostly absent in *S. cerevisiae*, except for some wine strains possessing a mutated *HXT3* allele enhancing fructose fermentation (Guillaume *et al.*, 2007). High glycerol producers sometimes are, unfortunately, high acetic acid producers too. Although acetic acid production by non-*Saccharomyces* has generated variable results and there is a high strain variability (Padilla,

Gil and Manzanares, 2016), some strains of *T. delbrueckii* (Bely *et al.*, 2008) and *S. bacillaris* (Englezos *et al.*, 2015) can produce low amounts of alcohol and acetic acid simultaneously.

Varietal aroma compounds are found in two forms in grape must, i.e. the free form, which is odour-active, and the bound form, which is odourless. Several non-*Saccharomyces* produce enzymes that allow the release of varietal aromas from their odourless precursors. *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima* (Rosi, Vinella and Domizio, 1994; Hernández-Orte *et al.*, 2008; Zott *et al.*, 2011) have been reported to show both β-glucosidase and carbon-sulfur lyase activities, which together promote the release of terpenol, norisoprenoids (Gunata *et al.*, 1988) and volatile thiols (Tominaga, Des Gachons and Dubourdieu, 1998) from their precursors.

Although the unique contribution of non-*Saccharomyces* to sensory complexity resides in varietal aroma release, their impact on the fermentative aroma is also significant. Many studies have reported increases in higher alcohol and ester production using non-*Saccharomyces*. For instance, *M. pulcherrima*, *Hanseniaspora* spp. and *Pichia* spp. have been reported as great acetate ester producers in several studies (Moreira *et al.*, 2008; Viana *et al.*, 2008; Tufariello *et al.*, 2021). However, this increase is usually associated with a high production of ethyl acetate, which imparts an unpleasant solvent-like aroma (Rojas *et al.*, 2001; Viana *et al.*, 2008). *T. delbrueckii* has also been shown to increase ester production, particularly ethyl octanoate (Viana *et al.*, 2008), compared to *S. cerevisiae*.

The main drawback for the industrial implementation of non-*Saccharomyces* is their fermentative capacities, which vary greatly between species. Some species are largely aerobic, and their contribution is limited to the first hours of fermentation. Examples are *Pichia* spp. and *Candida* spp. Others, such as the apiculate yeasts belonging to *Hanseniaspora* spp., have a low fermentative activity. Finally, other genera show a nearly entirely fermentative metabolism in oenological conditions. This last group includes the best-characterised species, as they can coexist for extended periods in a fermentation tank, alone or with *S. cerevisiae*. Some examples are *Torulaspora delbrueckii*, *Kluveromyces marxianus* or *Lachancea thermotolerans* (Jolly, Varela and Pretorius, 2014; Padilla, Gil and Manzanares, 2016).

Nevertheless, none of those species has shown a combination of fermentative power and stress tolerance comparable to those of *S. cerevisiae*, and most fail to deplete all sugars in the stressful winemaking environment. Consequently, *S. cerevisiae* strains generally outcompete non-*Saccharomyces*, minimising the contribution of the latter to wine composition. Co-cultures and sequential inoculations together with *S. cerevisiae* are a valid tactic, allowing to exploit the metabolic characteristics of non-*Saccharomyces* without the risk of stuck fermentation or contamination (Rollero *et al.*, 2021). However, those

approaches imply interactions between yeasts, including competition for nutrients such as nitrogen, which can impair fermentation (Rollero *et al.*, 2018). As a consequence, carrying out the entire process using a pure culture is the preferred approach in the field, as it allows tighter control over the outcome and the process itself. In this context, the development or discovery of yeasts with adequate fermentative power and distinct metabolic properties becomes crucial.

2.3. Non-*cerevisiae Saccharomyces*

The term *Saccharomyces* was introduced in the early nineteenth century to describe fermenting yeasts (Hittinger, 2013). The progressive discovery of different species eventually led taxonomists to establish the groups *Saccharomyces sensu stricto* and *Saccharomyces sensu lato*, a classification based on morphological, reproductive and physiological characteristics. The use of molecular biology techniques led to several re-classifications throughout the years and, only recently, sequencing has allowed taxonomists to establish the *Saccharomyces* genus as a monophyletic group, eliminating the *sensu stricto* and *sensu lato* terms (Dujon and Louis, 2017). The currently known biodiversity within the *Saccharomyces* genus recognises eight species (in order of discovery: *S. cerevisiae, S. uvarum*, *S. paradoxus*, *S. kudriavzevii*, *S. mikatae*, *S. arboricola*, *S. eubayanus* and *S. jurei*) as well as numerous interspecific hybrids between species of the clade (**Fig. I4**) (Alsammar and Delneri, 2020).

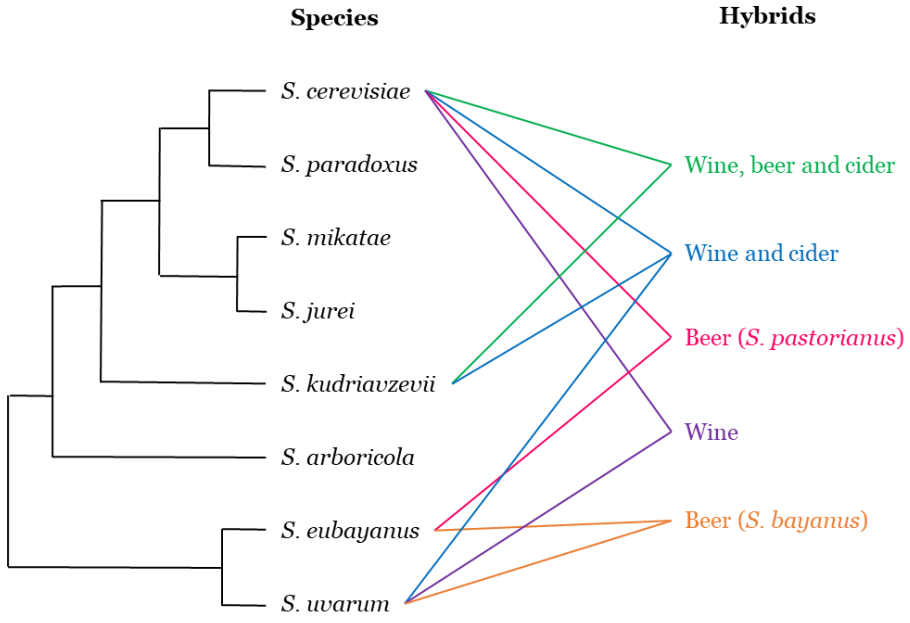


***Figure I4**. Schematic cladogram of the* Saccharomyces *genus and interspecific hybrids frequently isolated from fermentative environments (adapted from Boynton and Greig, 2014).*

2.3.1. Diversity of alternative *Saccharomyces* species

S. uvarum was first isolated in south Holland (Beijerinck, 1898). Besides *S. cerevisiae*, *S. uvarum* is the only *Saccharomyces* species that has been isolated from industrial beverage fermentations. In fact, *S. uvarum* has been repeatedly found to dominate in spontaneous fermentations for wine (Naumov *et al.*, 2002; Demuyter *et al.*, 2004; McCarthy *et al.*, 2021) but also for cider (Naumov *et al.*, 2001; González Flores *et al.*, 2019) production, showing excellent fermentative capacities. Additionally, *S. uvarum* displays a cryotolerant character, with a lower optimal growth temperature than *S. cerevisiae* (Salvadó *et al.*, 2011). Together, those two phenotypes (fermentative power and cryotolerance) make some *S. uvarum* strains especially interesting for white wine production, where fermentation temperatures are generally below 20 ºC. The most recent population survey for this species identified three lineages, two of them composed only of wild strains (South America B and Australasia) and a third one including both wild and domesticated isolates from the northern hemisphere (South America A/Holarctic) (Almeida *et al.*, 2014). That study provided the first phylogenetic evidence for domestication in this species. Interestingly, Almeida et al. (2014) also reported introgressions from *S. kudriavzevii* and *S. eubayan*us in the genome of *S. uvarum* wine strains. Recently, Macías *et al.* (2021) showed that some *S. uvarum* wine strains, similarly to *S. cerevisiae* wine strains, have suffered chromosomal translocations leading to overexpression of the *SSU1* gene conferring increased sulfite resistance, providing further domestication evidence. From a sensory perspective, different studies have confirmed the ability of *S. uvarum* to produce lower amounts of ethanol and acetic acid than *S. cerevisiae*, as well as higher levels of succinate, glycerol, and fermentative aromas such as 2-phenylethanol and 2-phenylethyl acetate (Gamero *et al.*, 2013; Stribny *et al.*, 2015; Minebois, Pérez-Torrado and Querol, 2020; Coral-Medina, Morrissey and Camarasa, 2022). To our knowledge, *S. uvarum* is also the only non-*cerevisiae* *Saccharomyces* available in the dry yeast market for wine production (Lallemand-Oenology, Velluto BMV58®).

S. paradoxus is the closest relative to *S. cerevisiae*. From its discovery in 1914 (Batshinskaya, 1914), *S. paradoxus* has been almost totally limited to wild environments, and it forms well-structured lineages related to geographical origin. Three main lineages have been established, namely Far Eastern, European (including strains from New Zealand) and North American, in addition to a Hawaiian lineage represented by a single strain (Liti *et al.*, 2009; Zhang *et al.*, 2010; He *et al.*, 2022). Those lineages are partially reproductively isolated (Liti, Barton and Louis, 2006), meaning that they can form hybrids between them but those produce spores with a very low viability. Despite a higher SNP variability, *S. paradoxus* is less phenotypically diverse than *S. cerevisiae*. This is probably due to the wider diversity of ecological niches colonised by *S. cerevisiae*, implying more frequent population bottlenecks (Liti *et al.*, 2009; Warringer *et al.*, 2011). The optimal growth

temperature of *S. paradoxus* is slightly lower than that of *S. cerevisiae*, but still higher than other *Saccharomyces* species (Salvadó *et al.*, 2011), and it is not considered cryotolerant. Although many *S. paradoxus* isolates have been found in the last decades, including strains isolated from vineyards (Redžepović *et al.*, 2002), only a few have been characterized in oenological conditions. Still, those strains have shown good fermentative behaviour, high ethanol resistance, and the ability to increase glycerol and reduce volatile acidity compared to *S. cerevisiae* (Redžepović *et al.*, 2002; Orlić *et al.*, 2007, 2009). Some studies also reported the ability of *S. paradoxus* to degrade malic acid, which could be interesting when fermenting unripe grapes in zones highly affected by climate change (Redzepovic *et al.*, 2003; Bovo *et al.*, 2016; Costantini *et al.*, 2021). Regarding aroma production, most studies did not report significant increases in volatile esters – which are the most desired group of odour-active compounds – compared to *S. cerevisiae* (Orlić *et al.*, 2007; Costantini *et al.*, 2021). However, some authors have reported a low higher alcohol production, which could help to enhance ester perception (Majdak *et al.*, 2002).

The first *S. kudriavzevii* isolate was found in decayed leaves in Japan (Naumov *et al.*, 2000), and it was later isolated in Portugal, Spain, France, Taiwan and Italy (Sampaio and Gonçalves, 2008; Lopes, Barrio and Querol, 2010; Erny *et al.*, 2012; Naumov, Lee and Naumova, 2012; Alsammar, 2018). Contrary to *S. cerevisiae*, *S. uvarum* or *S. paradoxus*, which show a global distribution, *S. kudriavzevii* has only been isolated in two continents, and only two lineages have been identified (i.e. Asian and European) (Peris *et al.*, 2016). To date, pure *S. kudriavzevii* strains have not been found in fermentative environments, despite the occurrence of interspecific hybrids of this species in such niches (Alsammar and Delneri, 2020). From an oenological perspective, *S. kudriavzevii* has several interesting properties. First, like *S. uvarum* and *S. eubayanus*, it is a cold tolerant species. The molecular mechanisms behind its cryotolerance are related to changes in membrane composition (Tronchoni *et al.*, 2012), translation efficiency (Tronchoni *et al.*, 2014) and a glycerol or acetaldehyde accumulation to compensate temperature-induced redox imbalances (Paget, Schwartz and Delneri, 2014). Second, *S. kudriavzevii* has been shown to reduce ethanol and increase glycerol, higher alcohol and MCFA ethyl ester production compared to *S. cerevisiae* (Stribny *et al.*, 2015; Peris *et al.*, 2016; Minebois, Pérez-Torrado and Querol, 2020; Pérez *et al.*, 2021). One of the genetic determinants of the high glycerol production by this species is an increased expression of the *GPD1* gene and the higher activity of its encoded enzyme (see Section 3.1) (Oliveira *et al.*, 2014). On the other hand, changes in aroma production are partially due to differences at the sequence level in genes coding enzymes leading to aroma formation, such as *ATF1*, *ATF2* or *ARO10* (see Section 3.3) (Stribny *et al.*, 2016; Stribny, Querol and Pérez-Torrado, 2016), and transcriptional regulators of those routes, such as *ARO80* (Tapia *et al.*, 2022). Finally, a recent study reported that some *S. kudriavzevii* strains can perform alcoholic fermentation at a similar pace than the wine *S. cerevisiae* strain EC1118 (Pérez *et al.* 2021). Although fermentations

in that study were carried out at laboratory scale and using synthetic grape must, those results might indicate a high potential of *S. kudriavzevii* for white wine production, which is carried out at temperatures around 20 ºC.

S. eubayanus is another widely known cryotolerant member of the *Saccharomyces* clade. Its isolation in Patagonia (Argentina) resolved the mystery of the non-*S. cerevisiae* founder species of the hybrid *S. bayanus* (see Section 2.3.2) (Libkind *et al.*, 2011). Later, *S. eubayanus* strains were isolated in North America (Peris *et al.*, 2014), Far East Asia (Bing *et al.*, 2014), New Zealand (Gayevskiy and Goddard, 2016) and Ireland (Bergin *et al.*, 2022). Currently, *S. eubaya*nus populations fit into three lineages, i.e. Patagonia A, Patagonia B/Holarctic (which includes the recently isolated Irish strains) and West Chinese (Peris *et al.*, 2016; Sampaio, 2022). The frequent isolation of this species in South America led to establish six subpopulations within the Patagonia A and B lineages (PA1, PA2, PB1, PB2, PB3 and Holarctic) as well as admixture populations (Langdon *et al.*, 2019). Regarding its fermentative behaviour in winemaking conditions, the main conclusion from literature is that *S. eubayanus* prefers very low fermentation temperatures, around 12 ºC (Magalhães, Krogerus, Castillo, *et al.*, 2017a; Su, Origone, *et al.*, 2019; Parpinello *et al.*, 2020). Other studies compared the fermentative capacities of *S. eubayanus* and *S. cerevisiae* at 20 ºC and did not find a clear differentiation between both species (Pérez *et al.*, 2021; Pérez, Denat, Heras, *et al.*, 2022). However, at temperatures above 26 ºC, where *S. cerevisiae* generally excels, strains of *S. eubayanus* displayed stuck fermentation profiles (Parpinello *et al.*, 2020). In terms of metabolite production, *S. eubayanus* is characterized by a similarly high production of aromas such as 2-phenylethanol and 2-phenylethyl acetate than *S. uvarum*, and several studies have reported higher glycerol and lower ethanol concentrations than *S. cerevisiae* (Magalhães, Krogerus, Castillo, *et al.*, 2017a; Parpinello *et al.*, 2020; Pérez *et al.*, 2021; Pérez, Denat, Heras, *et al.*, 2022).

To date, *S. arboricola* has only been isolated in China (Wang and Bai, 2008), Taiwan (Naumov, Lee and Naumova, 2012) and New Zealand (Gayevskiy and Goddard, 2016). Chinese and Taiwanese strains are closely related to each other, and New Zealander strains bear a genome divergence of 2.6% from the Chinese reference strain (Gayevskiy and Goddard, 2016; Alsammar and Delneri, 2020). Similarly, the isolation of *S. mikatae* has been limited to Japan (Naumov *et al.*, 2000) and China, while *S. jurei* has been found only in France (Naseeb *et al.*, 2017) and Germany (Hutzler *et al.*, 2021). Despite the interesting properties shown by the rest of the *Saccharomyces* species, *S. arboricola*, *S. mikatae* and *S. jurei* have not been phenotyped at the same level. Bellon *et al.* (2013) and Nikulin *et al.* (2017) studied the potential of *S. mikatae* in winemaking and brewing conditions, respectively, but only as parental strains to generate interspecific hybrids with *S. cerevisiae*. Bellon *et al.* (2013) reported that the *S. mikatae* strain was unable to grow in Chardonnay grape juice. Although Nikulin *et al.* (2017) observed a poor fermentative behaviour for the

single *S. mikatae* strain used, they also noticed a higher 2-phenylethanol, 2-phenylethyl acetate and 2-methylbutyl acetate production compared to *S. cerevisiae*. *S. arboricola* has been characterized for sake production (Winans *et al.*, 2020), but alike *S. mikatae*, the aim of the study was the generation of interspecific hybrids rather than the characterization of the species *per se*. Still, authors reported a high fermentative performance and ethanol production by the *S. arboricola* parental strain. From the three currently available *S. jurei* strains, D5095^{T} has shown resistance to ethanol concentrations up to 10 % (v/v) (Naseeb *et al.*, 2018), while TUM 629 consumed maltotriose and produced high amounts of ethyl hexanoate in brewing conditions (Hutzler *et al.*, 2021).

2.3.2. Wine yeast hybrids

Hybrids originate from the mating of two diverged lineages, resulting in individuals with chimeric genomes (Gabaldón, 2020). Hybridisation generates vastly more genetic diversity within just one generation than any kind of mutation (Stelkens and Bendixsen, 2022). The novel combination of genes (Hewitt *et al.*, 2014) and proteins (Piatkowska *et al.*, 2013) contributes to the emergence of unique characteristics, which are not necessarily intermediate between those present in progenitors. The new allelic combinations might be less fit than genotypes present in individual populations in a given environment, as they have not passed the filter of selection. However, other combinations might give rise to fitness advantages compared to the parental strains, a phenomenon known as hybrid vigour or heterosis (**Fig. I5**) (Gabaldón, 2020; Stelkens and Bendixsen, 2022). Heterosis often occurs because high fitness alleles are usually dominant over low fitness alleles (Ono, Greig and Boynton, 2020).

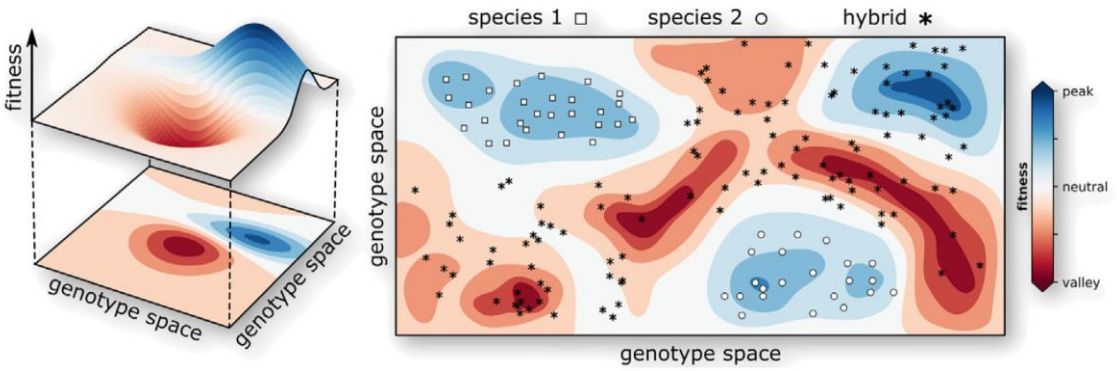


***Figure I5**. Theoretical fitness landscape indicating the evolutionary potential of yeast hybrids (From Stelkens and Bendixsen, 2022). Red valleys represent fitness reductions and blue peaks represent fitness improvements.*

Contrary to most *Saccharomyces* genuine species, interspecific hybrids between species of this genus have been frequently isolated from industrial fermentations. It is increasingly evident that, while *S. cerevisiae* dominates fermentations performed at warm temperatures (> 20 ºC), naturally occurring interspecific hybrids dominate lower temperature fermentations (Borneman and Pretorius, 2014). The best-known example of an industrial hybrid is *S. pastorianus*, resulting from the cross of *S. cerevisiae* and *S. eubayanus*. This hybrid has been used for centuries in low-temperature brewing for lager beer production (Sicard and Legras, 2011). *S. bayanus*, a hybrid between *S. eubayanus* and *S. uvarum* with a minimal contribution from *S. cerevisiae*, has also been isolated in brewing environments (Libkind *et al.*, 2011).

In wine fermentations, hybrids between *S. cerevisiae* and *S. uvarum* or *S. kudriavzevii* have been repeatedly isolated. The characterization of some of those hybrids has revealed an increased fermentative power at low temperatures and a higher production of several compounds of interest compared to the parental species (Masneuf-Pomarede *et al.*, 2002; González *et al.*, 2006; Erny *et al.*, 2012; Pérez-Torrado *et al.*, 2015). Additionally, it has been shown that the proportion of each parental genome retained in the final hybrid influences its fermentation performance. For instance, in hybrids between *S. cerevisiae* and *S. kudriavzevii*, a higher amount of the *S. cerevisiae* subgenome confers higher tolerance to fermentation stresses, while a higher proportion of the *S. kudriavzevii* subgenome allows more efficient low temperature fermentation (Belloch *et al.*, 2008; Peris *et al.*, 2012). The abovementioned examples indicate that interspecific hybridisation is a frequent natural mechanism that allows adaptation to stressful environments, such as alcoholic fermentation, in the genus *Saccharomyces*.

Given the interesting properties displayed by interspecific hybrids spontaneously present in industrial fermentations, many scientists have aimed to replicate the hybridisation process in the laboratory. Several research groups have successfully generated *de novo* interspecific hybrids for application in wine, beer, sake and cider fermentation (**Table I1**). In a winemaking context, interspecific hybridisation has been successfully used to improve fermentative capacities under low temperature and low nitrogen conditions, increase aroma and glycerol production, or reduce the production of ethanol and off-flavours such as H_2S (Bizaj *et al.*, 2012; Bellon *et al.*, 2013; Da Silva *et al.*, 2015; García-Ríos *et al.*, 2019; Su *et al.*, 2019; Lairón-Peris *et al.*, 2020). To date, most *de novo* hybrids have involved *S. cerevisiae*, with only two exceptions to our knowledge (Gyurchev *et al.*, 2022) (**Table I1**).

One of the main drawbacks in the generation of novel hybrids is their genome instability, which promotes drastic changes in the first generations of mitotic growth, altering the hybrid's phenotype (Kunicka-Styczyńska and Rajkowska, 2011). However, this can be used as an opportunity to further improve the physiology of the hybrid towards a

desired phenotype through experimental evolution (Krogerus, Holmström and Gibson, 2018). Pérez-través *et al.* (2014) showed that as few as 30 generations under fermentative stress are enough to reach genome stability in an interspecific hybrid.

During hybridisation, the mitochondrial DNA (mtDNA) can be inherited from either one or the other parental species (Giannakou, Cotterrell and Delneri, 2020). The mitochondrial genome is an important factor related to several characteristics of technological relevance, such as tolerance to ethanol (Jiménez and Benítez, 1988) and non-optimal temperatures (Li *et al.*, 2019). Albertin *et al.* (2013) showed that mitochondrial inheritance in *S. cerevisiae* x *S. uvarum* hybrids does not impact fermentation performance or its products. However, other studies have highlighted the importance of mtDNA from a cryotolerant species in the cryotolerance of interspecific hybrids (Rainieri *et al.*, 2008; Baker *et al.*, 2019). In a recent study by Pérez *et al.* (2022), hybrids inheriting mitochondria from a wine yeast showed increased fermentation performance, while those with mtDNA from a wild strain reduced ethanol and increased glycerol and organic acid production.

Table I1. Saccharomyces *interspecific hybrids developed for application in beverage fermentations.*

Parental species	Reference	GMO	Beverage	Highlights
S. cerevisiae x S. arboricola	Nikulin *et al.* (2017)	No	Beer	Maltotriose consumption, cryotolerance, improved fermentative capacities, increased ester formation
	Winans *et al.* (2020)	No	Sake	Increased ethanol and ethyl hexanoate, increased fermentation performance
S. cerevisiae x S. eubayanus	Hebly *et al.* (2015)	Yes	Beer	Maltotriose consumption, improved fermentative capacities
	Krogerus *et al.* (2015)	No	Beer	Maltotriose consumption, cryotolerance, flocculation
	Mertens *et al.* (2015)	No	Beer	Aroma profile diversification, robust fermentation performance
	Krogerus *et al.* (2016)	No	Beer	Maltotriose consumption, cryotolerance, improved fermentative capacities, increased ester formation
	Krogerus *et al.* (2017)	No	Beer	Maltotriose consumption, removal of phenolic off-flavour by sporulation
	Magalhães *et al.* (2017a)	No	Cider	Improved fermentative capacities (better than the S. cerevisiae parental) at low temperatures
	Magalhães *et al.* (2017b)	No	**Wine**	Robust fermentative performance, increased 2-phenylethanol production
	Su *et al.* (2019)	Yes	**Wine**	Improved fermentative capacities under low-temperature and low-nitrogen conditions
	Krogerus *et al.* (2021)	No	Beer	Increase acetate ester production
	Krogerus *et al.* (2022)	No	Beer	Varietal aroma release through β-lyase activity, improved fermentative capacities
S. cerevisiae x S. jurei	Giannakou *et al.* (2021)	No	Beer	Aroma profile diversification, robust fermentation performance
S. cerevisiae x S. kudriavzevii	Bellon *et al.* (2013)	No	**Wine**	Aroma profile diversification
	Bizaj *et al.* (2012)	No	**Wine**	Low H_2S and high volatile ester production
	Pérez *et al.* (2022)	No	**Wine**	Fruity and floral aroma increase (all hybrids), and improved fermentation performance or reduced ethanol, increased glycerol and organic acids depending on mitochondrial inheritance
S. cerevisiae x S. mikatae	Bellon *et al.* (2013)	No	**Wine**	Aroma profile diversification, robust fermentation performance
	Nikulin et al. (2017)	No	Beer	Maltotriose consumption, cryotolerance, improved fermentative capacities, increased ester formation
S. cerevisiae x S. paradoxus	Bellon *et al.* (2011)	No	**Wine**	Aroma profile diversification
S. cerevisiae x S. uvarum	da Silva *et al.* (2015)	Yes	**Wine**	Heterosis in ethyl ester production, slightly reduced ethanol yield, and improved fermentation performance compared to the *S. uvarum* parental strains
	Nikulin et al. (2017)	No	Beer	Maltotriose consumption, cryotolerance, improved fermentative capacities, increased ester formation
	Origone *et al.* (2018)	No	**Wine**	After genetic stabilization: low ethanol, high glycerol, aroma diversification
	Su *et al.* (2019)	Yes	**Wine**	Improved fermentative capacities under low-temperature and low-nitrogen conditions
	García-Ríos *et al.* (2019)	No	**Wine**	Improved fermentative capacities at low temperature
	Lairón-Peris *et al.* (2020)	No	**Wine**	Increased ethanol tolerance, high glycerol and aroma production
	Krogerus *et al.* (2022)	No	Beer	Varietal aroma release through β-lyase activity, improved fermentative capacities
	Pérez *et al.* (2022)	No	**Wine**	Fruity and floral aroma increase (all hybrids), and improved fermentation performance or reduced ethanol, increased glycerol and organic acids depending on mitochondrial inheritance
S. eubayanus x S. jurei	Gyurchev *et al.* (2022)	Yes	Beer	Maltose consumption, better growth on maltotriose in aerobic conditions than parent strains
S. eubayanus x S. mikatae	Gyurchev *et al.* (2022)	Yes	Beer	Maltose consumption, better growth on maltotriose in aerobic conditions than parent strains

3. Metabolism and its regulation during alcoholic fermentation

Saccharomyces yeasts are facultative anaerobic microorganisms, meaning that they can obtain energy from respiratory or fermentative metabolism depending on the oxygen availability. This allows them to survive in a wider range of environments when compared to strictly aerobic or anaerobic microbes. Additionally, they can implement a fermentative metabolism even in fully aerobic conditions, a phenomenon known as the Crabtree effect (Crabtree, 1929), by which the high fermentation rates at high glucose concentrations inhibit the synthesis of respiratory enzymes (De Deken, 1966). Even though anaerobic fermentation is energetically less efficient (2 ATP molecules produced against the 37 to 41 generated via respiration) and it decreases biomass production, it provides ethanol as a tool to outcompete other microorganisms (Hagman *et al.*, 2013). Thanks to the Crabtree effect, the metabolism of *Saccharomyces* yeasts in winemaking conditions is almost fully fermentative (**Fig. I6**).

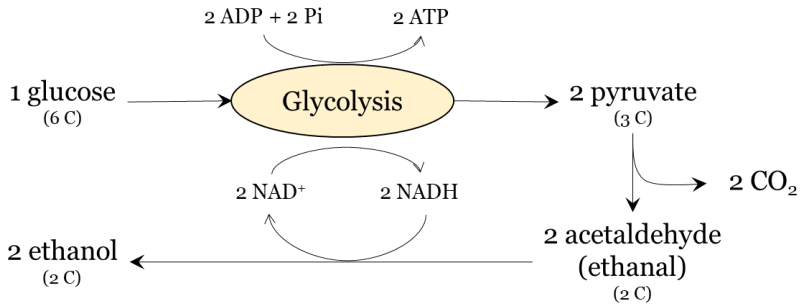


***Figure I6**. Simplification of the fermentative metabolism during anaerobic fermentation.*

The simplest way to summarise anaerobic fermentation in winemaking is the transformation of glucose and fructose into ethanol and carbon dioxide:

$$C_6H_{12}O_6 \rightarrow 2CO_2 + 2C_6H_5OH$$

The theoretical yield of this conversion is 0.51 g_{EtOH}/g_{sugars}. However, sugar is not entirely used for fermentative metabolism, as other cellular processes such as biomass synthesis and the maintenance of the redox balance also use sugar. Therefore, the experimental yield observed is usually around 0.47 g_{EtOH}/g_{sugars}, also depending on the yeast and the conditions used.

3.1. Central carbon metabolism

Prior to their catabolism, hexoses need to enter the yeast cell. In *S. cerevisiae*, glucose and fructose are transported by facilitated diffusion via the Hxt hexose permeases (Lagunas, 1993; Kruckeberg, 1996). Hxt permeases show higher affinity by glucose compared to fructose. For this reason, during wine fermentation, *S. cerevisiae* consumes glucose faster than fructose (a phenomenon known as glucophilia against the fructophilia presented by some other microorganisms). A proton-coupled fructose symporter was also reported in the wine *S. cerevisiae* strain EC1118 due to a horizontal gene transfer event (Galeote *et al.*, 2010).

Once inside the cell, glucose and fructose enter glycolysis, the first of the three main pathways of the CCM (**Fig. IX**). In this stage, each hexose molecule results in the formation of two molecules of pyruvate, yielding reduced NADH cofactors and energy in the form of ATP. From this point, the fate of pyruvate can be respiratory or fermentative. In anaerobic conditions, most of the pyruvate is transformed into acetaldehyde (ethanal) by a pyruvate decarboxylase (PDC), releasing carbon dioxide as a subproduct. Acetaldehyde is majorly transformed into ethanol by an alcohol dehydrogenase (ADH). To date, three *PDC* and seven *ADH* genes have been reported in *S. cerevisiae* (Cherry *et al.*, 2012). The most active isoforms of these enzymes during fermentation are *PDC1*, *PDC5* and *ADH1*. Alternatively, the acetaldehyde formed through pyruvate decarboxylation can be reduced into 2,3-butanediol, helping to equilibrate the redox balance of the cell, or oxidized into acetate. Acetate synthesis is the second source of NADPH in the yeast cell after the pentose phosphate pathway, accounting for approximately 40% of the NADPH required by the cell. Acetate can then be converted to acetyl-CoA via acetyl-CoA synthase (ACS). This acetyl-CoA can remain in the cytosol, acting as a precursor in fatty acid biosynthesis, or be incorporated into the mitochondria to enter the TCA cycle. If there is an excess of acetate in the previous step, this can be secreted to the medium, provoking a vinegarish off-flavour if found in too high concentrations.

Not all glucose is transformed into pyruvate: during glycolysis, a small fraction of glucose is used to produce glycerol via glycerol-3-phosphate dehydrogenase. Glycerol synthesis acts as an 'electron sink' to re-oxidize the NADH formed during biosynthesis (Dzialo *et al.*, 2017). It also acts as a precursor in phospholipid biosynthesis, contributing to resistance to cold temperatures and high osmotic stresses.

As mentioned, the Krebs (or TCA) cycle occurs in the mitochondria. A small fraction of the pyruvate formed during glycolysis enters the mitochondria, being transformed to acetyl-CoA by the PDH (pyruvate dehydrogenase) complex. This complex is composed of two subunits, i.e. Pda1p and Pdb1p. An alternative route for acetyl-CoA formation from pyruvate is the cytosolic PDH bypass (Remize, Andrieu and Dequin, 2000), after which

acetyl-CoA enters the mitochondria through the carnitine shuttle. The third way to feed the Krebs cycle from pyruvate is an anaplerotic reaction generating oxaloacetate, catalysed by pyruvate carboxylases (*PYC1*, *PYC2*) (**Fig. I7**).

The TCA cycle provides the cell with α-ketoglutarate, oxaloacetate and succinyl-CoA, required for the synthesis of some amino acids. Contrary to the initially described, the Krebs cycle does not operate as a cycle during fermentation, but rather as a branched pathway, which can be oxidative or reductive. The reductive branch leads to the production of succinate via fumarate reductase, while the oxidative branch generates α-ketoglutarate (Gombert *et al.*, 2001; Camarasa, Grivet and Dequin, 2003). α-ketoglutarate constitutes the link between the central carbon and the central nitrogen metabolism.

The third major route of the yeasts' central carbon metabolism is the pentose phosphate pathway (PPP). Although it does involve the oxidation of glucose-6-phosphate, its main role is anabolic rather than catabolic. It consists of two phases. The first phase, oxidative and irreversible, is catalysed by two dehydrogenases (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) and a 6-phosphogluconolactonase. This phase generates around 60% of the NADPH necessary for biosynthetic reactions in the cell (Frick and Wittmann, 2005; Cadière *et al.*, 2011). Therefore, this route is vital for protecting yeast from oxidative stress, since NADPH is an essential cofactor for glutathione- and thioredoxin-dependent enzymes that protect cells against oxidative damage (Minard and McAlister-Henn, 2001). The second phase, non-oxidative and reversible, involves transketolases and transaldolases. It leads to the production of erythrose-4-phosphate and ribose-5-phosphate, precursors of aromatic amino acids and nucleotides, respectively.

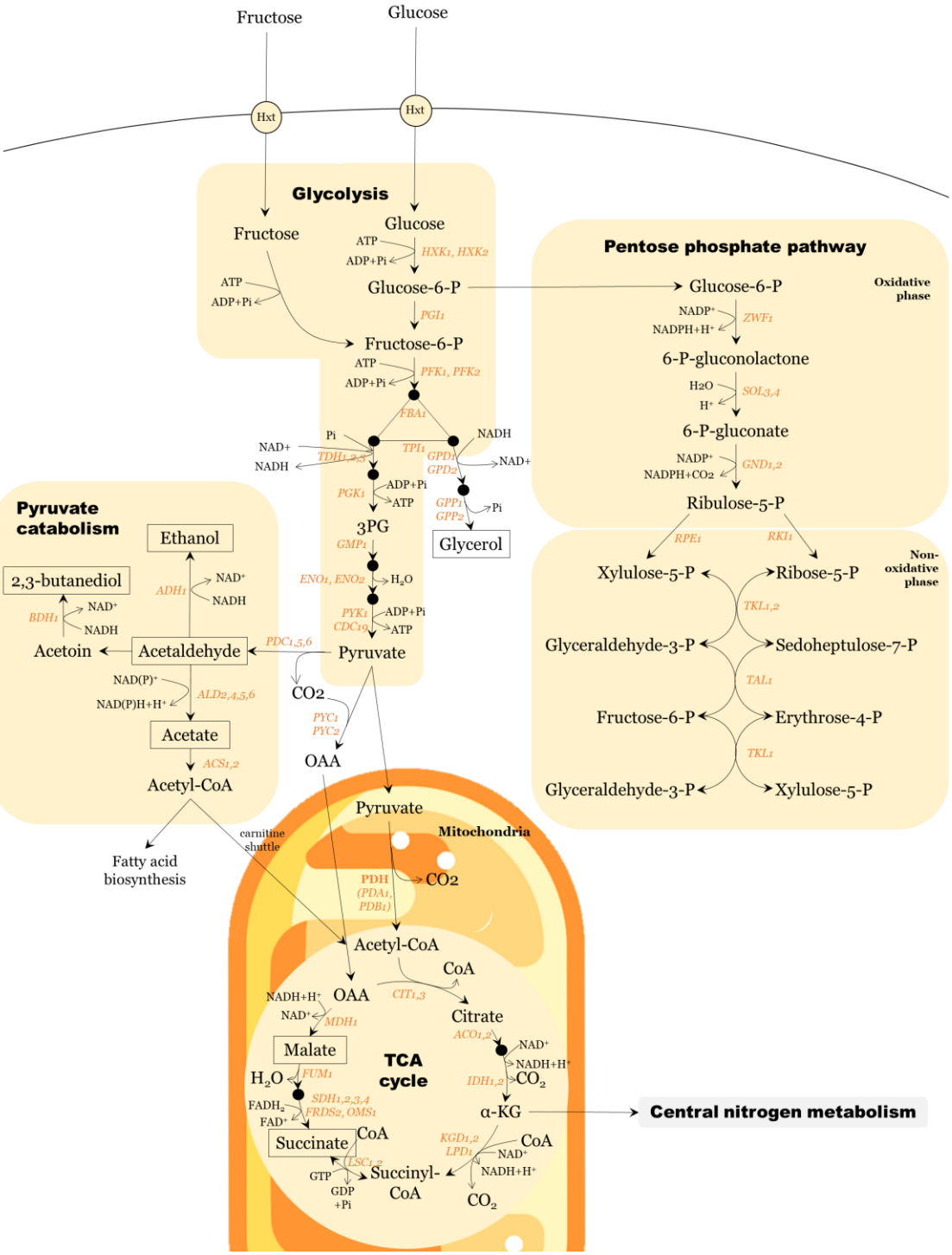


Figure 17*. Central carbon metabolism in fermentative conditions. The most relevant compounds for the wine industry are highlighted in black boxes.*

3.2. Nitrogen metabolism

3.2.1. Amino acid catabolism

During fermentation, yeasts consume the amino acids present in grape must. Like other nitrogen sources, amino acids are classified into 'preferred' and 'non-preferred' by yeasts. This classification has been made empirically by monitoring cell growth, observing that the so-called preferred amino acids (e.g. Glu, Gln, Asp) better support cell growth than the non-preferred (e.g. Pro, Leu, Met). When a mix of amino acids is available, yeast cells first consume the preferred amino acids, using mainly two mechanisms for their selection: the nitrogen catabolite repression (NCR) and the plasma membrane Ssy1-Ptr3-Ssy5 (SPS) sensor (Crépin *et al.*, 2012; Ljungdahl and Daignan-Fornier, 2012). The NCR represses the transcription of genes required for the use of non-preferred amino acids (Magasanik and Kaiser, 2002), while the SPS sensor specifically induces the expression of genes needed for the use of preferred amino acids (Ljungdahl, 2009).

Once inside the cell, amino acids can be directly used in biosynthetic reactions such as protein synthesis. Otherwise, they are temporarily stored in vacuoles or catabolized to provide building blocks for the *de novo* biosynthesis of other amino acids (Crépin *et al.*, 2017).

Amino acid catabolism implies their cleavage to release their amino group (in the form of NH_4^+ or glutamate) and generate carbon skeletons (i.e. α-keto acids). Most amino acids transfer their amino group to α-ketoglutarate by the action of transaminases or aminotransferases to form glutamate. Others, such as serine and threonine, possess a hydroxyl group on their β-carbon and can undergo direct deamination to release a free ammonium ion. This ammonium can be assimilated in two different reactions: the synthesis of glutamate from ammonium and α-ketoglutarate (catalysed by Gdh1p or Gdh3p), and the synthesis of glutamine from ammonium and glutamate (catalysed by Gln1p) (**Fig. I8**) (Magasanik, 2003; Crépin *et al.*, 2017).

Concerning the carbon skeletons, they are used differently depending on their nature. The utilization of 'preferred' amino acids as nitrogen source yields α-keto acids that are readily integrated in metabolism, such as pyruvate, α-ketoglutarate, succinate or oxaloacetate. On the contrary, the keto acids produced by the branched-chain amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), methionine and threonine, generally classified as 'non-preferred', enter the Ehrlich pathway (Ljungdahl and Daignan-Fornier, 2012).

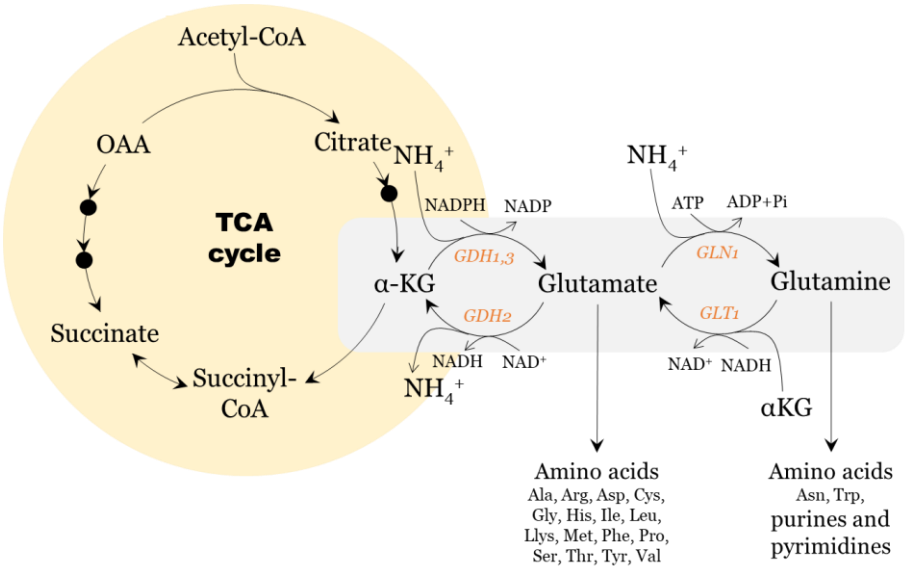


***Figure I8**. Central nitrogen metabolism, adapted from Ljungdahl and Daignan-Fornier (2012)*

3.2.2. Amino acid biosynthesis

If provided with an appropriate source of carbon and ammonium, yeast cells can synthesize *de novo* all the proteinogenic amino acids that they need (Jones and Flink, 1982). As explained above, ammonium can be incorporated during the formation of glutamate and glutamine in two reversible reactions known as the central nitrogen metabolism (**Fig. IX**). Glutamate and glutamine play a key role in amino acid biosynthesis, acting as donors of the amino group in the transamination reactions required for this process. In fact, around 85% of the total cellular nitrogen is derived from the amino nitrogen of glutamate, and the remaining 15% is incorporated through the amide nitrogen of glutamine (Cooper, 1982).

The carbon skeleton for the synthesis *de novo* of amino acids are provided by the central carbon metabolism. Ljungdahl and Daignan-Fornier (2012) classified the amino acids synthesised by yeast into different families depending on the carbon precursor they are derived from:

- Ribose-5-phosphate (PPP): His.
- Erythrose-4-phosphate (PPP): Phe, Tyr and Trp.
- 3-phosphoglycerate (glycolysis): Ser, Gly, Cys and Met.
- Pyruvate (glycolysis): Ala, Val, Leu and Ile.
- α-ketoglutarate (TCA cycle): Glu, Gln, Arg, Pro, and Lys.
- Oxaloacetate (TCA cycle): Asp, Asn, Thr, Cys and Met.

3.3. Fermentative aroma formation

The volatile molecules present in wine, often referred to as odour-active compounds (OAC), are incorporated in different stages of the winemaking process. From them, compounds produced *de novo* by yeast during alcoholic fermentation are known as fermentative aroma (Mina and Tsaltas, 2017). Depending on the metabolic pathways leading to their formation, the most relevant fermentative aromas can be classified as it follows:

- Ehrlich pathway derivatives:
  - Fusel alcohols (formed by reduction of a fusel aldehyde)
  - Acetate esters (formed by esterification of a fusel alcohol)
  - Fusel or branched acids (formed by oxidation of a fusel aldehyde)
  - Substituted ethyl esters (formed by esterification of a fusel acid)
- Fatty acid biosynthesis derivatives:
  - Short- and medium-chain fatty acids (S/MCFAs)
  - Linear ethyl esters (formed by esterification of S/MCFAs)

From them, the most relevant from a sensory perspective are higher (or fusel) alcohols, acetate esters and ethyl esters (Saerens *et al.*, 2010).

3.3.1. The Ehrlich pathway and its products

3.3.1.A. The Ehrlich pathway

Probably the best characterized metabolic route for fermentative aroma formation is the Ehrlich pathway. This route involves three enzymatic reactions: transamination, decarboxylation and reduction or oxidation (**Fig. I9**).

First, amino acids are converted to their respective α-keto acids by losing their amino group, which is transferred to a molecule of α-ketoglutarate (Hazelwood *et al.*, 2008). This reaction is catalysed by four different transaminases. Bat1p and Bat2p catalyse the transamination of branched-chain amino acids (Kispal *et al.*, 1996), while Aro8p and Aro9p are responsible for aromatic amino acid transamination. Additionally, the genes encoding those enzymes are expressed with different patterns during fermentation. While *BAT1* is highly expressed during the exponential phase and is localized in the mitochondria, *BAT2* is mostly expressed during the stationary phase and is produced in the cytoplasm (Eden, Simchen and Benvenisty, 1996). Similarly, *ARO8* is expressed constitutively, while the expression of *ARO9* is induced by the presence of aromatic amino acids (Iraqui *et al.*, 1998).

In a second step, the α-keto acid is irreversibly decarboxylated to form a fusel aldehyde. The same three PDCs used in the production of acetaldehyde from pyruvate (Pdc1p, Pdc5p and Pdc6p) are also responsible for this decarboxylation (Hazelwood *et al.*, 2008). However, two other genes (*ARO10* and *THI3*) in the *S. cerevisiae* genome share sequence similarity with PDCs (Hazelwood *et al.*, 2008). Although the function of Thi3p is assumed to be regulatory rather than catalytic (Nosaka *et al.*, 2005), Aro10p has shown to be the enzyme with the highest affinity for aromatic keto acids (Romagnoli *et al.*, 2012), and its expression levels suggest that it is the major decarboxylase of branched-chain, aromatic and sulfur-containing keto acids (Querol *et al.*, 2018).

A fraction of the α-keto acids decarboxylated in this step are in fact derived from the transamination reaction, using amino acids acquired from the medium. However, Crépin *et al.*, (2017) showed that the contribution of exogenous amino acids to this process remains low, and the highest flux of α-keto acid formation is their synthesis *de novo* from the central carbon metabolism. Consequently, most of the aromas produced through the Ehrlich pathway during fermentation derive from hexoses and not from amino acids.

Which reaction occurs in the final step of the Ehrlich pathway depends on the redox status of the cell (Styger, Jacobson and Bauer, 2011) and the culture conditions (Hazelwood *et al.*, 2008). If much reducing power in the form of NADH is available, the fusel aldehyde will be reduced to the corresponding higher alcohol, a reaction catalysed by an alcohol dehydrogenase (ADH). The genome of *S. cerevisiae* contains 16 of these enzymes (Hazelwood *et al.*, 2008), some of them also transforming acetaldehyde into ethanol during fermentation. On the contrary, low levels of NADH will likely lead to the production of the corresponding fusel acid by the action of an aldehyde dehydrogenase (ALD), the same enzymes that catalyse the conversion of acetaldehyde to acetate in the central carbon metabolism (Dzialo *et al.*, 2017). Regarding the culture conditions, it has been shown that during alcoholic fermentation, with high sugar amounts and almost complete anaerobiosis, reduction predominates over oxidation. As a consequence, aldehydes are mostly converted to higher alcohols and the formation of carboxylic acids plays only a minor role (Dickinson, Salgado and Hewlins, 2003).

The excretion of higher alcohols to the medium is performed by passive diffusion through the cell membrane, while acids are secreted by the action of a membrane transporter encoded by *PDR12* (Hazelwood *et al.*, 2006).

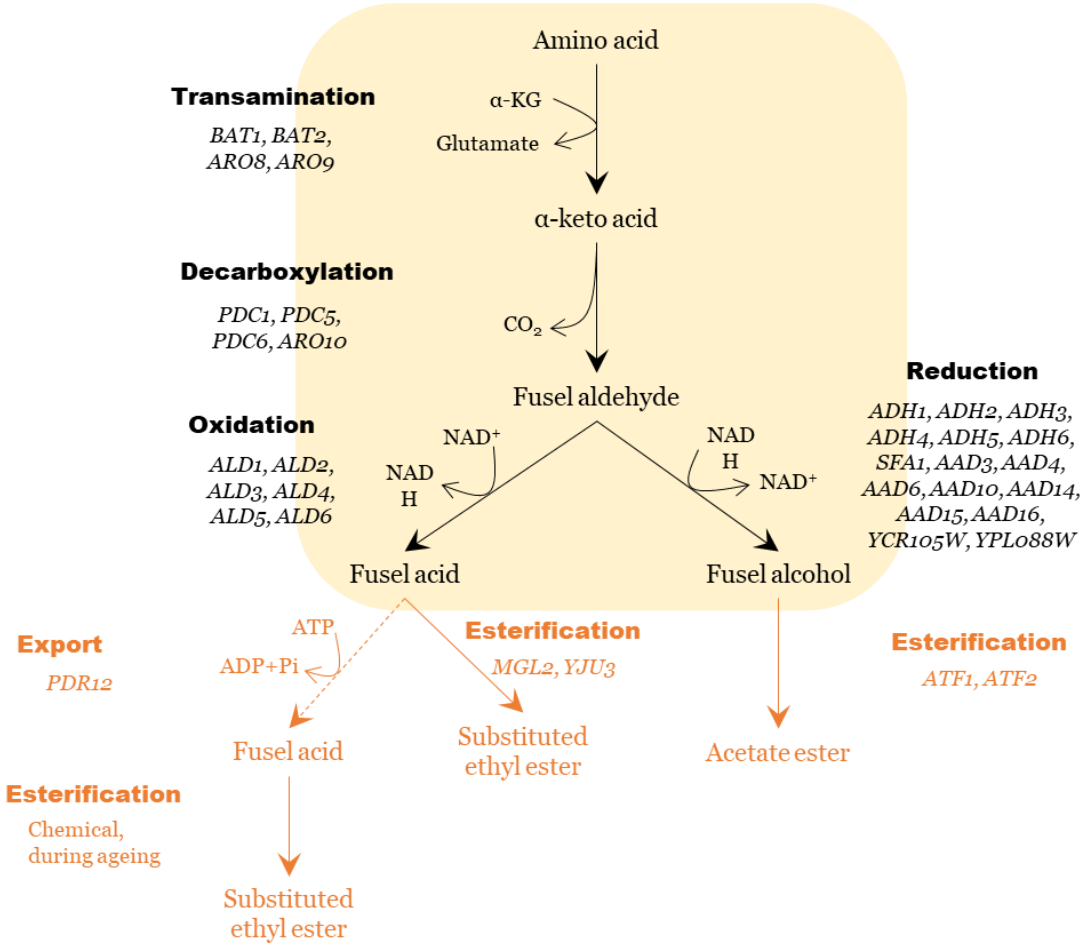


***Figure I9**. Ehrlich pathway (black) and the possible fates (orange) of its products.*

3.3.1.B. Products of the Ehrlich pathway: fusel alcohols and acids

The most relevant outcome of the Ehrlich pathway in fermentative conditions is the higher alcohols, both qualitatively and quantitatively. Generally, those compounds are considered positive for the organoleptic profile of wine only if they are present in amounts below 300 mg/L. Above 400 mg/L, however, they impart an unpleasant solvent-like aroma to the final product. The most relevant higher alcohols produced by yeast during alcoholic fermentation are shown in **Table I2**. From them, 2-phenylethanol is the most desirable, providing floral (rose-like) notes and being typically found in high concentrations compared to other higher alcohols (Lambrechts and Pretorius, 2000; Querol *et al.*, 2018). A desirable outcome is a high production of branched-chain and aromatic fusel alcohols in combination with low levels of off flavours (e.g. methionol) (Hazelwood *et al.*, 2008).

