Alternative yeasts for winemaking: *Saccharomyces* non-*cerevisiae* and its hybrids

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Running title: *S.* non-*cerevisiae* in winemaking

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Abstract

Wine fermentation has not significantly changed since ancient times and the most traditional aspects are seen by the market as elements that uplift wine nuances and quality. In recent years, new trends have emerged from the sector in line with consumer preferences, and due to the effects of global climate change on grape ripening. In the first cases the consumers are looking for wines with less ethanol and fruitier aromas and in the second cases the wineries want to reduce the wine alcohol levels and/or astringency. New yeast starters of alternative *Saccharomyces* species and their hybrids can help to solve some problems that wineries face. In this article we review several physiological and genetic aspects of *S. uvarum* and *S. kudriavzevii* and the hybrids, which are especially relevant during the winemaking process, such as their good fermentative capabilities at low temperatures, resulting in wines with lower alcohol and higher glycerol amounts.

Keywords

Winemaking; yeasts; *S.* non-*cerevisiae*; aroma; glycerol; cold fermentation

INTRODUCTION

Winemaking is one of the oldest food processing procedures that humans have performed since ancient civilizations. In fact, the earliest evidence of winemaking is traced to Iran at the Hajji Firuz Tepe site (5400-5000 BC) (This et al., 2006). The process of wine production is a conversion of grape juice made by microorganisms, mainly yeasts, where a huge number of compounds are metabolically consumed or produced. The result is one of the most widely consumed beverages. In the modern wine production process, selected yeasts are used to inoculate grape must in order to control the fermentation, reduce risk of contamination, increase the reproducibility and generate specific characteristics in the wine by selecting yeasts strains with specific abilities of fermentation. Only specific wine strains are able to outcompete and impose over other non-wine and spoilage microorganism and ensure a proper wine fermentation. Yeast starters companies have enormous interest in expand their strain catalogue with the objective to present wine strains with general wine strain type characteristics abut also with specific abilities that can be useful for wineries.

Wine yeasts are highly specialised organisms that evolved under restrictive environmental conditions created by human technology. During cellular adaptation to those manipulated environments, yeast strains were subjected to selective pressures generated by alcoholic fermentation (Querol et al., 2003), resulting in adaptative differences among them (Barrio et al., 2006). Most of commercial yeasts are *S. cerevisiae*, being the most frequently used in wine fermentations, as well as the most studied species. However other species of the *Saccharomyces* genus and even other species of the genus *Saccharomyces* (*S. uvarum*) and their interspecific hybrids (*S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii*) have shown their potential application to solve the new challenges of the winemaking industry, thus attracting high

63 interest in last years by researchers in the field (González et al., 2007; Gangl et al.,
64 2009; Tronchoni et al., 2009; Gamero et al., 2013). In addition, strain selection has been
65 extended in recent years to non-*Saccharomyces* yeasts such as those belonging to genera
66 *Candida*, *Kloeckera*, *Kluyveromyces Debaryomyces*, *Hanseniaspora*, *Hansenula*,
67 *Pichia*, *Metschnikowia*, *Schizosaccharomyces*, *Saccharomycodes*, *Starmerella*,
68 *Torulaspora* or *Rhodotorula*. Although non-*Saccharomyces* species lack
69 competitiveness under oenological conditions, mainly because they do not ferment so
70 vigorously and display lower stress resistance than *Saccharomyces*, employing mixed
71 starter cultures or sequential fermentations are using in the last years to enhanced
72 glycerol, improve the aroma complexity, reduced acetic acid and ethanol content
73 (Schuller and Casal, 2005; Comitini et al., 2011; Milanovic et al., 2012; Contreras et al.,
74 2015; Morales et al., 2015; Lencioni et al., 2016; Ciani et al., 2016 a and b; Varela,
75 2016; Wang et al., 2016). Besides the scientific interest for the use of these species, only
76 strains of the species *Kluyveromyces thermotolerans*, *Metschnikowia pulcherrima*,
77 *Pichia kluyveri*, *Torulaspora delbrueckii* are commercialized.

