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Minireview

Truth in wine yeast

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Summary

Evolutionary history and early association with anthropogenic environments have made *Saccharomyces cerevisiae* the quintessential wine yeast. This species typically dominates any spontaneous wine fermentation and, until recently, virtually all commercially available wine starters belonged to this species. The Crabtree effect, and the ability to grow under fully anaerobic conditions, contribute decisively to their dominance in this environment. But not all strains of *Saccharomyces cerevisiae* are equally suitable as starter cultures. In this article, we review the physiological and genetic characteristics of *S. cerevisiae* wine strains, as well as the biotic and abiotic factors that have shaped them through evolution. Limited genetic diversity of this group of yeasts could be a constraint to solving the new challenges of oenology. However, research in this field has for many years been providing tools to increase this diversity, from genetic engineering and classical genetic tools to the inclusion of other yeast species in the catalogues of wine yeasts. On occasion, these less conventional species may contribute to the generation of interspecific hybrids with *S. cerevisiae*. Thus, our knowledge about wine strains of *S. cerevisiae* and other wine yeasts is constantly expanding. Over the last decades, wine yeast research has been a pillar for the modernisation of oenology, and we can be confident that yeast biotechnology will keep contributing to solving any challenges, such as climate change, that we may face in the future.

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Introduction

Alcoholic fermentation is probably the most ancient biotechnological transformation in human history. Chemical analyses of archaeological specimens in China, Iran, Egypt, or Georgia trace the production of wine and other fermented beverages to the origins of agriculture (McGovern *et al.*, 1996, 2004, 2017). However, while we might assume that the domestication of vines and other crops was intentional, the domestication of the agents responsible for wine fermentation has been largely unconscious. The involvement of yeasts in alcoholic fermentation was not generally accepted until the XIX century (Barnett, 2003). Mastering fermentation processes using selected starter cultures is an even younger innovation in the history of winemaking (Kraus *et al.*, 1983); and it did not become a widespread practice until the 1970s. Up to the present, almost all the industrial starters used in winemaking belong to the species *Saccharomyces cerevisiae*.

This species was adopted early on as a model organism by various branches of biology; microbiology, biochemistry, physiology, genetics or genomics, among others (Barnett, 1998). From the first morphological descriptions or its contribution to the germ theory, *S. cerevisiae* was synergistically developed both as a biotechnological workhorse and a model organism. It was also the first eukaryotic organism to be completely sequenced (Goffeau *et al.*, 1996) and, with the popularization of NGS and whole-genome sequencing, it is now also a model for microbial ecology, population genetics and synthetic biology (Goddard and Greig, 2015; Peter *et al.*, 2018; Pretorius and Boeke, 2018).

Nevertheless, there are notable differences between the characteristics of the strains used in the laboratory and the industry, as well as in the growth conditions commonly found in both settings. Therefore, although wine yeast biotechnology can benefit from most of the advanced tools developed in other disciplines, knowledge transfer is not always straightforward.

In addition, it is currently recognized that other yeast species (known as non-*Saccharomyces* in the field) might be very relevant for the output of wine alcoholic fermentation (Ciani *et al.*, 2010). Only recently has this finding begun to be industrially exploited. The purpose of

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this article is to bring together, in a single document, the most relevant information available on the biology of wine strains of *S. cerevisiae*, including the most up-to-date information on genetic and genomic features.

The physico-chemical environment during wine fermentation

The many styles of wine that exist in the world today differ from each other in the *Vitis vinifera* cultivars employed, agricultural practices, fermentation conditions, and many different pre- and post-fermentation practices. From the point of view of microbial biotechnology, we must also consider secondary fermentation processes, involving yeasts or lactic acid bacteria. Anyway, most wines are produced following variants of two main workflows, one for red wines and a second one for white wines (Ribéreau-Gayon et al., 2006). The main difference between red and white fermentation is the presence of grape seeds and skins during most of the fermentation time, for red wines. This is accompanied by physical procedures aimed at improving the extraction of compounds from the solid parts; but also providing extra oxygen for some of the yeast's metabolic pathways. In contrast, white wine is usually fermented with pressed grape must, after the first racking, and in almost completely anaerobic conditions.

The sudden availability of nutrients caused by the crushing or pressing of the grape tends to be rapidly exploited by the epiphytic microbiota in the grape berries, together with the microorganisms already present in the equipment and atmosphere of the winery. Environmental conditions quickly become anaerobic due to the microbial metabolic activity and the use of relatively large containers (low surface-to-volume ratios). Nutrient availability is high for sugars, but often limiting for nitrogen sources and some vitamins, like thiamine (Labuschagne and Divol, 2021); a problem that winemakers often tackle by using specific yeast nutrients. The widespread use of sulphiting agents has a great impact on yeast physiology and ecology during wine fermentation. Other factors contributing to selective growth by some yeast species at the expense of others are osmotic pressure (ever-increasing due to global climate warming), a relatively high total acidity and low pH, or the ethanol released by fermentation. Specific fermentation styles have their own hallmark of factors potentially limiting microbial development (Novo *et al*., 2012), including suboptimal (low or high) temperatures, high polyphenol content, strong anaerobiosis, extremely high osmotic pressure (noble rot and ice wines), carbon dioxide overpressure (traditional sparkling wines), or aerobic growth on ethanol (biological ageing).

S. cerevisiae is well equipped to overcome many of these hurdles. For example, there are specific response mechanisms to osmotic stress. It involves the overproduction of glycerol as a compatible osmolyte and a dedicated MAP kinase cascade, the HOG pathway (Hohmann, 2015). However, tolerance to osmotic stress depends on many factors and the proper functioning of many cellular components such as mitochondria or the Golgi-endosome system (Gonzalez *et al*., 2016). Similarly, tolerance to ethanol or extreme temperatures depends on several cellular systems, including lipid composition and structure of the plasma membrane, antioxidant compounds and enzymes, chaperones, trehalose biosynthesis, plasma membrane proton ATPase, or mitochondrial functions among others (Swan and Watson, 1997; Piper, 1995; Torija *et al*, 2003; García-Rios *et al*., 2017). In addition, signalling pathways involved in the stress response show numerous examples of crosstalk and coordinated regulation (Gustin *et al*., 1998; Estruch, 2000). Indeed, *S. cerevisiae* displays an environmental stress response (ESR), which primes stressed cells to better respond to subsequent challenges through the action of the transcriptional regulators Msn2 and Msn4 (Berry and Gasch, 2008). The results of some early transcriptomic studies led several authors to define a coordinated response to fermentation stress (Rossignol *et al*., 2003). Marks *et al*. (2008), defined a fermentation stress response (FSR), involving more than two hundred genes showing a minimum of twofold positive change at the final time point. They concern many metabolic pathways. Around 20% of FSR genes are also part of the ESR, and others are common with other laboratory defined stress conditions, like nitrogen starvation, ethanol, osmotic, or oxidative stress.