Fusel acids impart negative notes, often described as fatty, cheesy, rancid and sour. It is worth mentioning that the accumulation of fusel oils can inhibit cell growth, as their formation requires large amounts of energy (Hazelwood *et al.*, 2008; Ljungdahl and Daignan-Fornier, 2012). From an oenological perspective, the production of fusel acids is desirable only as precursors for substituted ethyl ester synthesis. Ethyl ester production from fusel acids occurs mainly during wine ageing, in a chemical reaction involving ethanol (see Section 3.3.3.C).

***Table I2**. Main fermentative aromas derived from alpha-ketoacids.*

Amino acid		α-keto acid		Fusel aldehyde		Fusel alcohol / Fusel acid		Acetate ester / Ethyl ester
Leu	→	α-ketoisocaproate	→	3-methylbutanal	↗	3-methylbutanol (*isoamyl alcohol*)	→	3-methylbutyl acetate
					↘	3-methylbutanoic acid	→	Ethyl-3-methylbutanoate
Val	→	α-ketoisovalerate	→	2-methylpropanal	↗	2-methylpropanol (*isobutanol*)	→	2-methylpropyl acetate
					↘	2-methylpropanoic acid	→	Ethyl 2-methylpropanoate
Ile	→	α-ketomethylvalerate	→	2-methylbutanal	↗	2-methylbutanol (*active amyl alcohol*)	→	2-methylbutyl acetate
					↘	2-methylbutanoic acid	→	Ethyl 2-methylbutanoate
Phe	→	Phenylpyruvate	→	2-phenylethanal	↗	2-phenylethanol	→	2-phenylethyl acetate
					↘	2-phenylethanoic acid	→	Ethyl phenylacetate
Tyr	→	p-hydroxyphenylpyruvate	→	2-(4-hydroxyphenyl)-ethanal	↗	2-(4-hydroxyphenyl)ethanol (*tyrosol*)		
					↘	2-(4-hydroxyphenyl)ethanoic acid		
Trp	→	3-indole pyruvate	→	2-(Indol-3-yl)-acetaldehyde	↗	2-(Indol-3-yl)ethanol (*tryptophol*)		
					↘	2-(Indol-3-yl)ethanoic acid		
Met	→	α-keto-γ-(methylthio)butyrate	→	3-(methylthio)propanal	↗	3-(methylthio)propanol (*methionol*)	→	3-(methylthio)propyl acetate
					↘	3-(methylthio)propanoic acid	→	Ethyl-3-(methylthio) propanoate

3.3.2. Fatty acid biosynthesis

Like the fusel oils produced during the Ehrlich pathway, volatile fatty acids impart aromas often described as unpleasant. Saturated fatty acids with an even number of carbon atoms are relevant for the organoleptic profile of wine because they are precursors of ethyl esters. Although some authors have found positive correlations between fatty acid content and wine quality (Juan *et al.*, 2012), the reason behind those correlations is likely the subsequent ethyl ester formation.

Saturated fatty acids (SFA) in yeast cells have two possible origins: the biosynthesis via the fatty acid synthase complex, and the catabolism of fatty acids via β-oxidation (Mbuyane, Bauer and Divol, 2021).

The predominant pathway during wine fermentation is the biosynthetic, while the β-oxidation, which requires the presence of oxygen, is active only at the beginning of the fermentation. During their synthesis, saturated fatty acids are formed by successively adding two carbon atoms from malonyl-CoA to an acyl-CoA, with acetyl-CoA being the first substrate of this elongation cycle. In each step, one molecule of acetyl-CoA is consumed, and the fatty acid elongates by two carbon atoms. The elongation is catalysed by a multi-enzymatic complex known as fatty acid synthase (FAS) complex, composed by two subunits. The required acetyl-CoA can be obtained either by oxidative decarboxylation of pyruvate or by direct activation of acetate with ATP (see **Fig. I7** – central carbon metabolism) (Dufour, Malcorps and Silcock, 2003; Saerens *et al.*, 2010).

Oxygen availability affects the accumulation of MCFAs through the activity of acetyl-CoA carboxylase, an enzyme participating in their synthesis. Under oxygen-limited conditions (such as the fermentative environment), long-chain SFAs accumulate and inhibit the mentioned acetyl-CoA carboxylase, releasing acyl-CoAs under synthesis from the FAS complex. As a result, MCFAs accumulate. In the presence of oxygen, however, UFAs are synthesized and the acetyl-CoA carboxylase is active. Therefore, the elongation continues as normally, generating complete long-chain fatty acids and reducing the amount of MCFAs available for ethyl ester formation (Dufour, Malcorps and Silcock, 2003; Saerens *et al.*, 2010).

Alternatively, the β-oxidation pathway for fatty acid degradation consists of a series of reactions which release a molecule of acetyl-CoA from the fatty acid chain, generating a fatty acid reduced by two carbon atoms (Mbuyane, Bauer and Divol, 2021).

3.3.3. Esterification: adding fruitiness to wine

Volatile esters are found in low amounts in wine. However, they comprise the most relevant set of fermentative aromas, as they are responsible for a highly desired fruity, sweet, perfume-like character. They generally have low odour thresholds, the reason why even small changes greatly impact the perception of the final product. Additionally, the presence of different esters can have a synergic effect, where a mixture of compounds will enhance or mask the presence of others (Saerens *et al.*, 2010; Dzialo *et al.*, 2017).

Esters are formed by a condensation (esterification) reaction between acetyl- or acyl-CoA and an alcohol (**Fig. I10**), a reaction catalysed by an acyl transferase or ester synthase (Nordström, 1962). The energy required to form the new bond is provided by the thioester linkage of the acyl-CoA. The condensation of acetyl-CoA with a higher alcohol (or ethanol) will provide an acetate ester, whereas the esterification of ethanol with an acyl-CoA will produce an ethyl ester. The other acyl-CoAs are formed through acylation of free CoA with fatty acids, a reaction catalysed by acyl-CoA synthase.

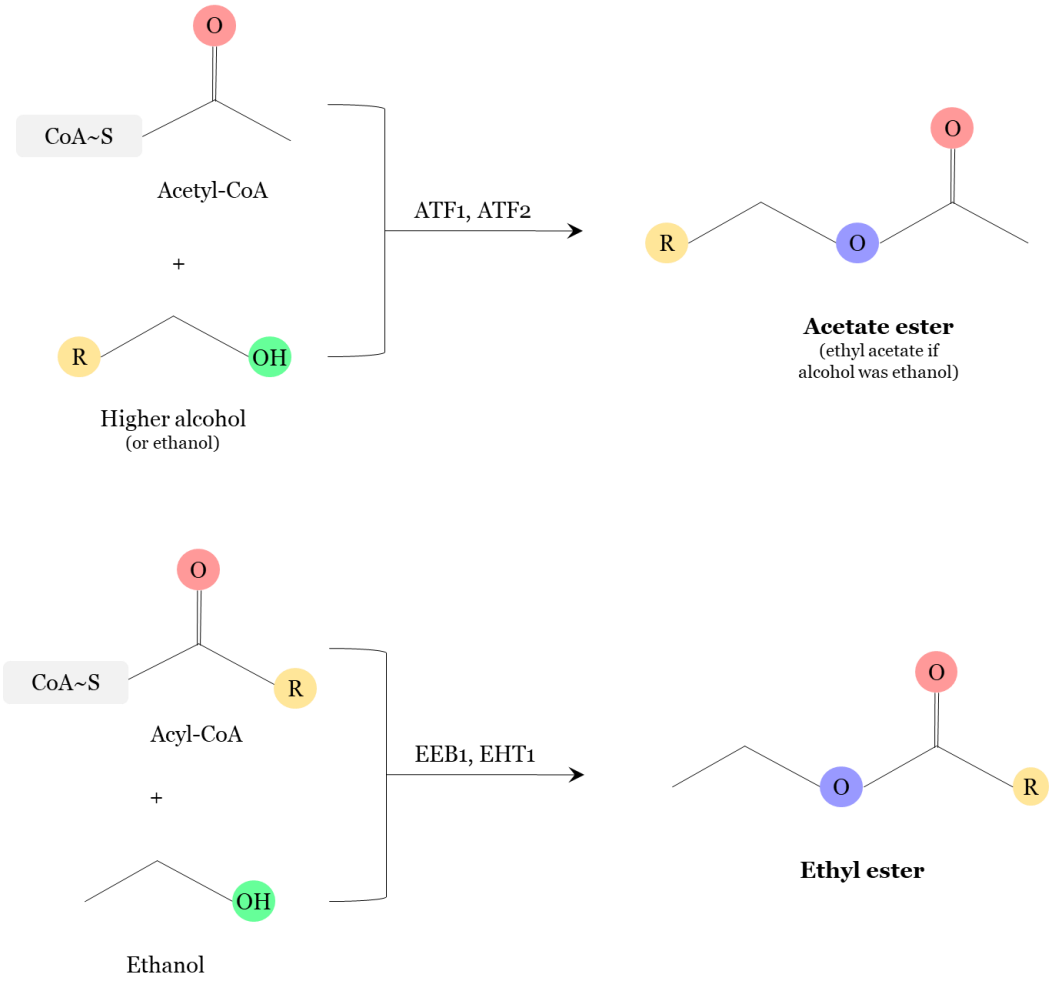


Figure I10. *Enzymatic ester synthesis in* S. cerevisiae *(adapted from Querol* et al. *2018)*

The accumulation of any given ester depends on three factors: the availability of the two co-substrates (the acyl-CoA and the alcohol) and the expression and activity of the enzymes involved in their synthesis and hydrolysis (Dzialo *et al.*, 2017).

Once synthesized inside the cell, most esters can diffuse through the membrane and reach the extracellular medium thanks to their lipophilic character. Acetate esters are excreted rapidly and almost in their totality. However, the diffusion of ethyl esters decreases as the chain-length of the molecule increases. The percentage of diffusion ranges from 100% for ethyl hexanoate to 54 – 68% for ethyl octanoate, and only 8 – 17% for ethyl decanoate (Nykänen, Nikkänen and Suomalainen, 1977; Saerens *et al.*, 2010).

3.3.3.A. Acetate esters

As explained above, a great increase in higher alcohol production during fermentation is not necessarily interesting from an organoleptic perspective. Instead, a high production of those is only desired if a high fraction is afterwards esterified into their corresponding acetate esters. Ethyl acetate is quantitatively the most important acetate ester, as intracellular pools of both co-substrates needed for its production (acetyl-CoA and ethanol) are the most abundant of all. When found in too high concentrations, ethyl acetate imparts a foul solvent-like aroma. Therefore, the aim of wine microbiologists is a reduction rather than an increase in its production, while increasing 'pleasant' acetate ester formation. Examples of relevant acetate esters are isoamyl acetate (fruity), isobutyl acetate (banana) or 2-phenylethyl acetate (rose, honey, fruity) (Lambrechts and Pretorius, 2000).

Acetate esters have received most attention in the past decades as they are produced in higher amounts compared to ethyl esters. As a consequence, the genes involved in their synthesis were discovered first, and the factors determining their formation are better characterized (Saerens *et al.*, 2010).

The formation of acetate esters is catalysed by alcohol O-acetyl- (or acyl-) transferases (AATases). In *S. cerevisiae*, only two genes encoding this type of enzyme, namely ATF1 and ATF2, have been identified to date (Querol *et al.*, 2018). Verstrepen *et al.* (2003) reported significant changes in acetate ester production by deleting or overexpressing those enzymes, confirming their importance in brewing conditions. Atf1p seemed to play a minor role compared to Atf2p. Although a double deletion of the *ATF1* and *ATF2* loci eliminated every trace of isoamyl acetate production, only a 50% reduction in ethyl acetate was achieved, indicating that other ester synthases were still beyond our knowledge. Seguinot *et al.* (2018) observed an overexpression of *ATF1* and *ATF2* due to nitrogen additions in winemaking conditions. Beyond *S. cerevisiae*, allelic variants of *ATF1* and *ATF2* from *S. kudriavzevii* and *S. uvarum* have been shown to increase the production

of various acetate esters when expressed in a *S. cerevisiae* host (Stribny, Querol and Pérez-Torrado, 2016). Kruis *et al.* (2017) identified a third enzyme, the ethanol acetyltransferase 1 (Eat1p in *Wickerhamomyces anomalus*, organism where it was discovered). The deletion of *YGR015C* (analogous of *EAT1* in *S. cerevisiae*) resulted in a 50% reduction in ethyl acetate formation, complementing the *ATF1* and *ATF2* production.

The substrate specificity of acyltransferases also plays an important role in ester production. Atf1p and Atf2p can use a broad range of alcohol co-substrates such as ethanol, propanol, isobutanol, hexanol and 2-phenylethanol, generating the corresponding acetate esters (Verstrepen *et al.*, 2003; Lilly *et al.*, 2006).

3.3.3.B. Ethyl esters of linear fatty acids

Ethyl esters are formed by condensation of ethanol and an acyl-CoA. Contrary to acetate esters, the factor with the higher impact in the production of ethyl esters is the availability of their precursors (Saerens *et al.*, 2008). The acyl-CoAs needed for ethyl ester production are formed through the acylation of free CoA with fatty acids, a reaction catalysed by an acyl-CoA synthase. The main linear ethyl esters and their fatty acids of origin are shown in **Table I3**.

ATF1 and *ATF2* are not involved in the synthesis of ethyl esters, as a double deletion of both genes did not impact their production in brewing conditions compared to the wild strain (Verstrepen *et al.*, 2003). Instead, their formation is catalysed by the acyl-CoA:ethanol O-acyltransferases (AEATases) Eht1p and Eeb1p. Saerens *et al.* (2006) studied the impact of *EHT1* and *EEB1* deletions in MCFA ethyl ester production. Their results suggested that *EHT1* plays only a minor role, catalysing exclusively the synthesis of ethyl hexanoate, while deletions in EEB1 significantly reduced the production of C4 to C10 ethyl esters. Still, strains with double deletions (*Δeht1Δeeb1*) were able to produce ethyl esters to some extent, indicating the existence of other enzymes still unknown. A third protein (Mgl2p), although sharing a high sequence homology with Eht1p and Eeb1p, had a slighter impact on ethyl ester production (Saerens *et al.*, 2006).

Saerens *et al.* (2006) also characterized the activity of Eht1p and Eeb1p in vitro, and reported that both enzymes, in addition to their AEATase activity, also catalysed ester hydrolysis. Particularly, a thio-esterase activity was observed for Eht1p, with the capacity of hydrolysing medium-chain acyl-CoA to release free fatty acids. In line with this discovery, overexpression of both *EHT1* and *EEB1* did not provide a significant increase in MCFA ethyl ester production, probably because of the mentioned esterase activity.

A QTL mapping study by (Steyer *et al.*, 2012) identified allelic variants of the *PLB2* gene, encoding a phospholipase with transacylase activity (Merkel *et al.*, 1999), affecting

ethyl ester production. The deletion of that gene, involved in lipid metabolism, resulted in a significant drop in the levels of ethyl octanoate and decanoate, and its impact was higher than that of the *EEB1* deletion.

***Table I3**. Main linear fatty acids and their ethyl esters.*

Fatty acid	Odour		Ethyl ester	Odour
Butanoic acid	Pungent, cheesy	→	Ethyl butanoate	Floral, fruity
Hexanoic acid	Sour, fatty, cheesy	→	Ethyl hexanoate	Apple, banana
Octanoic acid	Fatty, waxy, rancid	→	Ethyl octanoate	Pineapple, pear
Decanoic acid	Fatty, rancid, unpleasant	→	Ethyl decanoate	Floral, fruity
Dodecanoic acid	Mild fatty, coconut	→	Ethyl dodecanoate	Waxy, soapy

Source: Lambrechts and Pretorius (2000), and www.thegoodscentscompany.com

3.3.3.C. Ethyl esters of substituted (fusel) acids

Wine ageing has a strong impact in wine composition. The two described categories of esters (acetate esters of higher alcohols and ethyl esters of linear fatty acids) generally fade away during this process, being quickly hydrolysed. Their impact is therefore limited to young wines, which are bottled right after fermentation. However, there is a third group of esters whose concentrations are influenced by yeast metabolism: the substituted ethyl esters (hereby called SEE). This group of compounds, also providing fruity notes, has only gained in attention in the last decade, as they are produced in smaller quantities than ethyl esters during fermentation. However, their concentration during wine ageing steadily increases by chemical esterification of the corresponding fusel acid with ethanol (Díaz-Maroto, Schneider and Baumes, 2005; Denat *et al.*, 2021). Additionally, those compounds are also produced by flor yeasts during biological ageing of sherry wines (Cortes *et al.*, 1998). Therefore, substituted ethyl esters significantly contribute to the aroma of wines aged by oxidative (red wines) or biological (white wines) methods.

The biosynthesis of substituted esters has only recently been investigated. Eder *et al.* (2018) showed that the production of ethyl-2-methylpropanoate and ethyl-3-methylbutanoate depends on multigenic factors involving the biosynthesis of their metabolic precursors. The enzymatic activity controlling the esterification step was first described by Marullo *et al.* (2021). In a strain quadruple deleted for *AFT1*, *ATF2*, *EEB1* and *EHT1*, linear ester biosynthesis was strongly reduced, but the production of SEE was surprisingly enhanced. Conversely, they showed that two enzymes with MAGLase (mono-acyl glycerol lipase) activity, namely Mgl2p and Yju3p, were significantly involved in the production of SEE, as a double deletion of both genes decreased the production of those compounds by 50%. However, the production of 2-phenylethyl acetate was not affected by those deletions, denoting the existence of other enzymes still beyond our knowledge.

4. *Saccharomyces* hybrids as a tool for quantitative genetics

4.1. Yeast life cycle

Saccharomyces yeasts are capable of sexual and asexual reproduction. In nutrient-rich conditions, cells divide mitotically (**Fig. I11.A**), producing two clonal daughter cells. Cells can proliferate vegetatively both in haploid and diploid form, although the dominant form in nature is diploid (Bendixsen, Frazao and Stelkens, 2022). Under starvation conditions, diploid cells undergo meiosis (sporulation) (**Fig. I11.C**) and generate four haploid daughter cells inside a protective ascus (tetrad) (Roeder, 1997). Diploids possess one copy of each mating type locus *(*MAT*): MATa* and *MATα*. Gametes will inherit a single MAT locus and therefore behave as maters (i.e. capable of mating). These maters secrete mutually attractive pheromones ('a-factor' by *MATa* cells and 'α-factor' by *MATα* cells), which promote 'shmoo' formation and the conjugation between maters of the opposite mating type (**Fig. I11.B**) (Haber, 2012).

The fate of the spores generated by meiosis plays an important role in the maintenance and generation of genetic variation. A spore can mate with another spore from the same tetrad, a process known as intra-tetrad mating or automixis (**Fig. I11.D**). This process is the most common due to proximity, and it eliminates 33% of genetic variation (Hittinger, 2013). Alternatively, a gamete can mate with a spore from another tetrad. This second spore may be from the same strain or from a different strain, dispersed by, for example, an insect (**Fig. I11.F**). This phenomenon, known as outcrossing or amphimixis, is the primary route of creating diversity. Gametes are also capable of mating with a gamete from a different species, generating an interspecific hybrid. It is interesting to note that the intestine of fruit flies (Reuter *et al.*, 2007) and social wasps (Stefanini *et al.*, 2016) allows hybridisation between different yeast strains, increasing the outcrossing probability.

Homothallic strains (i.e. capable of switching mating type), which are the most common in nature, often undergo haplo-selfing. During this process, a haploid cell will divide mitotically, then undergo mating type switching, and then mate with its daughter cell to produce a diploid (**Fig. I11.E**). As this diploid is homozygous except for the MAT locus, haplo-selfing eliminates 100% of the genetic variation (Hittinger, 2013). Mating type switching is promoted by a DNA double-strand break catalysed by the HO endonuclease (encoded by the *HO* gene) on the active MAT locus. The MAT locus is present on the chromosome III but also on two other loci: *HML* (hidden MAT left) and *HMR* (hidden MAT right). The HO endonuclease catalyses the degradation of the active MAT locus, which is then repaired using the HML or HML locus as a template (Strathern *et al.*, 1982; Haber, 2012). On the other hand, heterothallic strains can multiply indefinitely as haploids, due to loss-of-function mutations in the *HO* gene. The deliberate deletion of the *HO* gene in the

laboratory allows to generate stable haploid strains, which can be useful to control mating (Cubillos, Louis and Liti, 2009).

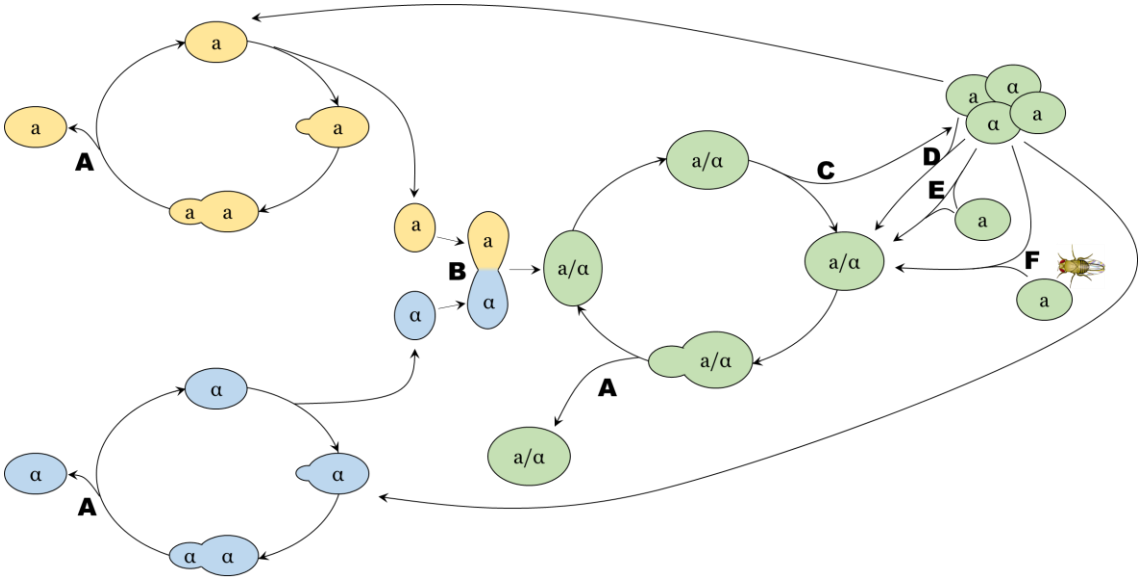


Figure I11. Saccharomyces *life cycle. **A**: Mitotic division. **B**: Conjugation. **C**: Meiotic division. **D**: Intra-tetrad mating. **E**: Mating-type switching and haplo-selfing. **F**: Outcrossing.*

4.2. Interspecific hybrid sterility

When given the choice, *Saccharomyces* spores preferentially mate with spores from the same species. This is because of differences in germination timing rather than species recognition (Maclean and Greig, 2008; Murphy and Zeyl, 2011). However, if no spores from the same species are available, germinated spores from different species readily mate, giving rise to an interspecific hybrid. The pre-zygotic barriers in the *Saccharomyces* genus are therefore said to be weak. However, the spores produced by those hybrids generally have very low viability (~ 1%). In other words, those hybrids are sterile, as sexual reproduction is not possible anymore.

Considerable effort has been made in the last decades to decipher the molecular basis of speciation and hybrid sterility. Nowadays, we know that mainly three mechanisms act independently, potentially causing post-zygotic reproductive isolation between *Saccharomyces* species: antirecombination, large-scale chromosomal differences, and genetic incompatibilities (Louis, 2011; Bozdag and Ono, 2022).

The primary mechanism leading to F1 hybrid sterility is the mismatch repair system (MMR) activity in response to sequence divergence during meiosis (Bozdag and Ono, 2022). The MMR detects and corrects errors that can arise during DNA replication and

recombination (Harfe and Jinks-Robertson, 2000). Homologous recombination, which takes place during meiosis, requires the formation of a DNA heteroduplex between complementary strands of the two recombining chromosomes. If these chromosomes differ in sequence, the heteroduplex will contain mismatches, and these will be detected by the MMR. When chromosomes contain too many mismatches, the MMR may abort recombination, a phenomenon called antirecombination (Borts *et al.*, 2000). When interspecific hybrids sporulate, their highly diverged chromosomes fail to recombine correctly, yielding gametes which are inviable because they lack essential chromosomes (Rogers *et al.*, 2018). It has been shown that deletion (Hunter *et al.*, 1996) and silencing during meiosis (Bozdag *et al.*, 2021) of MMR genes increase the viability of hybrid spores by 8 and 70-fold, respectively. The role of antirecombination in speciation was also demonstrated: Greig *et al.* (2003) generated intraspecific hybrids of either *S. cerevisiae* or *S. paradoxus* between partially reproductively isolated strains, and these also showed improved fertility upon MMR gene deletion. Additionally, there is a direct correlation between sequence divergence and reproductive isolation in both intraspecific and interspecific hybrids (Liti, Barton and Louis, 2006; Bendixsen, Frazão and Stelkens, 2022).

Chromosomal rearrangements have a high sterilising potential, as a single mutation can affect several genes. For instance, a single translocation involving a chromosome end will reduce spore viability by at least 25% if it contains at least one essential gene (Ono, Greig and Boynton, 2020). Inversions can interfere with recombination due to the abovementioned MMR, magnifying the effects of antirecombination. Delneri *et al.* (2003) engineered *S. cerevisiae* strains to make them collinear with *S. mikatae*. They found that collinearity (i.e. lack of gross chromosomal rearrangements) between strains of different species, although partially restoring spore viability, resulted in highly aneuploid gametes, confirming the much higher impact of antirecombination in hybrid sterility. At a higher scale, an abnormal number of chromosomes (i.e. aneuploidy) due to chromosome missegregation can also reduce spore viability, as some chromosomes fail to pair with their homologous counterparts during meiosis (Ono, Greig and Boynton, 2020).

In animal species, especially *Drosophila*, hybrid sterility is thought to be caused by genetic incompatibilities following the Bateson–Dobzhansky–Muller (BDM) model. This model attributes post-zygotic reproductive isolation to incompatibilities between genes from different species. Those genes, often called 'speciation genes' (Wu and Ting, 2004), function within each population but do not function properly when expressed in the same organism (Louis, 2011). Because of the high impact of antirecombination in hybrid sterility, the effect of potential genetic incompatibilities has been difficult to study (Kao, Schwartz and Sherlock, 2010). Greig *et al.* (2002) generated tetraploid versions of interspecific hybrids and compared their spore viability with their diploid counterparts. After genome

doubling, every chromosome had a perfectly homozygous pair to recombine with, eliminating the potential antirecombination and dominant gene incompatibility effects. They found that tetraploidisation restored fertility, achieving spore viabilities close to the non-hybrid diploids (Greig *et al.*, 2002). Incompatibilities between mitochondrial and nuclear DNA (i.e. cyto-nuclear) often decrease fitness of vegetative cells but do not cause spore inviability. However, as respiration is necessary for meiosis (Neiman, 2005), these incompatibilities can prevent sexual reproduction (Bozdag and Ono, 2022).

4.3. Overcoming hybrid sterility

Different mechanisms have been shown to restore hybrid fertility in recent years, and all of them involve an increase in sequence homology between homologous chromosomes.

The most intuitive way to achieve a high sequence homology is by means of whole-genome duplication (WGD). This phenomenon provides a perfectly homozygous partner for each chromosome, allowing accurate pairing during meiosis, a correct crossing-over, and thus a proper segregation. This has been attained both spontaneously (Marsit *et al.*, 2021) and deliberately by means of genetic engineering (Greig *et al.*, 2002), successfully restoring interspecific hybrid fertility. However, the diploid gametes produced by these tetraploids are sterile because they are heterozygous for the MAT locus, which represses mating. Additionally, it was shown that diploids carrying one *MAT* gene from each species cannot undergo mating-type switching and auto-diploidisation (Sipiczki, Antunovics and Szabo, 2020). This is known as the second sterility barrier (Sipiczki, 2018). This second barrier can be overcome by damage of the MAT locus or the loss of the chromosome carrying it (Sipiczki, Antunovics and Szabo, 2020), which restores their ability to mate and/or switch their mating type.

In a heterozygote, mitotic recombination can generate homozygous sequence blocks in events known as loss-of-heterozygosity (LOH). This reduction in sequence divergence also reduces antirecombination, thus increasing fertility. LOH has been shown to restore fertility in intraspecific (Dutta, Dutreux and Schacherer, 2021) and interspecific (D'Angiolo *et al.*, 2020) *Saccharomyces* hybrids.

A third mechanism to restore fertility is return-to-growth (RTG), where meiosis is aborted after genome duplication and mitotic growth continues with a diploid genome (Esposito and Esposito, 1974; Honigberg and Esposito, 1994). This event can restore fertility by inducing LOH and making the mating-type locus homozygous (Mozzachiodi *et al.*, 2021), thus overcoming the second sterility barrier as well.

4.4. QTL mapping: unveiling the genetic basis of complex traits

4.4.1. Principle, methodology and considerations

The variation of a phenotypic trait within a population can be categorized as qualitative or quantitative. When a change in a single locus alone is the cause for the observed phenotypic differences, the trait is said to be qualitative or Mendelian (Yeh, Jiang and Dunham, 2022). However, most phenotypes show a continuous distribution of a measurable character, being under the simultaneous control of several loci. Those are called quantitative or complex traits. Nevertheless, the distinction between Mendelian and quantitative traits is artificial, as quantitative traits can be seen as the cumulative effects of many qualitative traits and their interactions.

The multiple genome positions determining the phenotypic expression of a complex trait are known as quantitative trait loci or QTL. Each QTL can contain a single gene or a cluster of linked genes that contribute to the phenotype (Mackay, 2001), each of which can have a large effect (major QTL) or a small effect (minor QTL) in that phenotype (Swinnen, Thevelein and Nevoigt, 2012).

The QTL mapping method aims to simultaneously localise every locus with an effect on a given phenotypic trait (Swinnen, Thevelein and Nevoigt, 2012). In its most basic form, the first step of a QTL analysis is to select two strains which are genetically different from each other. Mating spores from those strains generates a hybrid (F1) whose homologous chromosomes differ in sequence to some extent. The sporulation of this hybrid produces a population of segregants, which are genetically different from each other due to meiotic recombination. As a consequence of this genetic variation, there will also be phenotypic variation. QTL mapping aims to find significant correlations between those two datasets: phenotypic information – in the form of a measurable character – and genotypic information – in the form of genetic marker frequency (Wu, Ma and Casella, 2007; Swinnen, Thevelein and Nevoigt, 2012).

The principle of QTL mapping resides in a phenomenon known as genetic linkage. When two loci are far from each other on a chromosome, one or more crossovers will probably occur between them. In this case, the recombination frequency (θ) between those loci will approach 50%, the same value as if those loci were located on different chromosomes. On the contrary, when two loci are located close to each other, it is improbable that a crossover will separate them during meiosis (i.e. their recombination frequency approaches 0%). Therefore, those loci tend to segregate together, and they are said to be linked. This correlation between recombination frequency and genetic distance is the basis of genetic map construction (Wu, Ma and Casella, 2007; Swinnen, Thevelein and Nevoigt, 2012). The mapping of a QTL relies on its co-segregation with genetic loci of

known positions, which are called genetic markers (Collard *et al.*, 2005). Those markers also allow to determine the parental origin of a given QTL. Currently, the most used genetic markers are single nucleotide polymorphisms (SNPs), which are often abundant and allow whole genome coverage. The location of such genetic markers can be done by sequencing, although other methods such as high-density oligonucleotide arrays have also been employed (Marullo, Aigle, *et al.*, 2007; Swinnen, Thevelein and Nevoigt, 2012).

Contrary to the common belief, phenotypic variation between parental strains is not a requirement for QTL mapping. This is because each parent strain may contain antagonistic alleles that reduce the phenotypic difference between them compared to the progeny, in which those loci are separated. Thus, phenotypically diverse progeny can be obtained from parents with similar phenotypes. In fact, the phenotypic variation in the segregant population is greater than that seen between the parental strains in virtually every cross (Liti and Louis, 2012), as exemplified by Cubillos *et al.* (2011) and Haas *et al.* (2019). However, parent strains do need to differ in genotype: if they are not polymorphic at a QTL, there will not be such QTL to detect; similarly, if they are polymorphic at a QTL but not at genetic markers nearby, that QTL will be impossible to detect.

Statistical methods determine whether a linkage between specific markers and the trait under study is significant. At a single-marker level, a simple analysis of variance (ANOVA) or t-test will answer this question by providing a probability value (Swinnen, Thevelein and Nevoigt, 2012). At a larger scale, interval mapping is often used. Simple interval mapping (SIM) (Lander and Botstein, 1989), which relies on genetic maps, allow a more accurate determination of the QTL locations. At each position, a LOD (logarithm base 10 of odds) score is calculated, indicating the probability that there is a QTL in such position. An improvement of this method is composite interval mapping (CIM) (Jansen and Stam, 1994; Zeng, 1994), which combine interval mapping and multiple regression, increasing resolution and reducing background noise.

Identifying QTLs is often tricky because several factors influence the genetic architecture of complex traits. For instance, the dissection of a trait can be impaired by epistatic effects (i.e. interactions between genes leading to a differential expression of a trait) (Carlborg and Haley, 2004). Epistasis implies that an allelic variant's effect on a phenotype differs on different genetic backgrounds. Similarly, the effect of a given allelic variant on a phenotype will not be the same in different environments (i.e. gene-environment interaction) (Liti and Louis, 2012); hence, the reproducibility of the phenotyping is critical. Another factor to consider is genetic heterogeneity, where different combinations of genetic determinants cause the same, indistinguishable phenotype (Risch, 2000).

In any case, the outcome of QTL mapping is a list of genomic regions significantly linked to the phenotype of interest, each containing one or several genes. It is then necessary to dissect those QTL to the gene level (QTG) and, ideally, to the nucleotide level (QTN). To this end, one must identify the genes present nearby the QTL which are potential candidates. First, the DNA sequence of each target gene is compared between the parental strains looking for polymorphisms, especially those resulting in changes in the promoter or the amino acid sequence of the protein. Publicly available Gene Ontology (GO) information can help narrow the number of candidate genes.

Finally, the impact of each target allele is tested through genetic engineering. A widely used strategy is reciprocal hemizygosity analysis (RHA). This method, developed by Steinmetz *et al.* (2002), involves the construction of a hybrid between both parental strains and the subsequent deletion of parent A and parent B alleles independently. The resulting strains are hemizygous for that locus (i.e. they only carry one allele). Their phenotypic characterization allows determining whether the candidate allele is beneficial for that trait.

4.4.2. Strategies and improvements

After generating a population of segregants, each can be genotyped individually. This approach, called individual segregant analysis (ISA), provides the highest information on genetic diversity, and it is useful when studying several non-selectable traits simultaneously (for example, metabolite production). However, until recently, sequencing has remained too expensive to perform ISA in a large population. A solution for this problem, called bulk segregant analysis (BSA), was developed by Michelmore, Paran and Kesseli (1991) (**Fig. I12.D**). In this approach, individuals with the most extreme trait expression (selected pool) are genotyped as a whole. Their allele frequencies are compared to those of an unselected pool or a pool of segregants of opposite phenotype. Using BSA also increases the mapping resolution compared to ISA (Swinnen, Thevelein and Nevoigt, 2012).

The number of segregants analysed is a crucial factor. A higher number of individuals increases the statistical power of the linkage analysis and shortens the QTL sizes (Bloom *et al.*, 2013). This was exemplified by the work of Nguyen Ba *et al.* (2022), who genotyped and phenotyped a population of 100,000 barcoded[1] (**Fig. I12.C**) F1 segregants and identified more than 100 QTLs for almost every trait under study. Conversely, when downscaling the sample size to 1000 segregants, they did not find more than 30 QTLs for any trait.

[1] Barcodes are short and unique DNA sequences that allow the identification of a strain or a species within a population.

Another way to improve the resolution of the analysis is by increasing the number of meiotic generations (**Fig. I12.B**). A population of segregants may be intercrossed and sporulated several times (generally up to the F12). The resulting population is an advanced intercrossed line (AIL) (Darvasi and Soller, 1995). Each generation further breaks up linkage groups, reducing the size of the potential QTLs and therefore increasing the mapping resolution (Parts *et al.*, 2011; Salinas *et al.*, 2012; Cubillos *et al.*, 2013).

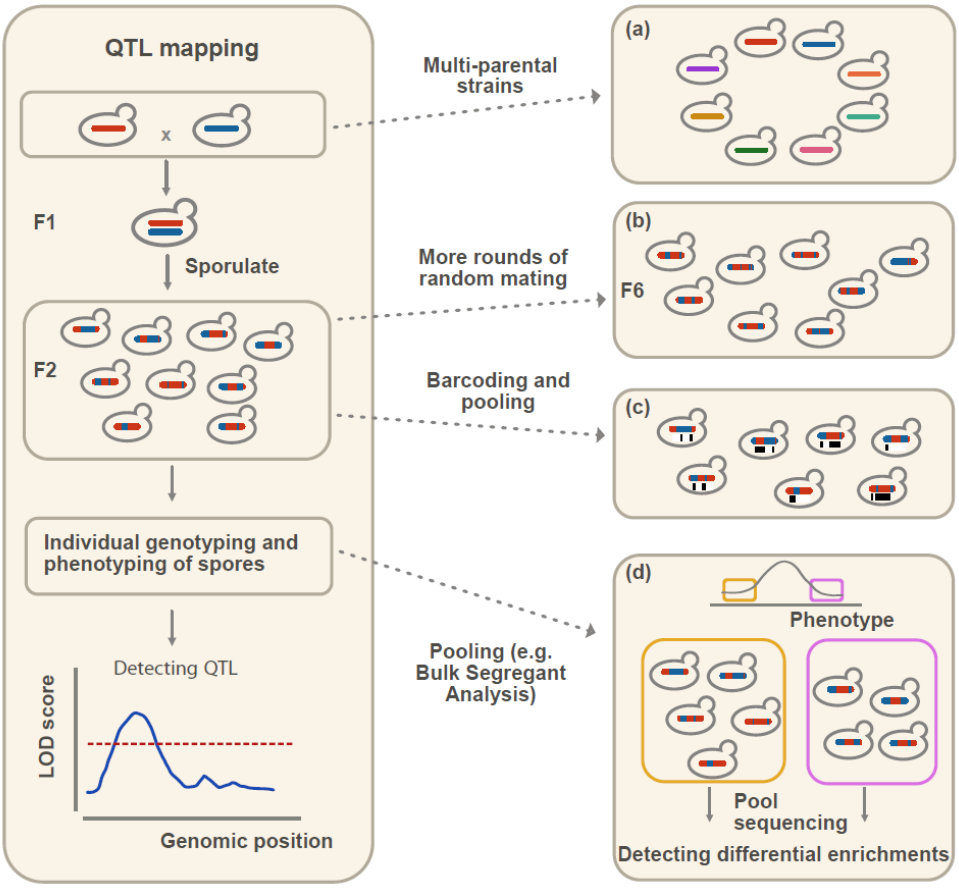


***Figure I12**. Traditional QTL mapping procedures (left) and recent advances (right), from Yeh, Jiang and Dunham (2022).*

It is possible to increase the phenotypic variation in the segregant population by incorporating more than two parent strains (**Fig. I12.A**). Those strains can be used to generate hybrids in pairwise combinations (Ehrenreich *et al.*, 2012; Treusch *et al.*, 2015) or, using more complex procedures, generate segregants with DNA from all parent strains simultaneously (Cubillos *et al.*, 2013; Linder *et al.*, 2020; Naseeb *et al.*, 2021). This type of construction is called a multi-parent population (MPP). In addition to the increased phenotypic variation, MPP approaches introduce the possibility of more than two alleles at a given locus and increases the potential epistatic interactions (Cubillos *et al.*, 2013).

Another recent advance is the use of interspecific hybrids for QTL mapping. As QTL mapping relies on meiotic recombination, it requires a hybrid capable of producing viable

spores. Thus, using interspecific hybrids for QTL analyses has remained unfeasible due to their sterility. Naseeb *et al.* (2021) successfully restored the fertility of interspecific hybrids between *S. cerevisiae* and four other *Saccharomyces* species using tetraploid intermediates. They investigated the genetic basis of complex traits in such organisms for the first time, identifying QTLs shared between species, species-specific, and even hybrid-specific. By controlling mitochondrial inheritance, they also identified mitotype-dependent and independent phenotypes.

4.4.3. Dissecting industrially relevant phenotypes

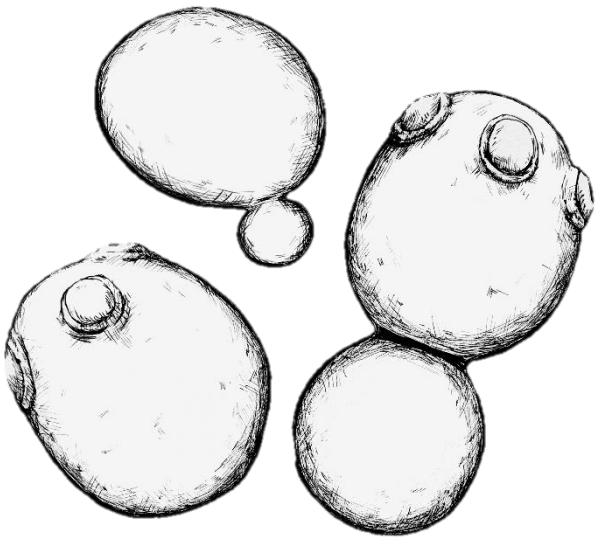
Over the last two decades, many studies have shed light on the genetic basis of complex traits in yeast. Several authors have studied the genetic determinants leading to differences in sporulation (Deutschbauer and Davis, 2005; Ben-Ari *et al.*, 2006), cell morphology (Nogami, Ohya and Yvert, 2007), flocculation (Brauer *et al.*, 2006; Wilkening *et al.*, 2014), thermotolerance (Steinmetz *et al.*, 2002; Sinha *et al.*, 2006; Cubillos *et al.*, 2011, 2013; Wang *et al.*, 2019; Naseeb et al., 2021), ethanol tolerance (Hu *et al.*, 2007; Haas *et al.*, 2019; Naaseb *et al.*, 2021), ethanol production (Hubmann *et al.*, 2013; Pais *et al.*, 2013), tolerance to various stresses (Greetham *et al.*, 2014) and resistance to different chemicals (Kim and Fay, 2007; Ehrenreich *et al.*, 2010, 2012; Cubillos *et al.*, 2013; Naseeb *et al.*, 2021).

Besides providing useful information about the genetic basis of those traits, the abovementioned studies paved the way for applying QTL mapping to other phenotypes of interest. In this way, QTL analysis has been successfully applied to investigate the genetic determinants of relevant traits in winemaking and brewing conditions, such as nitrogen assimilation (Gutiérrez *et al.*, 2013; Brice *et al.*, 2014; Jara *et al.*, 2014; Cubillos *et al.*, 2017; Kessi-Pérez, Molinet and Martínez, 2020), fermentation kinetics (Brion *et al.*, 2013; Kessi-Pérez *et al.*, 2019; Marullo *et al.*, 2019), low temperature fermentation (García-Ríos *et al.*, 2017), and the production of sulfur compounds (Noble, Sanchez and Blondin, 2015), central carbon metabolites (Marullo, Aigle, *et al.*, 2007; Salinas *et al.*, 2012; Eder *et al.*, 2018, 2020; Ho *et al.*, 2021) and fermentative aromas (Ambroset *et al.*, 2011; Steyer *et al.*, 2012; Den Abt *et al.*, 2016; De Carvalho *et al.*, 2017; Eder *et al.*, 2018; 2022; Ho *et al.*, 2021; Souffriau *et al.*, 2022).

The knowledge generated in those studies is directly applicable to the improvement of yeast strains. Generally, a QTL mapping study detects and validates one or more allelic variants improving a given phenotype. It is then possible to introduce this variant into another strain via breeding programmes without genetic engineering, allowing their use in the beverages sector. A technique widely used in plant breeding is marker-assisted selection (MAS), through which desired loci can be introgressed into a recipient strain (called ‘elite’

strain) by repeated backcrossing. The presence of the desired loci is tracked thanks to its genetic linkage with a known genetic marker. Using MAS, Marullo, Yvert, *et al.* (2007) mapped and introgressed three allelic variants into a strain derived from a commercial wine yeast, improving its H_2S production, lag phase, and POF (phenolic off-flavour) character. Similarly, Vion, Peltier, *et al.* (2021) used MAS to introgress 11 allelic variants known to enhance malic acid consumption, successfully improving this phenotype.

MATERIALS AND METHODS



1. Media and culture conditions

1.1. YPD (Yeast Extract Peptone Dextrose)

***Table M1**. Composition of YPD medium.*

Component	Amount for 1 L
Yeast Extract	10 g
Bacto Peptone	20 g
Glucose	20 g
Agar (for solid YPD)	20 g*

* For solid YPD (YPDA).

YPD medium was used for the daily maintenance of yeast strains. Solid and liquid cultures were incubated at 28 ºC, and liquid cultures were agitated at 250 rpm.

1.2. GNA (pre-sporulation medium)

Liquid GNA was used as a nutrient-rich medium for pre-sporulation of yeast strains. A single colony was inoculated into 5 mL of GNA and incubated at 28 ºC and 250 rpm overnight.

***Table M2**. Composition of GNA medium.*

Component	Amount for 1 L
Yeast Extract	10 g
Bacto Peptone	5 g
Glucose	100 g

1.3. spoMA (sporulation medium)

Using an overnight culture in pre-sporulation medium, liquid spoMA was used after two washing steps, at 22 ºC and 100 rpm, to induce sporulation.

***Table M3**. Composition of spoMA medium.*

Component	Amount for 1 L
Yeast Extract	1 g
Glucose	0.5 g
Potassium acetate (KAC)	10 g
Adenine	20 mg*

*From a stock solution 1000X in HCl 0.5 M

1.4. Micromanipulation medium

Micromanipulation medium was used for the dissection of tetrads generated through sporulation. Noble Agar was used instead of the classic agar to make the surface of the medium as flat as possible and facilitate micromanipulation.

***Table M4**. Composition of micromanipulation medium.*

Component	Amount for 1 L
Yeast Extract	2 g
Glucose	2 g
Noble Agar	20 g

1.5. YEPEG (Yeast Extract Peptone Ethanol Glycerol)

Petite strains lack functional mitochondria, and therefore they cannot grow if the only carbon sources available are non-fermentable (such as glycerol or ethanol). The absence of growth in YEPEG medium was used to confirm the *petite* character of mutants induced by contact with EtBr.

***Table M5**. Composition of YEPEG medium.*

Component	Amount for 1 L
Yeast Extract	10 g
Bacto Peptone	20 g
Glycerol	30 g
Ethanol	30 mL
Agar	20 g

1.6. Antibiotic selection medium

YPD was also used as the basis for media supplemented with antibiotics, when necessary (e.g. after DNA transformation). After autoclaving, media was cooled at approximately 50 ºC before the addition of either 300 µg/mL of Hygromycin B (HYG) or 200 µg/mL of Geneticin (G418).

1.7. Synthetic grape must (SM)

All fermentations in this study were carried out using a synthetic medium which mimics the composition of a standard grape juice (Bely, Sablayrolles and Barre, 1990). This medium is characterized by a high sugar amount (equimolar amounts of glucose and fructose) at a total concentration of 200 g/L, unless specified. Yeast assimilable nitrogen (YAN) was added in the forms of ammonium (NH_4Cl) and free amino acids, for a total YAN concentration of 200 mg/L. Lipids were added in the form of phytosterol (mainly β-sitosterol) for a final concentration of 2 mg/L, to satisfy yeast requirements during anaerobic growth. The medium was referred to as SM-200, SM-220 or SM-240, depending on the total sugar concentration. The final composition of SM-200 is detailed in **Table M6**. The composition of the stock solutions is detailed in **Tables M7** to **M11**. The stock solutions of trace elements, iron chloride and phytosterols were conserved at 4 ºC, and the

amino acid and vitamin solutions were conserved at -20 ºC. After adding all the components, pH was adjusted to 3.30 using NaOH 10 M. Once ready, synthetic must was conserved at -20 ºC until use.

***Table M6**. Composition of the **SM-200** medium.*

Component	Amount for 1 L
Glucose ($C_6H_{12}O_6$)	100 g
Fructose ($C_6H_{12}O_6$)	100 g
Malic acid ($C_4H_6O_5$)	6 g
Citric acid ($C_6H_8O_7$)	6 g
Potassium phosphate (KH_2PO_4)	0.75 g
Potassium sulphate (K_2SO_4)	0.5 g
Magnesium sulphate ($MgSO_4$) · 7 H_2O	0.25 g
Calcium chloride ($CaCl_2$) · 2 H_2O	0.155 g
Sodium chloride (NaCl)	0.2 g
Ammonium chloride (NH_4Cl)	0.46 g
Trace element solution (**Table M7**)	1 mL
Vitamin solution (**Table M8**)	10 mL
Amino acid solution (**Table M11**)	6.16 mL
Iron chloride solution (**Table M9**)	1 mL
Sodium hydroxide (NaOH) 10M	until pH 3.30

***Table M7**. Composition of the **trace element** stock solution.*

Component	Amount for 1 L
Manganese sulphate · H_2O	4 g
Zinc sulphate · 7 H_2O	4 g
Copper sulphate · 5 H_2O	1 g
Potassium iodide	1 g
Cobalt chloride · 6 H_2O	0.4 g
Boric acid	1 g
Ammonium heptamolybdate	1 g

***Table M8**. Composition of the **vitamin** stock solution.*

Component	Amount for 1 L
Myo-inositol	2 g
Calcium pantothenate	0.15 g
Thiamine hydrochloride	0.025 g
Nicotinic Acid	0.2 g
Pyridoxine	0.025 g
Biotin	0.3 mg

***Table M9**. Composition of the **iron chloride** stock solution.*

Component	Amount for 1 L
Iron chloride (III) · 6 H_2O	20 g

***Table M10**. Composition of the **phytosterol** stock solution.*

Component	Amount for 100 mL
Phytosterols	2 g
Tween 80	50 mL
Ethanol 100 %	qsp 100 mL

***Table M11**. Composition of the **amino acid** stock solution.*

Component	Amount for 1 L
Alanine	11.1 g
Arginine	28.6 g
Aspartic acid	3.4 g
Cysteine	1 g
Glutamic acid	9.2 g
Glutamine	38.6 g
Glycine	1.4 g
Histidine	2.5 g
Isoleucine	2.5 g
Leucine	3.7 g
Lysine	1.3 g
Methionine	2.4 g
Phenylalanine	2.9 g
Proline	46.8 g
Serine	6 g
Threonine	5.8 g
Tryptophan	13.7 g
Tyrosine	1.4 g
Valine	3.4 g

2. Yeast strains

All the yeast strains used in this study belong to the culture collections of the University of Leicester and INRAE Montpellier (**Table M12**). All the species belonging to the *Saccharomyces* genus were represented, accordingly to the availability of isolates in those collections. Two types of interspecific hybrids, i.e. *S. cerevisiae x S. kudriavzevii* and *S. cerevisiae x S. uvarum*, were also included. All yeasts were maintained at −80°C in 20 % glycerol before use.