78 One of the most important challenges in winemaking is the modification of the
79 composition and properties of the grape must due to climate change (Borneman et al.,
80 2013). Answers to these new demands require improvements in the enological practices,
81 among which the development of new yeast starters adapted to the fermentation
82 conditions imposed by climate change are of chief importance. Climate change impact
83 on the winemaking industry has stress out the importance of ethanol yield in the
84 fermentation (White et al., 2006). Temperature increases force early maturation of
85 grapes in Mediterranean regions and sugar contents in musts are higher and, as a
86 consequence, alcohol contents in the resulting wine increase (Jones et al., 2005). An
87 alternative is to harvest grapes earlier, when sugar content is optimum to obtain wines

with the appropriated alcohol content. However, in this case, the ripening of balanced fruits becomes more difficult because the maturation stage of grape tannins and phenols is not reached and the outcome is very astringent wine. These wines with high ethanol contents or with unpleasant young unripe tannins are not well accepted by consumers. To avoid this lack of competitiveness, wineries demand yeasts exhibiting lower ethanol yields as well as higher glycerol and mannoprotein productions, because these compounds help to balance wine astringency (White et al., 2006).

New yeast starters, of non-*cerevisiae* strains *(S. uvarum)* or hybrids (*S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii*) can contribute to solve some problems of the wineries related to climate change. Contrary to what happens with non-*Saccharomyces* species that have previously described, *S. uvarum* and hybrids among species of the genus *Saccharomyces* exhibit good fermentative capabilities at low temperatures, producing wines with lower alcohol and higher glycerol amounts, while fulfilling the requirements of the commercial yeasts, such as a good fermentative performance and aromatic profiles, of great interest for the wine industry (González et al., 2007; Tosi et al., 2009; Gamero et al., 2012). Moreover, some strains exhibit pectinolytic activity (Naumov et al., 2001) and, hence, produce more aromatic wines (Henschke and Rose, 1991).

Another important driving force in winemaking industry is the consumer's demands for new products with novel aromatic profiles. Nowadays, fermentations at low temperatures are a trend in winemaking because it improves the aromatic profile of the produced wines (Boulton et al., 1996). However, low fermentation temperatures have disadvantages because requires longer periods and the risks of halted or sluggish fermentations are bigger (Bisson, 1999). These problems can be avoided by providing yeasts better adapted to ferment at low temperatures.

BENEFICIAL ASPECTS OF *S. UVARUM*, *S. KUDRIAVZEVII* AND *SACCHAROMYCES HYBRIDS* FOR WINEMAKING

Low fermentation temperatures conducted by *S. cerevisiae* have disadvantages because longer processes are required, and the risks of halted or sluggish fermentations increase (Boulton et al., 1996; Bisson, 1999). These problems can be avoided with yeasts better adapted to ferment at low temperatures. Although supposedly "cryotolerant" *S. cerevisiae* yeasts are already available for the wine industry, e.g. QA23 from Lallemand Inc. or Fermol Cryophile and Fermol Reims Champagne from AEB, most of them do not offer desirable fermentation performances at low temperatures (10-15ºC). Physiological and enological works have indicated the potential benefits of the use of *S. uvarum*, *S. kudriavzevii* and their hybrids with *S. cerevisiae* fermenting at low temperature (Figure 1) and have also shown its well-established cryotolerant character (Tronchoni et al., 2012; Gamero et al., 2013; López-Malo et al., 2013). As *S. uvarum*, *S. kudriavzevii* and hybrids strains are able to finish fermentations in musts with 250 g/L sugars, producing lower alcohol levels and increased glycerol content without increasing acetic acid, especially at low temperatures (González et al., 2006; González et al., 2007; Lopandic et al., 2007; Tronchoni et al., 2009; Masneuf-Pomarede et al., 2010; Paget et al., 2014) which makes this species suitable to counteract problems derived from climate change. Furthermore, its sugar consumption rate, similar to that of *S. cerevisiae*, could make this organism a good candidate to compete for a place at low fermentation temperatures in the wine yeast industry (Figure 1).