Yeast ecology of spontaneous wine fermentation

Wine fermentation is a very competitive scenario for microorganisms. Despite the predominant status of *S. cerevisiae*, many other microorganisms are present and potentially contribute to the development of the sensory characteristics of the wines. Often neglected as spoilage microorganisms or, at most, irrelevant species, the growth of non-*Saccharomyces* species during the early stages of fermentation has been known for more than a hundred years. Our current view of the succession of yeast species from vine to wine is supported by decades of observation and yeast isolation efforts by many microbiologists, recently enriched with the help of metataxonomic studies (Setati *et al*., 2012; Bokulich *et al*., 2013; Bokulich *et al*., 2014; Pinto *et al*., 2015). The dominant yeast microbiota in healthy grapes at harvest is constituted by species that will not survive the first hours of fermentation, including basidiomycetous genera like *Cryptococcus* or *Rhodotorula*, and the ascomycetous species *Aureobasidium pullulans*. Other ascomycetous

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genera, such as *Hanseniaspora/Kloeckera*, *Candida*, *Pichia*, *Torulaspora*, *Kluyveromyces*, *Metschnikowia*, or *Starmerella* among others, can survive longer and jointly dominate the process for several hours until *S. cerevisiae* takes over the alcoholic fermentation. Indeed, these species largely outnumber *S. cerevisiae* cells in the surface of grape berries. After the initial contact with the grape sugars, they trigger the alcoholic fermentation. However, *S. cerevisiae* (initially in low numbers) takes advantage of its specific adaptive traits to quickly grow and become the main yeast species from the middle, tumultuous phase of fermentation. Most often, only *S. cerevisiae* can be readily isolated in the final stages of fermentation. Some physiological adaptations that allow *S. cerevisiae* strains to thrive during wine fermentation are their preference for fermentative metabolism, their ability to grow strictly anaerobically, and their high tolerance to sulphur dioxide. Moreover, *S. cerevisiae* is more tolerant to heat stress than other wine yeast species. For some authors, this could also contribute to the dominance of *S. cerevisiae* in this environment (Goddard, 2008).

Saccharomyces wine yeast starters

For almost the entire history of oenology, spontaneous fermentation has been the only way to transform the must into wine, modulated only by the eventual preparation of a "pied-de-cuve". This practice consists of adding a proportionally small volume of fermenting juice to a tank of fresh grape must. Spontaneous fermentation is a technically and conceptually very simple process, which often results in good quality wines. The microbiological control of the process used to be entrusted to sulphur dioxide (equally acting as an antioxidant), in concentrations that inhibit the development of most bacteria and non-*Saccharomyces* yeasts. However, this is not enough to prevent fermentation from sometimes going bad, leading to slow or stuck fermentation, excess acidity, or other sensory defects. On the other hand, the variability of the microbiota in the vineyard and cellar introduces a factor of unpredictability into the sensory characteristics of the wines produced each season. The possibilities opened by the discovery of yeasts as transformation agents, the development of pure cultures and, later on, the development of industrial yeast production techniques (especially for bakery and brewery use), did not begin to be exploited by the wine industry until many years later (Gonzalez *et al*., 2011). Nevertheless, by the end of the 20th-century inoculation of *S. cerevisiae* starters was a widespread practice, with dozens of different yeast strains on the market. While in spontaneous fermentation a succession of different yeasts takes place, the use of *S. cerevisiae* starter cultures usually results in a process dominated almost from the onset by the inoculated strain (Querol *et al*., 1992). Each strain of *S. cerevisiae* can contribute differently to the sensory characteristics of the wine, and not all of them are well adapted to all fermentation styles. Accordingly, yeast selection becomes an additional tool in the hands of winemakers to develop and differentiate their products. But the widespread assumption that the best starter cultures for a production region are those isolated from such region is lacking solid scientific support.

A minimal selection criterion for wine yeast starters is good fermentation kinetics, understood as fast fermentation onset and quick sugar consumption kinetics. These traits are directly related to tolerance to osmotic and ethanol stress, and efficiency in the use of available nitrogen sources. Indeed, a large proportion of sugar consumption and alcohol production during wine fermentation takes place in the absence of cell growth. The biomass produced during the initial growth phase must be sufficient to ensure correct fermentation kinetics, and this is highly dependent on nitrogen utilisation (Varela *et al*., 2004). Indeed, efficient nitrogen utilization is a selection criterion for wine yeast starters, despite the widespread use of nutrient supplementation. *S. cerevisiae* has developed an exquisite nitrogen source selection system (NCR, for nitrogen catabolite repression), involving nutrient sensing and different transcriptional and post-transcriptional control mechanisms (Zhang *et al*., 2018). Nitrogen metabolism by yeast during wine fermentation has a relevant impact on the production of several sensory active molecules, including acetic acid, higher alcohols, and esters (Martínez-Moreno *et al*., 2012; Styger *et al*., 2013; Rollero *et al*., 2017). It is also worth noting that NCR functioning under laboratory conditions could be very different from oenological fermentation conditions (Vallejo *et al*., 2020a; Vallejo *et al*., 2020b).

The killer phenotype is often also considered for starter selection (see below). Similarly, although most strains of wine origin show specific adaptations, it is advised to check the tolerance of candidate strains to sulphur dioxide. Furthermore, winemakers look for yeast starters that show tolerance to high or low temperatures (depending on the specific application); low production of volatile acidity (acetic acid), SO_2, or SH_2; and, in general, starters that do not contribute any off-flavours or negatively impact the perception of the wine. Indeed, tasting panels are unavoidable before new wine yeast starters can make their way to the market.