***Table M12**. Yeast strains used in this study.*

Code used	Species	Strain	Country	Ecological niche
SA01	*S. arboricola*	H-6 (T)	China	Tree
SA02	*S. arboricola*	ZX-15	China	Tree
SA03	*S. arboricola*	ZX-20	China	Tree
SK01	*S. kudriavzevii*	IFO 10990	Japan	Tree
SK02	*S. kudriavzevii*	IFO 10991	Japan	Tree
SK03	*S. kudriavzevii*	DBVPG6667	Unknown	Unknown
SK04	*S. kudriavzevii*	IFO 1802^T	Japan	Unknown
SK05	*S. kudriavzevii*	IFO 1803	Japan	Tree
SK06	*S. kudriavzevii*	ZP 542	Portugal	Tree
SK07	*S. kudriavzevii*	ZP 594	Portugal	Tree
SK08	*S. kudriavzevii*	ZP 629	Portugal	Tree
SK09	*S. kudriavzevii*	PB7	Spain	Wine
SCSK01	*Sc x Sk*	CR85	Spain	Tree
SK10	*S. kudriavzevii*	CA111	Spain	Tree
SK11	*S. kudriavzevii*	48BYC-4	China	Tree
SK12	*S. kudriavzevii*	JLFM8	China	Tree
SU01	*S. uvarum*	DBVPG6299	Spain	Insect
SU02	*S. uvarum*	L-1764	Chile	Unknown
SU03	*S. uvarum*	ZP 555	Canada	Tree
SU04	*S. uvarum*	ZP 556	Canada	Tree
SU05	*S. uvarum*	A4	New Zealand	Wine
SU06	*S. uvarum*	VKMY508	Czech Republic	Wine
SU07	*S. uvarum*	UWOPS99-807.1.1	Argentina	Tree
SU08	*S. uvarum*	A1var	New Zealand	Wine
SU09	*S. uvarum*	A4var	New Zealand	Wine
SU10	*S. uvarum*	A9var	New Zealand	Wine
SE01	*S. eubayanus*	CBS 12357 (T)	Argentina	Tree
SE02	*S. eubayanus*	LZSP32.1	China	Unknown
SE03	*S. eubayanus*	CDFM212.1	China	Unknown
SP01	*S. paradoxus*	UFRJ50791	Brazil	Insect
SP02	*S. paradoxus*	DBVPG6466	Denmark	Soil
SP03	*S. paradoxus*	YPS138	United States	Soil
SP04	*S. paradoxus*	UWOPS91-917.1	United States	Tree
SP05	*S. paradoxus*	N-43	Russia	Tree
SP06	*S. paradoxus*	Q74.4	United Kingdom	Unknown
SM01	*S. mikatae*	NBRC 10994	Japan	Tree
SM02	*S. mikatae*	NBRC 10998	Japan	Tree
SM03	*S. mikatae*	LSYS65-1	China	Tree
SM04	*S. mikatae*	CHSZ5L-2	China	Fruit
SM05	*S. mikatae*	IFO1815 (T)	Japan	Soil
SM06	*S. mikatae*	IFO1816	Japan	Decayed leaf
SC01	*S. cerevisiae*	RIB6003	Japan	Sake
SC02	*S. cerevisiae*	RIB6004	Japan	Sake
SC03	*S. cerevisiae*	DBVPG6765	Indonesia	Fruit
SC04	*S. cerevisiae*	DBVPG6044	Unknown	Wine
SC05	*S. cerevisiae*	YPS128	United States	Soil
SC06	*S. cerevisiae*	Y12663	Unknown	Wine
SC07	*S. cerevisiae*	UWOPS03-461.4	Malaysia	Tree
SC08	*S. cerevisiae*	R13_A5	France	Fruit
SC09	*S. cerevisiae*	UCD2120	United States	Wine
SC10	*S. cerevisiae*	DBVPG6040	Netherlands	Fruit
SC11	*S. cerevisiae*	DBVPG6254	Unknown	Wine
SC12	*S. cerevisiae*	DBVPG3051	Israel	Wine
SC13	*S. cerevisiae*	DBVPG1849	Ethiopia	Wine

***Table M12**. (Cont.)*

Code used	*Species*	Strain	Country	Ecological niche
SC14	*S. cerevisiae*	DBVPG6696	Burundi	Wine
SC15	*S. cerevisiae*	DBVPG6295	South Africa	Wine
SC16	*S. cerevisiae*	DBVPG1378	Italy	Wine
SCSK02	*Sc x Sk*	SOY3	Croatia	Wine
SK13	*S. kudriavzevii*	IFO 1802 (G)	None	None
SCSK03	*Sc x Sk*	Eg8	France	Wine
SCSK04	*Sc x Sk*	ALS 268	None	None
SCSK05	*Sc x Sk*	VIN 7	South Africa	Wine
SK14	*S. kudriavzevii*	CLIB 1504	Unknown	Unknown
SK15	*S. kudriavzevii*	CLIB 1503	Unknown	Unknown
SK16	*S. kudriavzevii*	ARD6.1	France	Tree
SCSK06	*Sc x Sk*	H10418	Hungary	Wine
SCSK07	*Sc x Sk*	Evo AD	None	None
SCSK08	*Sc x Sk*	H10423	Hungary	Wine
SCSK09	*Sc x Sk*	Eg6	France	Wine
SC17	*S. cerevisiae*	DBVPG1106	Australia	Fruit
SC18	*S. cerevisiae*	EC1118	Unknown	Wine
SU11	*S. uvarum*	BMV58	Spain	Unknown
SC19	*S. cerevisiae*	L-1374	Chile	Wine
SC20	*S. cerevisiae*	WE372	Unknown	Wine
SU12	*S. uvarum*	CLIB 251	Netherlands	Fruit
SCSK10	*Sc x Sk*	Eg8/93	France	Wine
SCSK11	*Sc x Sk*	Eg8/136	France	Wine
SCSK12	*Sc x Sk*	AM511	None	None
SCSK13	*Sc x Sk*	AM512	None	None
SCSK14	*Sc x Sk*	AM513	None	None
SCSK15	*Sc x Sk*	AM514	None	None
SCSK16	*Sc x Sk*	AM515	None	None
SCSK17	*Sc x Sk*	AM516	None	None
SCSK18	*Sc x Sk*	AM517	None	None
SCSK19	*Sc x Sk*	AM518	None	None
SCSK20	*Sc x Sk*	AM519	None	None
SCSK21	*Sc x Sk*	AM520	None	None
SCSU01	*Sc x Su*	H51	None	None
SCSU02	*Sc x Su*	H105	None	None
SCSU03	*Sc x Su*	H159	None	None
SJ01	*S. jurei*	D5088 (T)	France	Tree
SJ02	*S. jurei*	D5095	France	Tree

3. Phenotyping

Phenotyping experiments were performed in two sets, namely the screening (chapter 1) and the characterization of segregants (chapter 2), using the same methodology in both of them. Fermentations and chemical analyses were performed as explained hereby.

3.1. Fermentation in synthetic grape must

All fermentations were carried out in isothermal regime at 16 or 22 ºC, under an agitation of 250 rpm, and using synthetic grape must. Cylindrical 300 mL glassware fermenters were filled with 250 mL of medium. Fermenters were equipped with water-filled airlocks to allow CO_2 release while avoiding the entry of air from the environment, as well as a rubber septum that allows syringe-assisted sampling during fermentation. Pasteurization (15 min at 100 ºC) was employed instead of autoclaving to reduce the microbial charge in the fermenters while avoiding the degradation of thermolabile compounds (i.e. vitamins). Fermenters were injected with a filtrated air flux during 30 min and an agitation of 250 rpm, to equalize the amount of oxygen in all fermenters. The phytosterol solution was added after aeration to avoid excessive foaming.

Strains were recovered from cryopreserved cultures by streaking them onto YPD agar. Precultures of each strain were done in 30 mL of liquid YPD in a 50 mL Falcon tube, incubated at 28 ºC and 250 rpm overnight. The cell suspension was then centrifuged at 4500 rpm for 5 min, and cells were re-suspended in one volume of NaCl 0.9 %. After washing the cells twice with this solution to remove nutrient traces, the cell density was counted using a Multisizer™ 3 Coulter Counter (Beckman Coulter). After equilibrating the fermenters to the desired temperature, they were inoculated to an initial cell density of 10^6 cells/mL.

From this moment, fermentation kinetics was monitored by periodically measuring the weight loss of the fermenters, which was used to calculate the CO_2 production rate. To facilitate the fermentation monitoring, part of the experiments were performed using a custom-built robot (PlateButler®, Lab Services), which allowed automatic weight measurement for up to 90 fermenters as frequently as once per hour. Otherwise, weights were acquired manually. Fermentations were stopped when the CO_2 production rate dropped under 0.02 g/L/h.

3.2. Determination of kinetic parameters

Fermentation kinetics was determined from the weight loss of the fermenters. We assumed that all the weight loss was caused by CO_2 release, even though a minimal ethanol evaporation takes place as well. The weight loss between two points allow the calculation of the CO_2 released in that time frame (Eq. 1).

$$CO_{2\,(t)} = \frac{\Delta W}{V} = \frac{W_{(t-1)} - W_{(t)}}{V}\ [g/L] \qquad \textit{(Eq. 1)}$$

, where W is the weight of the fermenter and V is the volume. From this, the CO_2 production rate can be calculated using Eq. 2.

$$CO_{2\,rate} = \frac{dCO_2}{dt}\ [g/L/h] \qquad \textit{(Eq. 2)}$$

Fermentation curves were smoothed using the alfisStatUtilR (v1.0.0) R package based on a locally developed regression model (Duc *et al.*, 2020). This package allowed the calculation of several kinetic parameters for each curve, such as the maximum CO_2 production rate (R_{max}), the time to reach a given amount of CO2 released, or the lag time.

A novel parameter was calculated in this study to facilitate the comparison of kinetic capacities between different strains or conditions. This parameter was named *overall kinetic score*. For its calculation, the most relevant kinetic parameters were normalised using the whole dataset. It was calculated separately for chapters 1 and 2. Then, Eq. 3 or an equivalent equation[2] was applied.

$$Overall\ kinetic\ score\ = \left(R_{max,\,n} + R_{60,\,n} + t_{60,n} + t_{lag,n} + t_{ferm,n}\right) / 5 \qquad \textit{(Eq. 3)}$$

n indicates that a variable has been normalised. Normalisation was performed in a way that the highest value (i.e. 1) corresponds to the best behaviour, and the lowest value (i.e. 0) to the worst behaviour. For example, a value of 1 was assigned to the highest R_{max} and also to the lowest t_{lag} in a given dataset.

3.3. Biomass determination

The biomass production (dry matter) was determined for selected strains to complete a carbon balance. For that purpose, 10 mL samples were filtrated using previously dried 0.45 µm pore-size nitrocellulose filters (MF-Millipore, Merck), which were then washed three times with 50 mL of distilled water and dried for 24 h at 100 ºC. Dry matter was calculated as the average of three independent measurements.

3.4. Quantification of sugars and central carbon metabolites

3.4.1. HPLC

The amounts of sugars (glucose and fructose) and the main primary metabolites (ethanol, acetate, succinate, glycerol, α-ketoglutarate and pyruvate) were determined by high-performance liquid chromatography (HPLC), as described by Rollero *et al.* (2015).

[2] R_{60} and t_{60} were used for the screening (chapter 1), and R_{80} and t_{80} for the phenotyping of segregants (chapter 2), as sampling was performed at 60 and 80 g/L of CO_2 released, respectively.

200 uL of homogenised sample were diluted 1/6 into mobile phase (deaerated H_2SO_4 0.005 N). An HPLC 1290 Infinity (Agilent Technologies, Santa Clara, California, USA) device was used with a Rezex ROA column (Phenomenex, Le Pecq, France) at 60 ºC. The column was eluted with 0.005 N H_2SO_4 at a flow rate of 0.6 mL/min. Organic acids were quantified using a UV detector at 210 nm. For the rest of the compounds, a refractive index (RI) detector was used. All the data were treated using the ChemStation software (Agilent Technologies, Santa Clara, California, USA).

3.4.2. GC-FID

Acetoin and 2,3-butanediol were quantified for selected strains to complete a carbon balance. A simple extraction into chloroform was followed by gas chromatography coupled to a flame ionization detector (GC-FID), following the procedure used by Tilloy, Ortiz-Julien and Dequin (2014). Calibration points were made from an 8 g/L mother solution of both acetoin and butanediol. The internal standard solution was prepared by diluting 1 mL of hexanol into 1 L of EtOH 10% (v/v). All samples and calibration points were diluted 1/5 with distilled water before treatment, to compensate the matrix effect. 1 mL of diluted sample, 1 mL of the ISTD solution and 2.5 g of K_2CO_3 were added into a Pyrex-16 tube. After vortexing, 2 mL of chloroform were added to each sample. The mix was decanted for 1 h. Then, 1 mL of the organic (upper) phase was transferred to a 2 mL Eppendorf tube containing the drying agent (anhydrous sodium sulphate) to remove possible water traces. After gently vortexing for some seconds, 500 uL of the dehydrated organic phase were transferred to the final HPLC vial for injection into a 30-m Megabore column (DB-WAX, J&W Scientific) on a GC equipment (HP 6890).

3.5. Quantification of volatile aromas

The main fermentative aromas produced by yeasts during fermentation were quantified using gas chromatography-mass spectrometry (GC-MS), following the protocol described by Rollero et al. (2015). A standard solution was prepared for each group of compounds (ethyl esters, acetate esters, alcohols and acids), and calibration solutions were made by adding the appropriate amounts of the latter into synthetic wine. Synthetic wine was made by dissolving 6 g/L of L-malic acid into 1 L of 12 % ethanol solution and adjusting the pH to 3.4 with 1M NaOH. Then, a double liquid-liquid extraction with dichloromethane in presence of deuterated standards was followed by injection in a Hewlett Packard (Agilent™ Technologies, Santa Clara, CA, USA) 6890 gas chromatograph equipped with a CTC Combi PAL Autosampler AOC-5000 (Shimadzu™, Columbia, USA) and coupled to a Hewlett Packard 5973 Mass Spectrometry detector (Agilent™ Technologies, Santa Clara, CA, USA). For quantification, mass spectra were recorded in Selected Ion Monitoring (SIM) mode. The ions monitored in SIM runs are shown in **Table M13**. Data acquisition and

processing were done using the ChemStation software Agilent™ Technologies, Santa Clara, CA, USA).

***Table M13**. Parameters for the detection of fermentative aromas by GC-MS.*

Compound	RT (min)	Quantification ion (m/z)	Confirmation ion(s) (m/z)	Internal standard
Ethyl propanoate	3.69	57	102	n-butylacetate-d12
Ethy isobutanoate	3.79	71	88, 116	n-butylacetate-d12
Propyl acetate	3.95	61	73	n-butylacetate-d12
Isobutyl (2-methylpropyl) acetate	4.72	56	73	n-butylacetate-d12
Ethyl butanoate	5.18	71	88, 101	n-butylacetate-d12
1-propanol	5.23	59	42	n-butylacetate-d12
Ethyl-2-methylbutanoate	5.54	102	74	n-butylacetate-d12
n-butylacetate-d12	5.84	46	66, 78	-
Ethyl isovalerate (3-methylbutanoate)	5.98	88	60, 70	n-butylacetate-d12
2-methylpropanol	6.55	43	74	n-butylacetate-d12
2-methylbutyl acetate	7.42	57	72	n-butylacetate-d12
Isoamyl (3-methylbutyl) acetate	7.46	87	55, 61	n-butylacetate-d12
Ethyl pentanoate	7.83	88	85, 101	n-butylacetate-d12
3-methylbutanol	10.15	70	55	Ethyl hexanoate-d5
2-methylbutanol	10.15	70	56, 57	Ethyl hexanoate-d5
Ethylhexanoate-d5	10.83	93	106, 120	-
Ethyl hexanoate	10.94	88	99, 115	Ethyl hexanoate-d5
Hexyl acetate	12.21	56	69, 84	Ethyl hexanoate-d5
Ethyl lactate	14.53	45	75	Ethyl hexanoate-d5
1-hexanol	14.84	56	69, 84	Ethyl hexanoate-d5
Ethyloctanoate-d5	17.35	93	106, 127	-
Ethyl octanoate	17.46	88	101, 127	Ethyl octanoate-d5
Propanoic acid	21.08	74	45, 57	Ethyl octanoate-d5
Ethyl-3-methylthiopropionate	21.46	148	74, 103	Ethyl octanoate-d5
2-methylpropanoic acid	21.92	43	73, 88	Ethyl octanoate-d5
Ethyldecanoate-d5	23.5	106	162	-
Ethyl decanoate	23.61	88	101, 155	Ethyl decanoate-d5
Butanoic acid	23.68	60	73	Ethyl decanoate-d5
Diethyl succinate	24.74	101	129	Ethyl decanoate-d5
3-methylbutanoic acid	24.8	60	61, 87	Ethyl decanoate-d5
2-methylbutanoic acid	24.8	74	57, 87	Ethyl decanoate-d5
3-methylthiopropanol	25.76	106	61, 73	Ethyl decanoate-d5
Pentanoic acid	26.73	60	73	Ethyl decanoate-d5
2-Phenylethyl acetate	28.41	104	91	Ethyl decanoate-d5
Ethyl dodecanoate	29.25	88	101	Ethyl decanoate-d5
Hexanoic acid	29.52	60	73, 87	Ethyl decanoate-d5
Phenylethanol-d4	30.76	93	126	-
2-phenylethanol	30.9	91	92, 122	Phenylethanol-d4
Octanoic acid	34.46	60	73, 101	Decanoic acid-d5
Decanoic acid-d5	36.87	134	148, 177	-
Decanoic acid	36.9	73	60, 129	Decanoic acid-d5
Dodecanoic acid	38.9	73	60, 129	Decanoic acid-d5

4. Molecular biology and hybridisation

4.1. Primers

4.1.1. Primers from other studies

PCR was used to determine the mating type(s) and to verify the species of several yeast strains throughout this work. The primers employed for such purposes are shown in **Tables M14** and **M15**.

***Table M14**. Primers for determination of the mating type. FW: forward. RV: reverse.*

Primer name	Sequence (5' → 3')	Usage	Reference
MAT_FW	AGTCACATCAAGATCGTTTATGG	MAT locus amplification, FW	Internal (INRAe)
MATa_RV	ACTCCACTTCAAGTAAGAGTTTG	MAT locus amplification, RV	Internal (INRAe)
MATalpha_RV	GCACGGAATATGGGACTACTTCG	MAT locus amplification, RV	Internal (INRAe)

***Table M15**. Species-specific primers from other studies. FW: forward. RV: reverse.*

Primer name	Sequence (5' → 3')	Usage	Reference
Sarb_2011_FW	GGCACGCCCTTACAGCAGCAA	Specific for *S. arboricola*	Muir *et al.* (2011)
Sarb_2011_RV	TCGTCGTACAGATGCTGGTAGGGC	Specific for *S. arboricola*	Muir *et al.*, (2011)
Suva_2011_FW	GCTGACTGCTGCTGCTGCCCCCG	Specific for *S. uvarum*	Muir *et al.*, (2011)
Suva_2011_RV	TGTTATGAGTACTTGGTTTGTCG	Specific for *S. uvarum*	Muir *et al.*, (2011)
Scer_2011_FW	GCGCTTTACATTCAGATCCCGAG	Specific for *S. cerevisiae*	Muir *et al.*, (2011)
Scer_2011_RV	TAAGTTGGTTGTCAGCAAGATTG	Specific for *S. cerevisiae*	Muir *et al.*, (2011)
Skud_2011_FR	ATCTATAACAAACCGCCAAGGGAG	Specific for *S. kudriavzevii*	Muir *et al.*, (2011)
Skud_2011_RV	CGTAACCTACCTATATGAGGGCCT	Specific for *S. kudriavzevii*	Muir *et al.*, (2011)
Smik_2011_FW	ACAAGCAATTGATTTGAGGAAAAG	Specific for *S. mikatae*	Muir *et al.*, (2011)
Smik_2011_RV	CCAGTCTTCTTTGTCAACGTTG	Specific for *S. mikatae*	Muir *et al.*, (2011)
Spar_2011_FW	CTTTCTACCCCTTCTCCATGTTGG	Specific for *S. paradoxus*	Muir *et al.*, (2011)
Spar_2011_RV	CAATTTCAGGGCGTTGTCCAACAG	Specific for *S. paradoxus*	Muir *et al.*, (2011)
Seub_2013_FW	GTCCCTGTACCAATTTAATATTGCGC	Specific for *S. eubayanus*	Pengelly & Wheals (2013)
Seub_2013_RV	TTTCACATCTCTTAGTCTTTTCCAGACG	Specific for *S. eubayanus*	Pengelly & Wheals (2013)
Sjur_2021_FW	CTCAAATGGGAATGCCACCG	Specific for *S. jurei*	Naseeb *et al.* (2021)
Sjur_2021_RV	TCCTGATAGTGGTTGTTGCT	Specific for *S. jurei*	Naaseb *et al.* (2021)

4.1.2. Primers designed in this study

All primers used in this study for the deletion of the *HO* and *MAT* genes, as well as the verification of the *HO* deletion, were designed *de novo*. To that end, chromosome or gene sequences were downloaded from the SGD (*Saccharomyces* Genome Database) website. Benchling (https://www.benchling.com) was used for *HO* sequence multiple alignment, plasmid visualization, primer design and generation of virtual PCR products. Nucleotide BLAST (NCBI website) was used to check for possible homologies outside the target sequence, and primer dimers and self-complementarity for each oligo were tested using Multiple Primer Analyser (ThermoFisher). The lists of primers are shown in **Tables M16** and **M17**.

***Table M16**. Oligonucleotides designed for HO deletion and verification. FW: forward. RV: reverse. Capital letters indicate homology to the target gene, while small letters indicate homology to the plasmid.*

Primer name	Sequence (5' → 3')	Species	Usage	Length (bp)	% GC	Tm
HOdel_all_FW	CAACAATGTCAGACACTGGACGGAAGAATAATAACAATTCCCAAAAAttcgtacgctgcaggtcgac	all *Saccharomyces*	*ho::HYG* forward	67	43.28%	71.37°C
HOdel_all_RV	CAATATGACAGAACATTCTGTAATGTCGTTCCTCCAGCAACATTACAgcataggccactagtggatctg	all *Saccharomyces*	*ho::HYG* reverse	69	43.48%	70.90°C
HOdel_cer_par_FW	AATGCCACCAAATATAAAGTGAGATGGAGGAATCTGCAGCAATGTCAGACttcgtacgctgcaggtcgac	*S. cerevisiae* and *S. paradoxus*	*ho::HYG* forward	70	47.14%	73.63°C
HOdel_cer_par_RV	TCACTTCACGTGCTTCTGGTACATACTTGCAATTTATACAGTGATGTCCgcataggccactagtggatctg	*S. cerevisiae* and *S. paradoxus*	*ho::HYG* reverse	71	45.07%	72.30°C
HOdel_mik_jur_FW	GAATATGCGGCGAAGCGCTTTATAGAAGAAATGGAGCGCTCAAAAGGAGAttcgtacgctgcaggtcgac	*S. mikatae* and *S. jurei*	*ho::HYG* forward	70	50.00%	74.70°C
HOdel_mik_jur_RV	CCACGGACAGCATCAAACTGTAGAATTCCACCACATTTCAAACATTCTGgcataggccactagtggatctg	*S. mikatae* and *S. jurei*	*ho::HYG* reverse	71	46.48%	72.97°C
HOdel_eub_uva_FW	AACTTACAACAATGTCAGACACTTGACGGAAGAATAATAACAATTCCAAttcgtacgctgcaggtcgac	*S. eubayanus* and *S. uvarum*	*ho::HYG* forward	69	40.58%	70.70°C
HOdel_eub_uva_RV	TCACGTGCTTCTGGTACATATTTGCAGTTTATACAGTGATGGCCACTAgcataggccactagtggatctg	*S. eubayanus* and *S. uvarum*	*ho::HYG* reverse	70	45.71%	72.37°C
HOdel_arb_kud_FW	ACTTTGACATTGAAGTCAGAGATTTGGATTATCTTGATGCTCAGTTGAGttcgtacgctgcaggtcgac	*S. arboricola* and *S. kudriavzevii*	*ho::HYG* forward	69	43.48%	71.72°C
HOdel_arb_RV	ACAGCAGCCACCACATGACTTCACCTCTATTTTATTGCCTAATAAGAAgcataggccactagtggatctg	*S. arboricola*	*ho::HYG* reverse	70	44.29%	71.80°C
HOdel_kud_RV	TTGTGTTGTTCTCCTACACAACAGCTACCACATGATTTTACTTCTATTgcataggccactagtggatctg	*S. kudriavzevii*	*ho::HYG* reverse	70	41.43%	70.54°C
HOverif_par_FW	CAACTATTCTGATGGCCAGTGGT	*S. paradoxus*	*ho::HYG* check A (FW)	23	47.83%	56.62°C
HOverif_par_RV	CCACGAACAGCATCGAACTGTA	*S. paradoxus*	*ho::HYG* check D (RV)	22	50.00%	57.15°C
HOverif_cer_FW	CGACTATTCTGATGGCTAACGGT	*S. cerevisiae*	*ho::HYG* check A (FW)	23	47.83%	56.32°C
HOverif_cer_RV	GCGGACAGCATCAAACTGTA	*S. cerevisiae*	*ho::HYG* check D (RV)	20	50.00%	55.03°C
HOverif_mik_FW	CAACTATCCTGATGGCAAATGGT	*S. mikatae*	*ho::HYG* check A (FW)	23	43.48%	55.05°C
HOverif_jur_FW	CAACTATTCTGATGGCAAATGGTG	*S. jurei*	*ho::HYG* check A (FW)	24	41.67%	54.65°C
HOverif_mik_jur_RV	CACGGACAGCATCAAACTGTA	*S. mikatae* and *S. jurei*	*ho::HYG* check D (RV)	22	47.62%	54.92°C
HOverif_kud_FW	CCACTATTTTAATGGCCAATGGC	*S. kudriavzevii*	*ho::HYG* check A (FW)	23	43.48%	54.68°C
HOverif_kud_RV	CCACGAATAGCATCAAACTGCA	*S. kudriavzevii*	*ho::HYG* check D (RV)	22	45.45%	55.79°C
HOverif_arb_FW	CAACTATACTGATGGCCAATGGT	*S. arboricola*	*ho::HYG* check A (FW)	23	43.48%	54.55°C
HOverif_arb_RV	GCGAACAGCATCAAACTGCA	*S. arboricola*	*ho::HYG* check D (RV)	20	50.00%	56.53°C
HOverif_eub_FW	CAACTATATTGATGGCCAATGGC	*S. eubayanus*	*ho::HYG* check A (FW)	23	43.48%	54.26°C
HOverif_uva_FW	TGATGGCCAATGGTAAAATCGAAG	*S. uvarum*	*ho::HYG* check A (FW)	24	41.67%	55.85°C
HOverif_eub_uva_RV	ACGAACAGCGTCAAATTGCA	*S. eubayanus* and *S. uvarum*	*ho::HYG* check D (RV)	20	45.00%	55.82°C

***Table M17**. Oligonucleotides designed for MAT deletion. FW: forward. RV: reverse. Capital letters indicate homology to the target gene, while small letters indicate homology to the plasmid.*

Primer name	Sequence (5' → 3')	Species	Usage	Length (bp)	% GC	Tm
MATdel_ALL_FW	GATTTGAATGCGAGATAAACTGGTATTCTTCATTAGATTCTCTAGGCCCTccagctgaagcttcgtacgc	all *Saccharomyces*	*mat::KanMX FW*	70	44.29%	71.37 °C
MATdel_ALL_RV	AAGATAAACAACCTCCGCCACGACCACACTCTATAAGGCCAAATGTACAgcataggccactagtggatctg	all *Saccharomyces*	*mat::KanMX RV*	71	47.89%	73.56 °C
MATdel_par_FW	GATCCTTCACTTTCGTAGGGTCCTTTCTCACAGCAAAAGGCATCTACTTTccagctgaagcttcgtacgc	*S. paradoxus*	*mat::KanMX FW*	70	48.57%	73.64 °C
MATdel_par_RV	GCGTAATAAAGTTAAGATTCACTAAGTTCAACAGATGAGCGTTGGAAGCTGgcataggccactagtggatctg	*S. paradoxus*	*mat::KanMX RV*	73	43.84%	71.75 °C
MATdel_cer_FW	CTATGTCTGCAAACAGTTCTTGGTATTCATAATATTCAGCCAAGTGACTGTACCccagctgaagcttcgtacgc	*S. cerevisiae*	*mat::KanMX FW*	74	44.59%	72.26 °C
MATdel_cer_RV	GTCACATCAAGATCGTTTATGGTTAAGATAAGAACAAAGAATGATGCgcataggccactagtggatctg	*S. cerevisiae*	*mat::KanMX RV*	69	40.58%	69.75 °C
MATdel_mik_FW	AAGCCTTTGATATTCGTTATCGGTAGCCAAGTGGCTGTACCAAAAGGTAAGGATccagctgaagcttcgtacgc	*S. mikatae*	*mat::KanMX FW*	74	47.30%	74.02 °C
MATdel_mik_RV	CAGTCAGCAGAAAGTTCTATATATTGTGATCACTGAATTTTAATTCACTTCTGTGCgcataggccactagtggatctg	*S. mikatae*	*mat::KanMX RV*	78	39.74%	70.32 °C
MATdel_jur_FW	AAATTATTCAAACTTGTCTACTTTTCGACCATTTCATTCTCATTGGCccagctgaagcttcgtacgc	*S. jurei*	*mat::KanMX FW*	67	40.30%	70.70 °C
MATdel_jur_RV	ACCACAACATATAGGAATATCAGCTGACTGAAGATAAGATAGAACTACCgcataggccactagtggatctg	*S. jurei*	*mat::KanMX RV*	71	42.25%	70.09 °C
MATdel_kud_FW	CGTTGATTTTAATGTAAACAATACTTATAGTGAATGGACCCTCTGACTGCCGccagctgaagcttcgtacgc	*S. kudriavzevii*	*mat::KanMX FW*	72	44.44%	72.20 °C
MATdel_kud_RV	GGACTACTAACAACATATGTTCTTCGAATCAGCCGGAGAATAAAATACCGTCGgcataggccactagtggatctg	*S. kudriavzevii*	*mat::KanMX RV*	75	45.33%	72.14 °C
MATdel_arb_FW	AGTTTTGTCTTCTGGCACTTGGTCGGGTCGACCAATGTACTGGACTTTCAATTGCTTTATTCTTGTTTTATTTGTA AGTAGCTGGACAATAGCTGTAAAccagctgaagcttcgtacgc	*S. arboricola*	*mat::KanMX FW*	119	42.02%	72.09 °C
MATdel_arb_RV	AGAGGAATGGTATCGTTGTCCTCCGTCCCATATAGTCAGCATGTTCAAATTTCAAAAGTCACATCAAGATCGTTCA TTGTTAAGATAAAGACAAAGAAAGgcataggccactagtggatctg	*S. arboricola*	*mat::KanMX RV*	122	40.16%	71.45 °C
MATdel_eub_FW	AAAGAGGAGAATGTCGTTCAAGGTATGGTTGGTATCTAGCCAATAGGATTGTAGCCAGAGccagctgaagcttcgtacgc	*S. eubayanus*	*mat::KanMX FW*	80	47.50%	74.30 °C
MATdel_eub_RV	CGTCCTGCCGATCAGTCTTTCTTTGCCTTTATCTTGACCATAAATGATCTTGATGTGACgcataggccactagtggatctg	*S. eubayanus*	*mat::KanMX RV*	81	45.68%	71.22 °C
MATdel_uva_FW	ACGCTACATACAAAGAACGTGCTGCTACTCATCCTAGCCCAGTTGccagctgaagcttcgtacgc	*S. uvarum*	*mat::KanMX FW*	65	52.31%	75.27 °C
MATdel_uva_RW	CGTTACAGAAAAGCAGGCTGGGAAGCTTACTTGAAGAGATGCGGGgcataggccactagtggatctg	*S. uvarum*	*mat::KanMX RV*	67	52.24%	74.75 °C

4.2. Plasmids

***Table M18**. List of plasmids used in this study.*

Plasmid	Features	Use
pAG32	hphMX6 selectable marker conferring hygromicin resistance	HO deletion
pUG6	KanMX selectable marker, conferring geneticin (G418) resistance	MAT deletion

4.3. DNA extraction

4.3.1. Plasmid mini-preparation ("Mini-Prep")

DNA was extracted from *E. coli* strains containing one of the plasmids listed in **Table M18**. This was done by using the NucleoSpin Plasmid kit (Macherey-Nagel 740588.250) following the protocol for isolation of high-copy plasmid DNA.

4.3.2. Rapid DNA extraction

A rapid genomic DNA extraction protocol was used for verification and mating-type PCRs. This protocol was adapted from Lõoke, Kristjuhan and Kristjuhan (2017). Briefly, a single colony was suspended into 100 µL of lithium acetate solution (200 mM LiAc, 1% SDS) and incubated at 80ºC for 5-10 minutes to allow cell lysis. DNA was precipitated by adding 300 µL of 100% ethanol, centrifuged for 3 min at 13000 rpm and washed with 100 µL of 70% ethanol. The pellet was re-suspended in milli-Q water to dissolve nucleic acids. After a short centrifuge spin to precipitate cell debris, 1 – 2 µL of supernatant were used for PCR.

4.3.3. DNA extraction for genome sequencing

A phenol-chloroform protocol was used to extract genomic DNA for whole genome sequencing. A single colony was inoculated in 5 mL of YPD and grown for 36 h. Then, 2 mL were transferred to a 2 mL Eppendorf tube. Cells were washed twice with milli-Q water to remove nutrient traces and resuspended into 0.4 mL of lysis buffer (**Table M19**). This volume was transferred into a sterile screw tube to then add 0.4 g of acid-washed glass beads (425 – 600 µm, Sigma Aldrich) and 0.4 mL 25:24:1 phenol-chloroform-isoamyl alcohol solution (VWR Chemicals). This mix was vortexed for 4 minutes and then cooled down in ice. A 5-minute centrifugation at 10,000 x g allowed the separation of the organic and aqueous phases. Around 400 µL of the aqueous (upper) phase were carefully transferred into an Eppendorf tube containing 1 mL of 100% ethanol. This tube was inverted until thread-like strands of DNA formed a visible mass. Precipitation was enhanced by leaving the tubes at -20 ºC for 15 min. Then, tubes were centrifuged and the DNA pellet was washed twice with 70% ethanol, which was removed using a vacuum pump. The purified DNA was resuspended into 50 mL of Tris-HCl 10 mM pH 8.5 and re-dissolved

overnight at 4 ºC. After this time, RNase treatment was performed by adding 0.8 uL of RNase 10 mg/mL and incubating the mix 20 minutes at 37 ºC.

As phenol might interfere with some sequencing procedures, an extra precipitation and washing step was performed. For this, 10 µL of sodium acetate (2.5 M, pH 5.2, filtered) were added to each tube, followed by 250 µL of 100% ethanol. The protocol explained in the previous paragraph was then repeated from the DNA precipitation step until re-dissolution at 4 ºC in Tris-HCl 10 mM pH 8.5.

NanoDrop™ (ThermoScientific) was used to measure the absorbance ratios (260/280 and 260/230) indicating DNA purity, and the Quantus™ Fluorometer (Promega) was employed for DNA quantification.

Table M19. *Lysis buffer for genomic DNA extraction.*

Component	Concentration
Tris pH 8.0	10 mM
EDTA	1 mM
NaCl	100 mM
Triton	2 %
SDS	1 %

4.4. Polymerase chain reaction (PCR)

4.4.1. Amplification of deletion cassettes

High-fidelity PCR was used to obtain high copy number deletion cassettes for their transformation, using the plasmids listed in **Table M18** as DNA templates and the primers listed in **Tables M16** and **M17**. A thermostable *Taq* polymerase with proofreading activity (KAPA HiFi™ HotStart – KapaBiosystems KK2501) was used to limit errors during DNA replication. The PCR mix and thermocycling program used are shown in **Tables M20** and **M21**.

Table M20. *PCR reaction mix for the amplification of deletion cassettes.*

Component	Volume for 1 reaction
5x buffer	10 µL
10 mM dNTPs	1.5 µL
Primer FW	1.5 µL
Primer RV	1.5 µL
KAPA Taq pol.	1 µL
Nuclease-free H_2O	32.5 µL
Template DNA	2 µL (in each tube separately)
TOTAL	50 µL

***Table M21**. PCR program for the amplification of deletion cassettes.*

Step		T (ºC)	Time
Inicialisation		95	3 min
x 35	Denaturation	98	20 s
	Annealing	55	15 s
	Elongation	72	90 s
Final elongation		72	10 min
Conservation		4 - 10	∞

After amplification, the PCR product was purified using the Macherey-Nagel NucleoSpin kit.

4.4.2. Deletion verification

Successful deletions of the *HO* gene were confirmed using the PCR mix and program described in **Tables M22** and **M23**, with a "classic" *Taq* polymerase (Fermentas #EP0402 kit). Deletions of MAT genes were confirmed by mating-type PCR. The template DNA was obtained using the rapid method described in section 4.3.2.

***Table M22**. PCR mix for HO gene(s) deletion verification.*

Component	Volume for 1 reaction
Enzyme buffer	10 µL
10 mM dNTPs	1.5 µL
Primer FW	1.5 µL
Primer RV	1.5 µL
KAPA Taq pol.	1 µL
Nuclease-free H_2O	32.5 µL
Template DNA	2 µL (in each tube separately)
TOTAL	25 µL

***Table M23**. PCR program for HO gene(s) deletion verification.*

Step		T (ºC)	Time
Inicialisation		94	4 min
x 35	Denaturation	98	10 s
	Annealing	55	30 s
	Elongation	72	3 min
Final elongation		72	10 min
Conservation		4 - 10	∞

4.4.3. Mating-type PCR

To check the mating type(s) of a given yeast strain, the PCR mix and program described in **Tables M24** and **M25** were used, with a "classic" *Taq* polymerase (Fermentas #EP0402 kit), and DNA from the rapid extraction method.

***Table M24**. PCR mix for mating-type verification.*

Component	Volume for 1 reaction
10x buffer + KCl	2.5 µL
10 mM dNTPs	0.5 µL
Primer FW	1 µL
Primer RV (a)	1 µL
Primer RV (α)	1 µL
Taq polymerase	0.25 µL
$MgCl_2$	2.5 µL
Nuclease-free H_2O	15.25 µL
Template DNA	1 µL (in each tube separately)
TOTAL	25 µL

***Table M25**. PCR program for mating-type verification.*

Step		T (°C)	Time
Inicialisation		95	5 min
x 30	Denaturation	98	1 min
	Annealing	55	30 s
	Elongation	72	2 min
Final elongation		72	5 min
Conservation		4 - 10	∞

4.4.4. Species-specific multiplex PCR

Multiplex PCR was used to determine the species of a given yeast strain (e.g. for hybrid status confirmation). A working solution containing the 16 primers (0.625 µM each) was prepared from the initial mother solutions. The QIAGEN™ Multiplex PCR Kit was used with the PCR mix and program detailed in **Tables M26** and **M27**, using DNA from the rapid extraction protocol. Half of each volume indicated by the fabricant was used. PCR provided an amplicon of different molecular size depending on the species (**Table M28**).

***Table M26**. PCR mix for species-specific multiplex PCR.*

Component	Volume for 1 reaction
Master mix	6.25 µL
Working primer solution	4 µL
H_2O from kit	1.25 µL
Template DNA	1 µL (in each tube separately)
TOTAL	12.5 µL

***Table M27**. PCR program for species-specific multiplex PCR.*

Step		T (ºC)	Time
Inicialisation		95	5 min
x 30	Denaturation	95	30 s
	Annealing	57	90 s
	Elongation	72	30 s
Final elongation		60	30 min
Conservation		4 - 10	∞

***Table M28**. Molecular size of the amplicons generated by species-specific multiplex PCR.*

Species	Amplicon size (bp)
S. cerevisiae	150
S. eubayanus	228
S. jurei	~ 250
S. uvarum	275
S. arboricola	349
S. mikatae	508
S. kudriavzevii	660
S. paradoxus	739

4.4.5. Agarose gel electrophoresis

Electrophoresis in agarose gel was used to verify the molecular size and check for impurities or degradation in any given PCR product or DNA extraction outcome. Gels with 0.8 – 2.0 % (w/v) agarose in TAE (40 mM Tris-Acetate, 1 mM EDTA, 20 mM glacial acetic acid, pH 8.4) buffer with 0.2 µg/mL of ethidium bromide (Sigma E-1510) were used. The ExactLadder® DNA PreMix 2 log (Ozyme™), with a size range from 100 to 10.000 bp, was loaded for comparison. A transiluminator (Vilber Fusion Solo S) was used for gel visualization.

4.5. Transformation

Yeast transformation with the amplified and purified deletion cassettes was performed using the LiAc/SS carrier DNA/PEG method from Gietz and Schiestl (2007) with some adjustments. Cells were grown overnight in 5 mL of YPD. The next day, 50 mL of fresh YPD were inoculated to an initial OD_{600} of 0.5 using the first culture. When the exponential growth phase was reached (OD_{600} = 2.0 to 2.2), cells were recovered by centrifugation at 3000 rpm and washed twice with sterile water. Cell pellets were then transferred to a 1.5 mL Eppendorf tube and resuspended in 1 mL of sterile water. For each transformation reaction, 100 µL of cell suspension, containing approximately 10^8 cells, were transferred to a 2 mL Eppendorf tube. After a quick spin of 30 seconds at 13000 rpm to eliminate water, 360 µL of transformation mix (**Table M29**) were added to each cell pellet. A slight vortex touch followed by a heat shock (40 min at 42 ºC) allowed the

transformation into the cells. Cells were then recovered by centrifugation (30 seconds at 13000 rpm) and resuspended in water for spreading on the selective media plates, supplemented with the correspondent antibiotic. Growing colonies, if any, were confirmed or discarded as deletants by PCR. A schematic representation of the protocol is shown in **Fig. M1**.

***Table M29**. Transformation mix.*

Component	Amount for 1 transformation
PEG-4000 (50% w/v)	240 µL
LiAc 1.0 M (sterile)	36 µL
denatured ssDNA (2 g/L)	50 µL
DNA q.s. H_2O*	34 µL
Total	360 µL

* The amount of DNA depends on the nature of the fragment. For a PCR product for integration, 3 to 4 µg of purified PCR product were added.

4.6. Sporulation

For sporulation, 200 – 500 µL of an overnight preculture in GNA medium were washed three times with sterile water and used to inoculate a sterile 100-mL Erlenmeyer flask containing 15 mL of spoMA. Incubation was done at 22 ºC and 100 rpm. The formation of spores was periodically monitored under the optical microscope until tetrads were seen.

4.7. Micromanipulation

The micromanipulator MSM 300 (Singer Instruments) was used to dissect tetrads after sporulation and to perform marker-free cell mating.

4.7.1. Tetrad dissection

For tetrad dissection, 200 µL of a given sporulated culture were centrifuged for 1 min at 10 000 rpm. After discarding the supernatant, the pellet was resuspended in 100 µL of zymolyase solution (200 U/mL) (Sigma Aldrich) and incubated for 10 min at 37 ºC to partially digest the asci's cell walls. After this time, a sterile loop was used to acquire some biomass and spread it on the side of a micromanipulation plate. A micromanipulator was then used to separate each individual spore from the rest. When needed, the spore viability of a given strain was calculated as the percentage of surviving spores (i.e. colony–forming) from the total number of spores dissected (minimum of 40 spores).

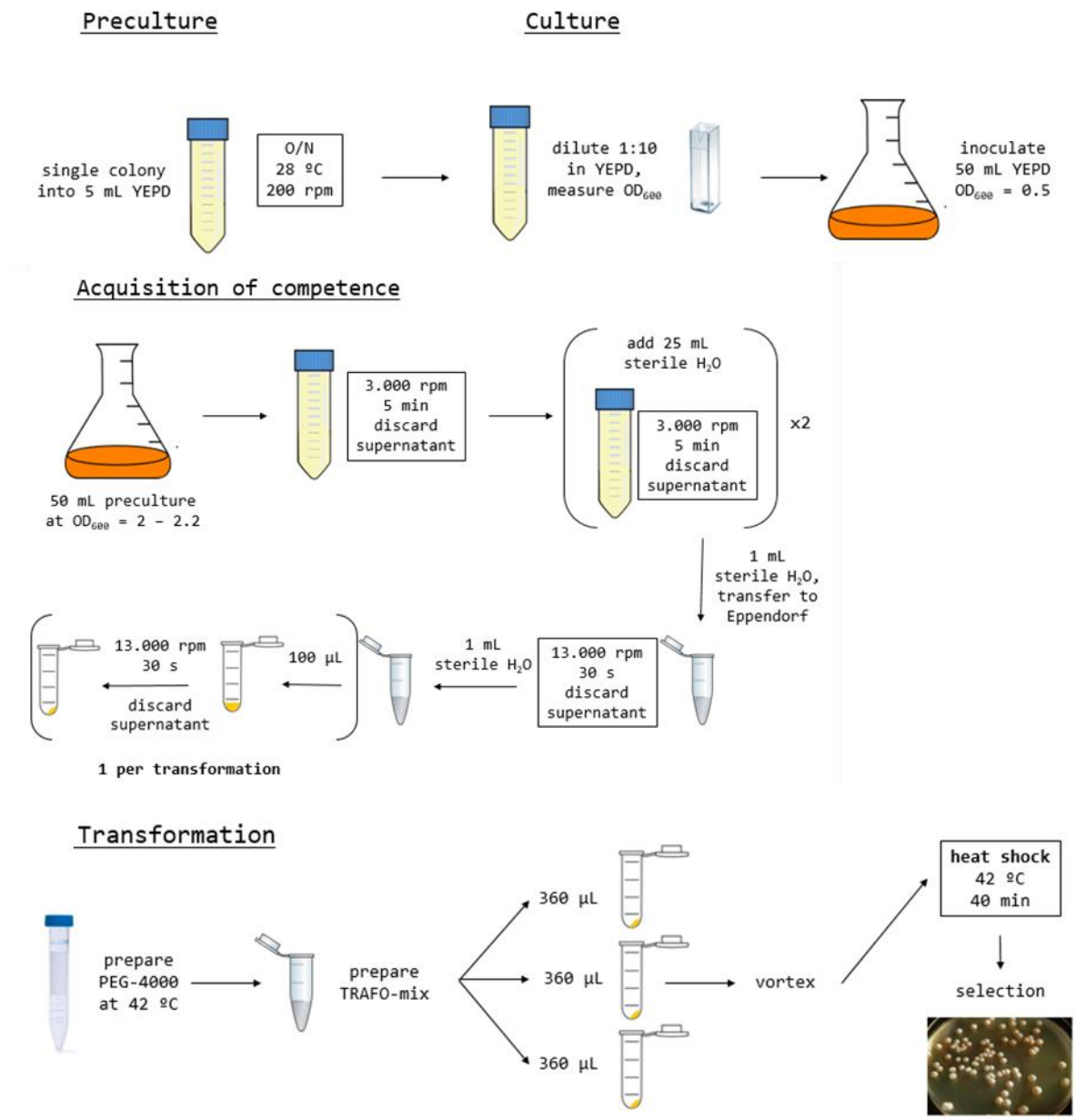


***Figure M1**. Schematic representation of the transformation protocol used in this study.*

4.7.2. Micromanipulation-assisted mating

Hybridisation of two strains of opposite mating types was performed without the use of selection markers. First, overnight YPD cultures of the strains to be mated were obtained. Then, a 5-µL drop of one strain was placed on a YPD plate. After drying (around 10 minutes), a 5-µL drop of the second strain was placed on top of the first one. Plates were then incubated for 1 to 3 hours, checking the status of the culture approximately every 30 minutes. For this, some biomass was placed on a microscope slide together with 10 µL of sterile water and then observed under the optical microscope. The observation of 'shmoos' (i.e. projections formed by attraction to pheromones of the opposite mating type) (**Fig.**

M2.b) indicated imminent mating. The micromanipulator was used to place zygotes (**Fig. M2.c**) on different positions of the plate, isolating them from the rest. After incubating the plates for 48 h at 28 ºC, each candidate was re-streaked onto YPD-agar to isolate single colonies. The hybrid status of those was confirmed by mating-type PCR and by verifying the sporulation ability of the candidate.

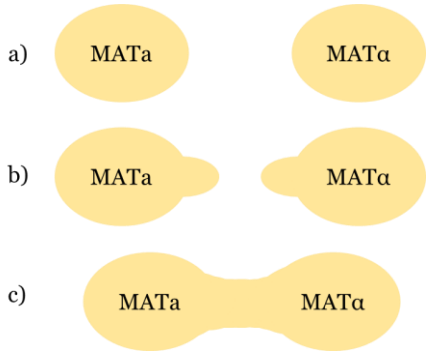


Figure M2. *Schematic representation of 'shmoo' (b) and zygote (c) formation during yeast cell mating.*

4.8. Generation of petite mutants

Petite strains (i.e. lacking functional mitochondria) were obtained by contact with ethidium bromide (EtBr). First, overnight YPD cultures of the strains of interest were obtained. The cell density of those cultures was measured using a Multisizer™ 3 Coulter Counter (Beckman Coulter). Then, around 300 cells were seeded on a YPD plate, and a 3-µL drop of EtBr solution (10 mg/mL) was placed on the centre of it. Plates were incubated at 28 ºC until colony formation. After this time, a "death" halo around the EtBr drop surrounded by small-size colonies could be observed. Those small colonies (i.e. the candidates) were re-streaked on YPD to isolate single colonies. The *petite* status of those was confirmed by their inability to grow on YEPEG.

4.9. Flow cytometry for ploidy estimation

The ploidy level of yeast strains was estimated by flow cytometry. First, a culture in exponential phase was obtained. For this, a single colony was used to obtain an overnight YPD culture, used to inoculate a second YPD culture at an initial OD_{600} of 0.05. This culture was incubated for 5 h at 28 ºC and 400 rpm. After this time, cells were fixed in ethanol. For this purpose, 750 µL of the culture were transferred into a 2 mL Eppendorf tube containing 1 mL of room temperature H_2O. The mixture was centrifuged for 1 min at 10 000 rpm, the supernatant was discarded, and the pellet was resuspended in 1 mL of H_2O. This suspension was deposited drop by drop into a Falcon tube containing 8 mL of 70% ethanol previously filtered, under constant vortexing. Those tubes were incubated at 4 ºC.

The next day, tubes were centrifuged and the supernatant discarded. Cell pellets were resuspended in 1 mL of filtered PBS and transferred to a new 1.5 mL Eppendorf. PBS was removed by centrifugation (10 000 rpm, 1 min) and cells were resuspended in 500 µL of RNase A (2 mg/mL in 10 mM Tris-HCl and 15 mM NaCl) and incubated at 37 ºC for 1 hour. The RNase was discarded using the centrifuge, the pellet was resuspended in 200 µL of proteinase K (1 mg/mL in PBS), and tubes were incubated for 1 h at 50 ºC. After the second enzymatic treatment, proteinase K was eliminated by centrifugation and cells were resuspended in 500 µL of PBS. The cell suspension was sonicated for 15 s at 50% capacity to separate cells from each other. Labelling was performed by transferring 200 µL of Sytox Green solution (1.25 µM in PBS) in a new 1.5 mL Eppendorf and adding 50 µL of the cell suspension.

A BD Accuri C6 Plus flow cytometer (BD Biosciences) was used to analyse the samples. Excitation was achieved with the blue laser (488 nm) with absorbance detected using a 533/30 standard filter. The membrane stain cell tracker DiD was measured in the APC channel, excitation using the red laser (640 nm) and absorbance detected using the 675/25 standard filter. Cell tracker DiD stain was used to gate cells, to exclude debris from the ploidy analysis. Histograms were produced for each sample of Sytox Green stain intensity against cell number. Control strains of known ploidy (n, 2n and 4n) were used for comparison.

4.10. Generation of advanced intercross lines (AILs)

Interspecific hybrids were pushed through multiple rounds of intercrossing (**Fig. M3**). (**A**) Sporulation was achieved as explained above. (**B**) The remaining vegetative cells were eliminated by adding one volume of diethyl ether (99.5 %, Sigma-Aldrich) to one volume of the sporulated culture resuspended in sterile H_2O, and vigorously vortexing the mixture for 10 minutes. (**C**) Then, the cell suspension was washed three times with water and asci's cell walls were digested by resuspension in zymolyase solution (10 mg/mL) and incubation for 30 min at 37 ºC. Zymolyase was eliminated by centrifugation, and the cell pellet was vortexed for 5 min to ensure inter-tetrad mating in the next step. (**D**) Finally, spores were plated onto YPD for 48 h to allow mating and germination. Ten percent of cell mass at each generation was collected in 25 % glycerol and stored at -80 ºC, ready for future investigation. The rest of the biomass was used for sporulation of that generation, and the process was repeated 11 times to reach the F12. Every three generations, after step (A), 200 µL of the sporulated culture were used to determine spore viability by micromanipulation.