S. kudriavzevii has been never found in winemaking probably due to low resistance to elevated concentrations of ethanol (Belloch et al., 2008), although its

137 contribution is very relevant as a part of the *Saccharomyces* hybrids. However, all the
138 published data has been obtained at the laboratory level, so the possibility of using *S.*
139 *kudriavzevii* in wine fermentations must be tested at the industrial scale. There are some
140 physiological properties of *S. kudriavzevii* that could explain the potential benefits in the
141 use of *S. kudriavzevii* in winemaking as the ability to perform complete wine
142 fermentations at laboratory scale with similar or even enhanced kinetics than *S.*
143 *cerevisiae* (Gonzalez et al., 2006; Combina et al., 2012). On the other hand, other works
144 have shown that *S. kudriavzevii* cannot outperform *S. cerevisiae* strains even at low
145 temperature in synthetic media growth (Arroyo-López et al., 2011). Thus, the study of
146 interaction among both species can be important to explain the absence of *S.*
147 *kudriavzevii* in wine fermentations conditions. In spite of these results, is not clear that
148 industrial wine fermentations inoculated with *S. kudriavzevii* selected strains will be
149 completed by them or they will be competed out at some point by *S. cerevisiae*
150 contaminant cells, which are present in different parts of the wineries. Thus, further
151 research al industrial scale must be conducted to finally implement the use of the
152 potentially beneficial species *S. kudriavzevii* in winemaking.

153 So far, it is known that *S. cerevisiae* and *S. kudriavzevii* natural hybrids are able
154 to dominate industrial wine fermentations performed at low temperatures in regions of
155 cold climates (Gonzalez et al., 2006; Gang et al., 2009; Erny et al., 2012). On the other
156 hand, mixed cultures between *S. kudriavzevii* and *S. cerevisiae* auxotrophic mutants
157 performed in synthetic must showed a clear *S. cerevisiae* imposition at high
158 temperatures (31 ºC) (Arroyo-López et al., 2011). But it is interesting to note that at low
159 temperatures (17 ºC), *S. kudriavzevii* is able to compete with *S. cerevisiae* in the first
160 part of the fermentation when they are inoculated 50:50. This suggests that in controlled
161 industrial wine fermentations at low temperatures, which contains very low levels of *S.*

cerevisiae cells coming from grape grains and winery equipment, the inoculation of *S. kudriavzevii* could contribute significantly to the final wine characteristics, by increasing glycerol production and enhancing its aroma profile (Figure 1) (Gonzalez et al., 2007; Tronchoni et al., 2009; Arroyo-López et al., 2011).

In the next sections we will analyze the molecular mechanisms related with the adaptation of *S. uvarum* and *S. kudriavzevii* to growth at low temperatures and the differences in the contribution to the aroma profile with respect to *S. cerevisiae* (Figure 1).

Adaptation to low temperatures

Several studies have focused in understand the cryophilic character of *S. uvarum* and *S. kudriavzevii* at the molecular level, including transcriptomic and metabolomic studies (Combina et al., 2012; López-Malo et al., 2013). Some aspects of these species have been highlighted in relation to cold resistance and winemaking as glycerol accumulation (Arroyo-López et al., 2010; Oliveira et al., 2014), membrane composition (Tronchoni et al., 2012), or translation efficiency (Tronchoni et al., 2014) and are addressed with more detail below.