The selection criteria shall not forget that wine yeast starters must be produced and distributed as active dry yeast (ADY). There are other alternatives, primarily for local markets (Fracassetti *et al*., 2020), but ADY can be produced throughout the year and stored stably until harvest time (typically distributed with a two-year self-life),

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which is a great advantage for a product whose consumption is inherently seasonal. To produce ADY, yeast is usually grown in a fed-batch process, using diluted molasses as a carbon source, under conditions that favour respiratory metabolism (to maximize biomass yield). The resulting biomass is then dehydrated by a combination of mechanical and thermal processes (Gonzalez *et al*., 2011). In the cellar, the ADY must be rehydrated to recover viable and metabolically active cells, in a process that is again stressful and deadly for them. Accordingly, the suitability of the selected yeast strains as industrial wine starters depends not only on their behaviour during wine fermentation, but also on their performance during the ADY production process (biomass yield on a substrate, tolerance to thermal and hydric stress), and rehydration (Matallana and Aranda, 2017; Rodriguez-Porrata *et al*., 2008).

Non-*Saccharomyces* starters

The notable predominance of *S. cerevisiae* in the inoculated fermentations, and the extensive use of some commercial strains, prompted oenologists and researchers to try to avoid a perceived tendency towards uniformity in the wines. Despite various precedents during the 20th century, it was not until the 21st century that this began to take hold. Currently, commercial non-*Saccharomyces* starter cultures are gradually gaining market share (Roudil *et al*., 2020). The use of non-*Saccharomyces* yeasts allows winemakers to recover some of the characteristics associated with spontaneous fermentation while maintaining microbiological control of it. The main recognised impact of non-*Saccharomyces* yeasts on wine quality is related to the aroma (Padilla *et al*., 2016). These yeasts can contribute to the enhancement of the primary aroma of wines, allowing the release of active molecules from precursors present in the must, especially through the production of enzymes with glycosidase activity. Many contributions to the primary aroma, including terpenes and aromatic alcohols, come from molecules mostly present in grapes as odour-inactive glycosidically-bound volatile precursors (Hjelmeland and Ebeler, 2015). An important impact on wine aroma is the release of varietal thiols (polyfunctional mercaptans), contributing to the characteristic fruity aroma of wines from Sauvignon Blanc and other aromatic white grape varieties. This depends on a β-lyase activity (Roncoroni *et al*., 2011). They also contribute to the complexity of secondary aromas, mainly through the production of aromatic alcohols and esters which add fruity notes (Rojas *et al*., 2001). Beyond the aroma, the genetic and metabolic diversity provided by this portfolio of yeast species has enabled other oenological applications to be considered, such as acidification or deacidification, higher glycerol or mannoprotein content, better colour stability, lower alcohol content (Charoenchai *et al*., 1997; Strauss *et al*., 2001; Rojas *et al*., 2003; Romano *et al*., 2003; Bely *et al*., 2008; Moreira *et al*., 2008; Viana *et al* 2008; Ciani *et al*., 2010; Manzanares *et al*., 2011; Viana *et al*., 2011; Gonzalez *et al*., 2013) or the biological control of spoilage microorganisms (Oro *et al*., 2014). As with *S. cerevisiae*, it should be noted that the actual metabolic and technological profile depends not only on the yeast species but also on the strain used.

Saccharomyces spp. are almost the only species in wine able of consuming all the sugars with suitable fermentation kinetics. This prevents stuck fermentation or the predominance of potential spoilage microorganisms. For this reason, alternative yeasts are most often used in combination with *S. cerevisiae* starter cultures, either sequentially or by simultaneous inoculation. From the point of view of process control, each of these alternatives presents its own challenges. Indeed, the interactions between different starter cultures might be critical for a successful fermentation and represent an interesting field of study (Ciani *et al*., 2010).

The first commercial wine starter cultures of alternative species belonged to *Torulaspora delbrueckii*, and this is still the non-*Saccharomyces* yeast species with the largest number of wine starters on the market. *Schizosaccharomyces pombe* is nowadays marketed for deacidifying must by means of malo-alcoholic fermentation. Other popular non-*Saccharomyces* species in wine yeast catalogues are *Pichia kluyveri*, when looking for improved secondary aroma; *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*), attending to lactic acid production; or *Metschnikowia pulcherrima*, for aroma and biocontrol (Roudil *et al*., 2020). Although *Hanseniaspora* isolates have been often related to excessive volatile acidity, *Hanseniaspora vineae* strains have been extensively studied as a potential wine starter for improved secondary aroma (Martin *et al*., 2018).

The physiology of *S. cerevisiae* wine yeast strains

Channelling most of the carbon flux towards fermentative ethanol production, independently of oxygen availability, is a metabolic signature of some yeast species. This is known as the Crabtree effect, and *S. cerevisiae* is the most prominent representative of the Crabtree-positive group. Several mechanisms contribute to the Crabtree effect in this species (Barnett and Entian, 2005), including carbon catabolite transcriptional repression of genes required for aerobic respiration. But the rate of sugar consumption plays a critical role since slowing down sugar uptake or a reduced capacity of glycolytic enzymes alleviate the Crabtree effect (Otterstedt *et al*., 2004; Jansen *et al*., 2005). High sugar uptake rates

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result in overflow metabolism at the pyruvate level (Holzer, 1961; Pronk, *et al*., 1996). The abundance and kinetic properties of mitochondrial pyruvate dehydrogenase complex are not enough to process all the pyruvate produced by glycolysis. Under these circumstances most of the pyruvate is metabolized to acetaldehyde by the activity of pyruvate decarboxylase, and then to ethanol by acetaldehyde dehydrogenase (Pronk, *et al*., 1996). The Crabtree effect is part of a combination of evolutionary adaptations that constitute a "make-accumulate-consume" strategy followed by several yeast lineages (Piskur *et al*., 2006). Some of the genetic traits that contribute to the Crabtree-positive character include duplication and specialization of alcohol dehydrogenase coding genes, multiple hexose transporters, or transcriptional control of respiratory and mitochondrial functions (Hagman *et al*., 2013). Although many yeast species are facultative anaerobes, only a few of them can support a sustained growth under anaerobic conditions. The ability for anaerobic pyrimidine biosynthesis is key to support anaerobic growth. Most eukaryotic dihydroorotate dehydrogenases, catalysing the fourth step of *de novo* pyrimidine biosynthesis (oxidation of dihydroorotate to orotate), are dependent on enzymes from the respiratory chain for their activity. However, some Saccharomycetaceae acquired a bacterial dihydroorotate dehydrogenase by horizontal gene transfer (HGT) (Hall *et al*., 2005), using fumarate (which is independent of a functional respiratory chain) as electron acceptor (Gojković *et al*., 2004). Biosynthesis of unsaturated fatty acids and sterols required by yeast cells is also oxygen dependent. However, the oxygen requirement, in this case, is lower (there is no need for an active electron transport chain); and wine yeasts can incorporate lipids from grape must or winemaking nutrients (Luparia *et al*., 2004). While the Crabtree effect seems to clearly confer a selective advantage to *S. cerevisiae* over most other microorganisms during alcoholic fermentation, it poses a problem when producing biomass for ADY preparation. In yeast production, the aim is to maximise the biomass yield. In a batch process, because of the Crabtree effect, yeasts would produce large amounts of ethanol at the expense of biomass. For this reason, yeast production is mostly carried out in fed-batch cultures, adjusting the substrate supply to yeast uptake, thus ensuring low sugar levels throughout the growth phase to minimise the Crabtree effect (Gonzalez *et al*., 2011).