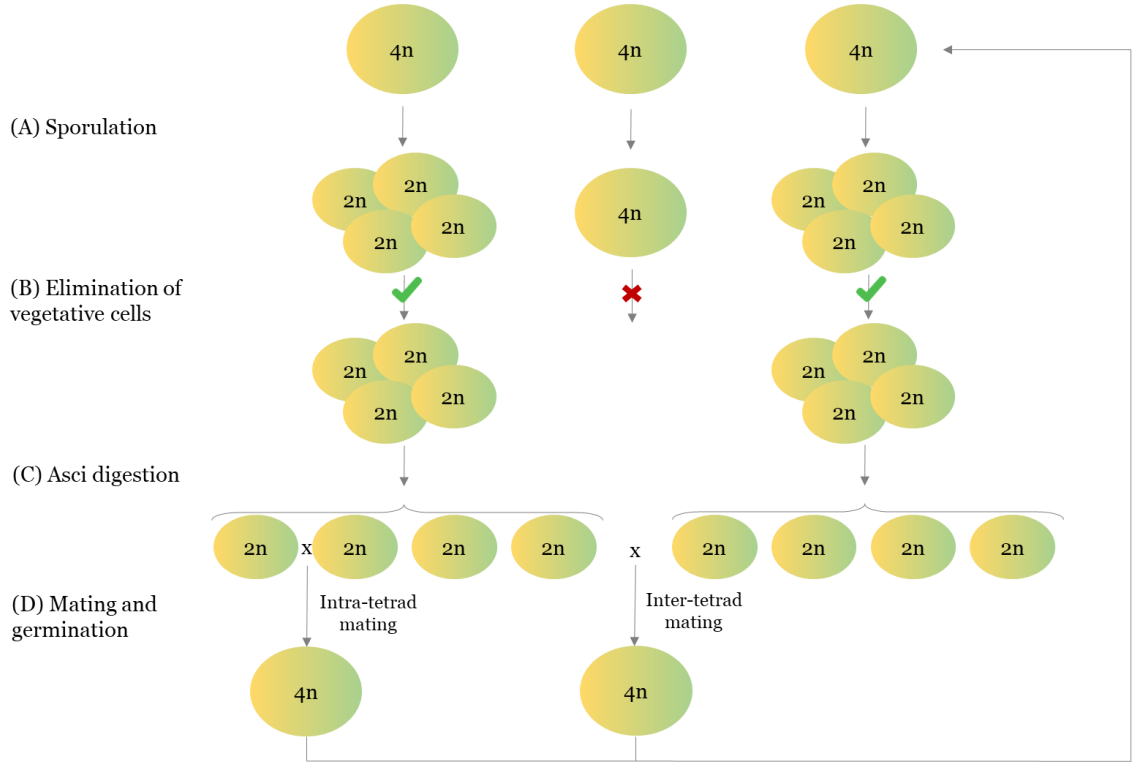


***Figure M3**. Schematic representation of the generation of AILs.*

5. Sequencing and bioinformatic analysis

5.1. Sequencing, mapping and variant calling

All strains were sequenced individually by the Earlham Institute (Norwich, UK). They used the LITE library prep to pool barcoded samples onto an Illumina NGS platform for short read sequencing.

Paired-end Illumina short reads were aligned to concatenated references by the hybrid sets and parental species (*S. uvarum* CBS7001 and *S. mikatae* IFO 1815^{T}) after quality check and trimming. Variant calling was applied on founder haploid strains and hybrid segregants. Variants were called separately by hybrid sets grouped by mitochondrion (i.e. *S. uvarum* or *S. mikatae*). Filters on SNPs were applied on the output of raw variant calling to obtain the variant table for the further analysis.

5.2. Genotyping and QTL analysis

Founder genotypes were obtained by the variant sites derived from two parental lines background under the same species. For hybrid segregants, bi-allele markers were then further aligned to founders to phase the genotypes (i.e. which base is the same in which parental background). The matched strain sets that both having phenotype and genotype records were used as the input dataset for QTL analysis. QTL Analyses were then

performed for each phenotype through r/qtl with marker regression. The significance level was set at 0.05 to determine the LOD threshold with 1000 permutation tests applied for each QTL scan.

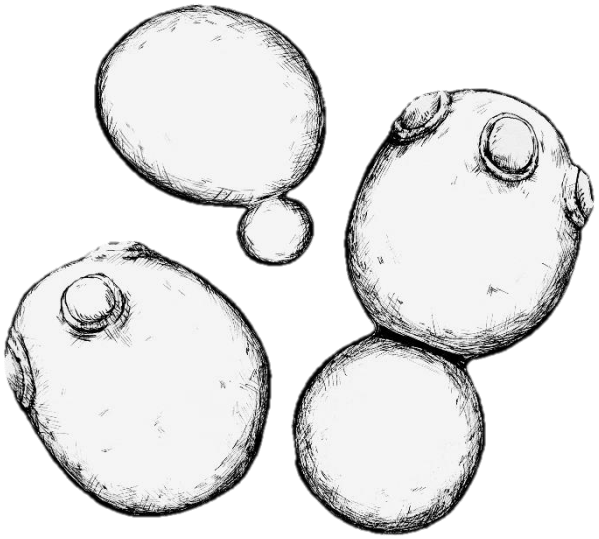
6. Data treatment and statistical analysis

The data acquired during this work was treated and analysed with R (R Core Team 2022) version 4.2.1, RStudio (RStudio Team) and the XLSTAT extension for Microsoft Excel (Addinsoft, Paris, France 2022). R packages used for specific purposes are detailed in **Table M30**.

***Table M30**. List of R packages used.*

R package	Usage
agricolae	Statystical analysis
alfisStatUtilR	Curve smoothing and determination of kinetic parameters
heatmaply	Heatmap generation
tidyverse	Data manipulation and visualisation
plotrix	Generation of 'beeswarm' plots
beeswarm	Generation of 'beeswarm' plots

CHAPTER ONE



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Beyond *S. cerevisiae* for winemaking: Fermentation-related trait diversity in the genus *Saccharomyces*

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ABSTRACT

Saccharomyces cerevisiae is the yeast of choice for most inoculated wine fermentations worldwide. However, many other yeast species and genera display phenotypes of interest that may help address the environmental and commercial challenges the wine industry has been facing in recent years. This work aimed to provide, for the first time, a systematic phenotyping of all *Saccharomyces* species under winemaking conditions. For this purpose, we characterized the fermentative and metabolic properties of 92 *Saccharomyces* strains in synthetic grape must at two different temperatures. The fermentative potential of alternative yeasts was higher than expected, as nearly all strains were able to complete fermentation, in some cases more efficiently than commercial *S. cerevisiae* strains. Various species showed interesting metabolic traits, such as high glycerol, succinate and odour-active compound production, or low acetic acid production, compared to *S. cerevisiae*. Altogether, these results reveal that non-*cerevisiae Saccharomyces* yeasts are especially interesting for wine fermentation, as they may offer advantages over both *S. cerevisiae* and non-*Saccharomyces* strains. This study highlights the potential of alternative Saccharomyces species for winemaking, paving the way for further research and, potentially, for their industrial exploitation.

1. Introduction

The wine industry has been facing important challenges during the last decades. First, global warming gradually accelerates grape ripeness, increasing berry sugar content while decreasing malic acid content. This eventually results in wines with excess alcohol and insufficient acidity, leading to undesired organoleptic imbalances (Mira de Orduña, R., 2010; Tilloy et al., 2015). Secondly, there is a growing demand for products with novel sensory profiles, which depend on both odour-active and odourless compounds that influence the taste, aroma and mouthfeel properties of the final product.

As far as we know, the history of winemaking has been a monologue starred by *Saccharomyces cerevisiae*. Most fermented beverages are currently obtained using *S. cerevisiae*, either as a pure species or as interspecific hybrids with other *Saccharomyces* species such as *S. eubayanus* or *S. kudriavzevii*. This model organism is known for its robust fermentative capacities, efficiently fermenting sugar-rich grape musts even in aerobic conditions (Crabtree effect). Despite their undeniable interest, commercially available strains of *S. cerevisiae* and its hybrids present limited variability, insufficient to effectively address the abovementioned challenges (Molinet and Cubillos 2020).

Different strategies have been proposed to tackle these challenges from a microbiological perspective. A number of authors aimed to improve *S. cerevisiae* strains by using non-GM methods that would allow their use in the beverages sector. For instance, backcrossing has been used to improve *S. cerevisiae* thermo-tolerance (Marullo et al., 2009) and enhance the release of volatile thiols (Dufour et al., 2013). Adaptive laboratory evolution (ALE) strategies have been successfully used to reduce ethanol synthesis while increasing glycerol production (Tilloy et al. 2014, 2015) and to increase the flux toward the pentose-phosphate pathway, improving fermentation rates and aroma production (Cadière et al., 2011). Interspecific hybridisation has also drawn increased attention over the last years as a promising tool to combine phenotypes of interest and, ideally, achieve heterosis. Several authors have generated hybrids to produce wines with improved aroma diversity (Bellon et al., 2011, 2013; Magalhães et al., 2017; Su et al., 2019), reduced ethanol and increased glycerol concentrations (Origone et al., 2018; Pérez et al., 2022a) as well as improved temperature robustness (Albertin et al., 2015) with respect to the parental strains.

Other researchers looked at the vast biodiversity found in vineyards

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and wineries, exploring the potential of non-*Saccharomyces* yeasts. These genera, long seen as contaminants, have revealed their potential to produce a diverse range of fermentation metabolites, including volatile compounds with positive sensory characteristics (Jolly et al., 2014). Some of these species also achieve substantially reduced ethanol yield, redirecting carbon fluxes towards other compounds of the central carbon metabolism or biomass. This is the case of *Lachancea thermotolerans*, which is commercially available to increase acidity due to a high production of lactic acid (Benito 2018). *Torulaspora delbrueckii*, also available on the market, has been shown to produce less acetic acid and more glycerol than *S. cerevisiae* (Fernandes et al., 2021). Additionally, many non-*Saccharomyces* strains are known by their significant contribution to varietal and fermentative aroma enhancement (Padilla et al., 2016). Despite their interesting capacities, most non-*Saccharomyces* are not fully fermentative species, which means they cannot complete fermentation by themselves. As a consequence, strains of *S. cerevisiae* naturally present in spontaneous fermentations generally outcompete non-*Saccharomyces* strains, thus minimising their contribution to wine composition (Jolly et al., 2014). Co-cultures and sequential inoculations of *S. cerevisiae* and non-*Saccharomyces* constitute a valid approach, although it can present some drawbacks related to negative interactions between species, mainly regarding competition for nutrients (Zilelidou and Nisiotou 2021). Therefore, carrying out the entire process using a pure culture is by far the preferred approach in the field, as it allows more control over both the outcome and the process itself. This context stresses the industrial need for novel strains capable of performing efficient fermentation while producing a diverse range of metabolites with eventually low ethanol concentrations.

In the general scheme of wine yeasts, non-*cerevisiae Saccharomyces* species appear as an intermediate, less studied group, however gaining interest over the last decade. Currently, there are eight recognised species in the genus (Dujon and Louis 2017). *S. cerevisiae* and *S. uvarum* have been found in industrial fermentations as pure species; *S. eubayanus* and *S. kudriavzevii* have been isolated in industrial settings, but only as a component of interspecific hybrids with *S. cerevisiae*; *S. mikatae*, *S. jurei*, *S. arboricola* and *S. paradoxus* have to date only been found in natural ecosystems (Alsammar and Delneri 2020) (except for some *S. paradoxus* strains isolated from vineyards) (Redžepović et al., 2002; Dashko et al., 2016). Interestingly, some non-*cerevisiae Saccharomyces* yeasts can ferment grape must until sugar exhaustion. For instance, populations of *S. uvarum* predominate in some spontaneous fermentations even when *S. cerevisiae* is present (Demuyter et al., 2004; McCarthy et al., 2021). Furthermore, different studies have identified acceptably fermenting strains from different *Saccharomyces* species, including *S. paradoxus* (Orlić et al., 2007), *S. eubayanus* (Parpinello et al., 2020) and *S. kudriavzevii* (Pérez et al., 2021). From a metabolic point of view, *S. kudriavzevii, S. eubayanus* and *S. uvarum* have been shown to produce wines with reduced ethanol, increased glycerol and high amounts of some esters and higher alcohols (Minebois et al., 2020; Pérez et al., 2021, 2022b), while *S. paradoxus* was also able to reduce volatile acidity, compared to *S. cerevisiae* (Orlić et al. 2007, 2009). In addition, other non-*cerevisiae Saccharomyces* are malic acid producers (Giuduci et al., 1995), which could be interesting in the context of climate change.

Several authors have drawn a picture of the phenotypic diversity within *S. cerevisiae* (Camarasa et al., 2011) and non-*Saccharomyces* (Rossouw and Bauer 2016) yeasts for winemaking, offering new perspectives based on microbial approaches to diversify wine sensory profile. However, despite the increasing availability of isolates from alternative *Saccharomyces* species, there is still little information on their phenotypes during wine fermentation. Moreover, available studies do not cover the entire evolutionary landscape of the genus, leaving aside species such as *S. arboricola, S. mikatae* and *S. jurei*. The main question to be answered is whether these and other *Saccharomyces* species can ferment as vigorously as *S. cerevisiae* while providing a range of metabolites comparable to that of non-*Saccharomyces*.

This work aimed to provide, for the first time, a systematic phenotyping of all the current species of *Saccharomyces* in a winemaking context, filling the evolutionary gap between these two groups, and highlighting the potential of alternative *Saccharomyces* species for the improvement of yeast starter cultures.

2. Materials and methods

2.1. Yeast strains

Ninety-two *Saccharomyces* strains (Table S1), originating from geographically and ecologically diverse environments, were used in this study. All the strains were selected from the University of Leicester (UK), INRAE Montpellier and CIRM-Levures (France) collections. Four commercial strains (*S. cerevisiae* EC1118 and DBVPG1106, *S. uvarum* BMV58 and *Sc x Sk* VIN7), provided by Lallemand (Montreal, Canada) were used as controls. All eight species that nowadays compose the *Saccharomyces* genus (viz. *S. cerevisiae, S. paradoxus, S. mikatae, S. jurei, S. kudriavzevii, S. arboricola, S. eubayanus* and *S. uvarum*) were represented, according to the availability of isolates in our collections. Two types of interspecific hybrids, i.e. *S. cerevisiae x S. kudriavzevii* and *S. cerevisiae x S. uvarum* were also included. All yeasts were maintained at −80 °C in 20% glycerol before use.

2.2. Fermentation in synthetic must

Fermentations were carried out at two different temperatures, i.e. 16 and 22 °C. A synthetic grape must (SM) was prepared as previously described (Rollero et al., 2015). Briefly, this medium contained 200 g L^{-1} sugars (equal amounts of glucose and fructose) and 200 mg L^{-1} assimilable nitrogen in the form of ammonium and free amino acids, as well as vitamins, trace elements and phytosterols, mimicking the composition of a standard grape juice. Fermentations were performed at least in duplicate, using cylindrical 300 mL glassware fermenters filled with 250 mL SM, under a 250 rpm agitation.

First, strains were recovered from cryopreserved cultures by streaking onto yeast peptone dextrose (YPD) agar. Precultures of all yeast strains were prepared by inoculating a single colony into 20 mL of YPD broth incubated at 28 °C and 180 rpm for 12–15 h. After this time, cell density was determined using a Multisizer™ 3 Coulter Counter (Beckman Coulter). Fermenters were filled with 250 mL SM and pasteurized to achieve asepsis without degrading vitamins or other thermolabile compounds. The medium was then saturated with oxygen by aerating the fermenters under agitation during 30 min. Phytosterols were added after aeration to avoid excessive foaming, using a stock solution composed of 4 g L^{-1} of phytosterols in Tween 80 and ethanol (1:1, v/v), for a final concentration of 5 mg L^{-1}. After equilibrating fermenters at the desired temperature, they were inoculated to an initial cell density of 1×10^6 cells·mL^{-1}. From this moment, fermentation kinetics were monitored by periodically measuring the weight loss of the fermenters, which was used to calculate the CO_2 production rate ($g{\cdot}L^{-1}h^{-1}$). Fermentations were stopped when the CO_2 production rate was lower than 0.02 g $L^{-1}h^{-1}$.

Three wild strains, namely SA03 (*S. arboricola*), SK06 (*S. kudriavzevii*) and SC03 (*S. cerevisiae*), were selected for fermentation experiments with higher sugar concentrations. The SM used had the same composition as the one previously described, but with varying amounts of sugars. Equal amounts of glucose and fructose were added to achieve final concentrations of 200, 220 and 240 g L^{-1}. Fermentations were performed in triplicate, using the commercial strains EC1118 (*S. cerevisiae*) and VIN7 (*Sc x Sk*) as controls.

2.3. Quantification of sugars and primary metabolites

Samples were collected from the fermenters at two different time points, i.e. at 60 g L^{-1} of CO_2 released and at the end of fermentation.

The amounts of sugars (glucose and fructose) and the main primary metabolites (ethanol, acetate, succinate, glycerol, α-ketoglutarate and pyruvate) were determined by high-performance liquid chromatography (HPLC), as described by Rollero et al. (2015). An HPLC 1290 Infinity (Agilent Technologies, Santa Clara, California, USA) device was used with a Rezex ROA column (Phenomenex, Le Pecq, France) at 60 °C. The column was eluted with 0.005 N H_2SO_4 at a flow rate of 0.6 mL min^{-1}. Organic acids were quantified using a UV detector at 210 nm. For the rest of the compounds, a refractive index (RI) detector was used. All the data were treated using the ChemStation software (Agilent Technologies, Santa Clara, California, USA).

To draw up carbon balances, biomass amounts were estimated, and the concentrations of α-hydroxyglutarate, acetoin, butanediol and acetaldehyde were determined. Hydroxyglutarate was quantified in the same way as other organic acids (HPLC). The amounts of acetoin and 2,3-butanediol were determined using gas chromatography with a flame ionization detector (GC-FID) after a simple extraction in chloroform, as described by Tilloy et al. (2014). To estimate biomass content, 10 mL samples were filtrated using previously dried 0.45 μm pore-size nitrocellulose filters (MF-Millipore, Merck), which were then washed three times with 50 mL of distilled water and dried for 24 h at 100 °C. Biomass was calculated as the average of three independent measurements.

2.4. Quantification of fermentative aroma

The main fermentative odour-active compounds (ethyl esters: ethyl acetate, ethyl propanoate, ethyl 2-methylpropanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and ethyl dodecanoate; acetate esters: 2-methylpropyl acetate, 2-methylbutyl acetate, 3-methyl butyl acetate and 2-phenylethyl acetate; higher alcohols: 2-methylpropanol, 2-methylbutanol, 3-methylbutanol, hexanol, 2-phenylethanol; and short- and medium-chain fatty acids: propanoic acid, butanoic acid, 2-methylpropanoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, hexanoic acid, octanoic acid decanoic acid and dodecanoic acid) were quantified using gas chromatography-mass spectrometry (GC-MS), following the protocol described by Rollero et al. (2015). Briefly, a double liquid-liquid extraction with dichloromethane in presence of deuterated standards was followed by injection into a Hewlett Packard (Agilent™ Technologies, Santa Clara, CA, USA) 6890 gas chromatograph equipped with a CTC Combi PAL Autosampler AOC-5000 (Shimadzu™, Columbia, USA) and coupled to a Hewlett Packard 5973 Mass Spectrometry detector (Agilent™ Technologies, Santa Clara, CA, USA). A standard solution was prepared for each group of compounds (ethyl esters, acetate esters, alcohols and acids), and calibration solutions were made by adding the appropriate amounts of the latter into synthetic wine.

2.5. Statistical analysis

All the fermentations were performed in duplicate or triplicate. In order to determine data reliability, the intraclass correlation coefficient (ICC) was calculated for every dataset at every condition. The irrICC package (R) was used for this purpose. Values of ICC above 0.75 and 0.90 are indicative of good and excellent reliability, respectively, while values below 0.75 and 0.50 indicate moderate and poor reliability (Koo and Li, 2016).

Fermentation curves were smoothed and kinetic parameters were determined, using the alfisStatUtilR (v1.0.0) R package based on a locally developed regression model (Duc et al., 2020). The kinetic parameters calculated were the maximum CO_2 production rate (R_{max}), the rate at 60 g L^{-1} of CO_2 released (R_{60}), the time necessary to reach 60 g L^{-1} of CO_2 released (t_{60}), the lag time (t_{lag}) and the fermentation time (t_{ferm}). To facilitate the comparison of the kinetic capacities between strains, a parameter encompassing the five variables determined, termed *overall kinetic score* (or simply *kinetic score*), was calculated for each fermentation curve. The values of the different variables were normalised, and the new parameter was calculated as follows:

$$Overall\ kinetic\ score = \left(R_{max,n} + R_{60,n} + t_{60,n} + t_{lag,n} + t_{ferm,n}\right) / 5$$

, where *n* indicates that a variable has been normalised. All the data collected for each variable were normalised together, to be able to compare between different conditions. Normalisation was performed in a way that the highest value (i.e. 1) corresponds to the best behaviour, and the lowest value (i.e. 0) to the worst behaviour. For example, a value of 1 was assigned to the highest R_{max} and also to the lowest t_{lag}.

A principal component analysis (PCA) was performed to determine the variables contributing the most to the total variation observed. Statistical tests were applied to each variable: 2-way ANOVA to determine the impact of the temperature and species factors, Tukey's and Dunnett's tests for multiple comparisons and *t*-test for pairwise comparisons.

3. Results

To investigate the phenotypic diversity within the *Saccharomyces* genus in wine fermentation, we phenotyped 92 strains using industrially relevant traits. The yeast strains used encompass all the current species of *Saccharomyces*, some of which have never been characterized under wine-making conditions to our knowledge (i.e. *S. mikatae, S. jurei* and *S. arboricola*), as well as *S. cerevisiae* interspecific hybrids with the cryotolerant species *S. kudriavzevii* and *S. uvarum*. Laboratory-scale fermentations in a synthetic must were carried out at 16 and 22 °C, two temperatures commonly used in winemaking, to evaluate temperature effect on the different parameters. Kinetic parameters, describing the fermentative dynamics over the entire process, were determined from weight loss. Samples were collected at two different time points (i.e. 60 g L^{-1} of CO_2 released and the end of the fermentation) and used to quantify the main primary and secondary metabolites of oenological interest. Volatile aromas were measured only at 60 g L^{-1} of CO_2 produced, corresponding to the mid-stationary phase, as strains were expected to leave different amounts of sugars at the end of the fermentation. Also, at a same amount of CO_2 released, the comparison is more precise from a metabolic perspective, as all strains share a similar physiological state. The full phenotypic description of each strain consisted of 41 kinetic and metabolic variables, each evaluated at two temperatures (raw data: https://doi.org/10.57745/TX96GG).

We found a striking diversity for most phenotypes under study (Fig. 1). As expected, strains from the same species tended to cluster together, exhibiting similar behaviours under the same conditions. This allowed us to identify phenotypic specificities for each species. Furthermore, when a large number of strains were characterised within a species, high intraspecific variations were often observed. In line with this, clear outliers were identified, sometimes going far beyond the phenotypes of the *S. cerevisiae* strains.

3.1. Evaluation of fermentative capacities

The majority of the strains successfully conducted wine fermentation, reaching the minimum level of dryness established by OIV (i.e. 4 g L^{-1} of residual sugars or less, out of the 200 g L^{-1} initially present) (OIV, 2021). Based on this criterion, 18 out of 92 strains could not complete the fermentation at 22 °C, while only 9 did not reach dryness at 16 °C (Table S2). Most isolates unable to ferment all sugars at 22 °C belonged to cryotolerant species (*S. kudriavzevii*, *S. uvarum* and *S. eubayanus*), while half of the strains not achieving dryness at 16 °C were *S. cerevisiae* isolates. The levels of CO_2 produced were in line with the amounts of residual sugars at the end of the fermentation, with fructose always in higher quantity. Correspondingly, the residual glucose/fructose ratio at 65% of the fermentation was always below 1, indicating the absence of any fructophilic strain.

Analysis of variance revealed a significant effect of both temperature

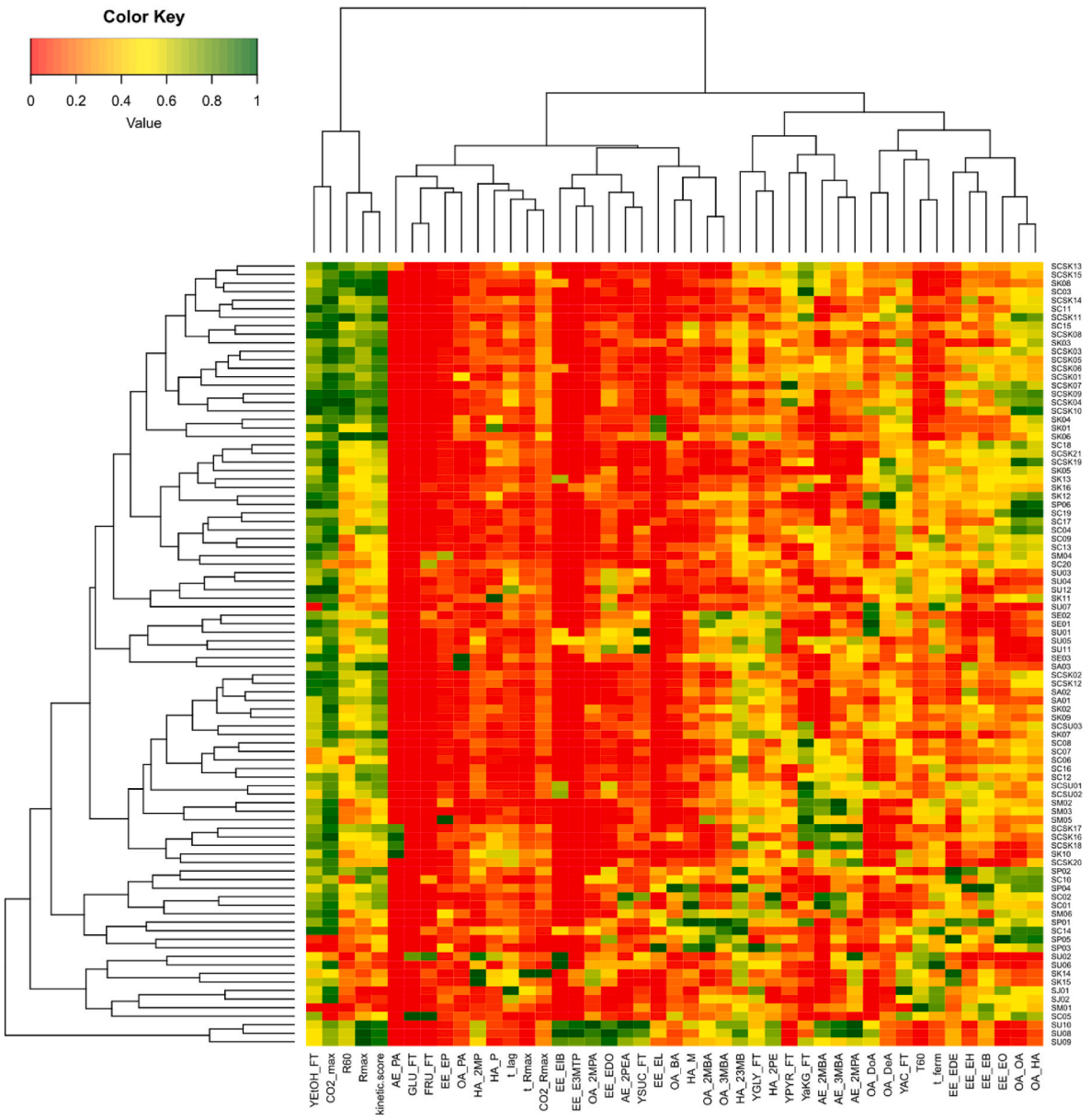

Fig. 1. Heatmap representing most of the variables determined at 16 °C, for the 92 strains. Each parameter is represented by a code, as follows: **Kinetic parameters**: Rmax (maximum CO_2 production rate), t_Rmax (time to reach Rmax), R60 (CO_2 production rate at 60 g/L of CO_2 released), T60 (time to reach 60 g/L of CO_2), CO_2_max (maximum CO_2 produced), t_lag (lag time), CO_2_Rmax (amount of CO_2 released when Rmax was achieved), t_lag (lag time), t_ferm (fermentation time), kinetic score. **CCM compounds** (FT for final time): FRU_FT (fructose), GLU_FT (glucose), YEtOH_FT (ethanol yield), YGLY_FT (glycerol yield), YAC_FT (acetate yield), YaKG_FT (α-ketoglutarate yield), YPYR_FT (pyruvate yield), YSUC_FT (succinate yield). **Aromas** (HA stands for higher alcohol, AE acetate ester, OA organic acid, and EE ethyl ester): EP (ethyl propanoate), EIB (ethyl isobutanoate), PA (propyl acetate), 2MPA (2-methylpropyl acetate), EB (ethyl butanoate), P (propanol), 2 MP (2-methylpropanol), 2MBA (2-methylbutyl acetate), 3MBA (3-methylbutyl acetate), 23 MB (2 and 3-methylbutanol), EH (ethyl hexanoate), EL (ethyl lactate), EO (ethyl octanoate), PA (propanoic acid), E3MTP (ethyl 3-methylthiopropionate), 2MPA (2-methylpropanoic acid), BA (butanoic acid), EDe (ethyl decanoate), 3MBA (3-methylbutanoic acid), 2MBA (2-methylbutanoic acid), M (methionol), 2PEA (2-phenylethyl acetate), HA (hexanoic acid), EDo (ethyl dodecanoate), 2 PE (2-phenylethanol), OA (octanoic acid), DeA (decanoic acid), DoA (dodecanoic acid).

and yeast species ($p < 0.05$) for all kinetic parameters, while the lag time also displayed a strong interaction effect. For some of these variables, remarkable differences were observed at the species level, revealed by multiple comparison tests (Tukey HSD and Dunnett's). For example, *Sc x Sk* hybrids showed significantly shorter fermentation times than *S. cerevisiae* at 16 °C (Fig. S3).

We determined a total of eight kinetic parameters from each fermentation curve. Although providing a complete characterization of the fermentation kinetics, this amount of data is often overwhelming and makes it difficult to identify the best and worst kinetic behaviours. For this reason, we decided to synthesise all the information into a single parameter, a general indicator of fermentation efficiency. We termed this new parameter the overall kinetic score, or simply kinetic score (Fig. 2). It was calculated as the average of the normalised values for five kinetic parameters, discarding those which provide redundant information. Kinetic scores ranged from 0.16 (SJ01) to 0.72 (SK06) at 16 °C, and from 0.41 (SU06) to 0.95 (SCSK09) at 22 °C, with every strain presenting higher values at the highest temperature. As expected, most of the strains isolated from wine fermentation showed high kinetic scores. However, some strains isolated from natural sources, a priori not domesticated, were also found among the best with regard to this parameter. Thus, strains isolated from fruits, trees and soil belonging to

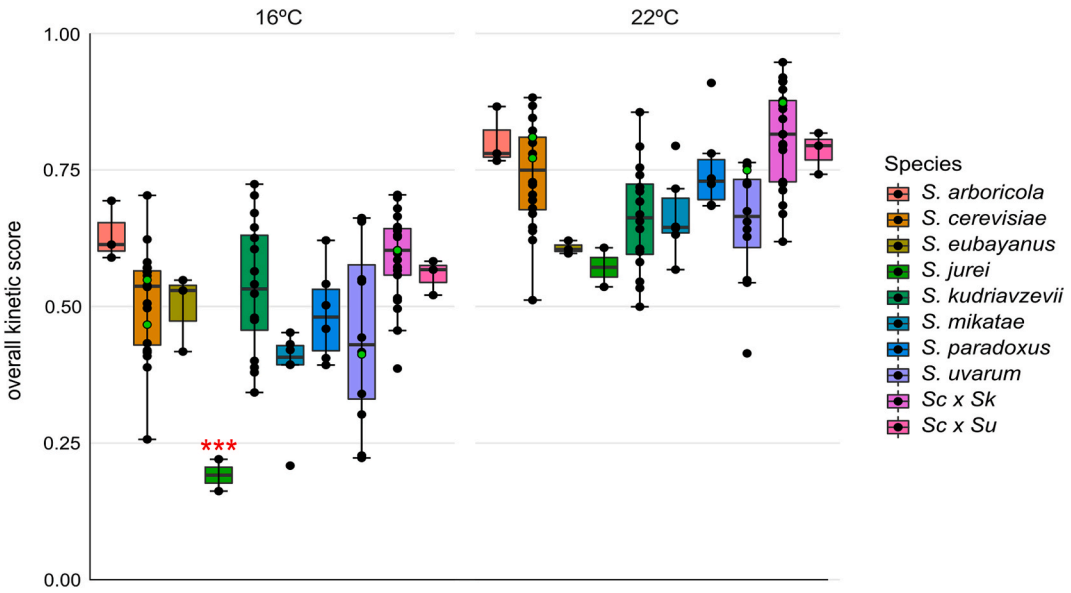

Fig. 2. Box plot showing the distribution of the overall kinetic score by the different species at each temperature. Commercial strains (*S. cerevisiae* EC1118 and DBVPG1106, *S. uvarum* BMV58 and *Sc x Sk* VIN7) are represented by green dots . Red asterisks on top of a box indicate a statistically significant difference (Dunnett's test) with respect to *S. cerevisiae*. Levels of significance: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

S. kudriavzevii, *S. arboricola*, *S. paradoxus* and *S. cerevisiae* displayed higher overall kinetic scores than commercial strains. For instance, SK06 and SA03 had a kinetic score 1.3-fold higher than the *S. cerevisiae* EC1118 at 16 °C (Fig. 3A). At species level, *S. jurei* showed the lowest values at both temperatures (Fig. 2). Although not statistically significant, both types of hybrids (*Sc x Sk* and *Sc x Su*), as well as *S. arboricola*, ranked higher than *S. cerevisiae* concerning this parameter at 16 and 22 °C.

Table S4 shows the average increment in the overall kinetic score for the different species with temperature. The species known to be cryotolerant (i.e. *S. kudriavzevii, S. eubayanus* and *S. uvarum*) were, as expected, less affected by the temperature decrease than other species. Surprisingly, *S. arboricola* was also found within this group, showing the highest kinetic score at 16 °C, thus suggesting a cryophilic character for this species as well. By contrast, the fermentation performance of *S. jurei* was greatly compromised at 16 °C.

Focusing on fermentations at 16 °C, lag times ranged from 16.9 (SK01) to 58 h (SJ01). *S. jurei* required more time than other species to start fermenting vigorously (Fig. S3), and displayed significantly lower values of R_{max} when compared to *S. cerevisiae*. On the contrary, three *S. uvarum* wine strains (SU08, SU09 and SU10), together with isolates of *S. kudriavzevii* (SK06 and SK08) and *S. arboricola* (SA03), showed R_{max} values 1.3-times higher than EC1118 at this temperature (Fig. 3A). Interspecific *Sc x Sk* hybrids showed an ability to keep fermenting at high rates during most of the stationary phase, as shown by their higher R_{60} values (Fig. S3). However, this phenotype was not specific to these hybrids, as some strains of *S. kudriavzevii* such as SK06 (Fig. 3A) displayed a similar capacity at low temperature. Fermentation times varied considerably, ranging from 208 (SCSK11) to 713 h (SU06). Many *Sc x Sk* hybrids were found among the fastest isolates, together with strains of *S. cerevisiae* and *S. kudriavzevii*.

Some tendencies observed at 16 °C were reproduced at 22 °C, such as the higher R_{60} of interspecific *Sc x Sk* hybrids with respect to *S. cerevisiae* (Fig. S3). Most *S. kudriavzevii* isolates, however, lost their superiority when fermenting at this temperature. The R_{60} value of some *Sc x Sk* strains exceeded the R_{max} value obtained with other strains, such as SK12, in the same conditions. Many of these hybrids performed a remarkably fast fermentation, requiring less than 180 h to achieve dryness, while others necessitated more than 500 h (e.g. SK12). The highest R_{max} values were observed for *Sc x Sk* strains, but also for *S. paradoxus* (SP02), *S. arboricola* (SA01), *S. uvarum* (SU11) and *S. cerevisiae* (several strains), all of them present within the top-ten concerning this parameter. Examples of the mentioned phenomena are shown in Fig. 3B.

Despite the tendencies mentioned above, intraspecific variation was substantial when a high number of strains were included for a species (e.g. *S. cerevisiae*, *S. kudriavzevii*, *S. uvarum* and *Sc x Sk*), unveiling a high diversity within the genus in terms of fermentative capacities (Fig. S3).

Interestingly, some strains isolated from natural environments, including SA03, SK06 and SC03, exhibited better fermentative capacities than commercial strains when fermenting in 200 g L^{-1} of initial sugars. However, sugar amounts up to 240 g L^{-1} are often present in natural grape juice. For this reason, we decided to investigate their behaviour in increasingly severe osmotic conditions (i.e. 200, 220 and 240 g L^{-1} of sugars). Fermentations were carried out at 16 °C using three replicates. The commercial strains *S. cerevisiae* EC1118 (SC18) and *Sc x Sk* VIN7 (SCSK05) were included as controls. All strains reached dryness when fermenting synthetic musts with 200 and 220 g L^{-1} sugar. However, at 240 g L^{-1}, *SA03* left more than 20 g L^{-1} of fructose when fermentation stopped. Concentrations above 220 g L^{-1} may therefore lead to stuck fermentations with this strain. SK06 did not consume all the sugars either, but the residual amount was much lower (5.7 g L^{-1}). After the exponential phase, the strain and sugar effects in fermentation kinetics were more evident (Fig. 3C and 3.D). For instance, SK06 and SCSK05 kept fermenting faster than the other strains during the stationary phase, showing an R_{60} value significantly higher than EC1118 at 200, 220 and 240 g L^{-1} of sugars (Fig. S5). Surprisingly, SK06 showed the highest R_{60} regardless of sugar availability, being 1.4-fold greater than EC1118. We can thus conclude that both SCSK05 and SK06 performed alcoholic fermentation more efficiently than the *S. cerevisiae* control strain for all conditions, as shown by their significantly higher overall kinetic score.

3.2. Production of central carbon metabolites

A high diversity was observed for the production of major fermentation metabolites (Fig. 4), with a significant effect of the species factor for most compounds measured. The temperature effect was also

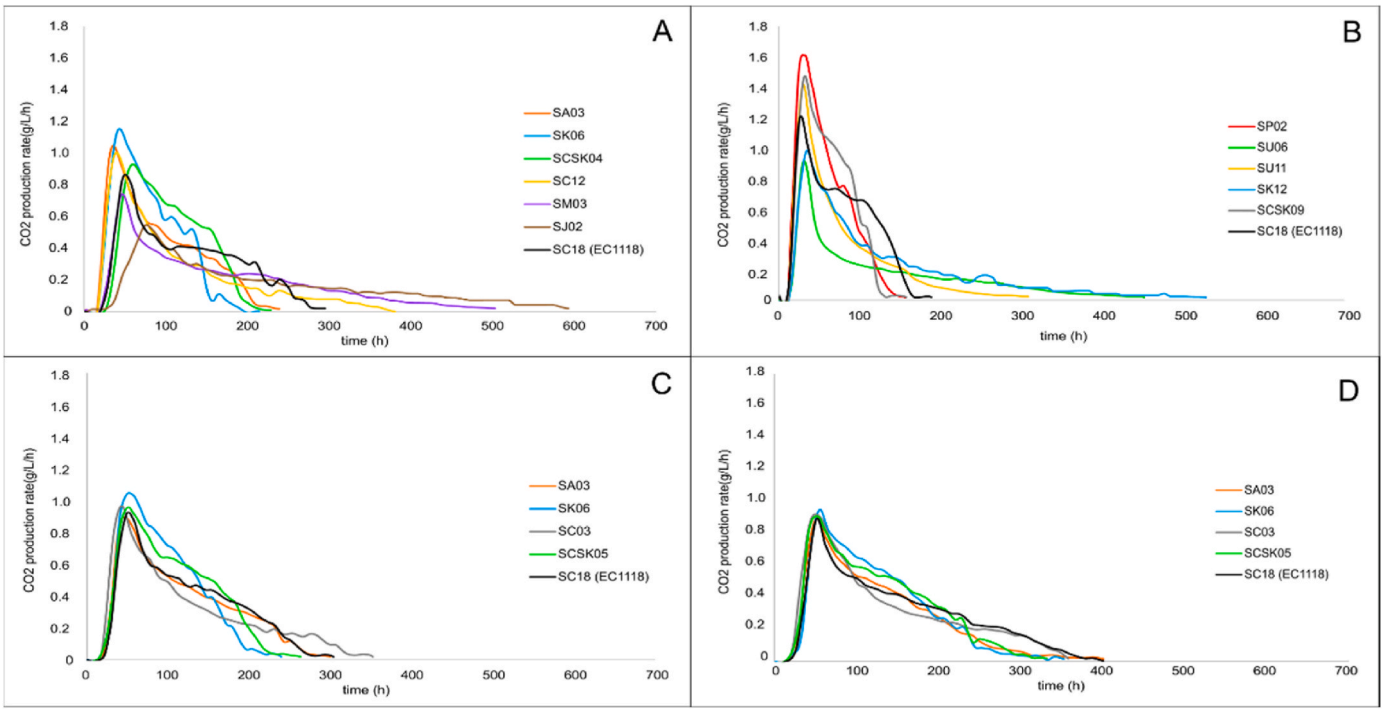

Fig. 3. Fermentation curves of different strains under different conditions. **A** and **B** illustrate the most dissimilar kinetic profiles at 16 and 22 °C, respectively. **C** and **D** represent curves generated using 220 and 240 g L^{-1} initial sugar, respectively. The control EC1118 was included in all graphs.

significant for ethanol, acetate and pyruvate.

Glycerol concentrations at 16 °C ranged from 5.5 to 10.3 g L^{-1}, *S. cerevisiae being* the species with the lowest capacity to secrete this compound. Indeed, four species had significantly higher glycerol yields compared to *S. cerevisiae* at this temperature, i.e. *S. arboricola*, *S. mikatae*, *S. jurei* and *S. eubayanus* (Fig. 4B). In the case of *S. jurei*, this high glycerol production was accompanied by a high production of acetic acid (Fig. 4C). Conversely, *S. mikatae* presented similarly high glycerol yields but produced the lowest amounts of acetic acid of all species, being the only one with a significantly lower acetate yield compared to *S. cerevisiae*. In fact, four of the six *S. mikatae* strains produced amounts of this compound below the limit of quantification of our method, together with one strain of *S. paradoxus* and one *Sc x Su* hybrid. Some *S. uvarum* strains produced remarkably high concentrations of succinic acid, up to 12.6 g L^{-1}. However, variation was quite high within this species (Fig. 4D). Two *S. cerevisiae* outliers (SC01 and SC02), both isolated from sake fermentation, also secreted particularly high amounts of succinic acid.

As ethanol depends directly on sugar consumption, we compared its production only between strains leaving less than 4 g L^{-1} of residual sugars. The species effect on ethanol production was not significant (Fig. 4A), while the temperature effect was the most striking. In fact, ethanol yield was reduced at 16 °C in 65 out of the 69 strains that completed fermentation at both temperatures (Fig. S6). This could be a consequence of redirecting carbon fluxes towards other cellular processes in order to cope with stress. Six strains (i.e. SP03, SP05, SC06, SC16, SCSU02 and SJ01) achieved ethanol reductions of more than 10 g L^{-1} when fermenting at 16 °C. For the rest of the main fermentation metabolites, the effect of temperature was strain-dependent, and most isolates produced similar amounts under both conditions.

To better understand the carbon fluxes of strains with extreme CCM profiles, carbon balances were performed at 16 °C using five selected isolates (Fig. 5). The *S. cerevisiae* strain EC1118 was used as a control, and strains showing high glycerol (SJ01), low acetate (SM02) and high succinate (SU01) yields in the first screening were included, as well as a strain with an intermediate production of these compounds (SCSK01). SJ01, SM02 and SU01 produced high glycerol, low acetate and high succinate amounts, respectively, confirming the results of the screening. Additionally, we observed that EC1118 produced higher biomass and less fermentation subproducts than any other strain, showing a more efficient metabolism to support growth. SCSK01 showed a similar profile but produced less biomass, more glycerol and more succinate than EC1118. SU01 used significantly less carbon than any strain for ethanol and CO_2 production, while the total amount of the other subproducts, excluding succinate, was similar to the other isolates. Therefore, SU01 may have used carbon for succinate overproduction at the expense of CO_2 and ethanol. Surprisingly, this strain directed a larger amount of carbon towards succinate production than towards glycerol production. SM02 and SJ01 were the strains using the most carbon for glycerol but differed in their production of other metabolites. For instance, SJ01 generated more acetic acid and 2,3-butanediol, while SM02 produced low amounts of these compounds and more succinate.

3.3. Production of fermentative aromas

Overall, only minor variations in the formation of volatile compounds were observed between fermentations conducted at 16 and 22 °C, with a low temperature effect on these parameters apart from a few exceptions. Strains of the same species generated relatively similar aroma profiles under the same conditions, forming clusters (Fig. 6A). The size of these clusters was a good indicator of the intraspecific variation. This figure also illustrates how the diversity in the aroma profile found for *S. cerevisiae* was extended when using alternative species. Interestingly, aromas generated from the same metabolic precursor tended to cluster in the variables chart (Fig. 6B). The already mentioned differentiation at species level could therefore be explained by differences in specific metabolic pathways activity.

3.3.1. Ehrlich pathway derivatives

We found an important variation at species and strain levels regarding the production of volatile compounds deriving from the Ehrlich pathway. For instance, *S. uvarum* and *S. eubayanus* synthesized

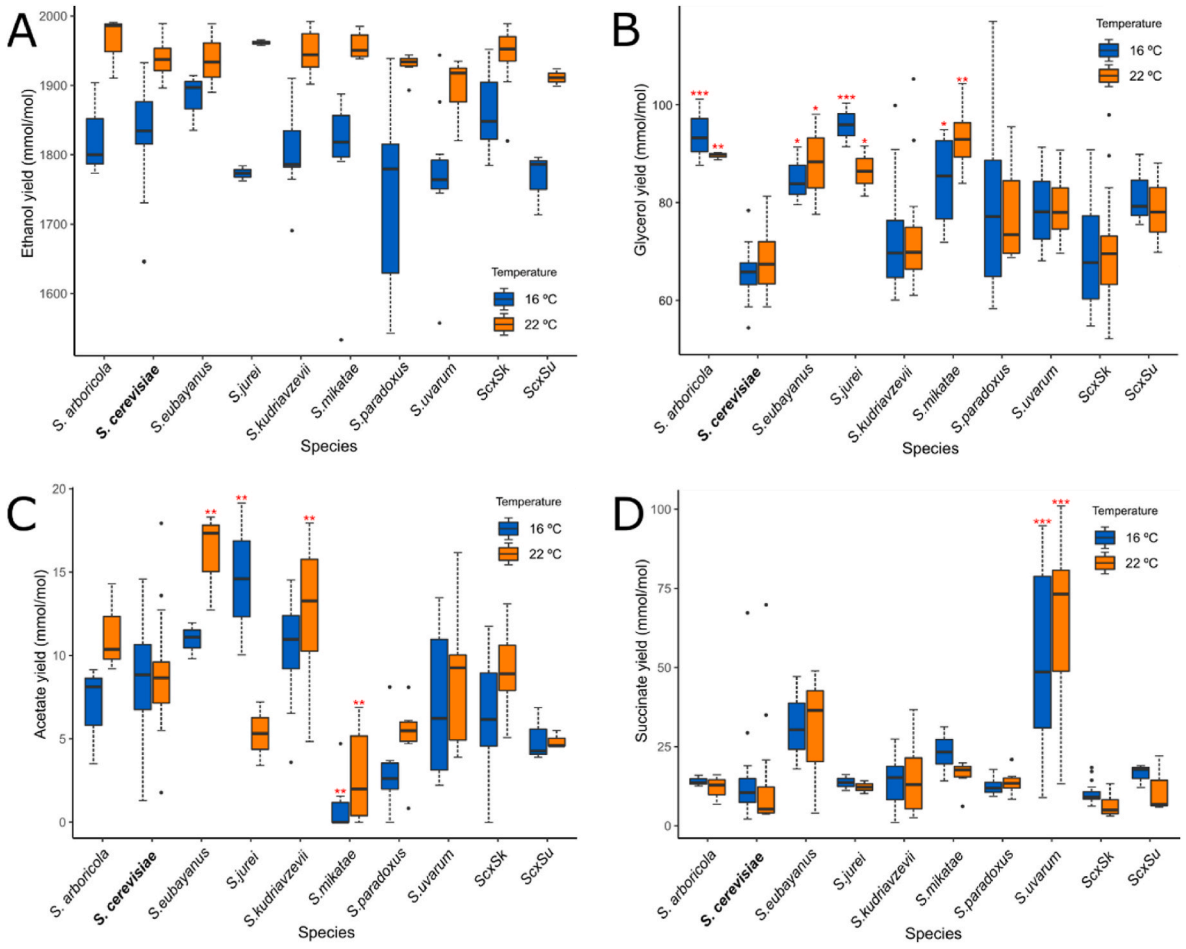

Fig. 4. Box plots representing the concentrations of the main fermentation metabolites at 16 and 22 °C by the different species, expressed in mmol of product per mol of sugar consumed. **A**: Ethanol yield. **B**: Glycerol yield. **C**: Acetate yield. **D**: Succinate yield. Asterisks on top of a box indicate a statistically significant difference (Dunnett's multiple comparison test) with respect to *S. cerevisiae*. Levels of significance: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

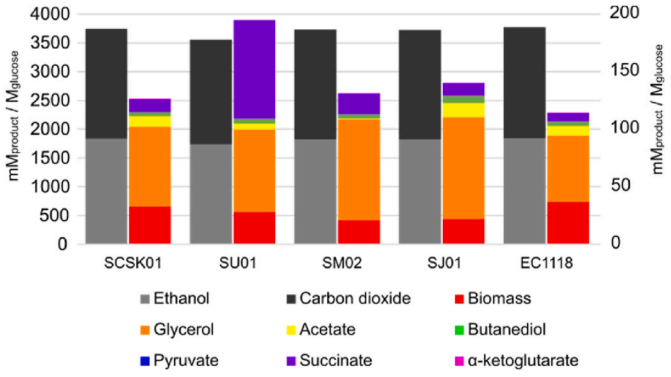

Fig. 5. Summary of the carbon balance performed for four selected strains and the EC1118 control. The main fermentation products (i.e. ethanol and CO_2) are shown in each of the left bars, referring to the left axis. The right bars (referring to the right axis) represent biomass and the main subproducts. All amounts are shown as the quantity of carbon (in mmol·L^{-1}) used for each product per mol·L^{-1} of consumed carbon (from sugar).

higher amounts of the two compounds derived from phenylpyruvate metabolism, i.e. 2-phenylethanol and 2-phenylethyl acetate (Fig. 7A and S9). However, the maximum concentrations of 2-phenylethanol were attained using two *S. cerevisiae* strains isolated from sake fermentation (SC01 and SC02), reaching 190.8 and 242.6 mg L^{-1}. On the contrary, the two *S. jurei* strains exhibited a deficient production of 2-phenylethanol at both temperatures, barely achieving 50 mg L^{-1}.

S. mikatae was the highest producer of 3-methylbutyl acetate, reaching amounts 5-fold higher than EC1118 (Fig. 7B and S10). By contrast, the highest concentrations of 3-methylbutanoic acid were achieved using *S. eubayanus*. This species and *S. jurei* were significantly superior to *S. cerevisiae* in producing 2-methylbutanoic acid. Regarding compounds derived from α-amino butyrate metabolism (Fig. S12), *S. kudriavzevii* reached the highest amounts of propanol at both temperatures, while ethyl propanoate was overproduced by *S. mikatae* (Fig. 7C). The maximum producer of this compound was SM02, reaching concentrations 10 times higher than EC1118 at 16 °C. The intraspecific variation displayed by *S. mikatae* for some aroma compounds was remarkable compared to other species despite the low number of isolates included (Fig. 7B and 7.C). Remarkably, *S. uvarum* produced the highest amounts of the four volatile aromas deriving from α-ketoisovalerate, i.e. 2-methylpropanol, 2-methylpropyl acetate, 2-methylpropanoic acid and ethyl isobutanoate (Fig. 7D and S11).