Glycerol accumulation

The cryophilic species *S. uvarum* and *S. kudriavzevii* produce higher amounts of glycerol compared to *S. cerevisiae* in must fermentations (Arroyo-López et al., 2010; Oliveira et al., 2014). Glycerol contributes to wine quality by generating sweetness and fullness (Ribereau-Gayon et al., 1977; Eustace and Thornton, 1987). Also increase

viscosity which can soften the astringency caused by unriped tannins present in early harvested grapes (Ishikawa and Noble, 1995, Llaudy et al., 2006). In addition, glycerol plays an important role in cold stress adaptation (Tulha et al., 2010), although other features have been related to yeasts adaptation to low temperatures, including synthesis of ribosomal proteins, changes in membrane lipid composition and a higher trehalose content (Aguilera et al., 2007). In *S. cerevisiae*, glycerol is synthesized after a cold shock via a regulatory mechanism involving the HOG (High Osmolarity Glycerol) pathway (Hayashi and Maeda, 2006; Panadero et al., 2006). Intracellular glycerol content was linked to cell survival in fermentations at low temperatures (Tulha et al., 2010) and also is involved in freeze/thawing stress resistance (Izawa et al., 2004).

Cryoprotectanct glycerol is synthesized by a branch of glycolysis involving two steps (Ansell et al., 1997; Norbeck et al., 1997; Pahlman et al., 2001), which in the case of *Saccharomyces* yeasts are catalyzed by two isoenzymes each step: GPD for glycerol-3-phosphate dehydrogenases (Gpd1p and Gpd2p) and GPP for glycerol-3-phosphatases (Gpp1p/Rhr1p and Gpp2p/Hor2p). Flux modelling calculations of metabolic control values of glycerol synthesis indicate that the glycerol-3-phosphate dehydrogenase reaction has a flux control coefficient of approximately 0.85, being the major responsible of the flux control of this pathway (Remize et al., 2001). Moreover, *GPD1* gene overexpression increases the glycerol levels produced while the overexpression of the other three enzymes does not (Nevoight and Stahl, 1996; Pahlman et al., 2001; Remize et al., 2001) whereas a reduction of *GPD1* expression leads to a reduced flux towards glycerol (Nevoight and Stahl, 1996; Hubmann et al., 2011). Furthermore, *GPD1* is activated in response to cold stress (Panadero et al., 2006). All this data supported the important role of *GPD1* in the cold stress response in *Saccharomyces.*

209 Recently, our research focused in deciphering the mechanisms by which the
210 cryophilic species *S. kudriavzevii* produce more glycerol and its putative role in the
211 better adaptation of this species to cold environments (Figure 1). Interestingly, it has
212 been shown that *S. kudriavzevii* has a higher *GPD1* expression and that Gpd1p presents
213 enhanced enzymatic parameters and an increased activity (Oliveira et al., 2014). All
214 these differences suggest that *S. kudriavzevii* has changed its metabolism to promote the
215 branch of the glycolytic pathway involved in the glycerol production to adapt to low
216 temperature environments (Figure 1) and maintain the NAD^+/NADH ratio in alcoholic
217 fermentations. In agreement with these results, new studies suggested that temperature-
218 induced redox imbalances could be compensated by increasing glycerol accumulation
219 but also by the production of cytosolic acetaldehyde through the deletion of *GUT2* or
220 *ADH3* respectively (Paget et al., 2014).

221

222 *Membrane lipid composition*

223 As previously indicated, the lipid composition of *S. kudriavzevii* exhibits some features
224 that may explain its adaptation to low temperatures (Tronchoni et al., 2012). Yeast
225 membranes are a lipid bilayer consisting of two amphipathic molecules, phospholipids
226 (PL) and sphingolipids, with an apolar phase of fatty acids (FA). The fatty acids of *S.*
227 *cerevisiae* mainly correspond both to unsaturated fatty acids (UFA), oleic acid and
228 palmitoleic acid, and saturated fatty acids (SFA), palmitic acid and stearic acid. The
229 medium chain fatty acids (MCFA) are found in lower proportions but their
230 concentrations increase when yeasts are grown under anaerobic conditions, such as the
231 wine fermentation. Sterols are also important, being ergosterol the main sterol in fungi
232 (Henschke and Rose, 1991). Changes in any of these lipids can significantly disturb the