The above-described features of *S. cerevisiae* seem to be common to most strains of the species, but sulphur dioxide tolerance is restricted to some strains, including most wine isolates. Chromosome rearrangements involving the promoter of *SSU1* (coding for a sulphite efflux pump), and resulting in higher levels of the Ssu1 permease, are usually responsible for the enhanced sulphite resistance shown by wine yeast strains (Goto-Yamamoto *et al*., 1998; Pérez-Ortín *et al*., 2002; Zimmer *et al*., 2014; García-Ríos *et al*., 2019).

Fermentation does more to grape must than just replacing sugars with alcohol, although ethanol does contribute to what is known as the wine's aromatic buffer (Escudero *et al*., 2004). The microorganisms involved in fermentation, and especially *S. cerevisiae* catalyse the transformation of some precursors of the primary aroma of grapes such as glycosylated precursors and polyfunctional mercaptans (Swiegers and Pretorius, 2007; Concejero *et al*., 2016); and above all, they release various volatile organic compounds that constitute the secondary aroma. These include fusel alcohols, which are related to amino acid metabolism via the Ehrlich pathway (Styger *et al*., 2013), as well as acetate and ethyl esters (Sumby *et al*., 2010). Glycerol, organic acids (including acetic acid), acetaldehyde, mannoproteins, SH_2 and SO_2 are further contributions of yeast metabolism to the plethora of compounds involved in the sensory complexity of wine (Styger *et al*., 2011). There is sufficient metabolic diversity among wine strains of *S. cerevisiae* for their differential contribution to wine quality to be appreciated by professionals and consumers, regardless of their relative genetic proximity. This justifies the high number of *S. cerevisiae* strains on the winemaking market, despite some redundancy in trade names (Fernández-Espinar *et al*., 2001; Borneman *et al*., 2016).

S.cerevisiae life cycle

S. cerevisiae is often referred to as "the budding yeast", even though this is the mode of vegetative growth for many other yeast species. Reproduction by budding makes it possible to distinguish mother cells from daughter cells. Mother cells have a finite replicative lifespan which depends on the genotype and the environment, but which is typically only a few tens of divisions (Austriaco, 1996). *S. cerevisiae* cells can proliferate vegetatively both in haploid and diploid form. However, the dominant form in nature is diploid, and no commercial haploid oenological strains are available. Budding is genetically regulated, and the selection of the budding site depends on the history and mating genotype of the cell (Madden and Snyder, 1998). In addition to the well-known unicellular ellipsoid form, *S. cerevisiae* can develop other morphologies (Voordeckers *et al*., 2012). Adhesins, or flocculins, encoded by the *FLO* family of genes play determinant roles in growth patterns of *S. cerevisiae*, as well as on technologically relevant aggregation phenotypes like vellum formation during sherry wine ageing (Fidalgo *et al*., 2006), or flocculation (Govender *et al*., 2010).

Under suitable conditions, typically involving starvation, diploid cells might experience meiosis, giving rise to

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an ascus with a tetrad of ascospores, two of them with an a, and two with an alpha mating-type. In homothallic strains, which are the most common in nature, a haploid mother cell undergoes a mating-type switch (starting from the second cell division) after each mitotic division (Haber, 2012). Thus, almost immediately after germination, a new homozygous diploid strain is generated by the fusion of two genetically identical cells. This process is known as haplo-selfing. In addition, cells can revert to the diploid state by automixis, or hybridisation between spores within the ascus; and amphimixis, or hybridisation between unrelated haploid cells (Knop, 2006). Mating-type switching is promoted by a DNA double-strand break catalysed by the HO endonuclease on the active mating-type locus (MAT), located on chromosome III. After each mitotic division, a different silent mating-type cassette (HMR or HML), located near one of the two edges of the same chromosome, is used as a template to generate the alternative mating-type (Haber, 2012).

Many laboratory strains are heterothallic, i.e. they can multiply indefinitely as haploids, due to loss-of-function mutations in the *ho* gene. Some wine strains are heterozygous for the *HO*/*ho* locus, and this has provided tools for generating haploid wine yeast derivatives as research tools (Mangado *et al*., 2018). Conservation of mating-type switching is in agreement with the "genome renewal" hypothesis for wine yeasts, which will favour homozygote diploids (Mortimer *et al*., 1994). Experimental evolution results also suggest diploidy as the most stable nuclear content for *S. cerevisiae* (Gerstein *et al*., 2006; Mangado *et al*., 2018). However, the current picture of *S. cerevisiae* wine yeast strains in nature is more complex than previously anticipated (Fischer *et al*., 2021). Indeed, one of the most popular industrial wine yeast strains, EC1118, is clearly heterozygous (Muñoz *et al*., 2009; Novo *et al*., 2009). There is evidence of mitotic recombination in wine yeasts, but sporulation events seem to be rare under winemaking conditions (Puig *et al*., 2000). In this context, it is interesting to note the recent discovery that the social wasp intestine is an environment that allows winter survival and hybridisation between yeast strains (Stefanini *et al*., 2016). Furthermore, *S. cerevisiae* can form interspecific hybrids with other *Saccharomyces* species. Hybrids are common for brewer's yeasts (Libkind *et al*., 2011) but were discovered more recently for wine yeasts (see below).