3.3.2. *Short- and medium-chain fatty acids and their ethyl esters*

In yeasts, all linear short- and medium-chain fatty acids have a common precursor, acetyl-CoA, which is used to sequentially elongate the lateral chain by 2 carbon units. For a more straightforward comparison of the production of short- and medium-chain fatty acids and their ethyl esters across the 92 *Saccharomyces* isolates, the equivalents in coenzyme A (CoA) used for the production of each of these molecules were calculated. Octanoic acid and ethyl hexanoate were respectively the fatty acid and ethyl ester preferentially produced by most species

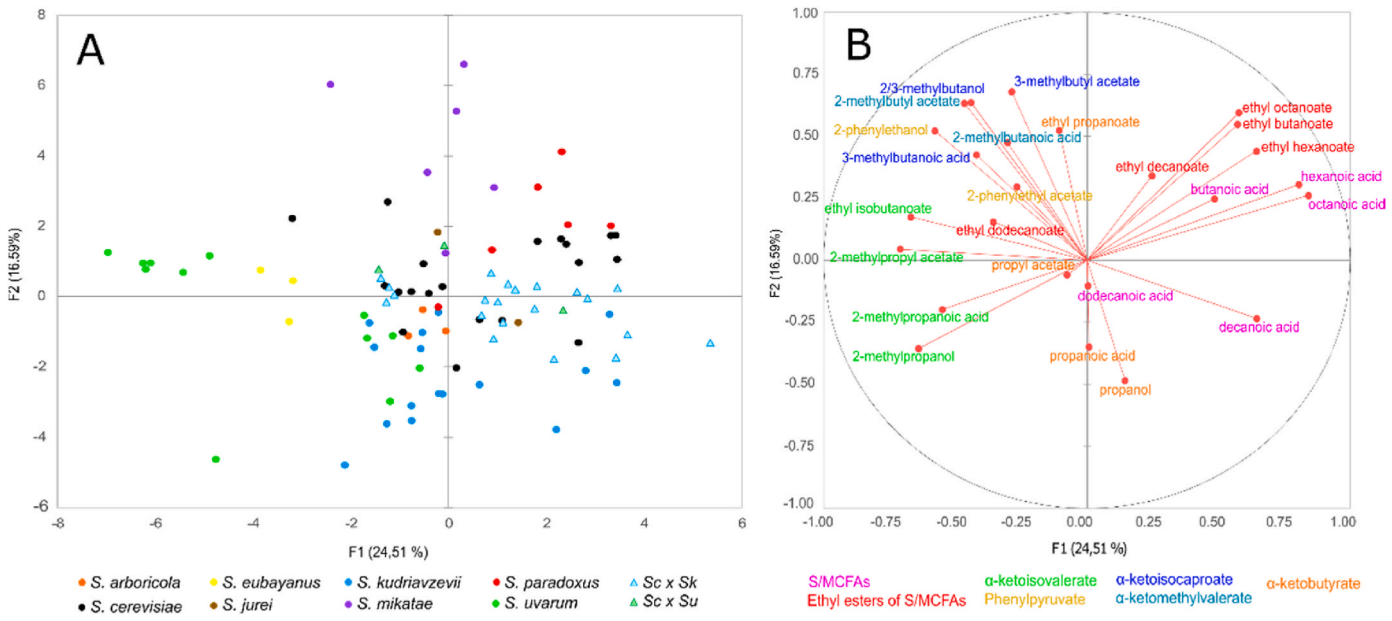

Fig. 6. PCA (Principal Component Analysis) of fermentative aroma production at 22 °C. **A**: Observations chart. Strains are coloured based on their species. **B**: Variables chart. Compounds are coloured based on their metabolic pathway or precursor. Aromas not originating from the Ehrlich pathway (i.e. short and medium-chain fatty acids or S/MCFAs and their ethyl ester derivatives) are shown in pink and red, respectively. The other aromas are coloured based on the α-keto acid associated to its metabolic pathway, i.e. α-ketoisovalerate, phenylpyruvate, α-ketoisocaproate, α-ketomethylvalerate and α-amino-butyrate. 2/3-methylbutanol represents 2- and 3-methylbutanol, which co-eluted and therefore were quantified together. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 8). However, *S. eubayanus* and *S. uvarum* used less CoA for short-chain fatty acid production (C4 – C8) than the other species while generating higher amounts of dodecanoic acid (Fig. 8A). At species level (Fig. 8B), *S. cerevisiae* and *S. eubayanus* produced the lower amounts of ethyl esters. Conversely, *S. uvarum* esterified high amounts of dodecanoic acid, secreting high quantities of ethyl dodecanoate (Fig. 8B). Some strains of this species produced 10 times more ethyl dodecanoate than the control EC1118 (Fig. S8). With a few exceptions, the esterification percentage was lower than 40%, and it was generally higher for higher chain lengths (Fig. 8C). *S. arboricola*, despite producing low amounts of hexanoic acid, excreted high amounts of ethyl hexanoate into the medium. This indicates a high efficiency of hexanoic acid esterification inside the cell. A similar phenomenon was observed for *S. mikatae* and the C10 and C12 ethyl esters.

4. Discussion

The industry of fermented beverages constantly endeavours to diversify its range of products to adapt to a rapidly changing world in terms of consumer demands while facing environmental challenges. The yeast strain used during alcoholic fermentation is one of the main tools available to tackle these problems, as it is a key factor influencing the chemical composition of wine and, therefore, its organoleptic profile. As the currently available set of commercial strains, mainly consisting of *S. cerevisiae*, has proved insufficient to tackle these problems (Molinet and Cubillos 2020), the discovery or development of novel yeasts with sufficient fermentative power and distinct metabolic properties has become critical.

S. cerevisiae is widely considered the best yeast species for alcoholic fermentation, thanks to its generally high fermentative power under oenological conditions. However, in recent years, there has been a growing interest in alternative species, aiming to better exploit the existing diversity to tackle issues such as aromatic profile diversification or the demand for low-alcohol wines. Recent studies have reported satisfying fermentative behaviours in other species of *Saccharomyces*, i.e. *S. uvarum* (McCarthy et al., 2021), *S. paradoxus* (Orlić et al., 2009), *S. eubayanus* (Parpinello et al., 2020) and *S. kudriavzevii* (Pérez et al. 2021). This context led us to address a tricky question: is it possible to replace *S. cerevisiae* in wine fermentation?

To answer that question, we carried out a systematic study of the fermentation and metabolic behavior of 92 strains of *Saccharomyces* during laboratory scale fermentations in synthetic must at two different temperatures.

Determination of kinetic parameters showed that strains belonging to all the *Saccharomyces* species, including the less studied *S. arboricola*, *S. mikatae* and *S. jurei*, successfully completed wine fermentation in the presence of 200 g L^{-1} sugars. Remarkably, strains of *S. kudriavzevii*, *S. arboricola* and *Sc x Sk* hybrids outperformed the *S. cerevisiae* control EC1118 at 16 and 22 °C. Those results suggest that the whole *Saccharomyces* genus, and not only *S. cerevisiae*, has promising potential for the development of yeast starter cultures.

The phenotypic and genetic diversity of *S. cerevisiae* has been widely studied during the last 15 years. It was shown that *S. cerevisiae* strains display substantial phenotypic variations, some of which are correlated with ecological niches (Camarasa et al., 2011; Warringer et al., 2011). *S. cerevisiae* wine strains, which form a separate cluster (Legras et al., 2018; Peter et al., 2018), have accumulated hallmarks of domestication (reviewed in Marsit and Dequin, 2015). These strains possess phenotypic traits conferring competitive advantages against other yeasts during alcoholic fermentation, such as copper (Almeida et al., 2015) or sulphite (Pérez-Ortín et al., 2002; García-Ríos et al., 2019) tolerance. Different studies showed that strains of *S. cerevisiae* isolated from winemaking environments are better adapted to ferment grape must at high rates (Camarasa et al., 2011; Pérez et al., 2021). However, other sugar-rich environments, such as fruits, are also a source of efficiently fermenting yeasts (Camarasa et al., 2011). In our study, most wine isolates displayed outstanding fermentative capacities. However, strains isolated from fruits (SC03) but also from trees (SA03, SK06, SK08) and soil (SP02) performed alcoholic fermentation more efficiently than *S. cerevisiae* wine strains. Remarkably, strains of *S. kudriavzevii, S. arboricola* and *Sc x Sk* hybrids outperformed commercial *S. cerevisiae* strains at 16 and 22 °C. Some isolates were further characterized in comparison to

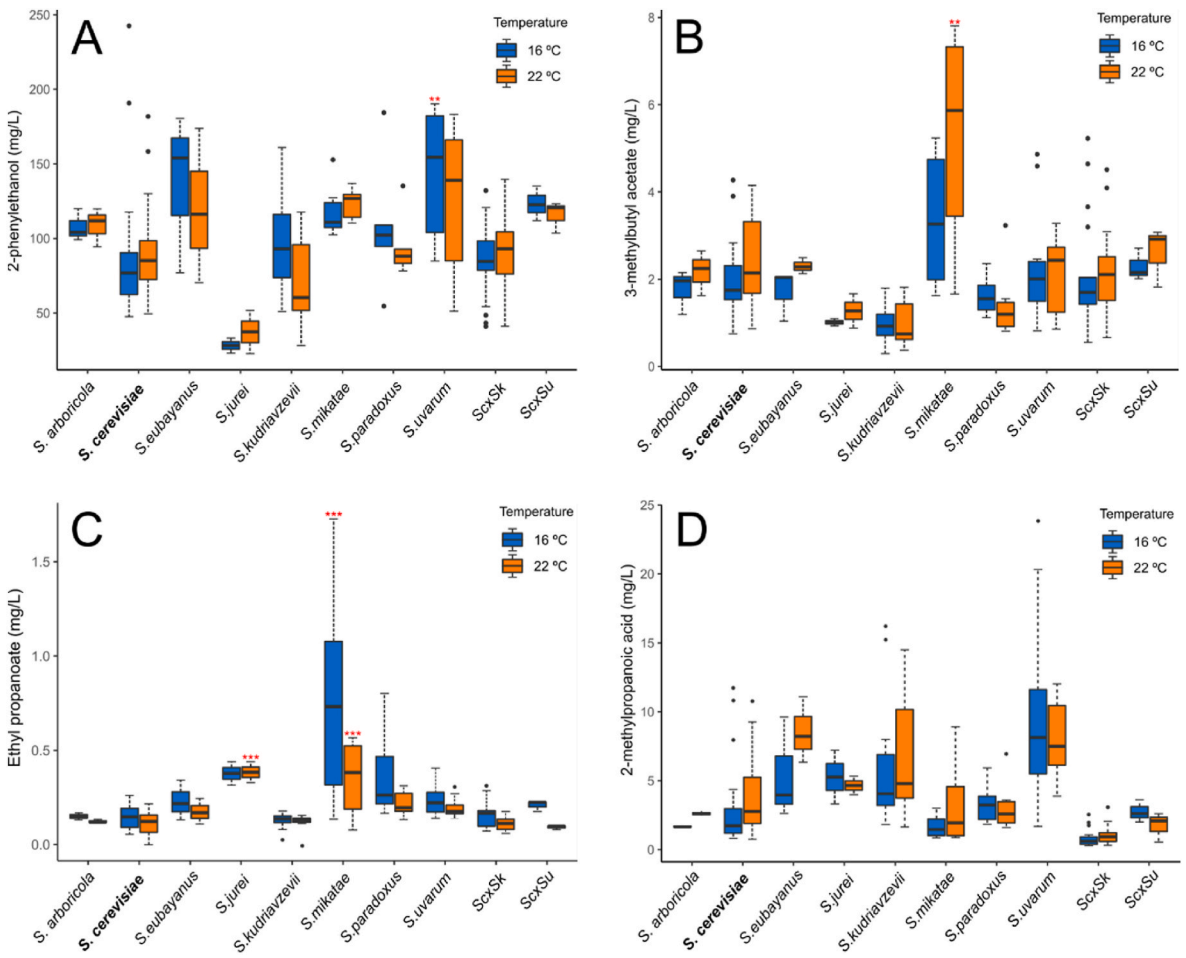

Fig. 7. Box plots of some Ehrlich pathway derivatives by the different species at each temperature. Asterisks on top of a box indicate a statistically significant difference (Dunnett's multiple comparison test) with respect to *S. cerevisiae*. Levels of significance: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). **A**: 2-phenylethanol. **B**: 3-methylbutyl acetate. **C**: ethyl propanoate. **D**: 2-methylpropanoic acid.

commercial strains in high-sugar synthetic musts (up to 240 g L^{-1}). Among them, SA03 displayed a stuck profile in the presence of 240 g L^{-1} of sugars. On the contrary, SC03 left an amount of residual sugars similar to EC1118 but required 50 h less to finish the fermentation. More surprisingly, SK06 showed an overall kinetic score significantly higher than EC1118 but similar to the interspecific hybrid VIN7 (named here SCSK05), two strains regularly used in the industry. Those findings are of high technological interest as some musts contain increasing amounts of sugar due to the effect of climate change on grape composition.

We also assessed the diversity within the *Saccharomyces* genus regarding metabolite production. To such end, we determined the capacity of each strain to produce compounds of the central carbon metabolism and fermentative aromas during wine fermentation.

Overall, the metabolic diversity within non-*cerevisiae Saccharomyces* was far beyond that of *S. cerevisiae* alone. For instance, *S. arboricola*, *S. mikatae*, *S. jurei* and *S. eubayanus* produced significantly higher amounts of glycerol than *S. cerevisiae*, which was the lowest glycerol producer of all species. In line with our results, recent studies have shown that other *Saccharomyces* species (*S. kudriavzevii*, *S. eubayanus* and *S. uvarum*) produce higher levels of this compound than *S. cerevisiae* (Pérez-Torrado et al., 2018; Pérez et al., 2021). Glycerol has been shown to facilitate yeast adaptation to low temperatures (Tulha et al., 2010). Therefore, the high glycerol production observed in this study could be related to cryotolerance.

Although we found two *S. cerevisiae* outliers producing high levels of succinate, *S. uvarum* displayed a much higher diversity in the production of this compound. Similarly, species such as *S. mikatae*, *S. paradoxus*, *S. kudriavzevii* or *S. uvarum* showed a substantially higher intraspecific diversity in the production of several aroma compounds compared to *S. cerevisiae*. These findings, together with the high interspecific variation for those and other traits, indicate a high potential of alternative *Saccharomyces* species for the improvement of wine quality and for achieving product diversification in terms of sensory attributes.

In line with the interspecies phenotypic variability, we observed that some species differ in their metabolism management during wine fermentation. For example, *S. paradoxus* is characterised by a lower acetate production than six of the other species. More interestingly, fermentations using *S. mikatae* led to non-detectable amounts of acetic acid at 16 °C. We can hypothesize that, in these species, acetic acid is either produced in very low amounts or consumed for other reactions, such as the formation of acetyl-CoA, a metabolic precursor in both lipids biosynthesis and formation of volatile compounds such as acetate esters by enzymatic esterification of higher alcohols. Thus, a more efficient conversion of acetate to acetyl-CoA could also explain the overproduction of some esters observed for *S. mikatae*. In agreement with this observation, Minebois et al. (2020) reported a similar trait for a *S. uvarum* strain that, after entering stationary phase, consumed the acetic acid previously produced during the growth phase. They hypothesized that this strain has a higher carbon flux towards acetyl-CoA for fatty acid biosynthesis, helping to remodel cell membranes as a mechanism to increase ethanol resistance in strains not adapted to alcoholic fermentation. This could also be the case for the *S. paradoxus* and *S. mikatae* strains studied here. In fact, we also found that *S. paradoxus* overproduced the C4, C6 and C8 fatty acids and their ethyl

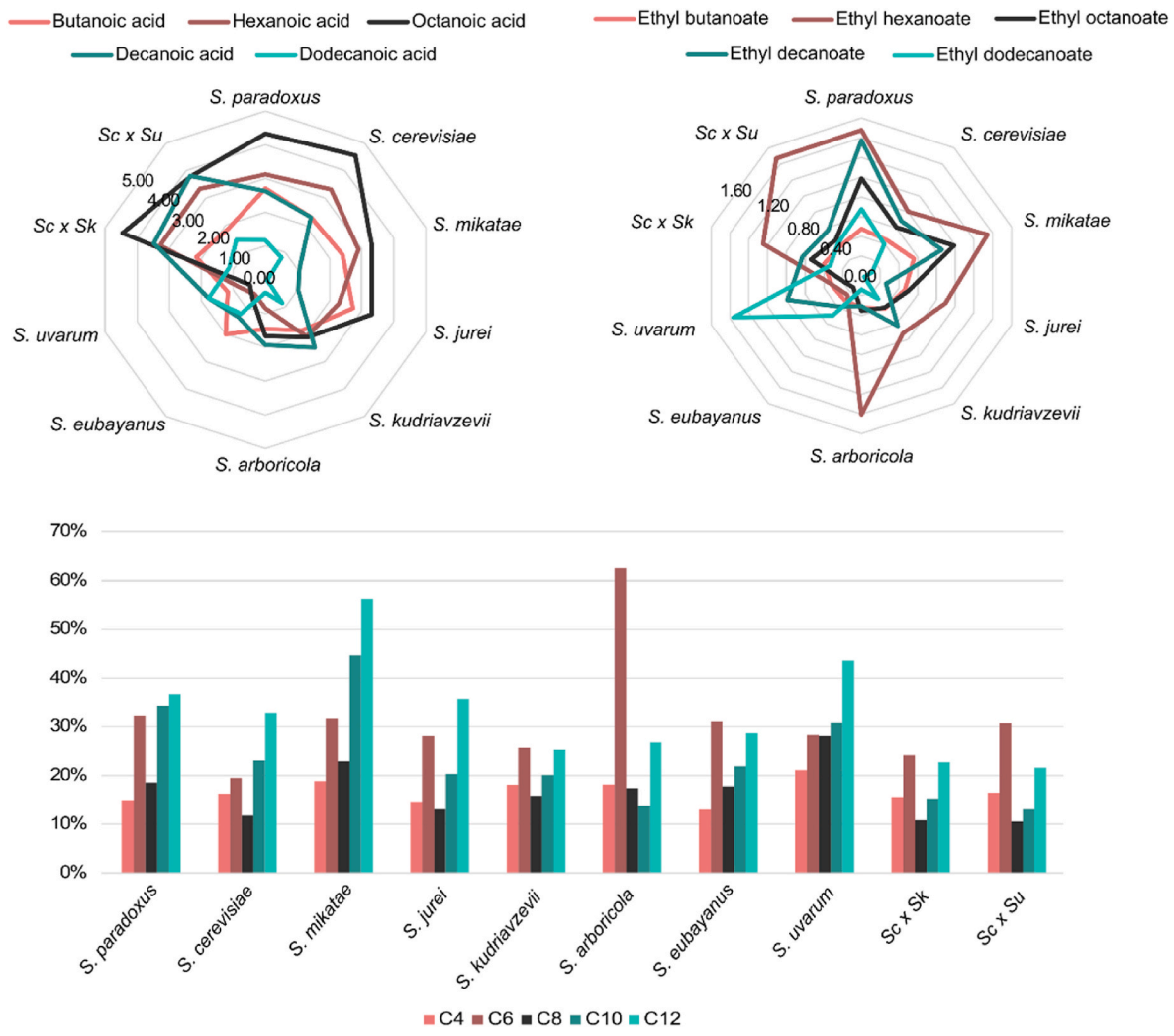

Fig. 8. Representation of the amounts of S/MCFAs (**A**) and their ethyl esters (**B**) secreted into the medium by each species, shown as the equivalent in CoA used for the production of each molecule, in mmol·L^{-1}. CoA equivalents were calculated from the concentration of a given compound, by dividing with the number of acetyl-CoA molecules used for its production (i.e. the number of carbon atoms in its chain divided by two). Subfigure **C** shows the proportion of each ester relative to its fatty acid counterpart, for each chain length (C4 – C12). All graphs correspond to fermentations at 22 °C.

esters, supporting this hypothesis.

S. uvarum was characterized by an enrichment in C10 and C12 fatty acids and their ethyl esters compared to their C4, C6 and C8 counterparts. Moreover, a high production of the four volatile aromas associated with α-ketoisovalerate metabolism was also observed for this species. In *S. cerevisiae*, it has been shown that the intracellular availability of acetyl-CoA can influence carbon flux distribution, especially the conversion of α-ketoisovalerate to α-ketoisocaproate (Rollero et al., 2017). Together, these findings suggest that, in *S. uvarum*, high amounts of acetate are required for the production of fatty acids via acetyl-CoA, limiting its availability for other metabolic pathways. The low levels of short-chain fatty acids could be explained by their incorporation into cell membranes. Tronchoni et al. (2012) reported a higher proportion of short- and medium-chain fatty acids in *S. kudriavzevii* membranes compared to *S. cerevisiae*, possibly contributing to membrane fluidity and ethanol tolerance. *S. uvarum*, being also a cryotolerant species, could display a similar mechanism. Furthermore, we observed a high succinic acid production by some *S. uvarum* strains. A high succinic acid production is, to some extent, a desirable trait in wine yeasts as it reduces pH and mitigates the loss of total acidity resulting from bitartrate precipitation. During wine fermentation, it can be produced by yeast from the reductive or oxidative branches of the tricarboxylic acid cycle (Camarasa et al., 2003). The high succinic acid production by *S. uvarum* observed in our study had been reported before (Minebois et al., 2020; Coral-Medina et al., 2022). The production of high levels of succinic acid is probably a mechanism to maintain redox balances, as it can serve as the final acceptor for reducing equivalents from NADH. However, other metabolites such as 2-phenylethanol from the shikimate pathway and erythritol from the pentose phosphate pathway – although being produced in much lower amounts than succinic acid – have been proposed as mechanisms to such end in *S. uvarum* (Minebois et al., 2020). In our study, a high production of 2-phenylethanol, 2-phenylethyl acetate and 2-methylpropanol was observed for various *S. uvarum* strains, supporting this hypothesis.

S. mikatae and *S. jurei*, two closely related species, showed nevertheless remarkable differences in central carbon metabolite production. If both species generated similarly high amounts of glycerol, *S. jurei*, however, produced more acetic acid and butanediol, while *S. mikatae* generated low amounts of both compounds and higher amounts of succinate. A possible explanation for this difference is that both species produce high intracellular acetate through the fermentative pathway, but, while *S. jurei* directly excretes this compound as a by-product, *S. mikatae* redirects most of the acetate produced towards the production of acetyl-CoA, feeding the TCA and resulting in a higher succinic acid production. This hypothesis is in line with the high esterification of C10 and C12 fatty acids and the high production of 3-methylbutyl

acetate observed for *S. mikatae*, as both processes require acetyl-CoA. Both species are much less characterized at a physiological level during wine fermentation, and their carbon fluxes during this process are little known. Therefore, it would be interesting to apply metabolic flux analysis using isotope-labelled substrates to better characterize the metabolic pathways towards which acetate is redirected in these species.

Further work is required to determine to what extent the observed phenotypic diversity is correlated with genetic diversity. Previous studies have revealed allelic variants of genes involved in the synthesis of aroma compounds in *S. uvarum* and *S. kudriavzevii,* conferring differences in enzymatic activities and possibly leading to aroma enhancement (Stribny et al., 2016a, 2016b; Tapia et al., 2022). Future research should focus on identifying more allelic variants of genes either directly or indirectly related to aroma formation pathways. A promising approach to this end is the generation of fertile *Saccharomyces* interspecific hybrids for QTL mapping (Naseeb et al., 2021).

5. Conclusions and perspectives

In conclusion, this study revealed that under the conditions tested and despite a high strain variability, all the *Saccharomyces* species showed adequate fermentative power. Remarkably, some strains of alternative species performed alcoholic fermentation more efficiently than wine *S. cerevisiae* strains. Further characterization in conditions closer to the industrial reality, where the stresses encountered are higher in number and intensity, is needed to confirm their industrial potential. This includes fermentations in natural grape must, non-aseptic conditions and larger volumes, and the assessment of tolerance to stresses such as copper or sulfites. The other major outcome of this study is the observation of striking interspecific variation in terms of metabolite production and of much higher diversity when using species alternative to *S. cerevisiae*. Different species have shown different ways to manage their metabolism in wine fermentation, resulting in significant differences in producing industrially relevant compounds. We believe that those specificities have the potential to improve wine quality and solve some of the challenges faced by the wine industry today. This study provides a solid basis for better exploitation of the *Saccharomyces* diversity, opening the way to the identification of strains with new phenotypic traits and, ultimately, to the diversification of yeast starters currently available.

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Declaration of competing interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2023.104270.

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Table S1. List of strains used in this study.

Code used	Species	Strain	Country	Origin
SA01	*S. arboricola*	H-6 (T)	China	Tree
SA02	*S. arboricola*	ZX-15	China	Tree
SA03	*S. arboricola*	ZX-20	China	Tree
SK01	*S. kudriavzevii*	IFO 10990	Japan	Tree
SK02	*S. kudriavzevii*	IFO 10991	Japan	Tree
SK03	*S. kudriavzevii*	DBVPG6667	Unknown	Unknown
SK04	*S. kudriavzevii*	IFO 1802^{T}	Japan	Unknown
SK05	*S. kudriavzevii*	IFO 1803	Japan	Tree
SK06	*S. kudriavzevii*	ZP 542	Portugal	Tree
SK07	*S. kudriavzevii*	ZP 594	Portugal	Tree
SK08	*S. kudriavzevii*	ZP 629	Portugal	Tree
SCSK01	*Sc x Sk*	PB7	Spain	Wine
SK09	*S. kudriavzevii*	CR85	Spain	Tree
SK10	*S. kudriavzevii*	CA111	Spain	Tree
SK11	*S. kudriavzevii*	48BYC-4	China	Tree
SK12	*S. kudriavzevii*	JLFM8	China	Tree
SU01	*S. uvarum*	DBVPG6299	Spain	Insect
SU02	*S. uvarum*	L-1764	Chile	Unknown
SU03	*S. uvarum*	ZP 555	Canada	Tree
SU04	*S. uvarum*	ZP 556	Canada	Tree
SU05	*S. uvarum*	A4	New Zealand	Wine
SU06	*S. uvarum*	VKMY508	Czech Republic	Wine
SU07	*S. uvarum*	UWOPS99-807.1.1	Argentina	Tree
SU08	*S. uvarum*	A1var	New Zealand	Wine
SU09	*S. uvarum*	A4var	New Zealand	Wine
SU10	*S. uvarum*	A9var	New Zealand	Wine
SE01	*S. eubayanus*	CBS 12357 (T)	Argentina	Tree
SE02	*S. eubayanus*	LZSP32.1	China	Unknown
SE03	*S. eubayanus*	CDFM212.1	China	Unknown
SP01	*S. paradoxus*	UFRJ50791	Brazil	Insect
SP02	*S. paradoxus*	DBVPG6466	Denmark	Soil
SP03	*S. paradoxus*	YPS138	United States	Soil
SP04	*S. paradoxus*	UWOPS91-917.1	United States	Tree
SP05	*S. paradoxus*	N-43	Russia	Tree
SP06	*S. paradoxus*	Q74.4	United Kingdom	Unknown
SM01	*S. mikatae*	NBRC 10994	Japan	Tree
SM02	*S. mikatae*	NBRC 10998	Japan	Tree
SM03	*S. mikatae*	LSYS65-1	China	Tree
SM04	*S. mikatae*	CHSZ5L-2	China	Fruit
SM05	*S. mikatae*	IFO1815 (T)	Japan	Soil
SM06	*S. mikatae*	IFO1816	Japan	Unknown
SC01	*S. cerevisiae*	RIB6003	Japan	Sake
SC02	*S. cerevisiae*	RIB6004	Japan	Sake
SC03	*S. cerevisiae*	DBVPG6765	Indonesia	Fruit
SC04	*S. cerevisiae*	DBVPG6044	Unknown	Wine
SC05	*S. cerevisiae*	YPS128	United States	Soil
SC06	*S. cerevisiae*	Y12663	Unknown	Wine
SC07	*S. cerevisiae*	UWOPS03-461.4	Malaysia	Tree
SC08	*S. cerevisiae*	R13_A5	France	Fruit
SC09	*S. cerevisiae*	UCD2120	United States	Wine
SC10	*S. cerevisiae*	DBVPG6040	Netherlands	Fruit
SC11	*S. cerevisiae*	DBVPG6254	Unknown	Wine
SC12	*S. cerevisiae*	DBVPG3051	Israel	Wine
SC13	*S. cerevisiae*	DBVPG1849	Ethiopia	Wine
SC14	*S. cerevisiae*	DBVPG6696	Burundi	Wine
SC15	*S. cerevisiae*	DBVPG6295	South Africa	Wine
SC16	*S. cerevisiae*	DBVPG1378	Italy	Wine
SCSK02	*Sc x Sk*	SOY3	Croatia	Wine
SK13	*S. kudriavzevii*	IFO 1802 (G)	None	None
SCSK03	*Sc x Sk*	Eg8	France	Wine
SCSK04	*Sc x Sk*	ALS 268	None	None
SCSK05	*Sc x Sk*	VIN 7	South Africa	Wine
SK14	*S. kudriavzevii*	CLIB 1504	Unknown	Unknown
SK15	*S. kudriavzevii*	CLIB 1503	Unknown	Unknown
SK16	*S. kudriavzevii*	ARD6.1	France	Tree
SCSK06	*Sc x Sk*	H10418	Hungary	Wine
SCSK07	*Sc x Sk*	Evo AD	None	None

Table S1 (cont.)

Code used	Species	Strain	Country	Origin
SCSK08	*Sc x Sk*	H10423	Hungary	Wine
SCSK09	*Sc x Sk*	Eg6	France	Wine
SC17	*S. cerevisiae*	DBVPG1106	Australia	Fruit
SC18	*S. cerevisiae*	EC1118	France	Champagne
SU11	*S. uvarum*	BMV58	Spain	Unknown
SC19	*S. cerevisiae*	L-1374	Chile	Wine
SC20	*S. cerevisiae*	WE372	Unknown	Wine
SU12	*S. uvarum*	CLIB 251	Netherlands	Fruit
SCSK10	*Sc x Sk*	Eg8/93	France	Wine
SCSK11	*Sc x Sk*	Eg8/136	France	Wine
SCSK12	*Sc x Sk*	AM511	None	None
SCSK13	*Sc x Sk*	AM512	None	None
SCSK14	*Sc x Sk*	AM513	None	None
SCSK15	*Sc x Sk*	AM514	None	None
SCSK16	*Sc x Sk*	AM515	None	None
SCSK17	*Sc x Sk*	AM516	None	None
SCSK18	*Sc x Sk*	AM517	None	None
SCSK19	*Sc x Sk*	AM518	None	None
SCSK20	*Sc x Sk*	AM519	None	None
SCSK21	*Sc x Sk*	AM520	None	None
SCSU01	*Sc x Su*	H51	None	None
SCSU02	*Sc x Su*	H105	None	None
SCSU03	*Sc x Su*	H159	None	None
SJ01	*S. jurei*	D5088 (T)	France	Tree
SJ02	*S. jurei*	France	None	Tree

Table S2. List of strains that did not complete fermentation at either temperature and amounts of residual sugars (RS). Amounts of RS higher than 4 g/L are highlighted in bold font.

Strain	Species	RS (g/L) at 16 ºC	RS (g/L) at 22 ºC
SK02	*S. kudriavzevii*	0.53	**4.95**
SK05	*S. kudriavzevii*	0.47	**5.12**
SK09	*S. kudriavzevii*	1.01	**4.37**
SK10	*S. kudriavzevii*	0.30	**6.29**
SU01	*S. uvarum*	1.57	**17.26**
SU02	*S. uvarum*	**22.62**	**44.35**
SU03	*S. uvarum*	**5.54**	**7.73**
SU04	*S. uvarum*	0.70	**15.27**
SU06	*S. uvarum*	2.13	**43.99**
SU07	*S. uvarum*	0.00	**7.13**
SE01	*S. eubayanus*	3.72	**14.64**
SE02	*S. eubayanus*	**6.89**	**35.49**
SE03	*S. eubayanus*	0.35	**30.08**
SM01	*S. mikatae*	1.87	**11.41**
SM04	*S. mikatae*	**5.81**	1.44
SM06	*S. mikatae*	0.79	**7.35**
SC05	*S. cerevisiae*	**27.26**	**10.50**
SC10	*S. cerevisiae*	**8.42**	0.48
SC13	*S. cerevisiae*	0.63	**4.65**
SC14	*S. cerevisiae*	**6.58**	0.56
SK14	*S. kudriavzevii*	**4.27**	3.80
SU11	*S. uvarum*	1.29	**4.54**
SC20	*S. cerevisiae*	**16.57**	0.37

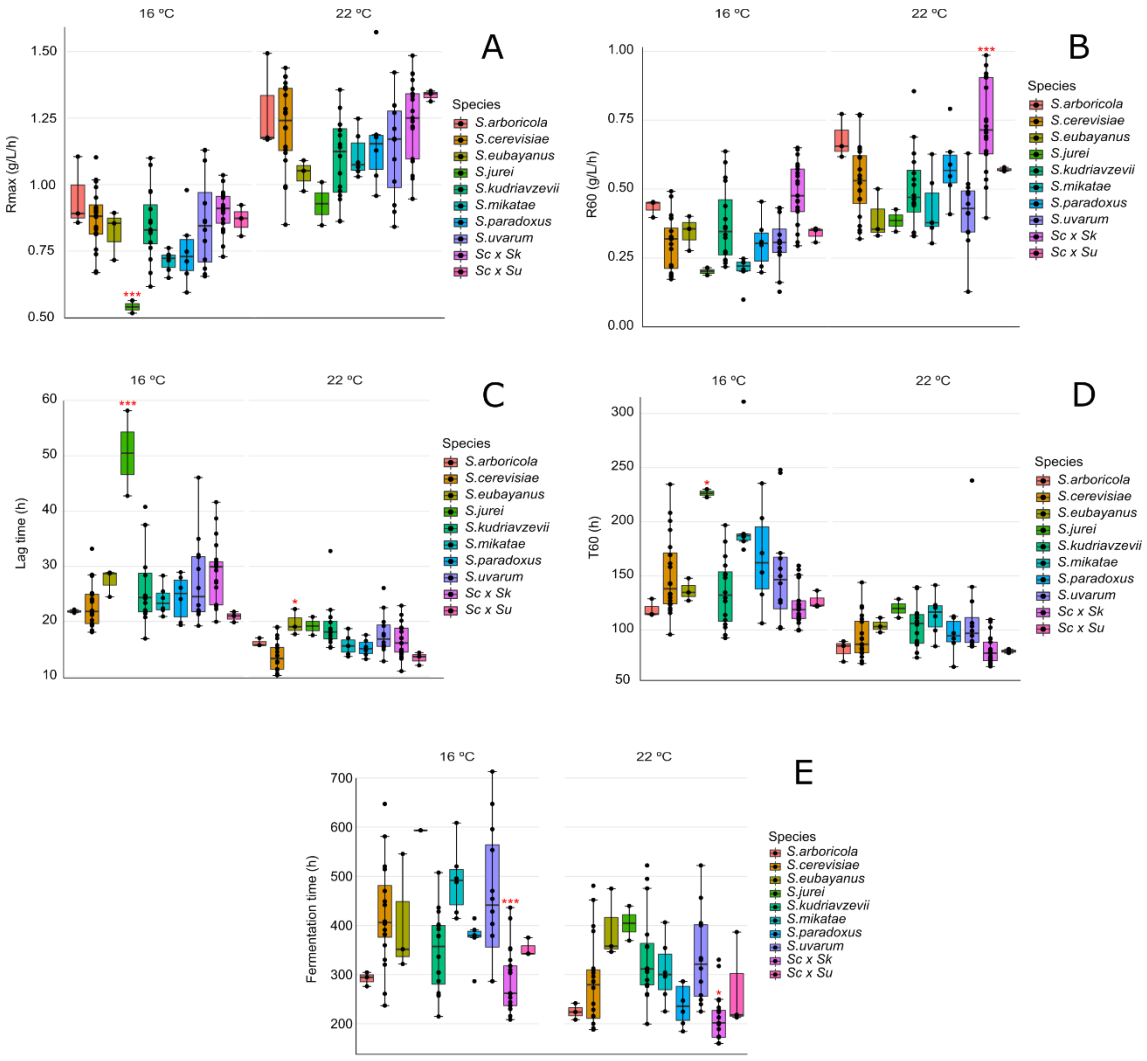


Figure S3. Boxplots of the main kinetic parameters at 16 and 22 ºC, grouped by species. (**A**) Rmax, (**B**) R60, (**C**) lag time, (**D**) T60 and (**E**) fermentation time. Asterisks on top of a box indicate a statistically significant difference (Dunnett's multiple comparison test) with respect to *S. cerevisiae*. Levels of significance: p<0.05 (*), p<0.01 (**) and p<0.001 (***).

Table S4. Average in the overall kinetic score (OKS) for each species at 16 and 22 ºC, and the increment between them.

Species	OKS at 16 ºC	OKS at 22 ºC	Increment
S. cerevisiae (n = 20)	0.51	0.74	0.24
S. paradoxus (n = 6)	0.49	0.75	0.27
S. mikatae (n = 6)	0.38	0.67	0.28
S. jurei (n = 2)	0.19	0.57	0.38
S. kudriavzevii (n = 16)	0.53	0.66	0.13
S. arboricola (n = 3)	0.63	0.80	0.17
S. eubayanus (n = 3)	0.50	0.61	0.11
S. uvarum (n = 12)	0.45	0.65	0.20
Sc x Sk (n = 21)	0.59	0.81	0.22
Sc x Su (n = 3)	0.56	0.78	0.23

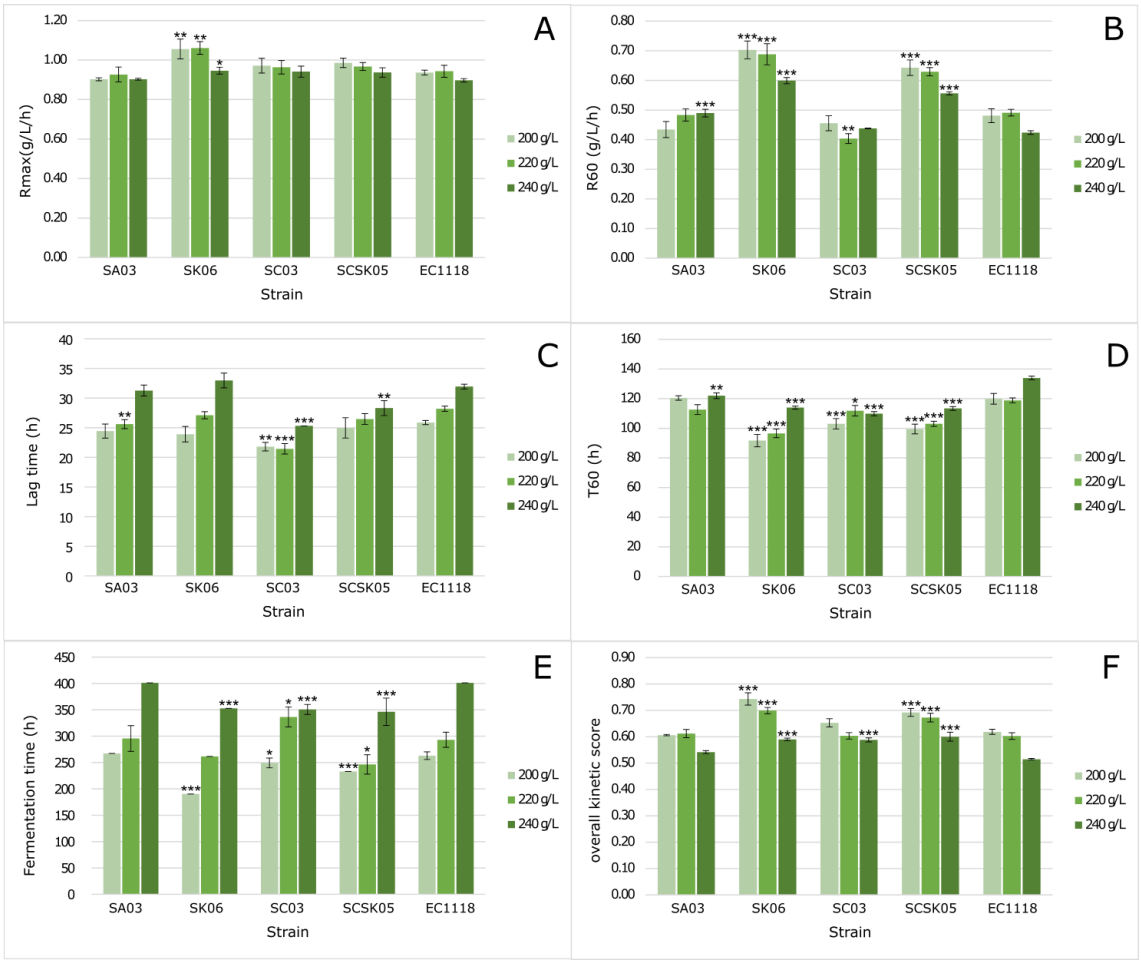


Fig. S5. Bar plots representing the kinetic parameters from fermentations using increased amounts of initial sugars (i.e. 200, 220 and 240 g/L). (**A**) Rmax, (**B**) R_{60}, (**C**) lag time, (**D**) T_{60}, (**E**) fermentation time, and (**F**) overall kinetic score. Asterisks on top of a box indicate a statistically significant difference (Dunnett's multiple comparison test) with respect to the control EC1118. Levels of significance: $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***)

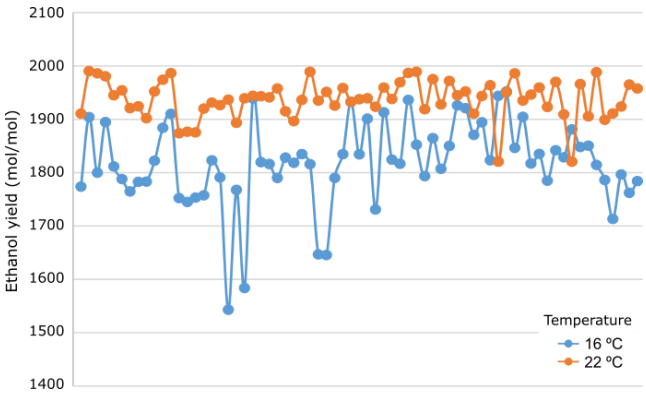


Fig. S6. Interaction plot of ethanol yield at 16 and 22 ºC, including only strains with less than 4 g/L of residual sugars at the end of fermentation.

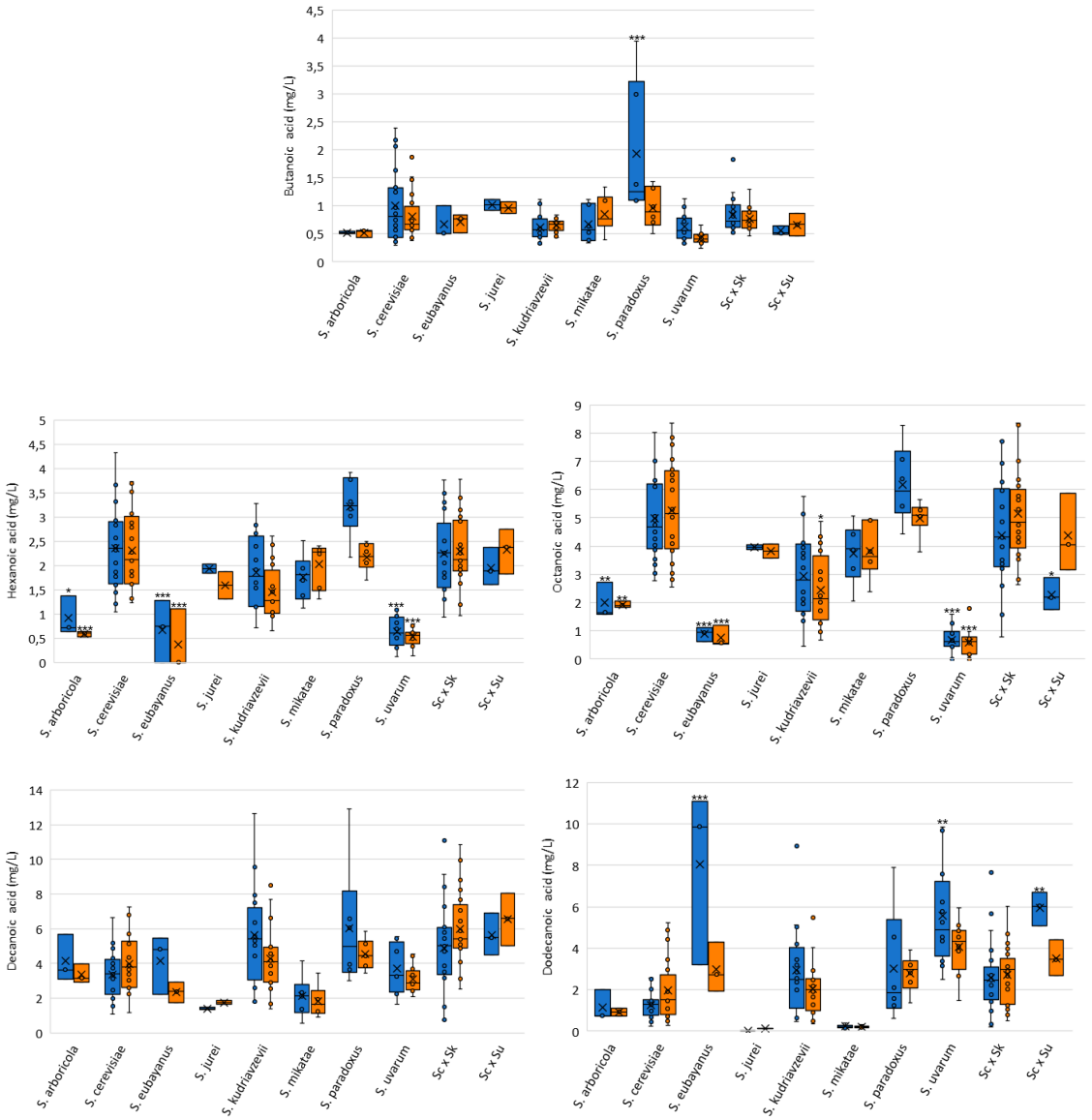


Fig. S7. Box plots of **short and medium-chain fatty acid** (S/MCFAs) production by the different species at each temperature: ■ 16 ºC and ■ 22 ºC. All amounts correspond to samples taken at 60 g·L^{-1} of CO_2 released (~ 65% of the fermentation). Asterisks on top of a box indicate a statistically significant difference (Dunnett's multiple comparison test) with respect to *S. cerevisiae*. Levels of significance: p<0.05 (*), p<0.01 (**) and p<0.001 (***).

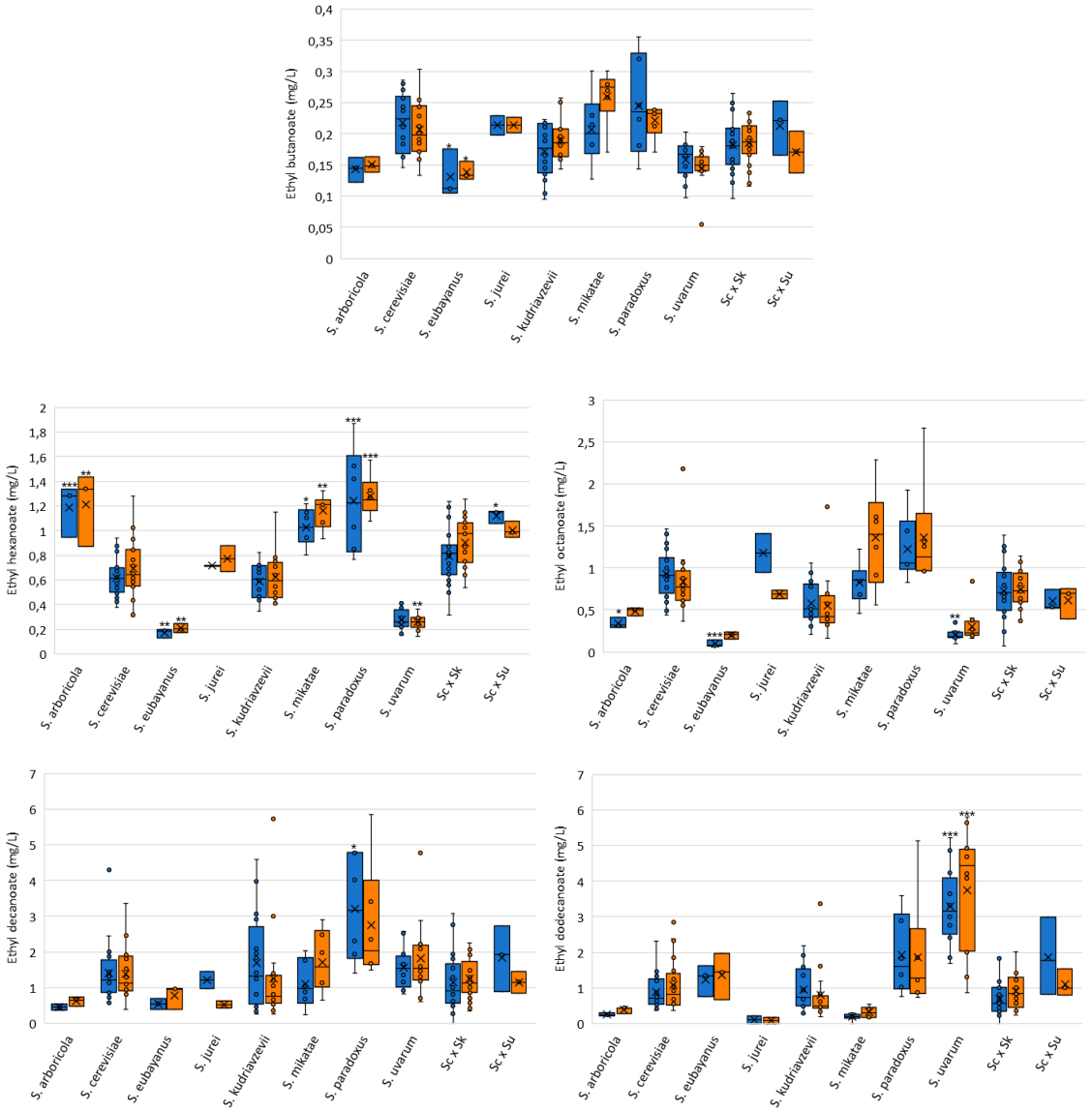


Fig. S8. Box plots of **fatty acid ethyl esters** production by the different species at each temperature: ■ 16 ºC and ■ 22 ºC. All amounts correspond to samples taken at 60 g·L^{-1} of CO_2 released (~ 65% of the fermentation). Asterisks on top of a box indicate a statistically significant difference (Dunnett's multiple comparison test) with respect to *S. cerevisiae*. Levels of significance: p<0.05 (*), p<0.01 (**) and p<0.001 (***).

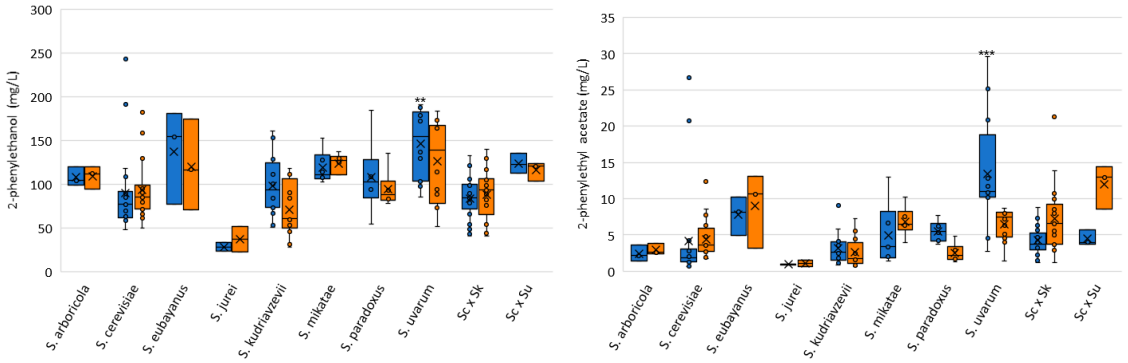


Fig. S9. Box plots of the production of aromas associated to **phenylpyruvate metabolism** by the different species at each temperature: ■ 16 ºC and ■ 22 ºC. All amounts correspond to samples taken at 60 g·L^{-1} of CO_2 released (~ 65% of the fermentation). Asterisks on top of a box indicate a statistically significant difference (Dunnett's multiple comparison test) with respect to *S. cerevisiae*. Levels of significance: p<0.05 (*), p<0.01 (**) and p<0.001 (***).