Genetic and genomic features of *S. cerevisiae* wine strains

The haploid genome of *S. cerevisiae* contains about 12 Mbp and is distributed over 16 chromosomes. The number of protein-coding genes is about 6000 (Goffeau *et al*., 1996). The current genome of *S. cerevisiae* (and a small set of yeast species known as post-WGD) is the result of a genome duplication event (WGD for whole-genome duplication) in an ancestral species, which resulted in a tetraploid cell (Wolfe and Shields, 1997). Most of the gene redundancy was subsequently lost, but still about 13% of the proteins of this species constitute pairs derived from this ancient duplication (Wolfe and Shields, 1997). In many cases, this event seems to have allowed the specialisation of at least one of the copies (Kellis *et al*., 2004). This duplication also seems to underlie an increase in glycolytic flux, which would eventually lead to the Crabtree effect (Conant and Wolfe, 2007; Hagman *et al*., 2013).

Recent findings suggest the all the *Saccharomyces* species originated in Asia, with a single out-of-China event as the origin of all non-Chinese *S. cerevisiae* strains (Peter *et al*., 2018). There is a strong genetic relationship among wine isolates, which fall in a Wine/European clade on one side of the *S. cerevisiae* phylogenetic tree. Comparative genomics also indicates that current wine isolates are monophyletic because of a population bottleneck during the domestication process (Borneman *et al*., 2016; Peter *et al*., 2018). Although the species can show variation in ploidy as well as aneuploidies, most wine yeast isolates are pure diploids. There are also a few haploid strains, with very few examples of higher ploidy. All those haploid strains are deleted for the *HO* locus. The authors also found several aneuploid strains (around 15% of wine isolates), most of them carrying an extra copy of a single chromosome (Peter *et al*., 2018). A summary of the main genomic characteristics of wine yeasts, according to data from Peter *et al*. (2018) is shown in Table 1.

Pan-genome analysis revealed more than 900 ORFs introgressed from *S. paradoxus*, which is the closest relative of *S. cerevisiae*. Most wine strains carry between 25 and 50 ORFs from this origin (Table 1). In addition, several contributions to the *S. cerevisiae* pan-genome from evolutionarily more distant species have been identified. The earliest examples were found in the wine strain EC1118, with three regions named A, B, and C (Novo *et al*., 2009). The source species of these fragments are, as far as known, *Zygosaccharomyces bailii*, *Torulaspora microellipsoides*, and *Torulaspora delbrueckii* (Novo *et al*., 2009; Marsit *et al*., 2015). In some cases, a correlation between HGT-acquired genes and specific adaptations to fermentation conditions, such as the limitation of nitrogen sources, has been established (Devia *et al*., 2020). *FSY1*, from region C, encodes a high-affinity active fructose transporter, whose expression is induced by ethanol, believed to confer a selective advantage in the final stages of wine fermentation (Galeote *et al*., 2010).

Many *S. cerevisiae* strains carry an extrachromosomal element, the 2-micron plasmid. This is a 6.3 kbp circular DNA with only four coding genes, involved in replication,

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Table 1. Main genomic features of wine strains of *S. cerevisiae*. Information summarised from the supplementary materials of Peter *et al.* (2018).

Genomic feature		Number of strains
HO deletion		27
Plasmid	A	204
	B	1
Ploidy	1 (euploid/homozygous)	28 (20/28)
	2 (euploid/homozygous)	199 (175/115)
	3 (euploid/homozygous)	2 (1/0)
	4 (euploid/homozygous)	1 (0/1)
		Copy number
Plasmid	A (if present)	1 to 210 (median 25)
Extra ORFs*	HGT regions (A, B or C)	Up to 30 (app.)
	S. paradoxus introgression	25–50
Ty copies*	Ty1	Up to 15 (app.)
	Ty2	Up to 30 (app.)
	Ty3–5	<10 each

Whenever possible, it refers to 229 strains labelled as “wine” for the “ecological origins” feature in that work. Otherwise, it refers to the 362 Wine/European clade strains used (*).

plasmid maintenance, and copy number control (Chan *et al.*, 2013). Three classes of this plasmid have been identified, A, C, and B, which is the result of recombination between the other two (Strope *et al.*, 2015). Almost all wine strains harbour a variable number of copies of the type A 2-micron plasmid (Table 1).

In *S. cerevisiae* spontaneous petit (respiration-deficient), or rho^0 (having lost the entire mitochondrial genome) mutants are found with some ease. However, mitochondria are involved in many essential functions of the cell (McBride *et al.*, 2006), and although the genes encoding most of the mitochondrial proteins have been transferred to the nucleus of *S. cerevisiae* during evolution (Karlberg *et al.*, 2000), the long-term survival of these mutants is compromised. They are also sporulation deficient since respiration is required for meiotic entry (Jambhekar and Amon, 2008). The mitochondrial genome of *S. cerevisiae* is about 86 kbp, is globally low in %GC, and has a large proportion of intergenic regions and group I and II introns (Foury *et al.*, 1998). Most of the genes it encodes are related to oxidative phosphorylation (Freel *et al.*, 2015). The mitochondrial genome has been related in wine yeasts to several characteristics of technological interest, such as tolerance to ethanol and high temperature (Jimenez and Benitez, 1988), cold temperature (Li *et al.*, 2019), or industrial drying to produce ADY (Picazo *et al.*, 2014). Interestingly, the difference in composition of the mitochondrial and nuclear genomes of *S. cerevisiae* has allowed the development of quick molecular fingerprinting systems for wine strains of this species (Querol *et al.*, 1992).

The killer phenotype in *S. cerevisiae* is due to the production of toxins against neighbouring microorganisms, mainly yeasts, with different mechanisms (Schmitt and Breinig, 2006). Although they were discovered in *S. cerevisiae*, it seems that killer toxins are common among different yeast species (Liu *et al.*, 2015). From the four types of killer toxins known for *S. cerevisiae*, K1, K2, K28 and Klus, (Schmitt and Breinig, 2006; Rodríguez-Cousiño *et al.*, 2011), mostly K2 and Klus have been found in wine isolates (Maqueda *et al.*, 2012). Indeed, the pH interval of K1 toxin activity falls outside the pH range (around 3.5) of grape juices and wines (van Vuuren and Jacobs, 1992). By the end in industrial wine fermentations prevalence of killer activity ranges from 0% to 100% (van Vuuren and Jacobs, 1992). Toxin production is due to infection by dsRNA viruses of the Totiviridae family, present in the cytoplasm as virus-like particles. Each killer strain carries a variant of the helper virus ScV-L-A, and one of the satellite viruses, specific for each of the known toxins: ScV-M1, ScV-M2, ScV-M28 and ScV-Mlus. The former encodes the capsid protein and the polymerase, while the latter encodes just for the toxin. Transmission of these viruses is vertical (mother to daughter cell) and through cell fusion (e.g. in mating). There is no known extracellular pathway of killer virus transmission. Apart from the competitive advantage over killer sensitive strains, the presence of these viruses is considered to be asymptomatic. However, recent studies indicate that a specific adaptation to the production of the toxin itself is required (Gier *et al.*, 2020). The exact mechanisms of immunity remain to be elucidated.