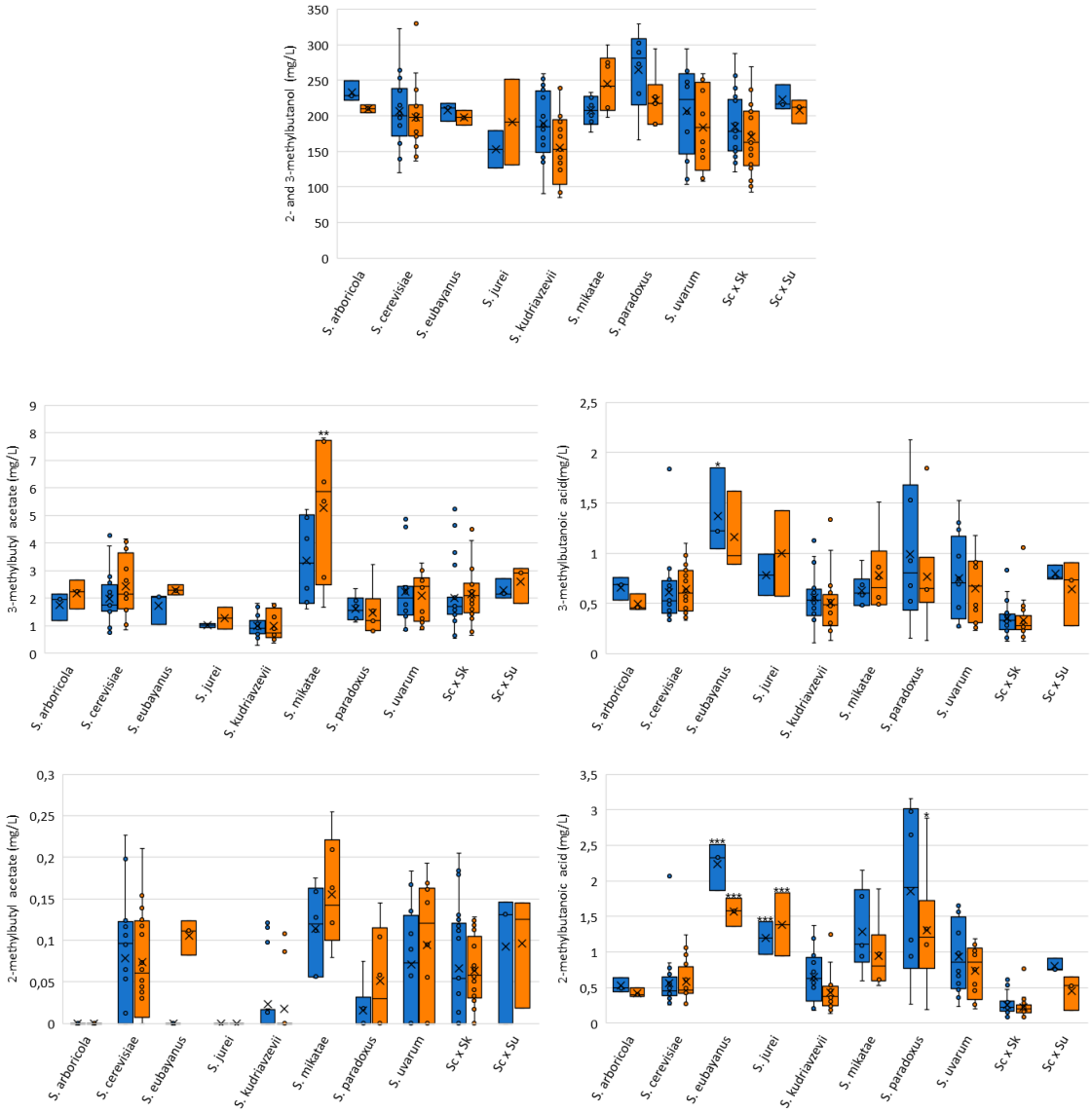


Fig. S10. Box plots of the production of aromas associated to **α-ketoisocaproate** and **α-ketomethylvalerate metabolism** by the different species at each temperature: ■ 16 ºC and ■ 22 ºC. All amounts correspond to samples taken at 60 g·L^{-1} of CO_2 released (~ 65% of the fermentation). Asterisks on top of a box indicate a statistically significant difference (Dunnett's multiple comparison test) with respect to *S. cerevisiae*. Levels of significance: p<0.05 (*), p<0.01 (**) and p<0.001 (***).

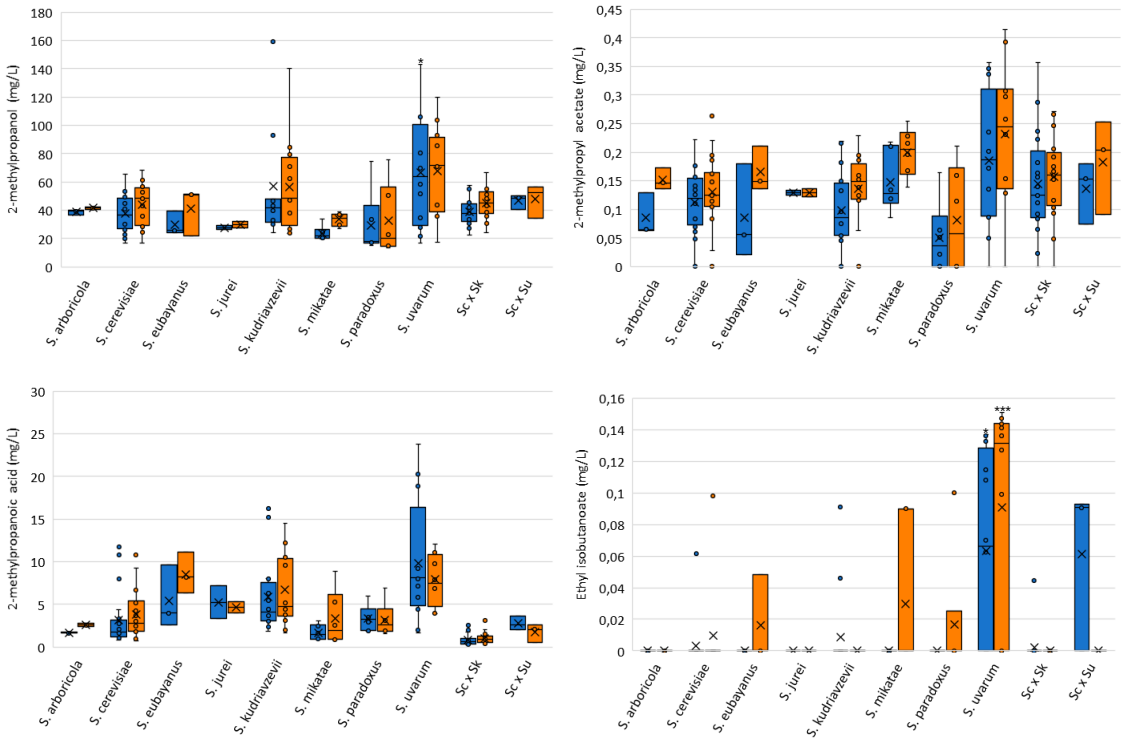


Fig. S11. Box plots of the production of aromas associated to **α-ketoisovalerate metabolism** by the different species at each temperature: ■ 16 ºC and ■ 22 ºC. All amounts correspond to samples taken at 60 g·L⁻¹ of CO_2 released (~ 65% of the fermentation). Asterisks on top of a box indicate a statistically significant difference (Dunnett's multiple comparison test) with respect to *S. cerevisiae*. Levels of significance: p<0.05 (*), p<0.01 (**) and p<0.001 (***).

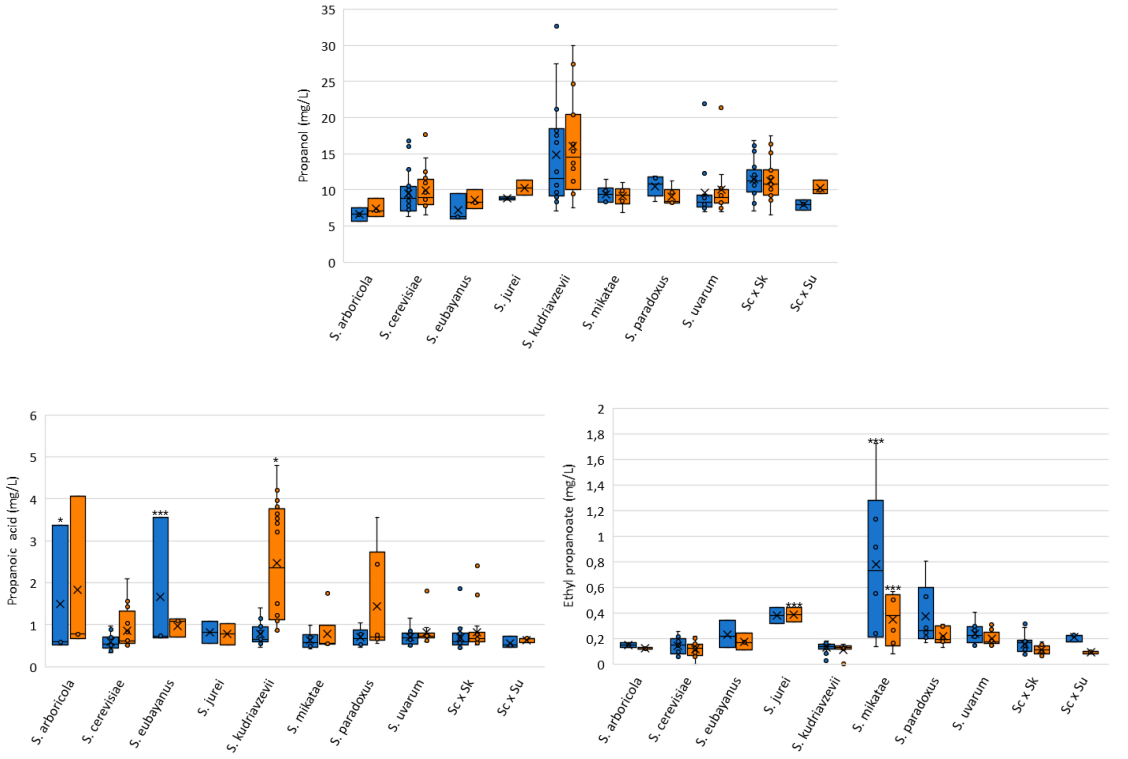
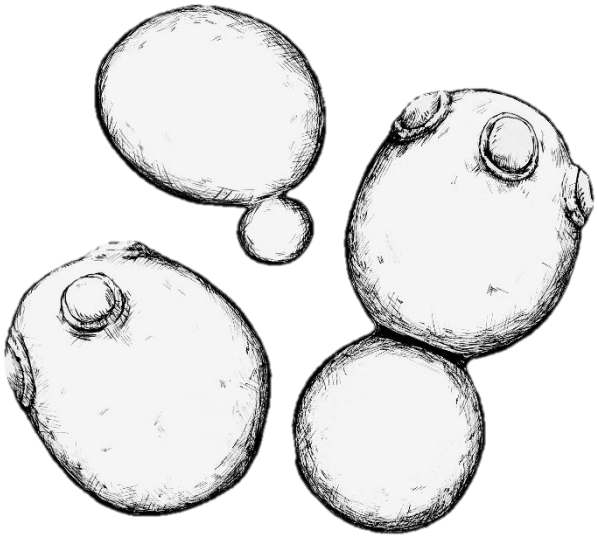


Fig. S12. Box plots of the production of aromas associated to **α-ketobutyrate metabolism** by the different species at each temperature: ■ 16 ºC and ■ 22 ºC. All amounts correspond to samples taken at 60 g·L⁻¹ of CO_2 released (~ 65% of the fermentation). Asterisks on top of a box indicate a statistically significant difference (Dunnett's multiple comparison test) with respect to *S. cerevisiae*. Levels of significance: p<0.05 (*), p<0.01 (**) and p<0.001 (***).

CHAPTER TWO



In Chapter 1, we showed a general picture of the existing phenotypic diversity in the genus *Saccharomyces* during wine fermentation. Given the high interest in the phenotypes revealed, we aimed to go further and study their genetic basis, aiming to generate new tools to generate artificial phenotypic diversity. One of the many tools available to expand yeast phenotypic diversity is the generation of interspecific hybrids. However, the sterility of these organisms hinders the application of quantitative genetics. In Chapter 2, we aimed to shed light on the genetic basis of oenologically relevant traits in interspecific hybrids via QTL mapping. For that purpose, we used a technique in which hybrid fertility is restored using tetraploid intermediates having two sets of chromosomes from each species. The details of this procrdure are explained in Chapter 2.

The hybridisation process started with 23 strains from six species: *S. cerevisiae*, *S. paradoxus, S. jurei, S. mikatae, S. arboricola* and *S. uvarum*. Strain selection was performed mainly based on metabolite production, where all selected strains stood out for different reasons. In most cases, *HO* deletion and sporulation to generate stable haploids were generally successful, as well as the hybridisation of the latter to generate intraspecific diploid hybrids. All strains generated during the hybridisation process are listed in Annexe 1 (**Tables A1** to **A6**). At this point, we decided to assess the fertility of the intraspecific hybrids by evaluating the viability of their spores. Indeed, 14 out of 23 hybrids were discarded in this step because of low spore viability (less than 50%). The subsequent generation of diploid maters through *MAT* deletion was also generally successful. Given this success, we selected only four species for the final steps – *S. paradoxus (Sp), S. jurei (Sj), S. mikatae (Sm)* and *S. uvarum (Su)* – aiming to generate all possible combinations between them. However, we could only obtain fertile *Sp x Su* and *Sm x Su* tetraploids. In the end, we decided to use the *Sm x Su* hybrids because we found the metabolic traits of their parent strains more appealing than the rest, in terms of both fermentative aroma and compounds of the central carbon metabolism. We were especially interested in studying the genetic basis of the low acetate and high glycerol, 2-methylbutanoate and S/MCFA ethyl esters by some *S. mikatae* strains, as well as the high succinate, 2-phenylethanol, 2-phenylethyl acetate and α-ketoisovalerate derivatives by some *S uvarum* isolates. Chapter 2 details the results obtained in a QTL mapping study with those *S. mikatae* x *S. uvarum* hybrids in a scientific publication format.

Genetic basis of oenological traits in an interspecific *Saccharomyces mikatae* x *S. uvarum* hybrid

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Abstract

In recent years, *Saccharomyces* species other than *S. cerevisiae* have shown to be promising alternatives to reduce ethanol and modulate organic acid and aroma production in wine fermentation. However, the genetic basis of those phenotypes remains largely unknown. *Saccharomyces* interspecific hybrids are usually sterile – which impedes their use in quantitative genetics – unless fertility is restored through engineering of the *MAT* locus and tetraploidisation. In this study, we generated interspecific *S. mikatae* x *S. uvarum* fertile hybrids, controlling their mitochondrial inheritance, to study the genetic basis of oenological traits in those species for the first time. We did not detect any mitotype-dependent QTL, neither QTLs in the *S. mikatae* subgenome. However, we found several genomic regions in the *S. uvarum* subgenome affecting the production of the main fermentation metabolites and, to a lesser extent, kinetic parameters and fermentative aromas. Several genes within the QTL regions have regulatory functions in glucose uptake and metabolism during alcoholic fermentation. The verification of those and other candidate genes will hopefully provide new tools for the improvement of *S. uvarum* and its hybrids in the context of wine fermentation.

INTRODUCTION

Today, the wine sector faces significant challenges. On the one hand, global warming leads to early industrial maturity coupled with suboptimal phenolic maturity, especially in countries with warm climates (Mira de Orduña, 2010). The main consequences of these phenomena in grape must composition are increased sugar content – leading to increased alcohol production – and decreased amounts of organic acids (Dequin *et al.*, 2017). This situation stresses the need for methods to increase wine acidity while reducing its ethanol content. On the other hand, consumer demands evolve towards wines with less alcohol and different aromatic profiles from what is offered nowadays, looking for novel organoleptic experiences. The market seeks wines with a marked fruity and floral character, providing wines with clear typicity. As the yeast strain used for alcoholic fermentation greatly impacts the final product's composition, microbiological approaches seem promising to tackle those challenges. The development of those strategies resides in the capacity of different yeasts to produce different amounts of metabolites in winemaking conditions while keeping adequate fermentative power in such a stressful environment. In this context, the study of yeast's natural diversity is vital.

The ubiquity, stress resistance and fermentative power of *Saccharomyces cerevisiae* have made out of this species the workhorse of beverage fermentations. In winemaking, most fermentations are conducted by inoculating known 'starter' strains, which are selected for properties of technological interest, mainly their capacity to complete fermentation in the presence of high sugar amounts. Although the genetic and phenotypic diversity of *S. cerevisiae* in nature is much broader than it was initially thought (Wang *et al.*, 2012; Peter *et al.*, 2018), the metabolic diversity of the commercially available *S. cerevisiae* starter cultures for winemaking is insufficient to cope with the wine sector's challenges (Molinet and Cubillos, 2020). The objective is then to improve and diversify the genetic stocks for the industry by using the natural diversity of *S. cerevisiae* (Molinet and Cubillos, 2020), isolating strains from different habitats and determining their kinetic and metabolic properties.

In addition to the interest of wild *S. cerevisiae* strains, other *Saccharomyces* species have received increased attention in the last decade. First, advances in sequencing technologies together with the massive isolation of *Saccharomyces* strains all around the world have shed light on the distribution and diversity of *Saccharomyces* species in natural habitats, making out of it a model genus in ecology and evolutionary biology (Hittinger, 2013; Peris *et al.*, 2023). Second, the phenotyping of *Saccharomyces* strains in winemaking conditions has generated interesting outcomes in recent years (Orlić *et al.*, 2007; Minebois, Pérez-Torrado and Querol, 2020; Pérez *et al.*, 2021; Coral-Medina, Morrissey and Camarasa, 2022). Recently, we showed that strains of all *Saccharomyces* species can

ferment synthetic grape must containing 200 g/L sugars, sometimes more efficiently than commercial *S. cerevisiae* strains (Álvarez *et al.*, 2023). We also reported a striking metabolic diversity within the *Saccharomyces* genus, with some species differing significantly from *S. cerevisiae* in the production of glycerol, acetic acid, succinic acid and fermentative aroma (Álvarez *et al.*, 2023). These phenotypic specificities potentially expand the possibilities we have to address the current challenges of the wine sector.

In order to improve any phenotypic trait, the understanding of its genetic basis is crucial. Most industrially relevant traits, such as stress tolerance, metabolite production or fermentative capacities, show continuous variations within a population (Marullo *et al.*, 2004; Swinnen, Thevelein and Nevoigt, 2012). Although the main metabolic pathways leading to the formation of oenologically-relevant metabolites have been described, investigating the genetic determinants leading to trait variations at the strain level remains challenging. The reason is that most traits involve multiple genetic loci (QTLs) with different contribution levels and often show complex genetic interactions (Peltier *et al.*, 2019). A widely used method to study the genetic determinants of complex traits is QTL mapping (Steinmetz *et al.*, 2002; Liti and Louis, 2012). This strategy relies on the co-segregation of loci of known positions (genetic markers) and the genetic determinants of the trait of interest during meiosis (Collard *et al.*, 2005). In *S. cerevisiae*, QTL mapping has been used to study the genetic basis of industrially relevant trait variations in winemaking conditions (Marullo *et al.*, 2007, 2019; Salinas *et al.*, 2012; Steyer *et al.*, 2012; Eder *et al.*, 2018). However, despite the interesting properties displayed by non-*cerevisiae* *Saccharomyces* in oenological conditions, the genetic basis of trait variations in those species has yet to be studied.

Saccharomyces species are post-zygotically isolated: they form interspecific hybrids which are viable, but also sterile, producing mostly unviable spores (Greig *et al.*, 2002). The impossibility to obtain viable, recombined progeny impedes their use in quantitative genetics. Recently, Naseeb *et al.* (2021) developed a strategy to obtain fertile tetraploid interspecific hybrids in which recombination occurs normally and leads to the production of viable progeny, allowing multigenerational breeding. The generation of advanced intercross lines allowed the generation of highly recombined progeny, suitable for the mapping of complex traits (Naseeb *et al.*, 2021). In this study, we generated fertile interspecific *S. mikatae* x *S. uvarum* hybrids for QTL mapping, intending to determine the genetic basis of variations in oenological traits. We genotyped and phenotyped a recombinant F12 population of segregants in winemaking conditions, and revealed different genomic regions in the *S. uvarum* subgenome suitable for potential improvement of this species and its hybrids in an oenological context.

MATERIALS AND METHODS

Yeast strains, culture conditions and sporulation

Nine *Saccharomyces* strains (Table 1), selected from the University of Leicester (UK) and INRAE Montpellier (France) culture collections, were used in this study. *S. mikatae* and *S. uvarum* strains were used for hybridisation, and *S. cerevisiae* EC1118 was used as a control in fermentation experiments. All strains were maintained at −80°C in 20% glycerol before use. Yeasts were routinely cultured in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 28 °C. To select for the drug resistance markers, YPD medium was supplemented with either 300 µg/mL of hygromycin B (HYG) or 200 µg/mL of geneticin (G418).

Sporulation was performed by pre-culturing cells into GNA medium (1% yeast extract, 0.5% bacto peptone and 10% glucose) overnight at 28 °C, washing the cell suspension twice using sterile water, and inoculating a small volume into a 100 mL Erlenmeyer flask containing 15 mL of spoMA (0.1% yeast extract, 0.05% glucose, 1% potassium acetate and 20 mg/L adenine). Flasks were incubated at 100 rpm and 22 °C for three weeks or until spores appeared.

Table 1. *Strains used in this study.*

Code used	Species	Strain	Country	Origin
SC18	S. *cerevisiae*	EC1118	France	Champagne
SM02	*S. mikatae*	NBRC 10998	Japan	Tree
SM05	*S. mikatae*	IFO1815 (T)	Japan	Soil
SM06	*S. mikatae*	IFO1816	Japan	Unknown
SU01	*S. uvarum*	DBVPG6299	Spain	Insect
SU03	*S. uvarum*	ZP 555	Canada	Tree
SU05	*S. uvarum*	A4	New Zealand	Wine
SU07	*S. uvarum*	UWOPS99-807.1.1	Argentina	Tree
SU11	*S. uvarum*	BMV58	Spain	Unknown

Generation of stable haploids

To generate genetically stable haploids, diploid strains were made heterothallic by deleting the *HO* gene using a PCR-mediated knockout strategy. Deletion cassettes conferring hygromycin resistance were amplified from the plasmid pAG32. Transformation was performed by using a standard PEG/LiAc heat shock protocol (Gietz and Schiestl, 2007). *HO* deletions were verified by PCR. The resulting heterozygote *HO/ho::HYG* strains were sporulated and tetrads were dissected using a micromanipulator (MSM 300, Sanger Instruments) to obtain *MATa* and *MATα* haploid strains. Their haploid status was verified by mating-type PCR (Huxley, Green and Dunbam, 1990) and lack of sporulation ability. All

the primers used for *HO* deletion and verification are shown in supplementary material (Table S1).

Generation of tetraploid hybrids

Tetraploid hybrids were constructed following the procedure described by Naseeb *et al.* (2021) (Fig. 1). Stable haploids of opposite mating type were mated using a micromanipulator (MSM 300, Sanger Instruments). Their hybrid status was confirmed by mating type PCR and renewed ability to sporulate. In diploid hybrids, one of the two *MAT* loci was deleted by transforming them with *mat::G418* deletion cassettes amplified from the plasmid pUG6. Mating-type PCR was used to verify *MAT* deletions. All the primers used for *MAT* deletion and verification are shown in supplementary material (Table S1).

Petite versions of each diploid hybrid were obtained by contact with ethidium bromide (EtBr). Briefly, around 300 cells were seeded on a YPD plate, and a 3-µL drop of EtBr solution (10 mg/mL) was placed on its centre. After incubation, small-size colonies were isolated, and their *petite* status was confirmed by inability to grow on YEPEG medium (1% yeast extract, 2% bacto peptone, 3% glycerol, 3% ethanol, 2% agar). Hybrids of different species and opposite mating type were mated to generate interspecific tetraploid hybrids. Their hybrid condition was confirmed by species-specific PCR, using the primers listed in Table S1 (Muir, Harrison and Wheals, 2011). Ploidy was estimated by flow cytometry after cell fixation in 70% ethanol, RNase A and proteinase K treatment, and DNA labelling with Sytox Green, using a BD Accuri C6 Plus flow cytometer (BD Biosciences). The spore viability of each intra- and interspecific hybrid was determined by dissecting ten tetrads of each cross and incubating the plates at 28 ºC for five days.

Generation of advanced intercrossed lines (AILs)

Tetraploid *S. uvarum* x *S. mikatae* hybrids were selected and pushed through multiple rounds of intercrossing. Briefly, hybrids were subjected to sporulation conditions, and the remaining vegetative cells were killed by adding diethyl ether (99.5 %, Sigma-Aldrich) and vortexing. The tetrad suspension was washed three times, treated with zymolyase solution (10 mg/mL), and incubated at 37 ºC for 30 min. Zymolyase was eliminated by centrifugation, and the spore pellet was vortexed to ensure inter-tetrad mating. Finally, spores were plated onto YPD at 28 ºC to allow mating and germination. The process was repeated 11 times to reach the F12. A population of 78 F12 diploid segregants was isolated using a micromanipulator. Their hybrid and diploid conditions were confirmed by species-specific PCR and flow cytometry, respectively, as described above.

Phenotyping

The population of segregants, the two *S. uvarum* and the two *S. mikatae* haploid parental strains, were phenotyped in oenological conditions. Fermentations were carried out at 22 ºC in 300 mL glassware fermenters containing 250 mL of SM200 synthetic grape must (Rollero et al., 2015). This medium contained 200 g/L sugars (equal amounts of glucose and fructose) and 200 mg/L assimilable nitrogen in the form of ammonium and free amino acids, as well as vitamins, trace elements and phytosterols, mimicking the composition of a standard grape juice. YPD precultures were incubated overnight at 28 ºC, and their cell density was determined using a Multisizer™ 3 Coulter Counter (Beckman Coulter). Fermenters containing SM200 were pasteurized and saturated with oxygen by injecting filtrated air for 30 min. 5 mg/L phytosterols (from a 4 g/L mother solution in Tween 80 and ethanol) were added, and fermenters were inoculated at an initial cell density of $1{\cdot}10^6$ cells/mL. From this moment, fermentation kinetics were monitored by periodically measuring the weight loss of the fermenters using the PlateButler® automated system (Lab Services). Fermentations were conducted in triplicate, using the commercial strain EC1118 as a control.

Kinetic parameters were determined from the CO_2 production rate using the alfisStatUtilR (v1.0.0) R package (Duc *et al.*, 2020). The parameters calculated were the maximum CO_2 production rate (R_{max}), the rate at 80 g/L of CO_2 released (R_{80}), the time necessary to reach 80 g/L of CO_2 released (t_{80}), the lag time (t_{lag}) and the fermentation time (t_{ferm}). The overall kinetic score (Álvarez *et al.*, 2023) was calculated to synthesize all the information into a single indicator of fermentation efficiency.

Samples were collected from each fermenter at 80 g/L of CO_2 released – equivalent to 87% of the maximum CO_2 release attainable – and at the end of the fermentation (FT). Residual sugars, primary and secondary fermentation metabolites were quantified as described by Rollero *et al.* (2015). The amounts of sugars (glucose and fructose) and the main primary metabolites (ethanol, acetate, succinate, glycerol, α-ketoglutarate, citrate and lactate) were determined by high-performance liquid chromatography (HPLC), while fermentative aromas (five higher alcohols, five acetate esters, nine organic acids and six ethyl esters) at 80 g/L of CO_2 released were quantified using GC-MS. The full phenotypic description of each segregant consisted of 50 kinetic and metabolic variables.

DNA extraction, genotyping and QTL analysis

Genomic DNA from segregants, haploid and diploid parent strains and tetraploids was extracted individually using a standard phenol-chloroform protocol. Briefly, cells from a 36 h preculture were washed twice with MilliQ water, then resuspended into 0.4 mL lysis buffer (10 mM tris pH 8.0, 1 mM EDTA, 100 mM NaCl, 2% triton and 1% SDS). After adding 0.4 g acid-washed glass beads (425 – 600 μm, Sigma Aldrich) and 0.4 mL 25:24:1

phenol-chloroform-isoamyl alcohol solution (VWR Chemicals), the mix was vortexed for 4 minutes and cooled down in ice. After centrifugation, the aqueous phase was recovered and mixed with 1 mL of ethanol for DNA precipitation. Pellets were washed with 70% ethanol. After rehydration in Tris-HCl 10 mM pH 8.5 buffer and RNase treatment, an extra re-precipitation and washing step was performed to remove possible phenol traces.

Whole genome DNA samples were sequenced by the Earlham Institute (Norwich, UK). Paired-end Illumina short reads were aligned to concatenated references by the hybrid sets and parental species (*S. uvarum* CBS7001 and *S. mikatae* IFO 1815 T) after quality check and trimming. Variant calling was applied on founder haploid samples and hybrid segregants. Variants were called separately by hybrid sets grouped by mitochondrion (i.e. *S. uvarum* or *S. mikatae*). Filters on SNPs were applied on the output of raw variant calling to obtain the variant table for the further analysis.

Founder genotypes were obtained by the variant sites derived from two parental lines background under the same species. For hybrid segregants, bi-allele markers were then further aligned to founders to phase the genotypes (i.e. which base is the same in which parental background). The matched strain sets that both having phenotype and genotype records were used as the input dataset for QTL analysis. QTL Analyses were then performed for each phenotype through r/qtl with marker regression. The significance level was set at 0.05 to determine the LOD threshold with 1000 permutation tests applied for each QTL scan.

Statistical analysis

Graphs were generated and statistical analyses were performed using R v4.2.3 (R Core Team, 2023) and XLSTAT v2023.1 (Addinsoft, Paris, France). The principal component analysis (PCA) was run only with the segregants – parent and control strains were added later as supplementary observations.

RESULTS AND DISCUSSION

Constructing fertile interspecific hybrids

In this study, we aimed to determine the genetic basis of oenological phenotypes in fertile *Saccharomyces* hybrids. We used three *S. mikatae* and five *S. uvarum* strains previously selected for their phenotypes in oenological conditions (Álvarez *et al.*, 2023). First, we generated stable haploids from those strains through *HO* deletion and subsequent sporulation. Then, we mated two strains of each species, aiming to generate intraspecific heterozygote hybrids with polymorphisms covering the entire genome. Using ethidium bromide allowed us to generate *petite* mutants (i.e. lacking functional mitochondria) of

each intraspecific hybrid. The deletion of a different *MAT* locus on each diploid restored their mating ability, which we used to construct interspecific tetraploid hybrids (Fig. 1).

A hybrid capable of producing viable spores is a prerequisite for QTL analysis. Although *Saccharomyces* species are post-zygotically isolated between each other, hybrids within the same species can also show decreased spore viability (Bendixsen, Frazão and Stelkens, 2022). For this reason, we evaluated the spore viability of intraspecific crosses, as we reasoned that this would translate to the spore viability of the tetraploids. The spore viabilities of all hybrids obtained are shown in Table 2.

Although the main sterilising mechanism in the *Saccharomyces* genus is antirecombination driven by sequence divergence, chromosomal rearrangements can also contribute to reproductive isolation (Ono and Greig, 2020), even within the same species. This is the case for some *S. paradoxus* strains, previously considered a different species (*S. cariocanus*) because they are partially reproductively isolated from other *S. paradoxus* strains (Louis, 2011). The reason for the low spore viability in the abovementioned *S. paradoxus x* (formerly) *S. cariocanus* hybrids is the presence of four reciprocal translocations (Liti, Barton and Louis, 2006). The commercial *S. uvarum* BMV58 (named here SU11) has a chromosomal translocation providing increased sulfite resistance (Macías *et al.*, 2021). In this study, the four intraspecific hybrids involving BMV58 had moderate to low spore viability, between 15 and 55%. The mentioned chromosomal translocation is likely the cause of the low fertility shown by those hybrids.

Three of the five *S. mikatae* hybrids constructed showed lower than 55% spore viability. This low viability is in agreement with previous observations for this species (Naumov, 1996; Naumov *et al.*, 2000). Still, two *S. mikatae* hybrids produced more than 90% viable spores, indicating that the sequence similarity between the strains used is enough to allow a proper chromosome segregation during meiosis. From all intraspecific crosses obtained, only those showing more than 80% viable spores, including six *S. uvarum* and two *S. mikatae* hybrids, were selected for further hybridisation.

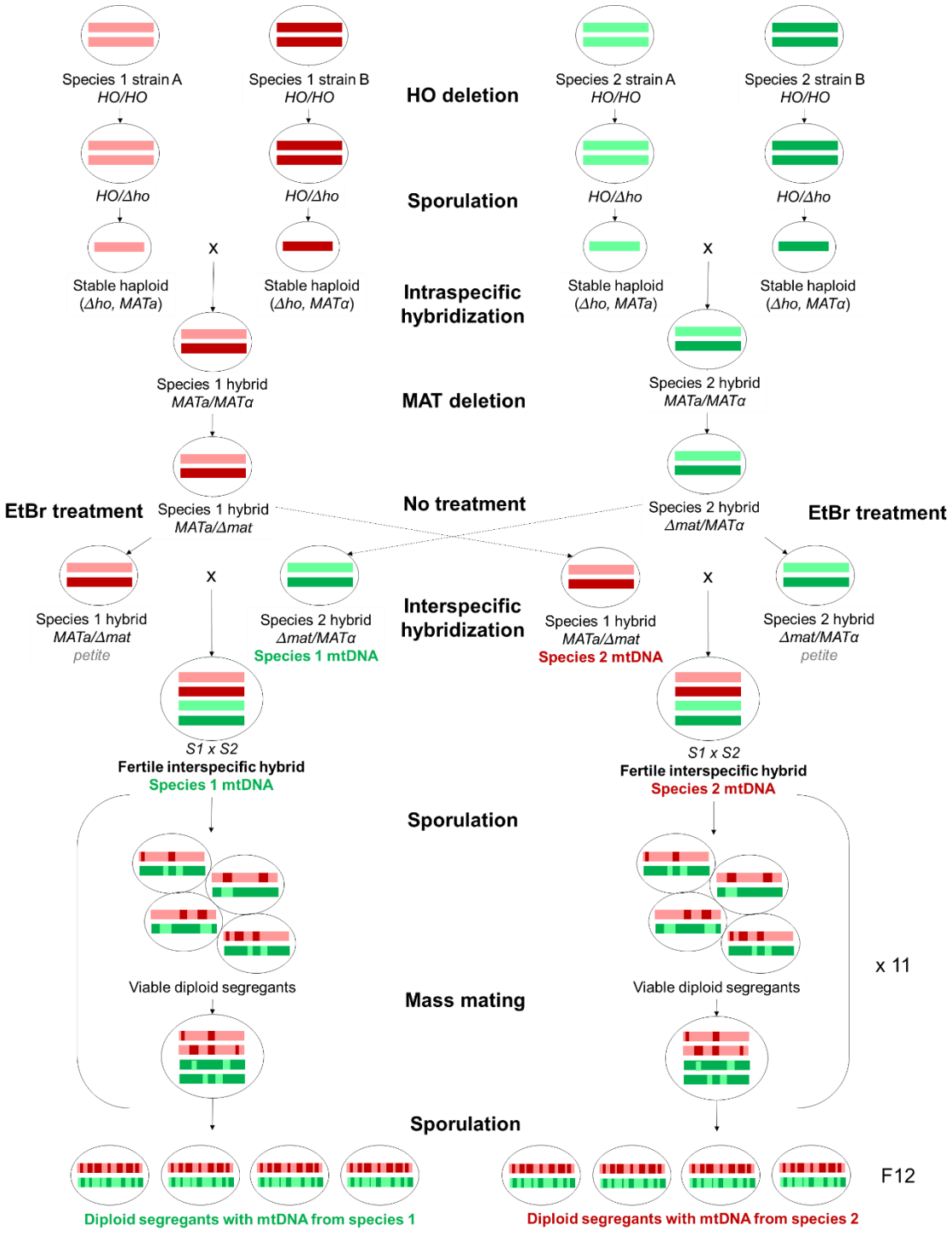

***Figure 1**. Scheme of the strategy used for the generation of fertile interspecific hybrids and advanced intercross lines (AILs).*

***Table 2**. List of intra- and interspecific hybrids generated in this study and their spore viability. Hybrids which eventually led to the generation of segregants for QTL mapping are highlighted in bold font.*

Intraspecific diploid hybrids					
Code used	Species	MATa parent	MATα parent	Spore viability	Mitotype
D04	*S. uvarum*	SU01-a	SU05-α	95.0%	*S. uvarum*
D09	*S. uvarum*	SU01-a	SU07-α	95.0%	*S. uvarum*
D10	*S. uvarum*	SU01-a	SU11-α	45.0%	*S. uvarum*
D11	*S. uvarum*	SU07-a	SU11-α	15.0%	*S. uvarum*
D14	*S. uvarum*	SU01-a	SU03-α	97.5%	*S. uvarum*
D15	***S. uvarum***	**SU03-a**	**SU05-α**	**100.0%**	***S. uvarum***
D16	*S. uvarum*	SU03-a	SU07-α	95.0%	*S. uvarum*
D17	*S. uvarum*	SU03-a	SU11-α	55.0%	*S. uvarum*
D18	*S. uvarum*	SU07-a	SU01-α	95.0%	*S. uvarum*
D19	*S. uvarum*	SU07-a	SU05-α	57.0%	*S. uvarum*
D20	*S. uvarum*	SU05-a	SU11-α	55.0%	*S. uvarum*
D22	*S. mikatae*	SM02-a	SM06-α	97.5%	*S. mikatae*
D23	*S. mikatae*	SM05-a	SM02-α	55.0%	*S. mikatae*
D24	*S. mikatae*	SM05-a	SM06-α	37.5%	*S. mikatae*
D25	***S. mikatae***	**SM06-a**	**SM02-α**	**92.5%**	***S. mikatae***
D26	*S. mikatae*	SM06-a	SM05-alpha	47.5%	*S. mikatae*
Interspecific tetraploid hybrids					
T16	*Su x Sm*	D14-a	D25-α	80.0%	mixed
T17	*Su x Sm*	D14-a	D25-α, ρ(-)	85.0%	*S. mikatae*
T18	*Su x Sm*	D14-a, ρ(-)	D25-α	90.0%	*S. uvarum*
T19	*Su x Sm*	D15-a	D25-α	87.5%	mixed
T20	***Su x Sm***	**D15-a**	**D25-α, ρ(-)**	**80.0%**	***S. mikatae***
T21	***Su x Sm***	**D15-a, ρ(-)**	**D25-α**	**82.5%**	***S. uvarum***

ρ(-) indicates *petite* status (i.e. with deficient mitochondria)

A total of six interspecific tetraploids were generated, belonging to two crosses: Sm^{D25} *x* Su^{D14} and Sm^{D25} *x* Su^{D15}. As expected, all tetraploids evaluated were fertile, showing spore viabilities higher than 80% (Table 2). However, the spore viability of tetraploids was not strictly correlated to that of the diploids used for their construction. For example, the hybrid T20 (Sm^{D25} *x* Su^{D15}) had lower spore viability than any of its founder strains. As those founders had spore viabilities of 100 and 92.5%, one might expect T20 having 92.5% viable spores, still it showed an 80%. Although negative epistatic interactions in *Saccharomyces* are quite rare (Dujon and Louis, 2017), they can contribute to hybrid sterility (Lee *et al.*, 2008; Chou *et al.*, 2010). Additionally, gene incompatibilities between *S. mikatae* and *S. uvarum* have not been studied yet. Thus, the slightly reduced fertility observed for the Sm^{D25} *x* Su^{D15} hybrid compared to its parent strains could be attributed to negative epistatic interactions between the *S. mikatae* and *S. uvarum* subgenomes. In line

with this observation, hybrids with the same exact nuclear DNA content (differing only in their mitochondrial genomes) showed slightly different spore viabilities. For instance, the spore viabilities of the *Sm*^{D25} *x Su*^{D14} hybrid ranged from 80 to 90%, depending on the mitotype (Table 2). As mitochondria are necessary for sporulation (Küenzi, Tingle and Halvorson, 1974), the presence of different mitochondria could contribute to the observed differences.

Analysis by flow cytometry confirmed the tetraploid status of all newly generated hybrids (example in Fig. S3). Thus, the two types of hybrid (i.e. *Sm*^{D25} *x Su*^{D14} and *Sm*^{D25} *x Su*^{D15}) were valid to follow the QTL mapping strategy. From them, we selected *S. mikatae*^{D25} *x S. uvarum*^{D15} because of the interesting phenotypes shown by its founder strains. For instance, the *S. mikatae* parent strains (SM02 and SM06) showed a high production of glycerol, 2-methylbutyl acetate, and several ethyl esters (including ethyl butanoate, pentanoate and octanoate), combined with the absence of acetic acid at the end of the fermentation. On the other hand, both *S. uvarum* strains produced high concentrations of succinate and variable amounts of glycerol and volatile aromas. More specifically, while SU03 stood out for high production of 2-phenylethyl acetate and dodecanoic acid, SU05 generated higher amounts of glycerol, 2-phenylethanol, and three aromas derived from α-ketoisovalerate metabolism (2-methylpropanol, 2-methylpropyl acetate and 2-methylpropanoic acid). All four parental strains showed adequate fermentative power in synthetic grape must (Álvarez *et al.*, 2023).

Phenotypic variation within 78 Sm x Su diploid segregants

We subjected the selected *S. mikatae*^{D25} *x S. uvarum*^{D15} tetraploid to 11 rounds of sporulation and mating. After sporulating the F11 tetraploids, we isolated 78 *Sm x Su* F12 segregants, 43 of which had mitochondria from *S. mikatae*, and 35 from *S. uvarum*. We phenotyped those segregants in synthetic grape must fermentation at 22 ºC, evaluating their fermentative capacities and ability to produce primary and secondary metabolites of oenological interest.

First, we assessed whether the different traits followed a normal distribution within the population. Most traits were roughly normally distributed (Fig. S4-S8), which indicates they are under polygenic control. However, some traits such as 3-methylbutanoic acid (Fig. 2.A) showed a biphasic distribution, probably indicating the presence of a major QTL. Acetic acid appears as a particular case, with 15 segregants producing 0 g/L at the first sampling point (i.e. 80 g/L of CO_2 released) (Fig. 2.B). Although most segregants kept producing acetic acid towards the end of the fermentation, very low amounts were found in most cases at final time (Fig. 2.C). This low acetate production was somewhat expected, as the two *S. mikatae* founder strains were selected for not secreting any acetic acid.

Transgressive phenotypes (i.e. outside the range of variation of the parent strains) indicate the presence of alleles of opposite effects in the founder strains, giving rise to new allelic combinations in the progeny (Ambroset *et al.*, 2011). Some traits, such as the production of glycerol (Fig. 2.D), acetate (Fig. 2.B and C), succinate, or volatile compounds including 2-methylpropanol (Fig. 2.E), 2-methylpropanoic acid, dodecanoic acid and its ethyl ester (Fig. 2.F), showed low or no transgression (less than 9%) (Fig. S4-S8). Many other phenotypes showed moderate transgression (12 – 50%), mostly negative (overall kinetic score) or mostly positive (e.g. decanoic acid and phenylethyl acetate). Other traits, such as the lag time and 2-phenylethanol production (Fig. 2.G), showed equally positive and negative transgression. Finally, several traits displayed an entirely positive transgression of more than 50%. The most striking examples were the CO_2 production rate in late stationary phase (R_{80}), the ethanol yield at the end of the fermentation, and the production of 3-methylbutanoic (Fig. 2.A) and hexanoic acids (Fig. 2.H). For those traits, almost every segregant performed better than any parent strain, displaying a clear heterotic effect (i.e. hybrid vigour).

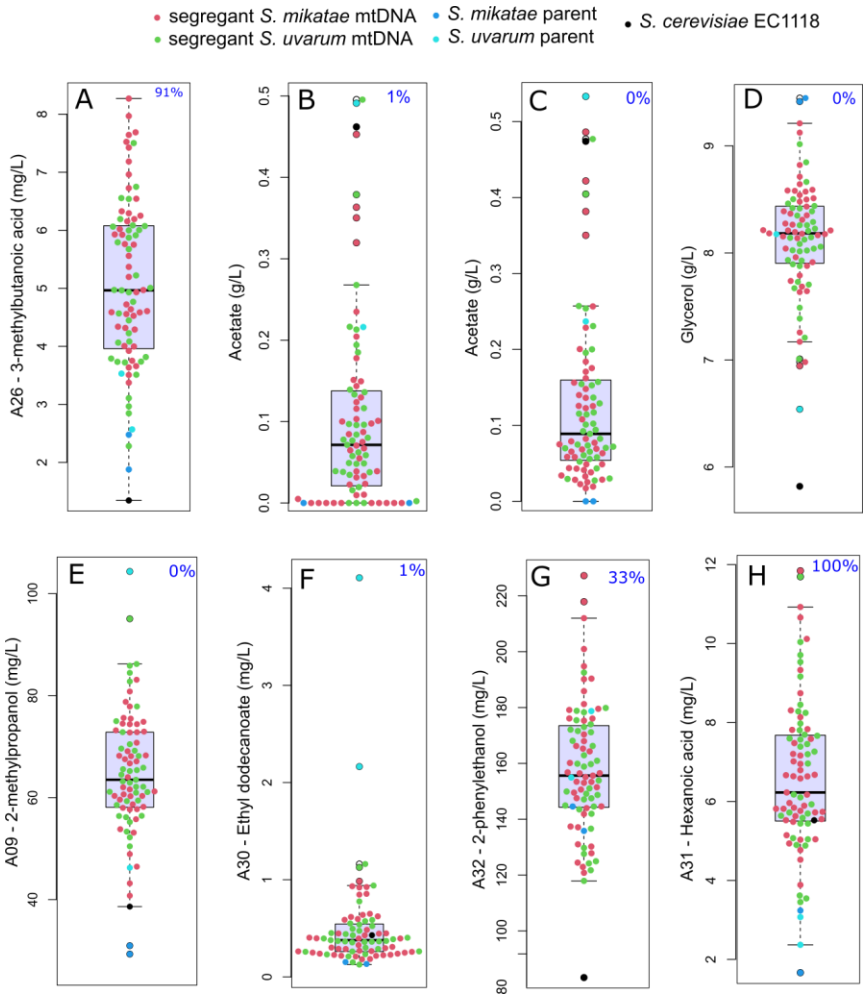

Figure 2. *Boxplots showing the distribution of different traits under study. The blue percentage indicates the proportion of segregants showing transgressive phenotypes (i.e. more than the best parent or less than the worst parent).*

It is important to note that the parent strains phenotyped were haploid, while segregants were diploid. It is still unclear how the ploidy level affects the mentioned phenotypes in wine fermentation, but it may impact both fermentation rate and metabolite production. In brewing experiments, Krogerus *et al.* (2016) showed that *S. cerevisiae* x *S. eubayanus* hybrids with higher DNA content fermented more efficiently and produced higher amounts of fruity esters, probably due to higher copy numbers of genes leading to their formation and changes in their expression level. In our study, however, the haploid *S. uvarum* parents performed alcoholic fermentation more efficiently than any segregant (Fig. 3). To what extent the phenotypic differences observed here are a consequence of heterosis or a higher ploidy would require further experiments. Constructing the four combinations of hybrid diploids between the four parent strains and their phenotyping would resolve to which extent transgression and heterosis are responsible for phenotypic variation.

The commercial *S. cerevisiae* strain EC1118 was used as a control in all experiments. Regarding fermentation kinetics, EC1118 had a shorter fermentation time, shorter t_{80} and higher overall kinetic score than most segregants. Its most differential parameter was its R_{80}, which was the highest of all strains (Fig. 3). EC1118 also left less residual sugars and had higher ethanol and acetic acid yields than most segregants and parent strains (Fig. S5 and S6). Regarding aroma production, EC1118 produced lower amounts of 11 metabolites derived from the Ehrlich pathway than any *Sm x Su* segregant (Fig. S7). The only fermentative aromas produced in higher quantities by EC1118 were propanol and octanoic acid, two compounds without particular organoleptic interest. Together, these results emphasise the interest in investigating alternative species such as *S. mikatae* or *S. uvarum* in wine fermentation, as they differ significantly from what is usually offered by commercial *S. cerevisiae* strains, yet showing adequate fermentative power.

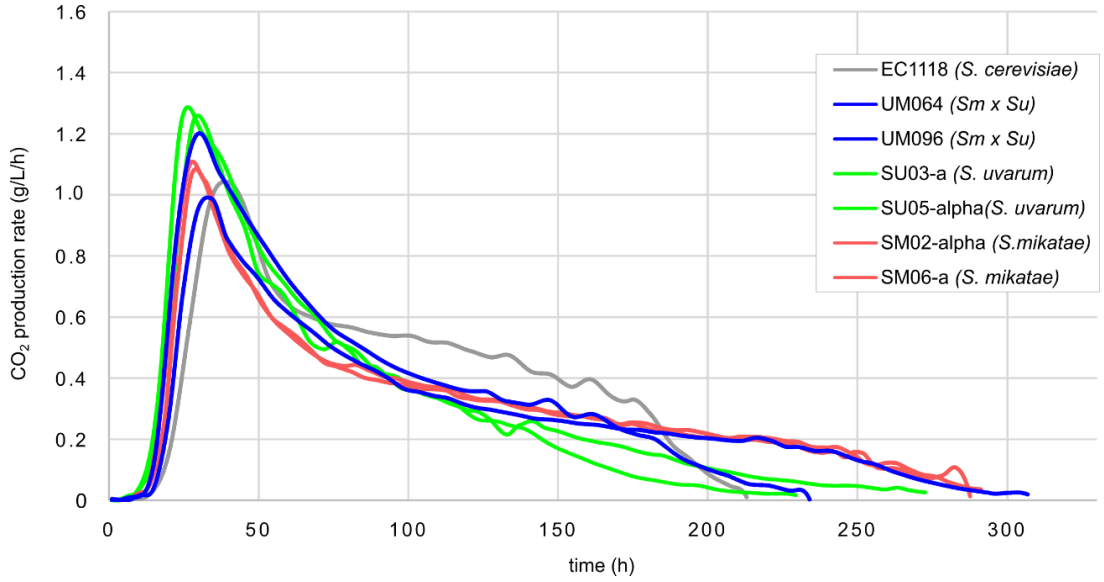

***Figure 3**. Range of phenotypic variation in terms of fermentation kinetics. The best and the worst segregants (blue), the four parent strains (green and red) and the control EC1118 (grey) are shown.*

We performed a principal component analysis (PCA) in order to visualise the whole dataset, reduce its complexity, and potentially identify common regulations (Fig. 4). The four parent strains and the *S. cerevisiae* control were included as supplementary observations (i.e. they were not used to generate the PCA). In general terms, there was not a clear differentiation between segregants carrying mitochondrial DNA from *S. mikatae* and *S. uvarum* (Fig. 4.A). However, one-way ANOVA revealed a significant effect of mitochondrial DNA for eight parameters (Table S8). The most significant mtDNA effect was found for the maximum rate (R_{max}), which was higher for segregants having mitochondria from *S. uvarum* (1.18 g/L/h in average, against the 1.14 g/L/h shown by segregants with mtDNA from *S. mikatae*). Two other kinetic parameters (i.e. t_{80} and overall kinetic score) were also significantly impacted by mitotype, with *S. uvarum*-mtDNA segregants showing a better performance. Albertin *et al.* (2013) showed that mitochondrial inheritance in *S. cerevisiae* x *S. uvarum* hybrids does not impact fermentation performance. However, other studies have highlighted the importance of mtDNA from a cryotolerant species in the cryotolerance of interspecific hybrids (Rainieri *et al.*, 2008; Baker *et al.*, 2019). Considering that the optimal growth temperature of *S. uvarum* is around 3 ºC lower than that of *S. mikatae* (Salvadó *et al.*, 2011), mitochondria may provide segregants with a better capacity to ferment at the relatively low temperature used here (i.e. 22 ºC).

The PCA also shows that most short- and medium-chain fatty acids and their ethyl esters formed a well-established cluster (Fig. 4.B). This can be explained by the biosynthesis of those compounds sharing a common step, i.e. fatty acid synthesis. Similarly, several aromas derived from the Ehrlich pathway formed a separate cluster. Within this second cluster, acetate esters appeared close to each other, which is logical, as the same enzymes (e.g. Atf1, Atf2) catalyse their formation. In all these cases, we can infer that segregants producing high amounts of all clustered compounds must have inherited allelic variants leading to higher production. This could be the case for the segregants UM095 and UM096 (Fig. 4.A), which produced the highest amounts of 2- and 3-methylbutyl acetate, phenylethanol, and phenylethyl acetate. Those segregants also showed the worst kinetic capacities (Fig. 3), displaying the lowest overall kinetic scores of all segregants. Interestingly, they were both isolated from the same tetrad. We can also deduce that a potential QTL detected for one of the compounds in a cluster will likely affect the production of other compounds in that cluster. This emphasises the interest of performing QTL mapping with multiple traits simultaneously.