Among the non-Mendelian heritable elements in *S. cerevisiae*, we also find prion-like elements (Wickner *et al.*, 2015) and the [GAR^+] element, one of the most recently discovered, raised expectations for its possible involvement in the adaptation of *S. cerevisiae* to alcoholic fermentation and interactions with bacteria present during winemaking (Brown and Lindquist, 2009; Jarosz *et al.*, 2014a). This element results in an alleviation of catabolite repression, which according to some authors could allow other carbon sources to be used during fermentation (apart from glucose and fructose) and may even lower alcohol production (Jarosz *et al.*, 2014b). But other authors concluded that the technological relevance of these yeast prions is rather residual and found no evidence of an appreciable decrease in alcoholic strength associated with the use of [GAR^+] strains (Gonzalez *et al.*, 2019).

The *S. cerevisiae* genome also harbours long terminal repeat-retrotransposons from five different families, Ty1 to Ty5 (Carr *et al.*, 2012), with known active elements for at least the first three types. The direct terminal repeats of Ty1 and Ty2 are known as delta elements, and can also be found in isolation, probably because of homologous recombination during ancient transposition

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events (Curcio *et al*., 2015). Delta elements have been used to develop molecular typing tools for *S. cerevisiae* wine strains (Legras and Karst, 2003).

With very few exceptions, such as translocations associated with the *SSU1* promoter and their impact on sulphur dioxide tolerance, many traits of technological interest in wine yeasts are quantitative and therefore difficult to deal with from a genetic point of view. In these cases, quantitative trait loci (QTL) mapping is a powerful but traditionally cumbersome tool. The possibility of bulk segregant analysis thanks to the popularisation, initially of genomic microarrays (Marullo *et al*., 2007), and subsequently of whole-genome sequencing and mass sequencing experiments, has changed the picture in recent years. Some authors have identified QTLs in crosses between two oenological yeasts, while in other cases the crosses also involve yeasts from other origins, to increase genetic diversity and to be able to identify QTLs that show little variability between wine yeasts, in part because of their relevance to thrive in the wine environment. Some authors have gone up to F13, to increase the resolution of the QTL mapping (García-Ríos *et al*., 2017). Examples of QTLs identified in recent years include traits of oenological importance such as acetic acid production (Marullo *et al*., 2007), several fermentation performance parameters (Ambroset *et al*., 2011; Marullo *et al*., 2019), production of various aroma components (Steyer *et al*., 2012; Eder *et al*., 2018), stress tolerance (Brion *et al*., 2013), nitrogen requirement (Brice *et al*., 2014; Cubillos *et al*., 2017), dehydration (Lopez-Martinez *et al*., 2015), or low-temperature fermentation (García-Ríos *et al*., 2017).

Other *Saccharomyces* species and natural hybrids in winemaking

Although most wine starters belong to the species *S. cerevisiae*, *S. uvarum,* previously known as *S. bayanus* var. *uvarum* (Naumov, 2000) has traditionally attracted much interest due to its ability to ferment at low temperatures (Masneuf-Pomarède *et al*., 2010). Among the cryophilic *Saccharomyces* species, we also find the more recently described *Saccharomyces kudriavzevii* (Naumov *et al*., 2000). Unlike *S. uvarum*, this species has not been found spontaneously in winemaking processes. Higher glycerol production and lower alcohol yields have been reported for strains of both species, as well as a differential contribution to the aromatic profile of wines (Pérez-Torrado *et al*., 2018). Both form natural hybrids with S. *cerevisiae*, which have been found in wine, especially in low-temperature fermentations (González *et al*., 2006; Boynton and Greig, 2014; Peris *et al*., 2018). These hybrids often combine the ethanol tolerance of *S. cerevisiae* with the low-temperature adaptation of *S. uvarum* (Querol *et al*., 2018). In addition to the metabolic particularities of the cryophilic strains, fermentation at low temperatures, especially white wines, allows better retention of volatile compounds and thus more aromatic wines. The combination of characteristics of interspecific hybrids within the genus *Saccharomyces* has increased the interest in using some of them as starter cultures (Pérez-Torrado *et al*., 2017). This has also prompted the development of breeding programmes relying on interspecific hybridisation (see below).

Genetic improvement of wine yeasts

Since the use of starter cultures became established as a common practice, the major source of starter improvement in oenology has been the natural diversity of the *S. cerevisiae* species. Despite the relative genetic homogeneity of wine strains, selection processes have allowed the development of starter cultures particularly suitable for different sensory profiles (neutral, terpenic, thiolic, and so on), and winemaking styles (white, red, young, aged, sparkling, and so on). However, genomic analysis of the strains currently on the market indicates that a saturation point has been virtually attained (Borneman *et al*., 2016).

Interest in the genetic improvement of previously selected wine strains began around the 1990s, driven by the rise of genetic engineering (Cebollero *et al*., 2007). However, the use of genetically modified organisms in wine production is unlikely to be commercially successful in the short term. The two recombinant strains of *S. cerevisiae* for oenological use that have come onto the market (mainly in the USA and Canada, due to regulatory issues in other countries) did not seem commercially successful, although reliable information on this issue is difficult to obtain. The first of these commercial strains catalyses the biotransformation of malic acid into lactic acid (Husnik *et al*., 2006). This frees winemakers from the uncertainty of malolactic fermentation, a process traditionally more difficult to control than alcoholic fermentation. The second one helps reduce the amount of urea released by yeasts during arginine metabolism (Coulon *et al*., 2006). Over time, excess urea reacts with ethanol leading to the formation of ethyl carbamate (Monteiro *et al*., 1989), considered a potential carcinogen. However, the control of malolactic fermentation, using bacterial starters, has steadily improved for the last decades; while the ethyl carbamate problem appears to be only occasional. The relatively low relevance of the problems they were meant to solve, together with marketing considerations and the limited genetic diversity available (one strain in each case), would explain the low impact of recombinant strains in real oenology.

Reducing alcohol yield is an increasingly common goal in oenological yeast biotechnology (Ciani *et al*.,

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2016; Dequin *et al*., 2017) and was first addressed in the 1990s by genetic engineering approaches, trying to divert carbon flux towards glycerol production or other metabolic endpoints (Dequin and Barre, 1994; Michnick *et al*., 1997; Cambon *et al*., 2006). Other authors addressed the expression of extracellular hydrolytic enzymes from different origins, mostly to help the release of primary aroma compounds from odourless precursors and improve mechanical properties of the juice and mass to ease extraction processing (Pérez-González *et al*., 1993; Ganga *et al*., 1999). Another common type of genetic modification was changing the expression levels of *S. cerevisiae* genes to achieve improvements in secondary aroma, autolysis, or mannoprotein release (Lilly *et al*., 2000; Lilly *et al*., 2006; Tabera *et al*., 2006; Cebollero *et al*., 2009; González-Ramos *et al*., 2009). But none of these efforts directly led to new commercial starter cultures. However, it is worth recognising that the generation of recombinant strains has allowed important advances in the knowledge of wine yeast physiology and the genetic determinants of some industrially relevant traits. Moreover, the information provided by these projects served as a guide to address genetic improvement processes using more conventional methods, some of which have resulted in the development of new commercial strains.

The quest for genetically improved wine strains free of the commercial and regulatory constraints of GMOs led to a burst of breeding efforts using conventional methods. They had been barely explored in this field before the 2000s. These include random mutagenesis, intra- and interspecific hybridisation, or adaptive laboratory evolution (ALE), also known as experimental evolution. Besides technical ease, the choice between these technologies must take into account the degree of control over the modification (at the genome sequence), the genetic determination of the trait, and the genetic variability that can be explored in each case (Fig. 1).

Induction of random mutagenesis, by physical or chemical agents, and phenotypic selection of mutants is one of the classical breeding techniques for industrial microorganisms. In *S. cerevisiae*, the most common mutagens are ethyl methane sulfonate, nitrosoguanidine, and UV radiation. Despite their apparent simplicity, the traits to be improved are not always easy to select phenotypically. There are some classic examples collected in a review by Snow (1983), and more recent examples of improvement, by indirect selection, of nitrogen source utilisation (Salmon and Barre, 1998; Long *et al*., 2018), mannoprotein release (Quirós *et al*., 2010), reduction of SH_2 and SO_2 production (Cordente *et al*., 2009; Walker *et al*., 2021), or reduction of volatile acidity (Cordente *et al*., 2013). Direct mutant selection has been used to improve autolysis (Gonzalez *et al*., 2003; Giovani and Rosi, 2007), or the release of mannoproteins (Gonzalez-Ramos *et al*., 2010). Diploidy of wine strains does not favour this approach, which in principle would allow the recovery of dominant or semi-dominant mutations, but hardly recessive mutations. However, Hashimoto *et al*. (2005) were able to obtain auxotroph mutants by UV mutagenesis of diploid sake strains; and other authors have been able to obtain spontaneous auxotrophic mutants, using positive selection strategies, from *S. cerevisiae* wine yeast strains (Pérez-Través *et al*., 2012). Auxotrophic strains are very useful for hybrid strain construction, as described below. Furthermore, the connections between amino acid metabolism and the biosynthesis of several secondary aroma compounds (Rollero *et al*., 2017), opens possibilities for genetic improvement by the selection of auxotrophic mutants. Random mutagenesis can be useful when the trait to be improved depends on one or two genes but may be limited for improving quantitative traits that show a continuous variation within the species.

Sexual hybridisation is an alternative to generate diversity on a genomic scale and is better suited for quantitative traits. Moreover, since there is no prezygotic isolation within the genus, it allows combining genomes of different species of *Saccharomyces* closely related genera (Santos *et al*., 2008; Su *et al*., 2019). Improvement of wine yeasts has sometimes been achieved by obtaining homozygous derivatives from homothallic strains (Ramírez *et al*., 1999), but the opposite output is also possible (Gimeno-Alcañiz and Matallana, 2001).

Genetic improvement by hybridisation can be approached by means of spore-spore crosses, rare mating and mass mating. In the first case, microdissection is used to position together pairs of haploid spores from the original strains. In this way, it is possible to obtain hybrids without selection markers such as auxotrophies or other directly selectable phenotypes, although molecular markers are still required to verify the hybrid nature of the primary zygotes. This technique has been used, for example, to obtain interspecific hybrids suitable for restarting stuck fermentations (Santos *et al*., 2008), or better adapted to low temperatures and secondary aroma (Kishimoto, 1994); as well as intraspecific hybrids with better fermentative characteristics and mannoprotein production (Pérez-Través *et al*., 2015), or improved thermotolerance (Marullo *et al*., 2009). For rare and mass mating, a selective growth medium is required, in which only the true hybrid strains will grow (Ramirez *et al*., 1998). Therefore, a first step in the breeding programme is usually to obtain spontaneous auxotrophic derivatives, by some positive selection method such as tolerance to alpha-aminoadipic acid or fluorotic acid, to obtain respectively auxotrophic strains for lysine or uridine (Pérez-Través *et al*., 2012). Spontaneous tolerance to

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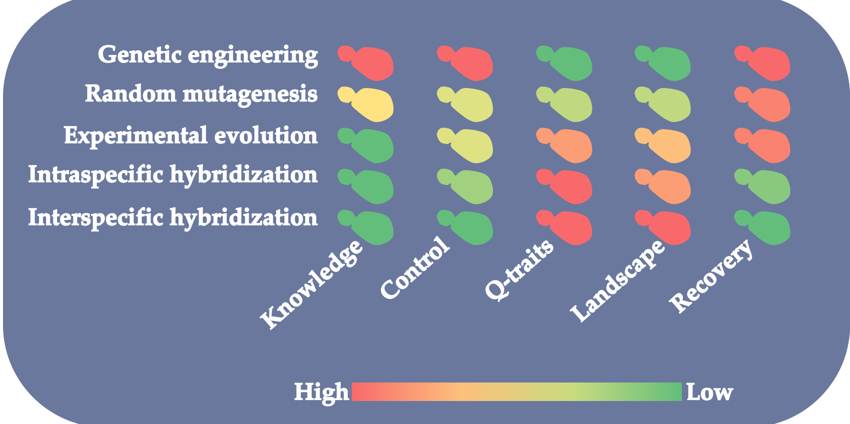