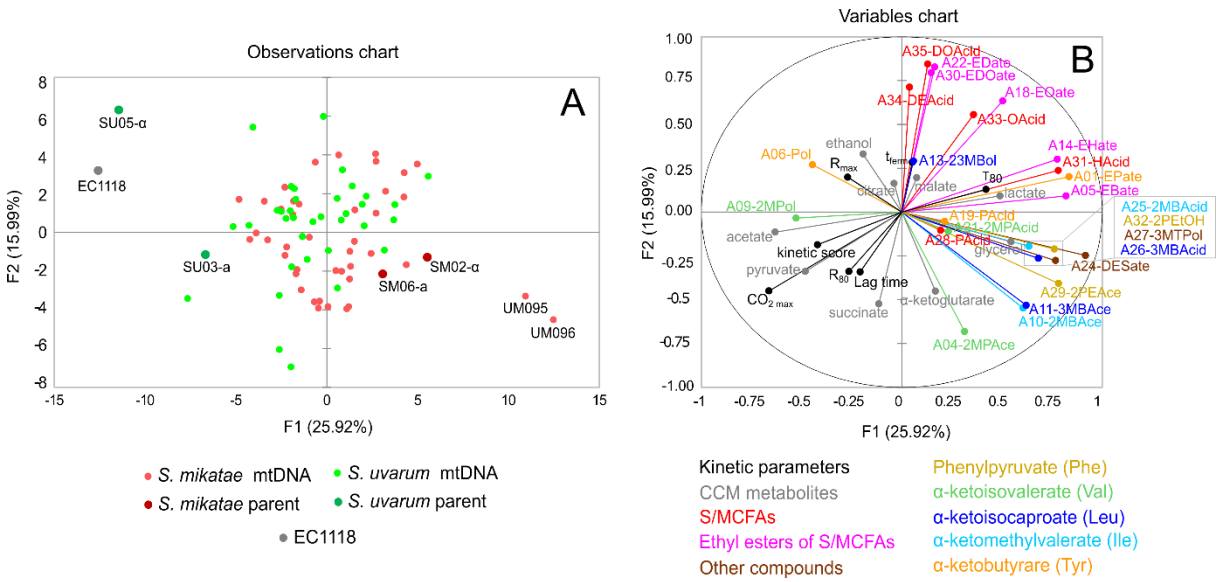

***Figure 4**. Principal component analysis (PCA) representing all kinetic variables and all metabolites quantified at 80 g/L of CO_2 released.*

Quantitative trait loci from S. uvarum *impact oenological traits*

To identify the genetic determinants of the phenotypic variation among the population of segregants, we performed QTL mapping using individual segregant analysis (ISA).

No QTLs were mapped in the subgenome of *S. mikatae*. Sequence analysis revealed that the two strains used were very similar, differing in only 1000 SNPs with the filter applied for variant calling. Nevertheless, a total of 38 QTLs were detected in the subgenome of *S. uvarum*, affecting 29 phenotypes (Table 3). Considering that some parameters are different ways to measure the same compound (i.e. a compound and its yield), those QTLs affected the production of 12 fermentative aromas, 6 central carbon metabolites and 3 kinetic parameters. Ethanol and glycerol had the most QTLs, with 3 and 4, respectively.

A high degree of pleiotropy was found for a QTL hotspot in chromosome V. This 24.6 kbp region was present in 7 QTLs affecting the production of four primary metabolites (i.e. glycerol [Fig. 6.A], ethanol [Fig. 6.B], acetate and lactate). Every QTL in that hotspot had peak LOD scores higher than 6, indicating high statistical significance (Fig. 5 and 6). Apart from that hotspot, five more QTLs were found in chromosome V, affecting the production of ethanol, ethyl hexanoate, 2-methylpropyl acetate, and the amount of CO_2 released at the R_{max}. Interestingly, another pleiotropic region was found in chromosome 7, affecting the production of three volatile compounds (i.e. ethyl butanoate, 2-methylpropanol and hexanoic acid). The rest of the QTLs were randomly distributed

throughout the genome, with only four chromosomes (i.e. I, IV, X and XIV) not showing any QTL.

Surprisingly, no QTLs were found for succinate production, despite the *S. uvarum* parent strains differing notably in its production. Similarly, despite the high number of fermentative aromas measured, only 12 QTLs were found for those compounds. This could indicate that the *S. uvarum* parent strains do not contain alleles with opposite effects on these phenotypes. However, the number of segregants has shown to be determinant in terms of the number of QTLs found (Nguyen Ba *et al.*, 2022). Therefore, another explanation could be that the number of segregants used here was not high enough to provide the statistical power required to map QTLs for those compounds.

The size of the mapped regions ranged from 1 bp (4 QTLs) to 55 kbp, with an average of 7.7 kbp. Seven of the mapped regions contained only one ORF, which will facilitate the dissection of those genetic determinants to the gene level. Given the small number of segregants employed (i.e. 78), the generally small size of the QTLs can be attributed to the use of an F12 advanced intercross population, highlighting the power of this strategy for the detection of narrow QTLs.

As mentioned above, we directed the mitochondrial inheritance of the segregants, which had mtDNA from either *S. uvarum* or *S. mikatae*. QTL analysis was run for each of those groups of segregants independently, aiming to assess whether the potential QTLs detected were specific to a given mitotype. However, we did not find any mitotype-dependent QTL. This could mean that the mitochondrial functions are well conserved between *S. mikatae* and *S. uvarum*, perhaps not having any important ecological difference, and thus not providing any change in terms of mitochondrial-nuclear interactions for the phenotypes measured. However, as we only used 35 and 43 segregants with mtDNA from *S. uvarum* and *S. mikatae* respectively, it is possible that the small sample size hindered their detection. The use of larger segregant populations bearing each mitotype would resolve this question.

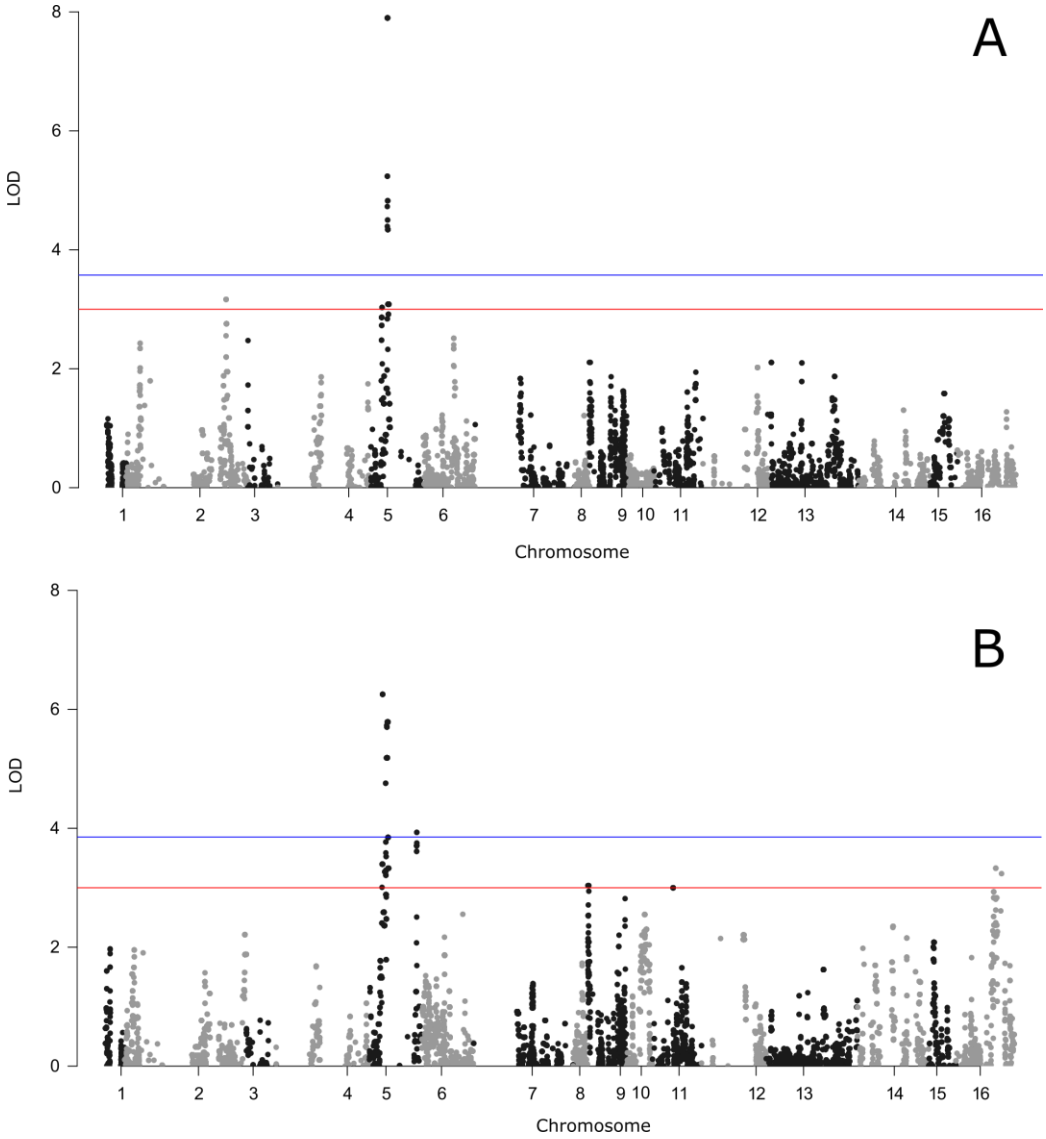

***Figure 5**. LOD score plots of the acetate (A) and ethanol (B) yields at 80 g/L CO_2 released in the whole genome. Colored lines indicate the LOD score thresholds: international standard (3.00, red line) and the one calculated here via Monte Carlo permutations (blue).*

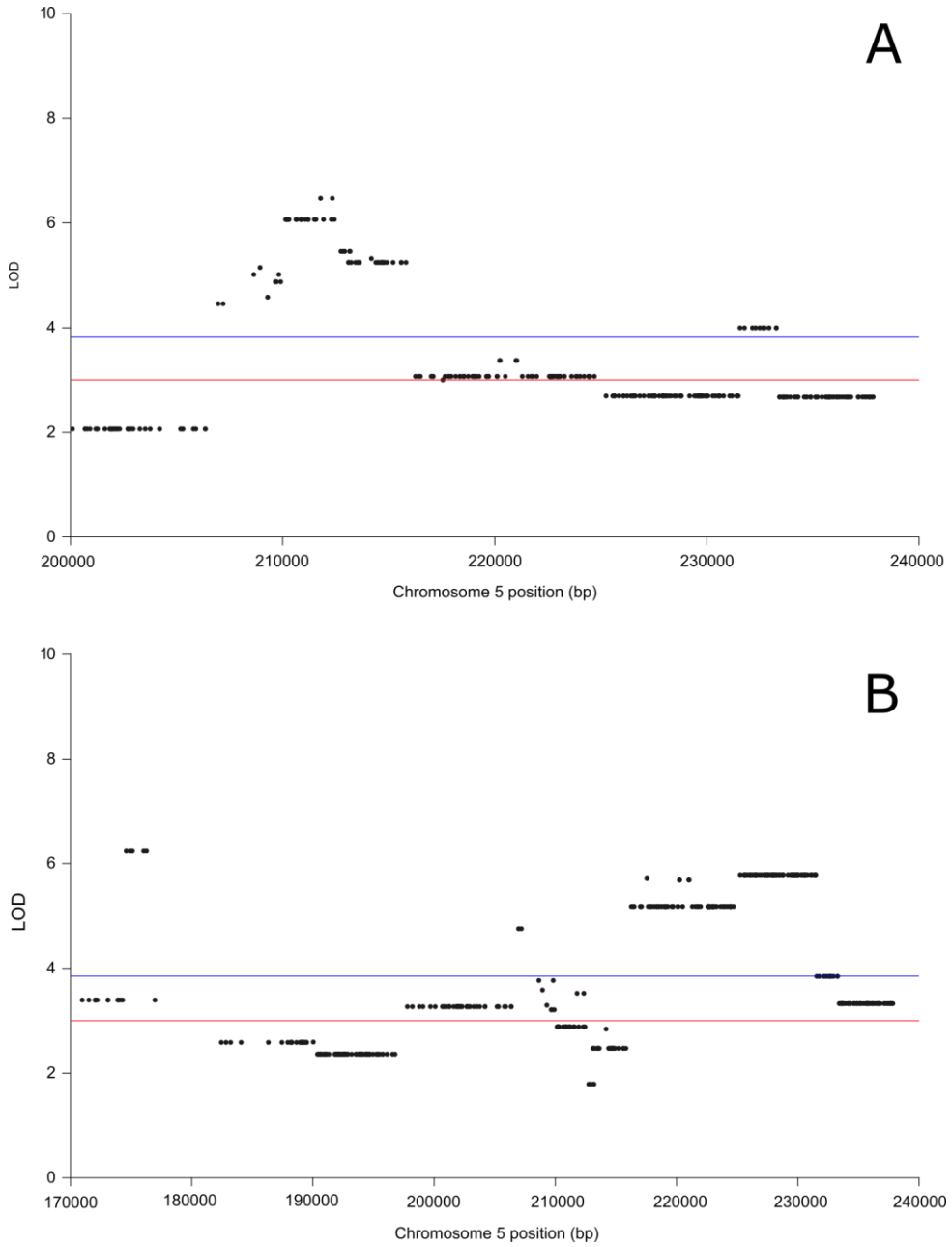

***Figure 6**. Regional LOD score plot showing the different QTLs found for the glycerol (**A**) and the ethanol (**B**) yields at 80 g/L CO_2 in chromosome V. Colored lines indicate the LOD score thresholds: international standard (3.00, red line) and the one calculated here via Monte Carlo permutations (blue).*

***Table 3**. Summary of the QTL analysis results. Genes in the peak of the QTL are highlighted in bold font.*

Chromosome	QTL	Peak LOD	Peak (bp)	Length (bp)	Phenotype	Gene features (ScanOne)
II	chr.II@824.8	3.82	824834	6183	A25_2MBAcid	*ZIP1 ;* ***INM2***
III	chr.III@147	3.90	147028	8327	Citrate (80)	***ADP1***
V	chr.V@148.6	4.23	148572	6997	A14_Ehate (80)	*EAF5 ;* ***MMS21*** *; PXP1 ; URA3*
V	chr.V@174.6	6.25	174595	1674	Ethanol yield (80)	*PMI40 ;* ***YND1***
V	chr.V@197.8	4.30	197789	8568	CO_2 at R_{max}	***HEM14 ; AFG3 ; FAA2 ; BIM1***
V	chr.V@207	4.76	206961	1677	Ethanol yield (80)	***AFG3*** *; ISC1*
V	chr.V@210.7	7.87	210661	6881	Acetate (80)	*ISC1 ; SBH2 ;* ***GPA2*** *;* ***RPN3*** *; SRB4*
V	chr.V@210.7	7.90	210661	6881	Acetate yield (80)	*ISC1 ; SBH2 ;* ***GPA2*** *;* ***RPN3*** *; SRB4*
V	chr.V@210.7	7.97	210661	6881	Acetate (FT)	*ISC1 ; SBH2 ;* ***GPA2*** *;* ***RPN3*** *; SRB4*
V	chr.V@210.7	8.03	210661	6881	Acetate yield (FT)	*ISC1 ; SBH2 ;* ***GPA2*** *;* ***RPN3*** *; SRB4*
V	chr.V@211.8	6.47	211789	8855	Glycerol (80)	*AFG3 ; ISC1 ; SBH2 ;* ***GPA2*** *; RPN3 ; SRB4*
V	chr.V@211.8	6.57	211789	8855	Glycerol yield (80)	*AFG3 ; ISC1 ; SBH2 ;* ***GPA2*** *; RPN3 ; SRB4*
V	chr.V@211.8	5.96	211789	7178	Glycerol yield (FT)	*ISC1 ; SBH2 ;* ***GPA2*** *; RPN3 ; SRB4*
V	chr.V@211.8	6.24	211789	7178	Glycerol (FT)	*ISC1 ; SBH2 ;* ***GPA2*** *; RPN3 ; SRB4*
V	chr.V@212.7	6.55	212748	14554	Lactate (80)	*GPA2 ;* ***RPN3*** *; SRB4 ; PRO3 ; YAT2 ; GCD11 ; CHO1*
V	chr.V@213.1	7.01	213099	26904	Lactate (FT)	*HEM14 ; AFG3 ; FAA2 ; BIM1 ; AFG3 ; ISC1 ; SBH2 ; GPA2 ;* ***RPN3*** *;* ***SRB4*** *; PRO3 ; YAT2 ; GCD11 ; CHO1*
V	chr.V@225.2	5.79	225247	15216	Ethanol yield (80)	*SRB4 ; PRO3 ; YAT2 ; GCD11 ; CHO1 ;* ***GAL83*** *;* ***MIG3*** *;* ***SMB1*** *;* ***CHZ1*** *;* ***YPT31*** *;* ***FIR1***
V	chr.V@231.6	4.00	231559	1715	Glycerol (80)	***FIR1***

Legend**: A01_Epate (ethyl propanoate), 04_2MPAce (2-methylpropyl acetate), A05_EBate (ethyl butanoate), A09_2MPol (2-methylpropanol), A13_23Mbol (2/3-methylbutanol), A14_EHate (ethyl hexanoate), A18_EOate (ethyl octanoate), A21_2MPAcid (2-methylpropanoic acid), A25_2MBAcid (2-methylbutanoic acid), A32_2PetOH (2-phenylethanol). Sampling points:* ***80 *(80 g/L CO2 released) and* ***FT*** *(final time).*

***Table 3**. (Cont.)*

Chromosome	QTL	Peak LOD	Peak (bp)	Length (bp)	Phenotype	Gene features (ScanOne)
V	chr.V@531.9	4.80	531943	49	A04_2MPAce (80)	***CCA1***
VI	chr.VI@152.4	4.38	152443	1	Glycerol (80)	***GCN20***
VI	chr.VI@152.4	4.07	152443	1	Glycerol yield (80)	***GCN20***
VII	chr.VII@473.8	5.00	473773	19132	A05_EBate (80)	*ALK1 ; GET1 ; CKB1 ; JAC1 ; ATE1 ; KAP122 ; BIL2 ; PUF4 ; PDR1 ; ERG4 ;* ***SCL1***
VII	chr.VII@474.5	4.91	474460	1716	A09_2MPol (80)	***SCL1 ; MPO1***
VII	chr.VII@475.8	3.79	475778	6238	A31_HAcid (80)	*SCL1 ; MPO1 ;* ***LEU1***
VIII	chr.VIII@71.1	4.23	71124	1160	Fructose (80)	***AIM17*** *; OPI1*
IX	chr.IX@172.7	4.05	172654	6738	A25_2MBAcid (80)	*PFK26 ; MOB1 ; SHQ1 ;* ***SLM1***
IX	chr.IX@380.4	5.05	380429	12318	A13_23MBol (80)	*YAP5 ; FLO11 ; MRS1 (potentially counted with 2 peaks)*
IX	chr.IX@420.9	3.82	420915	54839	Glycerol (80)	*EGH1 ; STS1*
XI	chr.XI@331.3	7.20	331283	5629	α-ketoglutarate (FT)	*MDM35 ; SFK1 ;* ***YKL050C***
XI	chr.XI@356.2	3.97	356208	5510	Citrate (FT)	***RGT1*** *; UGP1 ; TUL1*
XII	chr.XII@205.8	3.59	205776	1	A01_EPate (80)	***UBR2***
XII	chr.XII@442.1	3.57	442119	13254	R_{80}	***YLR149C ; SPE4 ; PEP3 ; RMP1 ; DPH6 ; ACF2***
XIII	chr.XIII@356.5	3.88	356463	753	R_{max}	***MSN2***
XIII	chr.XIII@359.3	4.15	359341	91	Lactate (80)	***CCS1***
XIII	chr.XIII@359.3	4.82	359341	91	Lactate (FT)	***CCS1***
XIII	chr.XIII@436.2	3.96	436238	1	A32_2PEtOH (80)	*no genes annotated (adjacent to ADH3)*
XVI	chr.XVI@349.8	4.19	349841	3281	A18_EOate (80)	***GDE1*** *; MCO76*
XVI	chr.XVI@507.3	3.94	507305	9776	A21_2MPAcid (80)	*MET12 ;* ***RAD1*** *; ECM23 ; VTC3*

Legend**: A01_Epate (ethyl propanoate), 04_2MPAce (2-methylpropyl acetate), A05_EBate (ethyl butanoate), A09_2MPol (2-methylpropanol), A13_23Mbol (2/3-methylbutanol), A14_EHate (ethyl hexanoate), A18_EOate (ethyl octanoate), A21_2MPAcid (2-methylpropanoic acid), A25_2MBAcid (2-methylbutanoic acid), A32_2PetOH (2-phenylethanol). Sampling points:* ***80 *(80 g/L CO2 released) and* ***FT*** *(final time).*

Identification of potential candidate genes

We investigated all annotated genes within each QTL 95% confidence interval to identify candidate genes affecting each trait. On average, we found 3.5 annotated genes per confidence interval, with a minimum of one gene (seven QTLs) and a maximum of 14. We used SGD (*Saccharomyces* Genome Database, https://www.yeastgenome.org/) to compile information about each annotated gene in each QTL and GeneMANIA (https://genemania.org/) to identify possible genetic interactions between genes affecting the same trait. From all candidate genes, only those containing SNPs between the *S. uvarum* parent strains were considered (Table 4).

Within all QTLs affecting kinetic parameters, three genes caught our attention because of their function and the available information in the bibliography. First, *MSN2*, the only gene in QTL 13@356.5 influencing the maximum fermentation rate (R_{max}), is a potential candidate. *MSN2* encodes a general stress-responsive transcriptional activator regulating the expression of more than 200 genes (Estruch and Carlson, 1993). Disruption of this gene results in higher stress sensitivity (Martínez-Pastor *et al.*, 1996) and increases the peak fermentation rate during ethanol fermentation (Watanabe *et al.*, 2011). Also, *MSN2* overexpression has been shown to decrease cell growth and proliferation (Estruch and Carlson, 1993). Therefore, polymorphisms between the *MSN2* alleles of the *S. uvarum* parent strains (Table 4) reducing its activity would increase cell growth and, consequently, the CO_2 production rate in the early fermentation stage, where cells divide exponentially. A superior *MSN2* allele would have the opposite effect.

SPE4, in QTL 12@442.1 affecting R_{80}, encodes a spermine synthase which is also involved in pantothenic acid biosynthesis (Hamasaki-Katagiri *et al.*, 1998). The synthetic grape must used here contains 1.5 mg/L of pantothenic acid, which is usually enough to maintain a high cellular activity during the whole process. Jimenez-Lorenzo *et al.* (2021) showed that pantothenic acid, when scarce, can limit the fermentation rate. As R_{80} is measured at the end of the stationary phase (it is equivalent to 87% of the fermentation process), deficiencies in pantothenic acid biosynthesis might impact the fermentation rate at this stage. Finally, *RMP1*, encoding a subunit of the enzyme RNase MRP (Salinas *et al.*, 2005), was found in the same QTL. RNase MRP is required for the progression of the cell cycle at the end of mitosis, and mutations lead to exit-from-mitosis defects caused by increased CLB2 mRNA levels (Gill *et al.*, 2004). Therefore, polymorphisms leading to defects in *SPE4* or *RMP1* could cause part of the observed variations in terms of R_{80}.

From the 12 QTLs found for fermentative aromas, only two contained genes that attracted our attention because of their function. First, QTL 5@148.6 affecting ethyl hexanoate production contained *EAF5*, which encodes a non-essential subunit of the NuA4 acetyltransferase complex (Krogan *et al.*, 2004). The last step in ethyl ester biosynthesis is

catalysed by acyl-CoA:ethanol O-acyltransferases (AEATases). Although the NuA4 complex catalyses the acetylation of histones, some acetyltransferases have a wide substrate specificity. For instance, Mgl2 was characterised for its monoacylglycerol lipase (MAGLase) activity (Selvaraju *et al.*, 2016), and its role in substituted ethyl ester formation was confirmed later (Marullo *et al.*, 2021). Additionally, some of those enzymes preferentially catalyse the esterification of certain fatty acids. This is the case for Eht1, which catalyses exclusively ethyl hexanoate synthesis (Saerens *et al.*, 2006). Although Eaf5 is not the catalytic subunit of the NuA4 acetyltransferase complex, it could be implicated in the esterification of hexanoic acid to form ethyl hexanoate. Second, QTL 13@436.2 impacting 2-phenylethanol formation did not contain any annotated ORF, but it was adjacent to a *ADH3*. The last step in the Ehrlich pathway for higher alcohol production is the reduction of fusel aldehydes, a reaction catalysed by alcohol dehydrogenases, including Adh3 (Hazelwood *et al.*, 2008). If QTL 13@436.2 is confirmed to be in the promoter region of *ADH3*, it could explain some of the differences observed in 2-phenylethanol production.

The QTL hotspot on chromosome V contained several genes regulating glucose uptake and metabolism (Table 5). *GPA2*, which encodes a G-protein α-subunit necessary for the stimulation of PKA upon the presence of glucose (Kraakman *et al.*, 1999), was found in three QTLs impacting the production of acetate, glycerol and lactate. The primary role of PKA in response to glucose is the inactivation of Rgt1, a repressor of hexose transporters (HXTs) (Kim and Johnston, 2006; Bisson, Fan and Walker, 2016). Within QTL 5@225.2, affecting ethanol production, we also found *GAL83*, encoding one of the three alternative β-subunits of the Snf1 complex (Wiatrowski *et al.*, 2004). This complex is responsible for glucose catabolite repression, which represses genes necessary for utilising alternative carbon sources, as well as respiration and gluconeogenesis upon sufficiently high hexose concentrations (Crabtree effect) (Bisson, Fan and Walker, 2016). Polymorphisms in both *GPA2* and *GAL83* could affect the production of central carbon metabolites through alterations in the regulation of glucose uptake.

One of the roles of the Snf1 complex is the inactivation of Mig1, a zinc finger transcription factor repressing genes whose transcription is shut off in the presence of glucose, such as the enzymes needed for the catabolism of alternative sugars (Carlson, 1999). We found *MIG3*, encoding another zinc finger transcription factor (Dubacq, Chevalier and Mann, 2004), in QTL 5@225.2. The role of Mig3 in glucose sensing was considered minor compared to that of Mig1 (Westholm *et al.*, 2008). However, Lewis and Gasch (2012) observed that Mig3 function had been lost in S228c-derived laboratory strains but was active in wild strains. There is little overlap between targets of Mig1 and Mig3, indicating those transcription factors play different roles (Lewis and Gasch, 2012). For instance, *MIG3* deletion reduced the expression of genes involved in mitochondrial function and respiration (Lewis and Gasch, 2012). Therefore, its role is considered to be

opposite to that of Mig1, which represses respiratory genes in favour of a fermentative metabolism. We hypothesise that mutation in *MIG3* lading to a change in its activity level or function could cause some of the differences observed in ethanol yield between segregants.

***Table 4**. List of SNPs found for some genes with a possible implication in the traits of interest.*

Chromosome	Position	SU03	SU05	Gene	Chromosome	Position	SU03	SU05	Gene
suva_chr05	147179	T	C	*EAF5*	suva_chr05	218566	G	A	*YAT2*
suva_chr05	147588	C	T	*EAF5*	suva_chr05	218749	G	A	*YAT2*
suva_chr05	147630	A	G	*EAF5*	suva_chr05	218944	C	A	*YAT2*
suva_chr05	208638	C	T	*ISC1*	suva_chr05	219001	T	C	*YAT2*
suva_chr05	208935	A	G	*ISC1*	suva_chr05	219086	G	A	*YAT2*
suva_chr05	209292	C	T	*ISC1*	suva_chr05	219106	C	T	*YAT2*
suva_chr05	209652	G	A	*ISC1*	suva_chr05	219129	A	C	*YAT2*
suva_chr05	210661	G	A	*GPA2*	suva_chr05	219265	T	C	*YAT2*
suva_chr05	210849	A	G	*GPA2*	suva_chr05	219610	G	A	*YAT2*
suva_chr05	210868	G	T	*GPA2*	suva_chr05	219624	C	T	*YAT2*
suva_chr05	210881	G	A	*GPA2*	suva_chr05	219713	A	T	*YAT2*
suva_chr05	211060	A	T	*GPA2*	suva_chr05	220099	C	T	*YAT2*
suva_chr05	211198	T	C	*GPA2*	suva_chr05	220120	A	G	*YAT2*
suva_chr05	211207	A	G	*GPA2*	suva_chr05	220225	G	A	*YAT2*
suva_chr05	211499	G	C	*GPA2*	suva_chr05	220250	A	C	*YAT2*
suva_chr05	211573	T	C	*GPA2*	suva_chr05	225247	G	A	*GAL83*
suva_chr05	211789	C	T	*GPA2*	suva_chr05	225561	C	T	*GAL83*
suva_chr05	211927	T	C	*GPA2*	suva_chr05	225641	T	C	*GAL83*
suva_chr05	212435	C	T	*RPN3*	suva_chr05	225647	T	C	*GAL83*
suva_chr05	212748	C	T	*RPN3*	suva_chr05	225847	C	T	*GAL83*
suva_chr05	212844	A	G	*RPN3*	suva_chr05	226495	G	C	*MIG3*
suva_chr05	212925	G	C	*RPN3*	suva_chr05	226550	A	T	*MIG3*
suva_chr05	213099	A	G	*RPN3*	suva_chr05	226628	C	T	*MIG3*
suva_chr05	213123	C	T	*RPN3*	suva_chr05	226834	C	A	*MIG3*
suva_chr05	213159	G	A	*RPN3*	suva_chr05	227025	A	G	*MIG3*
suva_chr05	213180	T	C	*RPN3*	suva_chr05	227247	C	T	*MIG3*
suva_chr05	213231	T	C	*RPN3*	suva_chr05	227269	C	T	*MIG3*
suva_chr05	213433	T	C	*RPN3*	suva_chr05	227396	C	A	*MIG3*
suva_chr05	213534	C	T	*RPN3*	suva_chr05	227462	T	C	*MIG3*
suva_chr05	213549	C	T	*RPN3*	suva_chr05	227537	T	C	*MIG3*
suva_chr05	213621	G	A	*RPN3*	suva_chr05	227558	T	C	*MIG3*
suva_chr05	217816	T	C	*YAT2*	suva_chr13	355710	T	C	*MSN2*
suva_chr05	217867	T	C	*YAT2*	suva_chr13	356463	A	C	*MSN2*
suva_chr05	217951	C	T	*YAT2*	suva_chr13	357117	C	T	*MSN2*
suva_chr05	218160	G	A	*YAT2*	suva_chr13	357171	A	G	*MSN2*
suva_chr05	218355	C	A	*YAT2*	suva_chr13	357281	C	T	*MSN2*
suva_chr05	218360	C	G	*YAT2*	suva_chr13	357597	T	A	*MSN2*
suva_chr05	218508	C	T	*YAT2*					

In the Snf3/Rgt2 glucose sensing pathway, when glucose is present, the membrane proteins Snf3 and Rgt2 activate the Yck1/2 kinases. Those enzymes phosphorylate the transcription factors Mth1 and Std1, marking them for degradation via the ubiquitin-proteasome pathway (Schmidt *et al.*, 1999; Bisson, Fan and Walker, 2016). The decrease in Mth1 and Std1 prevents Rgt1 from repressing hexose transporters (Kakykci and Nielsen, 2015). Similarly, in the PKA-cAMP cascade, PKA phosphorylates gluconeogenesis and respiration enzymes and marks them for proteolytic degradation (Rodicio and Heinisch,

2017). Therefore, proteasome activity plays a key role in glucose sensing and the regulation of its metabolism. We found two genes involved in proteasome activity within QTLs impacting central carbon metabolite production (Table 5). For instance, *RPN3*, encoding a regulatory subunit of the 26S proteasome lid (Kominami *et al.*, 1997), was found in three QTLs in the chromosome V hotspot. Similarly, *STS1*, encoding a protein required for localising proteasomes to the nucleus (Tabb *et al.*, 2000), was found on QTL 9@420.9 affecting glycerol production. Allelic variations in those genes could contribute to the differences observed in acetate, glycerol and lactate production within the segregants.

Finally, two genes involved in the maintenance of acetyl-CoA homeostasis were found within the chromosome V hotspot (Table 5): *ISC1*, which encodes an inositol phosphosphingolipid phospholipase C (Sawai *et al.*, 2000; Galdieri *et al.*, 2013), and *YAT2*, encoding a carnitine acetyltransferase (Swiegers *et al.*, 2001). Variations in cytosolic acetyl-CoA pools directly influence carbon fluxes within the cell. Therefore, variations in the nucleotide sequence of both *ISC1* and *YAT2* could also influence the phenotypic variation observed in terms of primary metabolite production.

***Table 5**. Candidate genes potentially affecting the production of compounds of the CCM.*

Gene	QTL	Compound	Description (SGD)	Metabolic pathway	Possible role in trait variation
GPA2	5@210.7 5@211.8 5@212.7	Acetate Glycerol Lactate	Nucleotide binding alpha subunit of the heterotrimeric G protein	Snf3/Rgt2 glucose sensing pathway	Glucose sensing (together with Gpr1)
GAL83	5@225.2	Ethanol	β-subunit of the Snf1 complex	Snf1/Mig1 repression pathway	Snf1 complex efficiency
MIG3	5@225.2	Ethanol	Zinc finger transcriptional regulator	Several	Activation / repression of genes implicated in glucose metabolism
RPN3	5@210.7 5@211.8 5@212.7	Acetate Glycerol Lactate	Essential non-ATPase regulatory subunit of the 26S proteasome lid	Ubiquitin-proteasome pathway	Degradation of respiration enzymes and Mth1 and Std1 transcriptional factors
STS1	9@420.9	Glycerol	Protein required for localizing proteasomes to the nucleus	Ubiquitin-proteasome pathway	Degradation of respiration enzymes and Mth1 and Std1 transcriptional factors
ISC1	5@207.0 5@210.7 5@211.8 5@213.1	Ethanol Acetate Glycerol Lactate	Inositol phosphosphingolipid phospholipase C	Sphingolipid metabolism	Involved in acetyl-CoA homeostasis
YAT2	5@212.7 5@213.1 5@225.2	Lactate Lactate Ethanol	Carnitine acetyltransferase	Carnitine shuttle	Involved in acetyl-CoA homeostasis

CONCLUSIONS AND PERSPECTIVES

In conclusion, fertility was successfully restored in allotetraploid *S. mikatae x S. uvarum* hybrids, allowing multigenerational breeding and mapping oenological traits for the first time in non-*cerevisiae Saccharomyces* species. QTL mapping in the *S. mikatae* subgenome was not possible due to the high sequence similarity between the parent strains. The use of alternative *S. mikatae* strains in the future may provide more allelic variants contributing to the interesting traits shown by this species in wine fermentation. In the case of *S. uvarum*, several candidate genes were identified, mainly affecting the production of compounds of the central carbon metabolism and, to a lesser extent, fermentative aromas and kinetic parameters. The validation of those allelic variants in the near future will likely provide targets which could be used to improve *S. uvarum* and its hybrids for wine fermentation. Although no mitotype-dependent QTLs were found, segregants containing mitochondria from *S. uvarum* fermented more efficiently and produced significantly different amounts of some fermentation metabolites. This observation highlights the interest of studying the mitochondrial inheritance in interspecific *Saccharomyces* hybrids for industrial applications. Overall, this work lays the foundations for mapping oenological traits in non-*cerevisiae Saccharomyces* species and provides valuable lessons for future studies.

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SUPPLEMENTARY MATERIAL – CHAPTER 2

***Table S1**. Primers used for HO and MAT gene deletion, verification, and species determination.*

FW: forward. RV: reverse. Capital letters indicate homology to the target gene, while small letters indicate homology to the plasmid.

Primer name	Sequence (5' → 3')	Species	Usage	Reference
HOdel_all_FW	CAACAATGTCAGACACTGGACGGAAGAATAATAACAATTCCCAAAAAttcgtacgctgcaggtcgac	all *Saccharomyces*	*ho::HYG*	this study
HOdel_all_RV	CAATATGACAGAACATTCTGTAATGTCGTTCCTCCAGCAACATTACAgcataggccactagtggatctg	all *Saccharomyces*	*ho::HYG*	this study
HOdel_mik_jur_FW	GAATATGCGGCGAAGCGCTTTATAGAAGAAATGGAGCGCTCAAAAGGAGAttcgtacgctgcaggtcgac	*S. mikatae* and *S. jurei*	*ho::HYG*	this study
HOdel_mik_jur_RV	CCACGGACAGCATCAAACTGTAGAATTCCACCACATTTCAAACATTCTGgcataggccactagtggatctg	*S. mikatae* and *S. jurei*	*ho::HYG*	this study
HOdel_eub_uva_RV	TCACGTGCTTCTGGTACATATTTGCAGTTTATACAGTGATGGCCACTAgcataggccactagtggatctg	*S. eubayanus* and *S. uvarum*	*ho::HYG*	this study
HOverif_mik_FW	CAACTATCCTGATGGCAAATGGT	*S. mikatae*	*ho::HYG* check	this study
HOverif_mik_jur_RV	CACGGACAGCATCAAACTGTA	*S. mikatae* and *S. jurei*	*ho::HYG* check	this study
HOverif_uva_FW	TGATGGCCAATGGTAAAATCGAAG	*S. uvarum*	*ho::HYG* check	this study
HOverif_eub_uva_RV	ACGAACAGCGTCAAATTGCA	*S. eubayanus* and *S. uvarum*	*ho::HYG* check	this study
MATdel_ALL_FW	GATTTGAATGCGAGATAAACTGGTATTCTTCATTAGATTCTCTAGGCCCTccagctgaagcttcgtacgc	all *Saccharomyces*	*mat::KanMX*	this study
MATdel_ALL_RV	AAGATAAACAACCTCCGCCACGACCACACTCTATAAGGCCAAATGTACAgcataggccactagtggatctg	all *Saccharomyces*	*mat::KanMX*	this study
MATdel_mik_FW	AAGCCTTTGATATTCGTTATCGGTAGCCAAGTGGCTGTACCAAAAGGTAAGGATccagctgaagcttcgtacgc	*S. mikatae*	*mat::KanMX*	this study
MATdel_mik_RV	CAGTCAGCAGAAAGTTCTATATATTGTGATCACTGAATTTTAATTCACTTCTGTGCgcataggccactagtggatctg	*S. mikatae*	*mat::KanMX*	this study
MATdel_uva_FW	ACGCTACATACAAAGAACGTGCTGCTACTCATCCTAGCCCAGTTGccagctgaagcttcgtacgc	*S. uvarum*	*mat::KanMX*	this study
MATdel_uva_RW	CGTTACAGAAAAGCAGGCTGGGAAGCTTACTTGAAGAGATGCGGGgcataggccactagtggatctg	*S. uvarum*	*mat::KanMX*	this study
MAT_FW	AGTCACATCAAGATCGTTTATGG	all *Saccharomyces*	*MAT* locus amplification	none
MATa_RV	ACTCCACTTCAAGTAAGAGTTTG	all *Saccharomyces*	*MAT* locus amplification	none
MATalpha_RV	GCACGGAATATGGGACTACTTCG	all *Saccharomyces*	*MAT* locus amplification	none
Smik_2011_FW	ACAAGCAATTGATTTGAGGAAAAG	*S. mikatae*	Species determination	Muir *et al.*, (2011)
Smik_2011_RV	CCAGTCTTCTTTGTCAACGTTG	*S. mikatae*	Species determination	Muir *et al.*, (2011)
Suva_2011_FW	GCTGACTGCTGCTGCTGCCCCCG	*S. uvarum*	Species determination	Muir *et al.*, (2011)
Suva_2011_RV	TGTTATGAGTACTTGGTTTGTCG	*S. uvarum*	Species determination	Muir *et al.*, (2011)

***Table S2**. Strains generated in this study for the construction of fertile tetraploid hybrids.*

Species	Strain	Type	Genotype	Original strain
S. uvarum	SU01-ho	HO deletant	*HO/ho::HYG*	SU01
S. uvarum	SU03-ho	HO deletant	*HO/ho::HYG*	SU03
S. uvarum	SU05-ho	HO deletant	*HO/ho::HYG*	SU05
S. uvarum	SU07-ho	HO deletant	*HO/ho::HYG*	SU07
S. uvarum	SU11-ho	HO deletant	*HO/ho::HYG*	SU11
S. mikatae	SM02-ho	HO deletant	*HO/ho::HYG*	SM02
S. mikatae	SM05-ho	HO deletant	*HO/ho::HYG*	SM05
S. mikatae	SM06-ho	HO deletant	*HO/ho::HYG*	SM06
S. uvarum	SU01-a	Stable haploid	*ho::HYG, MATa*	SU01-ho
S. uvarum	SU01-α	Stable haploid	*ho::HYG, MATα*	SU01-ho
S. uvarum	SU03-a	Stable haploid	*ho::HYG, MATa*	SU03-ho
S. uvarum	SU03-α	Stable haploid	*ho::HYG, MATα*	SU03-ho
S. uvarum	SU05-a	Stable haploid	*ho::HYG, MATa*	SU05-ho
S. uvarum	SU05-α	Stable haploid	*ho::HYG, MATα*	SU05-ho
S. uvarum	SU07-a	Stable haploid	*ho::HYG, MATa*	SU07-ho
S. uvarum	SU07-α	Stable haploid	*ho::HYG, MATα*	SU07-ho
S. uvarum	SU11-a	Stable haploid	*ho::HYG, MATa*	SU11-ho
S. uvarum	SU11-α	Stable haploid	*ho::HYG, MATα*	SU11-ho
S. mikatae	SM02-a	Stable haploid	*ho::HYG, MATa*	SM02-ho
S. mikatae	SM02-α	Stable haploid	*ho::HYG, MATα*	SM02-ho
S. mikatae	SM05-a	Stable haploid	*ho::HYG, MATa*	SM05-ho
S. mikatae	SM05-α	Stable haploid	*ho::HYG, MATα*	SM05-ho
S. mikatae	SM06-a	Stable haploid	*ho::HYG, MATa*	SM06-ho
S. mikatae	SM06-α	Stable haploid	*ho::HYG, MATα*	SM06-ho
S. uvarum	D04-a	Intraspecific diploid mater	*ho::HYG/ho::HYG, MATa/mat::G418*	D04
S. uvarum	D04-α	Intraspecific diploid mater	*ho::HYG/ho::HYG, mat::G418/MATα*	D04
S. uvarum	D09-a	Intraspecific diploid mater	*ho::HYG/ho::HYG, MATa/mat::G418*	D09
S. uvarum	D09-α	Intraspecific diploid mater	*ho::HYG/ho::HYG, mat::G418/MATα*	D09
S. uvarum	D14-a	Intraspecific diploid mater	*ho::HYG/ho::HYG, MATa/mat::G418*	D14
S. uvarum	D14-α	Intraspecific diploid mater	*ho::HYG/ho::HYG, mat::G418/MATα*	D14
S. uvarum	D15-a	Intraspecific diploid mater	*ho::HYG/ho::HYG, MATa/mat::G418*	D15
S. uvarum	D15-α	Intraspecific diploid mater	*ho::HYG/ho::HYG, mat::G418/MATα*	D15
S. mikatae	D22-a	Intraspecific diploid mater	*ho::HYG/ho::HYG, MATa/mat::G418*	D22
S. mikatae	D22-α	Intraspecific diploid mater	*ho::HYG/ho::HYG, mat::G418/MATα*	D22
S. mikatae	D25-a	Intraspecific diploid mater	*ho::HYG/ho::HYG, MATa/mat::G418*	D25
S. mikatae	D25-α	Intraspecific diploid mater	*ho::HYG/ho::HYG, mat::G418/MATα*	D25
S. uvarum	D14-a, ρ(-)	Petite intraspecific diploid mater	*ho::HYG/ho::HYG, MATa/mat::G418, ρ(-)*	D14-a
S. uvarum	D14-α, ρ(-)	Petite intraspecific diploid mater	*ho::HYG/ho::HYG, mat::G418/MATα, ρ(-)*	D14-α
S. uvarum	D15-a, ρ(-)	Petite intraspecific diploid mater	*ho::HYG/ho::HYG, MATa/mat::G418, ρ(-)*	D15-a
S. uvarum	D15-α, ρ(-)	Petite intraspecific diploid mater	*ho::HYG/ho::HYG, mat::G418/MATα, ρ(-)*	D15-α
S. mikatae	D22-a, ρ(-)	Petite intraspecific diploid mater	*ho::HYG/ho::HYG, MATa/mat::G418, ρ(-)*	D22-a
S. mikatae	D22-α, ρ(-)	Petite intraspecific diploid mater	*ho::HYG/ho::HYG, mat::G418/MATα, ρ(-)*	D22-α
S. mikatae	D25-a, ρ(-)	Petite intraspecific diploid mater	*ho::HYG/ho::HYG, MATa/mat::G418, ρ(-)*	D25-a
S. mikatae	D25-α, ρ(-)	Petite intraspecific diploid mater	*ho::HYG/ho::HYG, mat::G418/MATα, ρ(-)*	D25-α

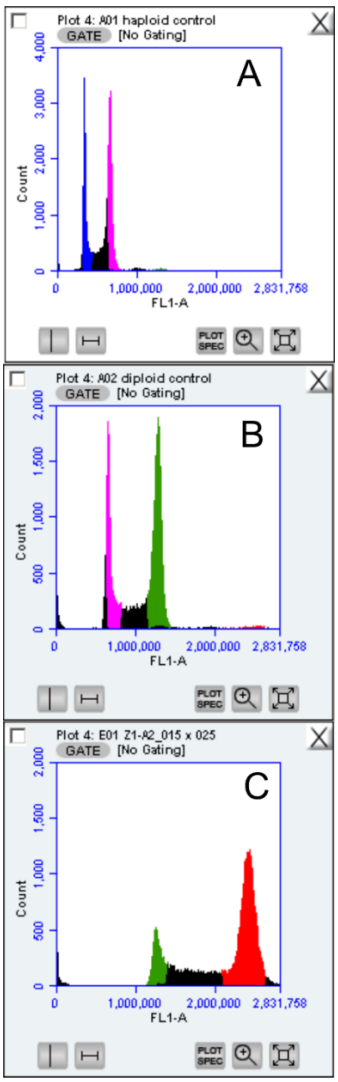


***Figure S3**. Example of ploidy estimation using flow cytometry.*
***A**: haploid S. cerevisiae control. **B**: diploid S. cerevisiae control. **C**: hybrid T20.*

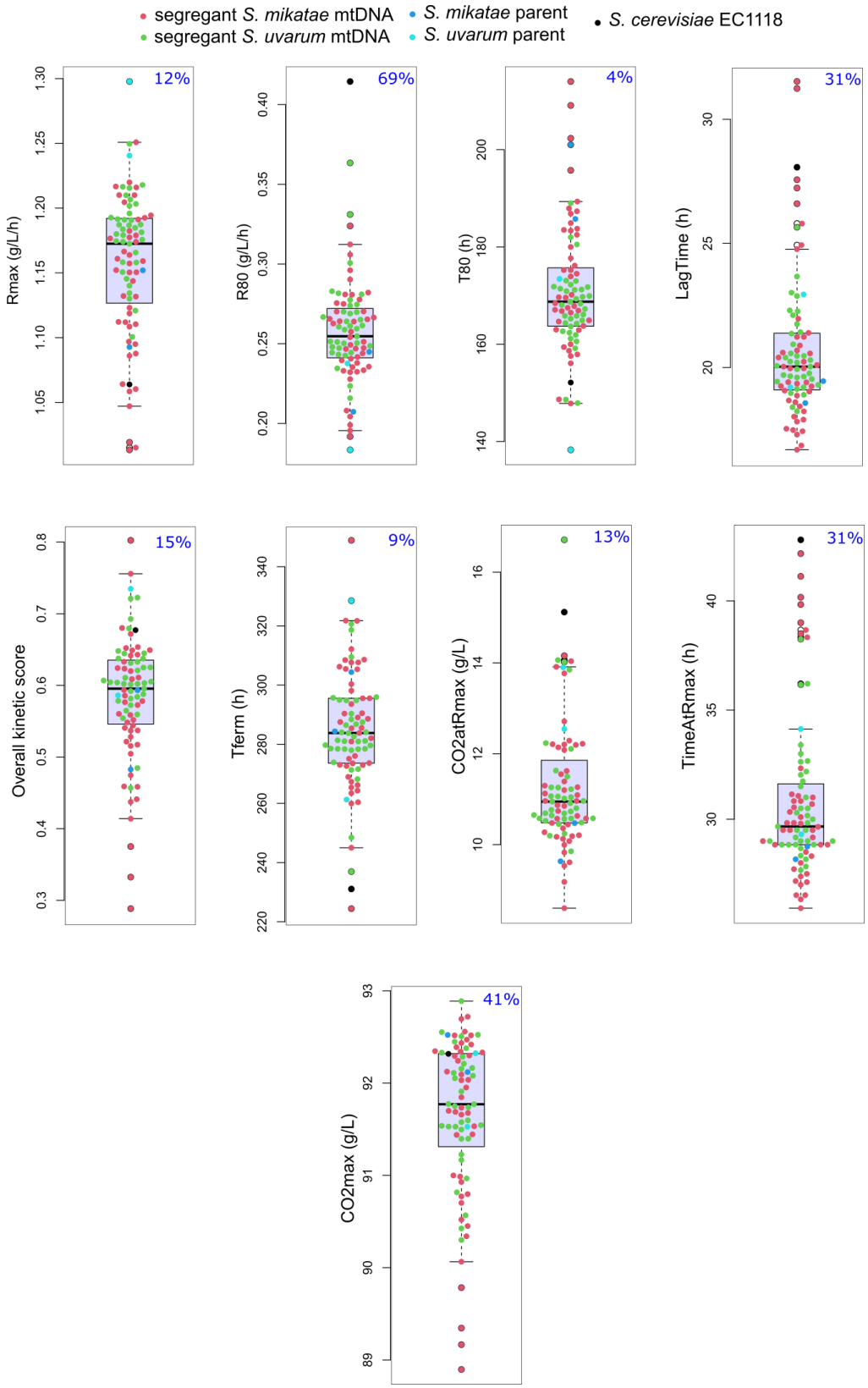


Figure S4. *Boxplots showing the distribution of kinetic parameters. The blue percentage indicates the proportion of segregants showing a transgressive phenotype.*

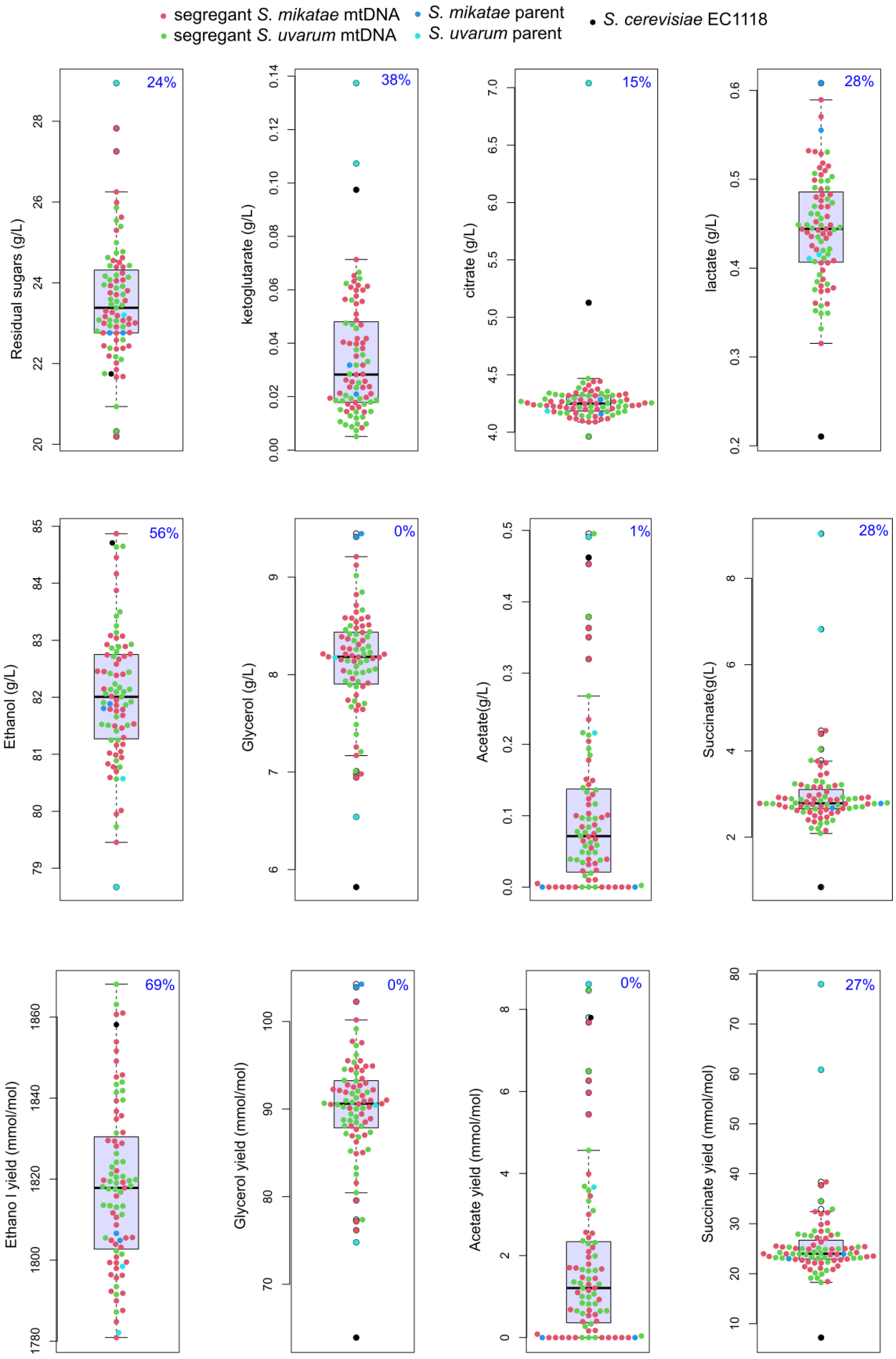


Figure S5. *Boxplots showing the distribution of central carbon metabolites, at 80 g/L CO_2 released. The blue percentage indicates the proportion of segregants showing a transgressive phenotype.*