Fig. 1. Main characteristics of different technologies available for the genetic improvement of wine yeasts. Knowledge: required level of knowledge about the genetic determination of the trait of interest. Control: degree of control of the technology at the DNA sequence level. Q-traits: utility for quantitative trait improvement. Landscape: the range of genotypes and phenotypes that could be reached. Recovery: ease of recovering the traits of industrial interest from the original strain.

antibiotics or killer factors (Ramirez *et al*., 1998), ability to use certain carbon sources, or to grow under particular conditions (e.g. thermotolerance) can also be used as hybridisation markers. For mass mating, spores from the two original strains are combined in large numbers on the same medium and allowed to mate. This approach has been used, for example, to obtain interspecific (Bizaj *et al*., 2012) or intraspecific (Agarbati *et al*., 2020) hybrids with better aromatic characteristics, especially in relation to hydrogen sulphide production. Rare mating uses vegetative cells and is not dependent on sporulation. With a very low frequency, diploid cells can eventually undergo mating-type switching and conjugate with other cells. A powerful selection method is required to recover those hybrids. Bellon *et al.* (2011, 2013) were able to generate wine yeast strains with improved aroma profile by interspecific rare mating. In turn, Pérez-Través *et al.* (2015) used intraspecific rare mating to improve mannoprotein release. Another way of combining genomes is the artificial fusion of protoplasts, but in this case, improved strains would fall under GMO regulations according to European legislation. Primary zygotes from spore-spore and mass-mating crosses can be relatively stable, depending on the genomic compatibility of the original strains. But allotetraploid strains that are often generated by protoplast fusion or rare mating must become stable through recombination and chromosome loss (Sipiczki, 2008; Pérez-Través *et al*., 2012). The advantages of sexual hybridisation are that strains with traits derived from both parental strains can be obtained. In addition, transgressive phenotypes can be obtained for quantitative traits (Marullo *et al*., 2006). However, it is more difficult to ensure that all the original traits of the parental strain are retained, unless engaging in multiple backcrossing cycles. Availability of genetic markers, like microsatellites or those derived from QTL mapping of the target traits, would be invaluable in those cases (Marullo *et al*., 2007, 2009).

Adaptive laboratory evolution (ALE) involves maintaining an industrial strain over many generations under a carefully designed selective pressure. The principle is the same as for natural evolution: among the variants that arise spontaneously in each generation, those that leave more offspring will increase their relative frequency in the population, while the negative variants will become replaced after a few generations. To avoid unwanted genetic drift, the evolutionary conditions should mimic those under which the strains are meant to be used, but this is not always feasible. Design is relatively straightforward to improve some traits, such as ethanol tolerance, as done by Novo *et al.* (2014). But in other cases, indirect selection strategies are required. For example, Tilloy *et al.* (2014) used osmotic stress as a proxy to select strains with higher glycerol production. ALE shares advantages and limitations with hybridisation and mutagenesis. Like hybridisation, it can be used to improve quantitative traits, as it potentially affects the whole genome, although it does not generate all the variability of a genetic cross, nor does it allow overcoming the species barrier. On the other hand, it shares with mutagenesis a greater potential to preserve the original traits of the strain. However, good design of experimental conditions and final verification of the improved strains are necessary to ensure the recovery of the crucial traits of the original strain. A summary of the merits and drawbacks of the main tools available for the genetic improvement of wine yeasts is shown in Fig. 1.

This revival of traditional methods is not a simple step backwards, as the progress of knowledge and analytical tools for yeast biotechnology, including high-throughput

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phenotyping and genotyping tools, renders techniques that were very laborious a few decades ago much easier nowadays. Despite the loss in precision, as compared to genetic engineering, these techniques may be superior when it comes to improving quantitative traits or those for which the genetic basis are not fully elucidated (Fig. 1). For example, in the case of ALE, improved strains may have accumulated mutations in genes not previously linked to that phenotype, due to metabolic trade-offs or genetic drift.

Finally, some authors see considerable potential in new genome edition techniques (CRISPR/Cas-based) and synthetic biology as tools for the genetic improvement of wine yeasts in the future (Pretorius, 2017; Vigentini *et al*., 2017). Tools based on CRISPR/Cas will help solve some of the bottlenecks of conventional genetic engineering, like stacking multiple genetic modifications, or targeting simultaneously all homologous alleles in diploid or aneuploid strains. However, for the time being, it seems that these techniques will continue to face the same challenges that conventional genetic engineering has encountered in most countries so far.

Future perspectives

Our knowledge of *S. cerevisiae* wine yeast strains keeps increasing in quantity and quality. However, it seems we have reached near saturation regarding the exploitation of natural diversity. Therefore, the tailoring of wine yeast starters to the demands of an ever-changing market requires the generation of new genotypes. Commercial and regulatory constraints for genetic engineering have pushed wine biotechnology towards alternative techniques, including random mutagenesis, sexual hybridization, or experimental evolution. The power of these technologies has been boosted by recent advances in genomics, NGS based technologies, and systems biology approaches to yeast metabolism. These can help guide backcrossing, for introgression into target starter yeasts of traits from mutant or unrelated strains, as well as the rational design of selection strategies for experimental evolution or random mutagenesis. Strains improved in this way will take advantage of our extensive scientific knowledge on wine yeasts, while benefiting from a non-GMO status. They are likely to be a major source of innovation in winemaking over the next few years.

Genome edition is, somehow, halfway between these techniques and traditional genetic engineering. It can be considered “cleaner” than any of them, as the genetic modification can be fully targeted while avoiding any unwanted DNA sequences in the final strains. Anyway, its potential cannot be fully exploited without a thorough knowledge of the genetic determination of wine yeast technological traits. There are high expectations among wine biotechnologists that new regulations in Europe, and other wine producing countries, will be less restrictive for CRISPR/Cas-derived strains. But the multiple regulatory layers affecting the global production and marketing of wine do not warrant easy market entry for genome-edited strains.

Finally, despite most of our knowledge on wine yeasts comes from studies under axenic conditions, yeasts have been evolutionarily shaped within microbial communities. Moreover, non-*Saccharomyces* starters are increasingly used in combination with conventional ones. These considerations have led to a growing interest in the social life of wine yeasts, with the aim of better understanding the different wine yeast species and their ecological context (Jouhten *et al*., 2016; Conacher *et al*., 2021). In the short term, the development of multi-species starter cultures and the study of interactions between wine microorganisms are also likely to become key drivers of oenological innovation.

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Conflict of interest

The authors have no conflict of interest to disclose.

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