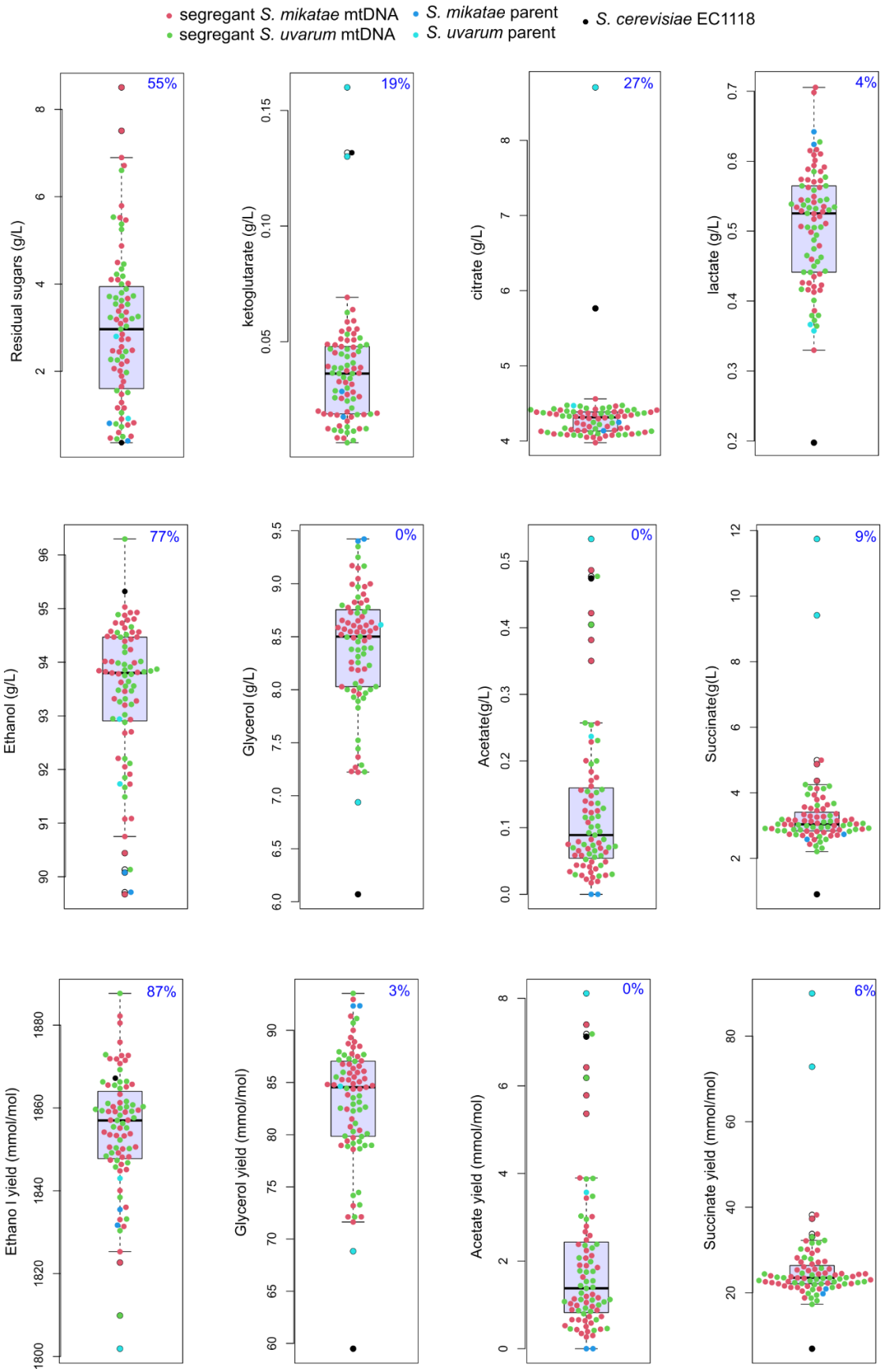


Figure S6. *Boxplots showing the distribution of central carbon metabolites at final time. The blue percentage indicates the proportion of segregants showing a transgressive phenotype.*

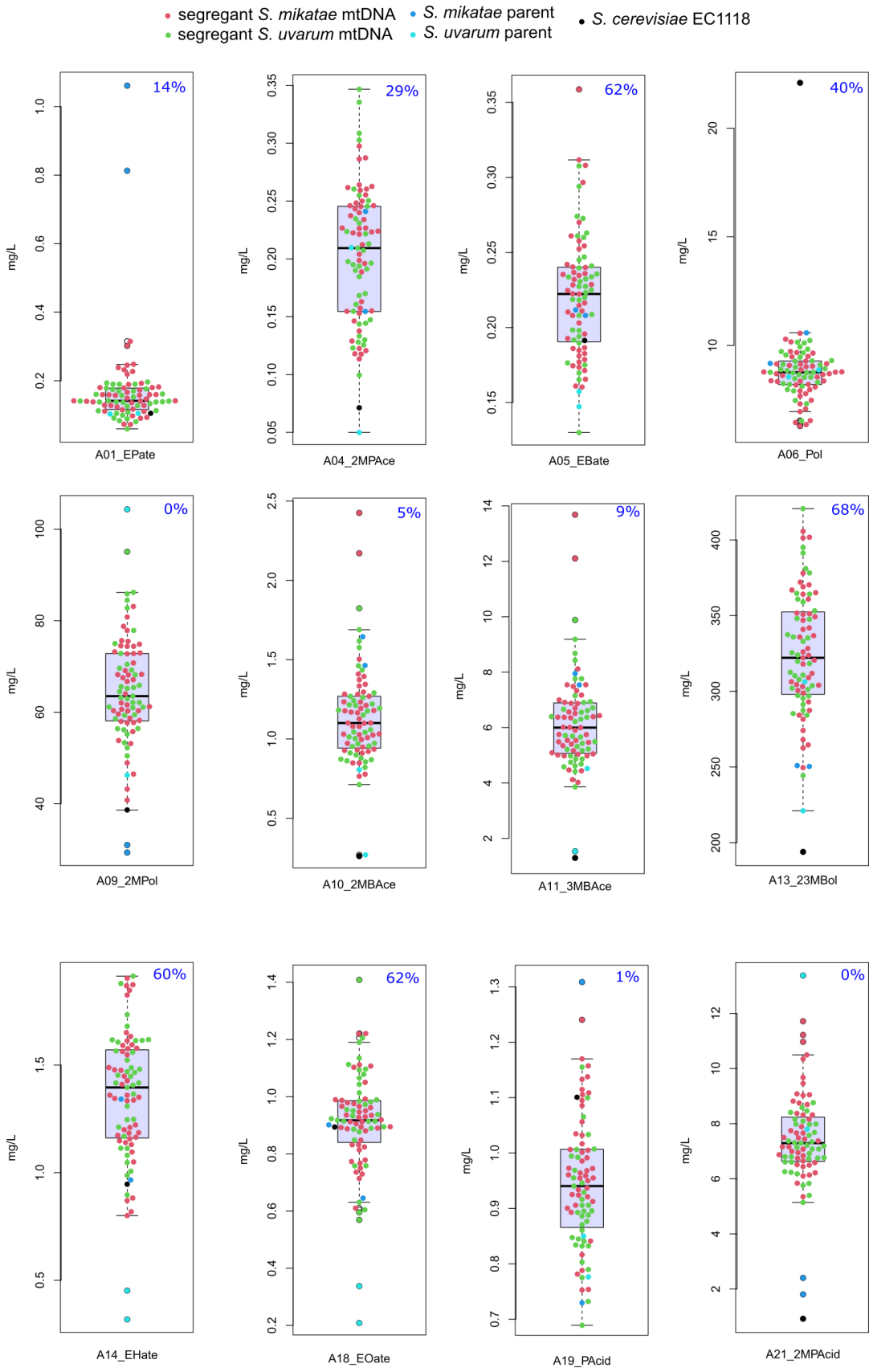


Figure S7*. Boxplots showing the distribution of fermentative aromas at 80 g/L CO2 released. The blue percentage indicates the proportion of segregants showing a transgressive phenotype.*

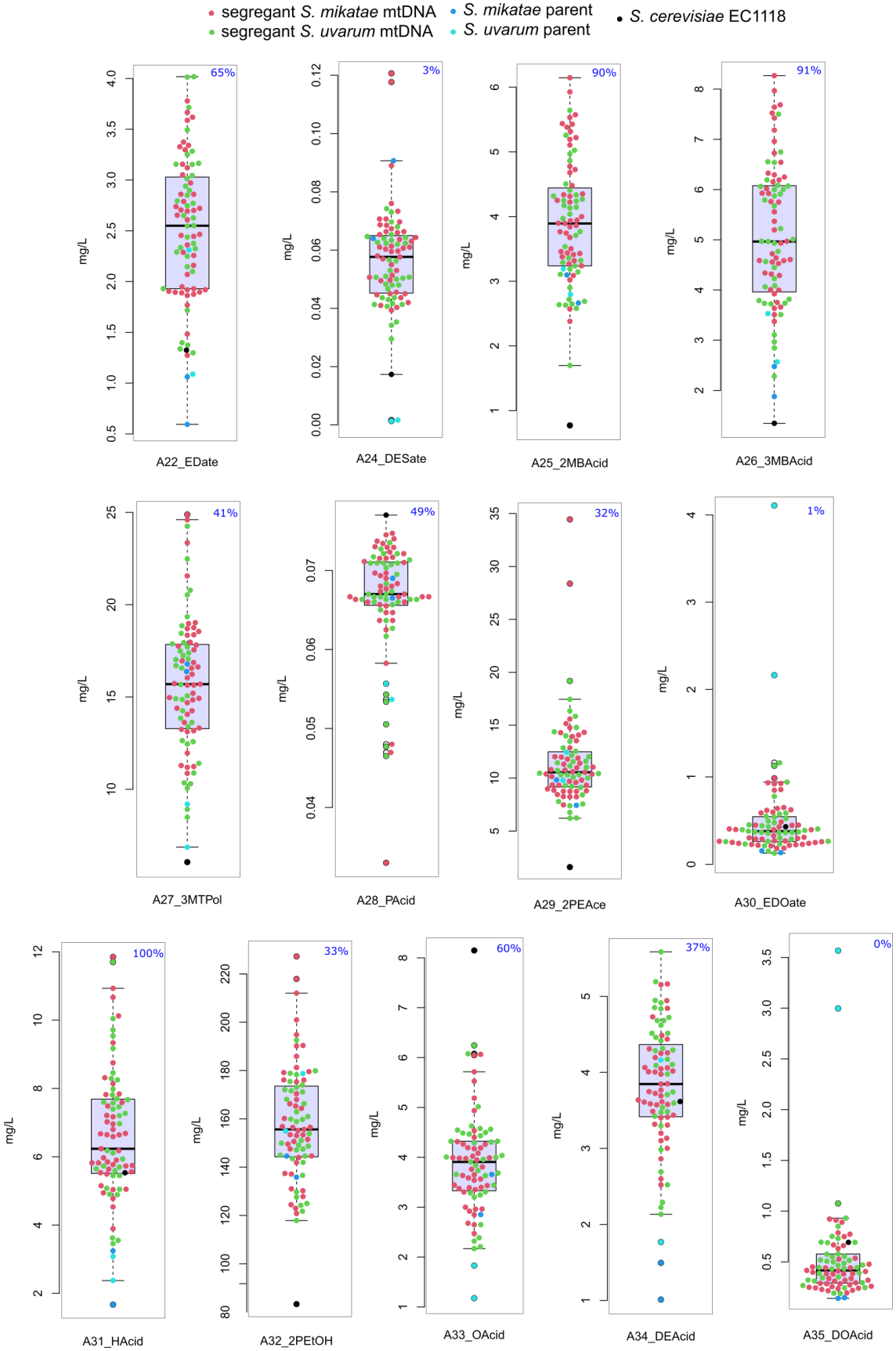


Figure S7. *(cont.)*

One-way ANOVA tests were performed to compare the origin of mitochondrial DNA's effect (*S. uvarum* or *S. mikatae*) on every parameter. Only statistically significant results are shown in **Table S8**.

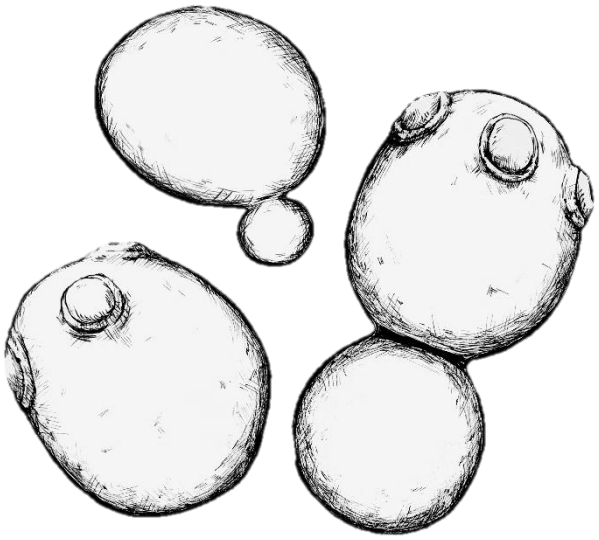
***Table S8**. Summary of the results of one-way ANOVA.*
*Signification codes: 0 < *** < 0.001 < ** < 0.01 < * < 0.05.*

t_{80}	DF	Sum of squares	Mean square	F	Pr > F	Signification
Model	1	730.839	730.839	4.973	**0.029**	*
Residual	76	11169.578	146.968			
Total	77	11900.416				
R_{max}	DF	Sum of squares	Mean square	F	Pr > F	Signification
Model	1	0.030	0.030	12.480	**0.001**	***
Residual	76	0.182	0.002			
Total	77	0.212				
kinetic score	DF	Sum of squares	Mean square	F	Pr > F	Signification
Model	1	0.047	0.047	6.576	**0.012**	*
Residual	76	0.540	0.007			
Total	77	0.587				

α-ketoglutarate	DF	Sum of squares	Mean square	F	Pr > F	Signification
Model	1	0.003	0.003	8.834	**0.004**	**
Residual	76	0.024	0.000			
Total	77	0.026				

A19, propanoic acid	DF	Sum of squares	Mean square	F	Pr > F	Signification
Model	1	0.105	0.105	9.059	**0.004**	**
Residual	76	0.878	0.012			
Total	77	0.982				
A21, 2-methylpropanoic acid	DF	Sum of squares	Mean square	F	Pr > F	Signification
Model	1	11.247	11.247	6.862	**0.011**	*
Residual	76	124.566	1.639			
Total	77	135.813				
A24, diethyl succinate	DF	Sum of squares	Mean square	F	Pr > F	Signification
Model	1	0.001	0.001	5.989	**0.017**	*
Residual	76	0.017	0.000			
Total	77	0.018				
A25, 2-methylbutanoic acid	DF	Sum of squares	Mean square	F	Pr > F	Signification
Model	1	4.134	4.134	4.968	**0.029**	*
Residual	76	63.236	0.832			
Total	77	67.370				

GENERAL DISCUSSION



Today, the main goal of wine microbiologists is to diversify the set of commercially available active dry yeasts (ADY) in an increasingly competitive wine sector, which is hard-pressed by evolving market trends and the effects of global warming in grape must composition (Mira de Orduña, 2010; Dequin *et al.*, 2017). In general terms, we seek to discover or create yeast strains able to produce wines with different flavour and aroma, higher organic acid and lower ethanol amounts than the currently available ADY. Within the wide toolset available for that purpose, the study of the natural yeast biodiversity in winemaking conditions is fundamental. It allows the discovery of new phenotypic traits whose genetic bases have to be determined thereafter for a more accurate strain improvement. The characterisation of both *S. cerevisiae* and non-*Saccharomyces* wild isolates has allowed their improvement using genetic engineering, laboratory evolution, mutagenesis or hybridisation, sometimes leading to commercialisation. Although the study of the *Saccharomyces* genus in ecology and evolution is on the rise, only a little fraction from all available non-*cerevisiae Saccharomyces* strains have been phenotyped in winemaking conditions, hindering their usage. This thesis project was dedicated to expand the knowledge on the oenological traits and their genetic bases in *Saccharomyces* species and interspecific hybrids, with a special focus on non-*cerevisiae Saccharomyces*.

In a first stage, we phenotyped 92 strains belonging to the eight known *Saccharomyces* species in synthetic grape must fermentation at 16 and 22 ºC. We included 20 *S. cerevisiae* strains isolated from wine fermentations and wild habitats, and strains from *S. cerevisiae*, *S. uvarum* and *Sc x Sk* commercially available as ADY.

Regarding fermentative capacities, perhaps the most surprising finding was the fact that strains from every *Saccharomyces* species could ferment synthetic must with 200 g/L sugars until dryness.

A fermentation profile is described by several parameters, including the lag phase, the maximum fermentation rate and the time needed to reach it (which reflect the fermentation efficiency at the beginning of the process), the time required to produce 60 g/L of CO_2 and the rate at that time (which reflect the activity of the yeasts at the end of the process), and finally, the fermentation time and the final amount of CO_2 produced. For a general comparison of the kinetic capacities between strains, we developed the *overall kinetic score*, a general indicator of fermentation efficiency. Using this parameter, we observed that some wild *S. cerevisiae*, *S. paradoxus*, *S. kudriavzevii* and *S. arboricola* strains fermented more efficiently than commercial strains under the conditions used. This and other studies (Orlić *et al.* 2007; Parpinello *et al.* 2020; Pérez *et al.* 2021) suggest that fermentative capacities are not a phenotype unique to *S. cerevisiae*, but rather extended to the whole *Saccharomyces* genus. It is possible that, if given the opportunity, *Saccharomyces* species other than *S. cerevisiae* could have developed similar domestication hallmarks and thrive in industrial wine fermentations. This is exemplified by

some strains of *S. uvarum* – phylogenetically the most distant species from *S. cerevisiae* – dominating spontaneous fermentations in some regions (Demuyter *et al.* 2004), and having domestication signatures such as a chromosomal translocation conferring increased sulfite resistance (Macías *et al.*, 2021). Adaptive laboratory evolution (ALE) experiments mimicking the domestication process could be a promising approach to achieve similar characteristics in other *Saccharomyces* species.

We should, however, be cautious when interpreting our results regarding fermentative capacities, as the conditions employed here were mild compared to those used in industrial settings. For instance, the natural grape musts used by winemakers can have higher sugar amounts, different ratios between sugars, lipids, nitrogen, vitamins and dissolved oxygen, and more complex nutrient sources such as oligosaccharides and oligopeptides. Additionally, the scaling-up of the process to higher volumes and the fermentation in the presence of solid particles can impact fermentation kinetics. Evaluating the effect of those parameters on the fermentative capacities of non-*cerevisiae* *Saccharomyces* is necessary before considering their commercialisation. Indeed, we evaluated the behaviour of three wild strains in grape musts with increased sugar amounts. From them, SA03 was negatively affected by sugar amounts higher than 220 g/L, while SC03 and SK06 performed better than the *S. cerevisiae* control EC1118 in the presence of 240 g/L sugars. Future research with these two strains should focus on finding their limitations, if any, in industrial conditions.

Regarding metabolite production, we observed far greater diversity within non-*cerevisiae* *Saccharomyces* than within *S. cerevisiae* alone. We found some specificities at the species level, with certain species differing significantly from *S. cerevisiae* in the production of aromas and compounds of central carbon metabolism. For instance, strains of non-conventional *Saccharomyces* species stood up for low acetic acid (*S. mikatae* and *S. paradoxus*), high glycerol (*S. arboricola, S. jurei, S. mikatae* and *S. eubayanus*), high succinic acid, 2-phenylethanol and 2-phenylethyl acetate (*S. uvarum*), high propanol (*S. kudriavzevii*), or high 2-methylbutyl acetate and ethyl propanoate (*S. mikatae*) production. This metabolic diversity has the potential to solve some of the challenges faced by today's wine sector. However, the fact that those differences in metabolite production are statistically significant does not imply that they significantly impact wine flavour and aroma. As the overall organoleptic experience depends on the interaction between thousands of compounds, many of which we did not quantify, sensory analysis is the most straightforward approach to determine the actual impact of those strains. Moreover, natural grape musts have additional aroma precursors, absent in the synthetic must used here, and whose impact (positive or negative) in fermentative and varietal aroma production we did not evaluate. Fermentations in natural grape must will answer this question in the future.

Finally, we did not assess some other important characteristics required by the wine industry, such as resistance to sulfites, pesticide residues, efficient respiratory growth for biomass production, or the survival of yeast cells after the drying process used for ADY production. Those properties should be evaluated as well prior to industrialisation.

In a second stage, we sought to determine the genetic basis of some of the mentioned phenotypes by generating fertile interspecific hybrids and applying QTL mapping on their progeny. We successfully generated fertile *S. mikatae* x *S. uvarum* tetraploids, controlling their mitochondrial inheritance, and performed QTL analysis on 78 diploid segregants.

To our knowledge, ours was the first QTL mapping study performed entirely with non-*cerevisiae Saccharomyces* species. The fact that we could generate fertile interspecific hybrids without *S. cerevisiae* was not surprising, as the mating capacity and the post-zygotic reproductive barriers are common to the whole *Saccharomyces* genus. Still, our results suggest that this strategy can be employed with every pair of *Saccharomyces* species. A straightforward perspective is to use the same technique with different species in the future.

Regarding the strain choice, we used four strains isolated from wild environments as we were interested in the genetic basis of their metabolic traits. Conversely, previous QTL mapping studies on *S. cerevisiae* have crossed strains from different locations and niches, including wine strains (Cubillos *et al.*, 2011). As mentioned above, there are examples of *S. uvarum* strains adapted to industrial wine fermentation. Therefore, future studies could aim to construct fertile hybrids using wine strains of *S. uvarum* and *S. cerevisiae*, for example, applying the strategy used here. This would allow the mapping of QTLs specific to domesticated isolates, some of which may be interesting for application in oenology.

Contrary to the expected, we did not detect any mitotype-dependent QTL (this is, a QTL present only in segregants with a given mitotype and absent in segregants with the other one). However, we found significant differences between segregants having *S. mikatae* and *S. uvarum* mitochondria for some phenotypes. Particularly, segregants with *S. uvarum* mitotype fermented more efficiently at 22 ºC than those having mitochondria from *S. mikatae*. This is an interesting finding, given that *S. uvarum* strains generally fermented more efficiently than *S. mikatae* in Chapter 1, which was confirmed in Chapter 2 using their haploid derivatives. Moreover, the mtDNA origin significantly affected the production of some metabolites (i.e. diethyl succinate and propanoic, 2-methylpropanoic and 2-methylbutanoic acids). This highlights the interest in further investigating mitochondria's role in fermentation kinetics and other oenologically relevant traits, using our approach or

simply constructing interspecific hybrids with different mitotypes and comparing their phenotypes.

We could not detect any QTL for the *S. mikatae* subgenome, due to the use of two very similar *S. mikatae* strains. This highlights the importance of a previous genetic marker screening in addition to the phenotypic screening. Although whole genome sequencing is increasingly cheap, it is not necessary for the genetic marker screening, as other techniques such as high-density oligonucleotide arrays can be used for SNP detection.

Conversely, we detected several QTLs in the *S. uvarum* subgenome. Thanks to the use of an F12 advanced intercross population, those QTLs were generally narrow – many of them containing one or two ORFs – despite the relatively low number of segregants used. In future studies, the use of larger segregant populations will allow mapping minor QTLs with small effects in the traits of interest, approaching the sources of missing heritability. Nevertheless, the reproducible phenotyping of a very large number of strains in wine fermentation is still a challenge, and improving the current high-throughput automated phenotyping systems is still necessary to accomplish that goal.

Within the *S. uvarum* QTLs, we found some candidate genes whose role in trait variation is worth investigating. For instance, the effect of *MSN2* allelic variants on the maximum fermentation rate has been reported in *S. cerevisiae* strains in similar conditions (Watanabe *et al.*, 2011), and it would be interesting to validate its function in a different species. Regarding aroma production, the role of *EAF5* in ethyl ester production has not been reported, but its acetyltransferase activity might indicate an implication in the biosynthesis of those compounds. Interestingly, we found a QTL hotspot on chromosome V, with a marked pleiotropic effect on compounds of the CCM. Pleiotropic polymorphisms are of high interest, as their use can allow the improvement of several traits simultaneously. Mainly within this hotspot, but also in other regions of the genome, we identified various candidate genes with regulatory functions, such as the regulation of glucose uptake and metabolism (*GPA2*, *GAL83*, *MIG3*), proteasome activity (*RPN3*, *STS1*) and genes involved in acetyl-CoA homeostasis (*ISC1*, *YAT2*). Nevertheless, the actual impact of allelic variants of the mentioned genes is yet to be determined. Applying reciprocal hemizygosity analysis (RHA) (Steinmetz *et al.*, 2002) will shortly answer this question. If confirmed, the role of those genes would emphasise the key role of regulatory functions in oenological trait variations, as previously reported in *S. cerevisiae* (Salinas *et al.*, 2012; Eder *et al.*, 2018). Moreover, it would be interesting to find out whether the presence of the *S. mikatae* subgenome affects those QTLs through epistatic interactions, which could be ascertained by repeating the whole process with the *S. uvarum* intraspecific hybrid.

Taken together, the array of possibilities that this research opens up for the diversification of *Saccharomyces* wine yeasts is enormous:

1. The isolation and characterisation of more *Saccharomyces* strains will probably lead to the discovery of other strains (and most likely other species as well) with adequate fermentative power and diverse metabolic properties.

2. Both intra- and interspecific QTL mapping will allow to discover allelic variants responsible for other phenotypes of interest in non-conventional *Saccharomyces* species and hybrids.

3. Virtually, every technique currently used to study and improve *S. cerevisiae* can be applied to other *Saccharomyces* species. As an example, the use of ^{13}C metabolic flux analysis (MFA) has revealed useful information for strain improvement in *S. cerevisiae*. Its application on other *Saccharomyces* species would allow studying the mechanisms behind some of the metabolic traits showed in Chapter 1.

4. Researchers will likely attempt many interspecific hybrid combinations using non-GMO generating methods (e.g. rare mating) to increase fitness in wine fermentation while improving metabolite production. These hybrids do not necessarily need to include *S. cerevisiae*. Moreover, the isolation of petite strains from one or the other parent species would allow generating hybrids with a selected mitotype.

5. As the genome of recently formed interspecific hybrids is generally unstable, scientists could use this situation to further improve the hybrids mentioned in point 4 towards a desired phenotype through ALE strategies (for example, using increasing sulfite concentrations).

6. Perhaps less attractive from an industrial perspective, it would be the sequential or co-inoculation of a non-*Saccharomyces* and a non-*cerevisiae Saccharomyces* strain. If the coexistence of non-*Saccharomyces* and *S. cerevisiae* in the same fermentation tank has provided promising results, alternative *Saccharomyces* species might yield similar or even better outcomes.

7. Going even further, the *Saccharomyces* strain in the latter combination could be the laboratory-evolved interspecific hybrid mentioned in point 5.

All those possibilities are promising ways tackle the challenges faced by today's wine industry. As usually, the solution for most human problems is in nature. We just need to look deep enough.

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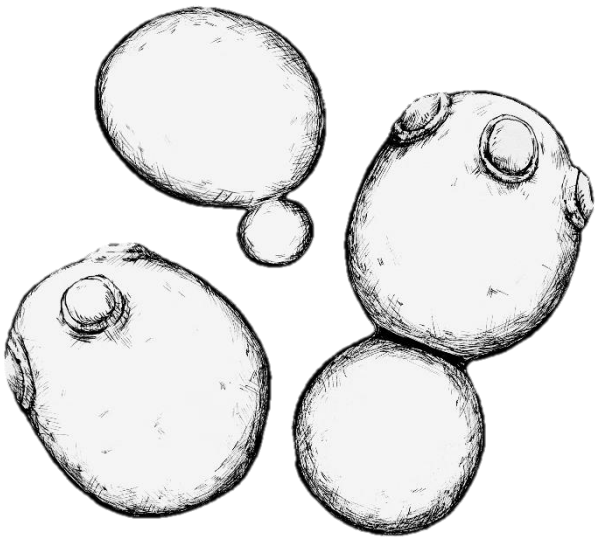

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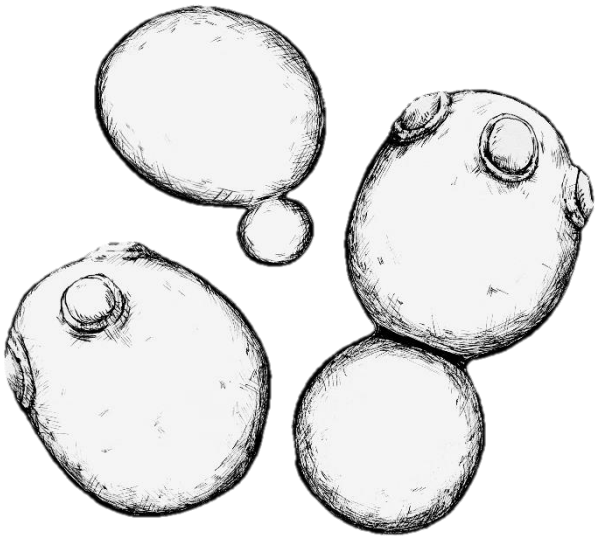
ANNEXES



Annexe 1: Strains generated throughout the hybridisation process

Different yeast strains were generated in this work for the construction of interspecific hybrids and the generation of a segregant progeny from those. HO deletant strains (**Table A1**), stable haploids (**Table A2**), intraspecific diploid hybrids (**Table A3**), diploid maters (**Table A4**), petite versions of diploid maters (**Table A5**) and interspecific tetraploid hybrids (**Table A6**) are shown in this annexe, together with their genotypes and mitotypes, when required. The final population of diploid segregants is shown in **Table A7**. In tables A1 to A6, the strains which eventually led to the generation of segregants are highlighted in bold font.

Table A1*. HO deletant strains generated in this study.*

Species	Strain	Original strain	Genotype
S. arboricola	SA01-ho	SA01	*HO/ho::HYG*
S. arboricola	SA02-ho	SA02	*HO/ho::HYG*
S. arboricola	SA03-ho	SA03	*HO/ho::HYG*
S. uvarum	SU01-ho	SU01	*HO/ho::HYG*
S. uvarum	**SU03-ho**	**SU03**	***HO/ho::HYG***
S. uvarum	SU04-ho	SU04	*HO/ho::HYG*
S. uvarum	**SU05-ho**	**SU05**	***HO/ho::HYG***
S. uvarum	SU07-ho	SU07	*HO/ho::HYG*
S. uvarum	SU08-ho	SU08	*HO/ho::HYG*
S. uvarum	SU10-ho	SU10	*HO/ho::HYG*
S. paradoxus	SP01-ho	SP01	*HO/ho::HYG*
S. paradoxus	SP02-ho	SP02	*HO/ho::HYG*
S. paradoxus	SP03-ho	SP03	*HO/ho::HYG*
S. paradoxus	SP05-ho	SP05	*HO/ho::HYG*
S. paradoxus	SP06-ho	SP06	*HO/ho::HYG*
S. mikatae	**SM02-ho**	**SM02**	***HO/ho::HYG***
S. mikatae	SM05-ho	SM05	*HO/ho::HYG*
S. mikatae	**SM06-ho**	**SM06**	***HO/ho::HYG***
S. cerevisiae	SC03-ho	SC03	*HO/ho::HYG*
S. cerevisiae	SC15-ho	SC15	*HO/ho::HYG*
S. uvarum	SU11-ho	SU11	*HO/ho::HYG*
S. jurei	SJ01-ho	SJ01	*HO/ho::HYG*
S. jurei	SJ02-ho	SJ02	*HO/ho::HYG*

***Table A2**. Stable haploid strains generated in this study.*

Species	Strain	Original strain	Genotype
S. arboricola	SA01-a	SA01-ho	*ho::HYG, MATa*
S. arboricola	SA01-alpha	SA01-ho	*ho::HYG, MATα*
S. arboricola	SA02-a	SA02-ho	*ho::HYG, MATa*
S. arboricola	SA02-alpha	SA02-ho	*ho::HYG, MATα*
S. arboricola	SA03-a	SA03-ho	*ho::HYG, MATa*
S. arboricola	SA03-alpha	SA03-ho	*ho::HYG, MATα*
S. uvarum	SU01-a	SU01-ho	*ho::HYG, MATa*
S. uvarum	SU01-alpha	SU01-ho	*ho::HYG, MATα*
S. uvarum	**SU03-a**	**SU03-ho**	***ho::HYG, MATa***
S. uvarum	SU03-alpha	SU03-ho	*ho::HYG, MATα*
S. uvarum	SU04-a	SU04-ho	*ho::HYG, MATa*
S. uvarum	SU04-alpha	SU04-ho	*ho::HYG, MATα*
S. uvarum	SU05-a	SU05-ho	*ho::HYG, MATa*
S. uvarum	**SU05-alpha**	**SU05-ho**	***ho::HYG, MATα***
S. uvarum	SU07-a	SU07-ho	*ho::HYG, MATa*
S. uvarum	SU07-alpha	SU07-ho	*ho::HYG, MATα*
S. uvarum	SU08-a	SU08-ho	*ho::HYG, MATa*
S. uvarum	SU08-alpha	SU08-ho	*ho::HYG, MATα*
S. uvarum	SU10-a	SU10-ho	*ho::HYG, MATa*
S. uvarum	SU10-alpha	SU10-ho	*ho::HYG, MATα*
S. uvarum	SU11-a	SU11-ho	*ho::HYG, MATa*
S. uvarum	SU11-alpha	SU11-ho	*ho::HYG, MATα*
S. paradoxus	SP02-a	SP02-ho	*ho::HYG, MATa*
S. paradoxus	SP02-alpha	SP02-ho	*ho::HYG, MATα*
S. paradoxus	SP05-a	SP05-ho	*ho::HYG, MATa*
S. paradoxus	SP05-alpha	SP05-ho	*ho::HYG, MATα*
S. paradoxus	SP06-a	SP06-ho	*ho::HYG, MATa*
S. paradoxus	SP06-alpha	SP06-ho	*ho::HYG, MATα*
S. mikatae	SM02-a	SM02-ho	*ho::HYG, MATa*
S. mikatae	**SM02-alpha**	**SM02-ho**	***ho::HYG, MATα***
S. mikatae	SM05-a	SM05-ho	*ho::HYG, MATa*
S. mikatae	SM05-alpha	SM05-ho	*ho::HYG, MATα*
S. mikatae	**SM06-a**	**SM06-ho**	***ho::HYG, MATa***
S. mikatae	SM06-alpha	SM06-ho	*ho::HYG, MATα*
S. jurei	SJ01-a	SJ01-ho	*ho::HYG, MATa*
S. jurei	SJ01-alpha	SJ01-ho	*ho::HYG, MATα*
S. jurei	SJ02-a	SJ02-ho	*ho::HYG, MATa*
S. jurei	SJ02-alpha	SJ02-ho	*ho::HYG, MATα*

***Table A3**. Intraspecific hybrid strains generated in this study.*

Species	Strain	Parent strain A	Parent strain B	Genotype
S. arboricola	D01	SA01-a	SA02-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. arboricola	D02	SA01-a	SA03-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. arboricola	D03	SA02-a	SA03-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. uvarum	D04	SU01-a	SU10-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. uvarum	D05	SU10-a	SU01-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. paradoxus	D06	SP02-a	SP05-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. paradoxus	D07	SP02-a	SP06-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. paradoxus	D08	SP05-a	SP06-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. uvarum	D09	SU01-a	SU07-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. uvarum	D10	SU01-a	SU11-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. uvarum	D11	SU07-a	SU11-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. jurei	D12	SJ01-a	SJ02-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. jurei	D13	SJ02-a	SJ01-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. uvarum	D14	SU03-a	SU01-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. uvarum	**D15**	**SU03-a**	**SU05-alpha**	***ho::HYG/ho::HYG, MATa/MATα***
S. uvarum	D16	SU03-a	SU07-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. uvarum	D18	SU07-a	SU01-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. uvarum	D21	SU05-a	SU01-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. mikatae	D22	SM02-a	SM05-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. mikatae	D23	SM05-a	SM02-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. mikatae	D24	SM05-a	SM06-alpha	*ho::HYG/ho::HYG, MATa/MATα*
S. mikatae	**D25**	**SM06-a**	**SM02-alpha**	***ho::HYG/ho::HYG, MATa/MATα***
S. mikatae	D26	SM06-a	SM05-alpha	*ho::HYG/ho::HYG, MATa/MATα*

***Table A4**. Diploid mater strains generated in this study.*

Species	Strain	Original strain	Genotype
S. arboricola	D01-a	D01	*ho::HYG/ho::HYG, MATa/mat::G418*
S. arboricola	D01-alpha	D01	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. arboricola	D02-a	D02	*ho::HYG/ho::HYG, MATa/mat::G418*
S. arboricola	D02-alpha	D02	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. arboricola	D03-a	D03	*ho::HYG/ho::HYG, MATa/mat::G418*
S. arboricola	D03-alpha	D03	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. paradoxus	D06-a	D06	*ho::HYG/ho::HYG, MATa/mat::G418*
S. paradoxus	D06-alpha	D06	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. paradoxus	D07-a	D07	*ho::HYG/ho::HYG, MATa/mat::G418*
S. paradoxus	D07-alpha	D07	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. paradoxus	D08-a	D08	*ho::HYG/ho::HYG, MATa/mat::G418*
S. paradoxus	D08-alpha	D08	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. jurei	D12-a	D12	*ho::HYG/ho::HYG, MATa/mat::G418*
S. jurei	D12-alpha	D12	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. jurei	D13-a	D13	*ho::HYG/ho::HYG, MATa/mat::G418*
S. jurei	D13-alpha	D13	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. uvarum	D14-a	D14	*ho::HYG/ho::HYG, MATa/mat::G418*
S. uvarum	D14-alpha	D14	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. uvarum	**D15-a**	**D15**	***ho::HYG/ho::HYG, MATa/mat::G418***
S. uvarum	D15-alpha	D15	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. uvarum	D16-a	D16	*ho::HYG/ho::HYG, MATa/mat::G418*
S. uvarum	D16-alpha	D16	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. uvarum	D17-a	D17	*ho::HYG/ho::HYG, MATa/mat::G418*
S. uvarum	D17-alpha	D17	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. uvarum	D18-a	D18	*ho::HYG/ho::HYG, MATa/mat::G418*
S. uvarum	D18-alpha	D18	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. mikatae	D22-alpha	D22	*ho::HYG/ho::HYG, mat::G418/MATalpha*
S. mikatae	D24-a	D24	*ho::HYG/ho::HYG, MATa/mat::G418*
S. mikatae	D25-a	D25	*ho::HYG/ho::HYG, MATa/mat::G418*
S. mikatae	**D25-alpha**	**D25**	***ho::HYG/ho::HYG, mat::G418/MATalpha***

***Table A5**. Petite diploid mater strains generated in this study.*

Species	Strain	Original strain
S. arboricola	D01-a, ρ(-)	D01-a
S. arboricola	D01-alpha, ρ(-)	D01-alpha
S. arboricola	D03-a, ρ(-)	D03-a
S. arboricola	D03-alpha, ρ(-)	D03-alpha
S. paradoxus	D07-a, ρ(-)	D07-a
S. paradoxus	D07-alpha, ρ(-)	D07-alpha
S. jurei	D13-a, ρ(-)	D13-a
S. jurei	D13-alpha, ρ(-)	D13-alpha
S. uvarum	D14-a, ρ(-)	D14-a
S. uvarum	D14-alpha, ρ(-)	D14-alpha
S. uvarum	**D15-a, ρ(-)**	**D15-a**
S. uvarum	D15-alpha, ρ(-)	D15-alpha
S. uvarum	D16-a, ρ(-)	D16-a
S. uvarum	D16-alpha, ρ(-)	D16-alpha
S. uvarum	D18-a, ρ(-)	D18-a
S. uvarum	D18-alpha, ρ(-)	D18-alpha
S. mikatae	D25-a, ρ(-)	D25-a
S. mikatae	**D25-alpha, ρ(-)**	**D25-alpha**

***Table A6**. Interspecific tetraploid strains generated in this study.*

Species	Strain	Parental strain A	Parental strain B	Mitotype
Sp x Su	T01	D07-a	D14-alpha	mixed
Sp x Su	T02	D07-a	D14-alpha, ρ(-)	*S. paradoxus*
Sp x Su	T03	D07-a, ρ(-)	D14-alpha	*S. uvarum*
Sp x Su	T04	D07-a	D15-alpha	mixed
Sp x Su	T05	D07-a	D15-alpha, ρ(-)	*S. paradoxus*
Sp x Su	T06	D07-a, ρ(-)	D15-alpha	*S. uvarum*
Sp x Sm	T07	D07-a	D25-alpha	mixed
Sp x Sm	T08	D07-a	D25-alpha, ρ(-)	*S. paradoxus*
Sp x Sm	T09	D07-a, ρ(-)	D25-alpha	*S. mikatae*
Sj x Su	T10	D13-a	D14-alpha	mixed
Sj x Su	T11	D13-a	D14-alpha, ρ(-)	*S. jurei*
Sj x Su	T12	D13-a, ρ(-)	D14-alpha	*S. uvarum*
Sj x Su	T13	D13-a	D15-alpha	mixed
Sj x Su	T14	D13-a	D15-alpha, ρ(-)	*S. jurei*
Sj x Su	T15	D13-a, ρ(-)	D15-alpha	*S. uvarum*
Sm x Su	T16	D14-a	D25-alpha	mixed
Sm x Su	T17	D14-a	D25-alpha, ρ(-)	*S. mikatae*
Sm x Su	T18	D14-a, ρ(-)	D25-alpha	*S. uvarum*
Sm x Su	T19	D15-a	D25-alpha	mixed
Sm x Su	**T20**	**D15-a**	**D25-alpha, ρ(-)**	***S. mikatae***
Sm x Su	**T21**	**D15-a, ρ(-)**	**D25-alpha**	***S. uvarum***

Table A7*. Diploid segregants generated in this study.*

Species	Strains	Original strain	Mitotype
Sm x Su	UM001 – UM060	T21	*S. uvarum*
Sm x Su	UM061 – UM120	T20	*S. mikatae*
Sm x Su	UM121 – UM132	T21	*S. uvarum*
Sm x Su	UM133 – UM144	T20	*S. mikatae*

Annexe 2: Supplementary QTL mapping results

During the experiments conducted for Chapter 2, we phenotyped a total of 99 segregants in synthetic must fermentation. Those fermentations were run in four batches using the PlateButler® robotic system (Lab Services), which allows performing up to 90 fermentations simultaneously. From them, the three first batches showed a high reproducibility, with both the *S. cerevisiae* control and the average of the segregants showing very similar results. They were therefore included in Chapter 2. The fourth batch showed statistically significant differences for kinetic parameters and compounds of the central carbon metabolism compared to the three first ones (one-way ANOVA, $p > 0.05$; data not shown). Aiming to use them for QTL analysis, we applied batch compensation using the data from the EC1118 control, which was used in all four batches. For that purpose, we calculated the difference between the average of EC1118 in the first three batches, the percentage of difference with EC1118 in the fourth batch, and applied this percentage to compensate the results of the fourth batch. Using these results, we performed QTL analysis with those 99 segregants (**Table A8**). Despite the high interest of these results obtained, we did not use them, as we still obtained good results with only 78 segregants (detailed in Chapter 2) and had higher confidence on this second analysis.

This first QTL analysis provided even shorter QTLs, some of which were absent in the second one. Within those QTLs we found some candidate genes, not discussed in this work, but perhaps worth investigating in the future (**Table A8**).

***Table A8**. QTL mapping results using 4 robot batches (99 segregants). Genes in the peak of the QTL are highlighted in bold font.*

Phenotype	Chromosome	QTL	Peak (bp)	Peak LOD	Length (bp)	Similar ORFs in S288c (ScanOne)
A28_Pentanoic acid	IV	chr.04@796889	796889	4.18	1819	***YBR197C*** *; TAF5*
Acetate yield at 80	V	chr.05@210868	210868	8.44	2296	***GPA2*** *; RPN3 ; SRB4*
Acetate (g/L) at FT	V	chr.05@211060	211060	8.36	2296	***GPA2***
T_{ferm}	V	chr.05@211789	211789	3.68	2296	***GPA2*** *; RPN3 ; SRB4*
Glycerol yield at 80 g/L	V	chr.05@211789	211789	7.16	2296	***GPA2*** *; RPN3 ; SRB4*
Acetate yield at FT	V	chr.05@212295	212295	8.47	2296	***GPA2*** *; RPN3 ; SRB4*
Glycerol (g/L) at 80 g/L	V	chr.05@212343	212343	7.22	4044	*GPA2 ;* ***RPN3*** *; SRB4*
Lactate (g/L) at 80 g/L	V	chr.05@212925	212925	5.93	3068	***RPN3*** *; SRB4*
Lactate (g/L) at FT	V	chr.05@213159	213159	7.48	5677	*GPA2 ;* ***RPN3*** *; SRB4*
Glycerol yield at FT	V	chr.05@213159	213159	6.80	5677	*GPA2 ;* ***RPN3*** *; SRB4*
A27_2-methylthiopropanol	V	chr.05@221039	221039	4.02	8216	*SRB4 ; PRO3 ; YAT2 ;* ***GCD11***
A28_Pentanoic acid	V	chr.05@221039	221039	5.58	8432	*SRB4 ; PRO3 ; YAT2 ;* ***GCD11***
Ethanol yield at 80 g/L	V	chr.05@221039	221039	8.21	8432	*SRB4 ; PRO3 ; YAT2 ;* ***GCD11***
Ethanol yield at FT	VII	chr.07@468063	468063	9.67	5065	***PUF4***
Ethanol (g/L) at FT	VII	chr.07@473180	473180	8.32	1538	***ERG4***
A05_Ethyl butanoate	VII	chr.07@473610	473610	4.06	821	*ERG4 ; SCL1*
R_{max}	VIII	chr.08@040792	40792	4.11	9898	*VMR1 ; SBP1 ;* ***RPL8B*** *; GUT1 ; GOS1*
A09_2-methylpropanol	VIII	chr.08@117808	117808	4.41	204	***STP2***
Ethanol yield at FT	IX	chr.09@038320	38320	4.05	4189	*none annotated*
A13_2/3-methylbutanol	IX	chr.09@380669	380669	5.90	250	*none annotated*
Ethanol yield at FT	X	chr.10@114065	114065	4.04	3918	***PET130*** *; BBC1*
A24_Diethyl succinate	XI	chr.11@229501	229501	4.03	1144	***AAT1***
Glycerol yield at 80 g/L	XI	chr.11@368895	368895	3.92	1385	***IXR1***
R_{max}	XI	chr.11@424358	424358	4.90	2734	***BYE1*** *; AUR1 ; MRP17*

Table A8 *(Cont.)*

Phenotype	Chromosome	QTL	Peak (bp)	Peak LOD	Length (bp)	Similar ORFs in S288c (ScanOne)
A26_3-methylbutanoic acid	XI	chr.11@470629	470629	4.12	1	***ALY1***
A31_Hexanoic acid	XI	chr.11@470629	470629	4.05	1	***ALY1***
R_{80}	XII	chr.12@444410	444410	4.08	13254	*DPH6 ; ACF2 ; RMP1 ; SPE4 ; YLR146W-A ; SMD3 ; PEP3 ; YLR149C ; STM1 ; PCD1 ; YLR152C ; ACS2 ; RNH203*
A31_Hexanoic acid	XIII	chr.13@136794	136794	3.82	2671	*BET5 ;* ***WAR1***
T_{80}	XIII	chr.13@227829	227829	3.79	1658	***USA1***
Overall kinetic score	XIII	chr.13@229487	229487	4.90	10327	*SRC1 ; RAD52 ; NDC1 ; RCF1 ;* ***USA1***
R_{80}	XIII	chr.13@230027	230027	4.26	3741	***USA1*** *; TSA1*
Ethanol yield at FT	XIII	chr.13@298973	298973	3.95	2688	***HXT2***
R_{max}	XIII	chr.13@356463	356463	4.04	753	***MSN2***
R_{max}	XIV	chr.14@093311	93311	4.75	1	*none annotated*
Ethanol (g/L) at FT	XVI	chr.16@282148	282148	4.00	16555	*PEP4 ; RAD53 ; RRD2 ; PRP46 ; YPL150W ; ATG5 ; PPT2 ;* ***PXA1***
Ethanol yield at FT	XVI	chr.16@282148	282148	3.95	5844	*YPL150W ; ATG5 ; PPT2 ;* ***PXA1***
R_{80}	XVI	chr.16@458281	458281	3.76	1	***KTR6***
A09_2-methylpropanol	XVI	chr.16@563417	563417	4.44	8435	***YPR003C*** *; AIM45 ; HAL1 ; ICL2 ; REC8*
A28_Pentanoic acid	XVI	chr.16@700575	700575	4.50	3525	***YPR078C*** *; MRL1*

Abstract

Wine fermentation has long been conducted using *Saccharomyces cerevisiae*. This species is the workhorse of wine, beer, cider, sake and bread production all around the globe, as a pure species or, to a lesser extent, as a part of interspecific hybrids with other *Saccharomyces* species. However, *Saccharomyces* species other than *S. cerevisiae* have shown a promising potential to diversify the organoleptic properties of wine and tackle the environmental challenges that the wine industry has been facing in recent years.

In this work, we sought to shed light on the phenotypic diversity in the genus *Saccharomyces* for winemaking. We phenotyped 92 yeast strains belonging to all the current *Saccharomyces* species in synthetic grape must fermentation. Unexpectedly, all *Saccharomyces* species fermented efficiently under the conditions used. Remarkably, strains of *S. kudriavzevii* and *S. arboricola* and interspecific *Sc x Sk* hybrids fermented more efficiently than wine *S. cerevisiae* strains. Regarding metabolite production, we observed a high strain variability for some species. Even more interestingly, we observed specificities at the species level: some non-*cerevisiae Saccharomyces* produced high amounts of industrially relevant compounds such as glycerol, succinate and fermentative aromas, or extremely low amounts of acetic acid, compared to *S. cerevisiae*. Overall, the potential of alternative *Saccharomyces* was higher than expected. They constitute a promising alternative to diversify the current set of commercially available yeast strains, either as pure species or following interspecific hybridisation.

Given the interest of these phenotypes, we aimed to determine their genetic basis through QTL mapping in *Saccharomyces* interspecific hybrids. Hybrids between different *Saccharomyces* species can be easily obtained in the lab thanks to the weak pre-zygotic barriers in this genus. Although viable, those hybrids are sterile, as the high genetic divergence between homologous chromosomes prevents their correct segregation during meiosis. However, allotetraploid hybrids are fertile, as recombination in these organisms occurs between chromosomes of the same species, minimising sequence divergence and facilitating correct segregation during sporulation. We used this phenomenon to construct fertile *S. uvarum* x *S. mikatae* hybrids. After genotyping and phenotyping its F12 progeny in oenological conditions, we performed the first QTL mapping study in non-*cerevisiae Saccharomyces* species. We found several genomic regions in the *S. uvarum* subgenome affecting the production of central carbon metabolites and, to a lesser degree, fermentative aromas and kinetic parameters. Verifying some of the candidate genes in these loci will shortly provide new tools for improving *S. uvarum* and its hybrids in a winemaking context.

Overall, this study uncovered the potential of non-*cerevisiae Saccharomyces* species to tackle the current challenges of the wine industry, establishing innovative lines of action for future research.

Title: Genetic basis of oenological traits in *Saccharomyces* interspecific hybrids

Keywords: Non-*cerevisiae Saccharomyces*; phenotypic diversity; wine fermentation; fermentative aroma; interspecific hybrid; QTL mapping.