membrane function and alter the role of membrane-associated proteins. Thus, many organisms have developed mechanisms to keep the appropriate membrane fluidity regardless of the temperature (Cossins and Prosser, 1978). It has been described that low temperatures increase in the degree of fatty acid (FA) unsaturation in *S. cerevisiae* (Sakamoto and Murata, 2002), producing a higher membrane fluidity. Another way to increase membrane fluidity is to reduce the FA chain length (Torija et al., 2003). Beltran et al. (2006) reported some of these effects in *S. cerevisiae* strains during an industrial wine fermentation. Redón et al. (2011) also reported common changes in the lipid composition of different industrial species and strains of *Saccharomyces* after growth at low temperature showing that the MCFA and triacylglyceride content increased, whereas the phosphatidic acid content and the phosphatidylcholine/phosphatidylethanolamine ratio decreased, as occurs in *S. uvarum* compared to *S. cerevisiae* (Figure 1). A similar response was observed in *S. kudriavzevii*, although sterol esters and squalene were also increased (Tronchoni et al., 2012). These results indicate that the membrane of *S. kudriavzevii* has a composition that allows its adaptation to growth at low temperatures due to two mechanisms already observed in the other species (Figure 1). Regardless the growth temperature, the *S. kudriavzevii* strains had higher medium-chain fatty acids and squalene percentages and also shorter chain lengths (Tronchoni et al., 2012). This differential lipid content has a significant impact in the enhanced fitness of *S. kudriavzevii* at low temperatures. However, more research must be done in order to clarify the role of the different membrane components in the physiology of these yeasts.

Translation efficiency

At low temperatures, ribosome maturation may be compromised due to a reduction in RNA structure plasticity by RNA hyperstabilization (Zavanelli et al., 1994; Fortner et al., 1994; Li and Brow, 1996; Staley and Guthrie, 1999; Hilliker et al., 2007; Perriman and Ares, 2007; Kurata et al., 2013). A transcriptome comparison of *S. kudriavzevii* with *S. cerevisiae* shed light on the response of this cryotolerant yeast to low temperature (Tronchoni et al., 2014). A similar low temperature stress response for both species is the presence of up-regulated genes related to translational machinery, although *S. kudriavzevii* shows a higher response compared to *S. cerevisiae*. Tronchoni et al (2014) postulated that this higher response could be the result of changes in the stability of a functional RNA conformation in relation to a competing structure (Zavanelli et al., 1994). However, the different paromomycin susceptibility (Tronchoni et al., 2012) suggests that other mechanisms can also be involved related to *S. kudriavzevii* translation machinery at low temperatures. Also, paromomycin resistance can be the result of enhanced translation efficiency (Perriman and Ares, 2007). This study suggests that *S. kudriavzevii* has increased translation efficiency due to higher ribosome availability after adaptation to cold shock (Figure 1) (Tronchoni et al., 2012). García-Rios et al (2016) analyzing the proteome profiling of *S. cerevisiae* and cryotolerant species *S. uvarum* and *S. kudriavzevii* during low-temperature wine fermentationwe by iTRAQ-based showed that the main differences among the proteomic profiling of the three *Saccharomyces* strains grown at 12 °C and 28 °C lay in translation, glycolysis and amino acid metabolism. These data corroborate previous transcriptomic results (Tronchoni et al., 2014) , which suggest again that *S. kudriavzevii* is better adapted to grow at low temperature as a result of enhanced more efficient translation.

Metabolic balance

Although no similar transcriptomic data is available for *S. uvarum,* the metabolome comparison of *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* growing at 12 ºC revealed that the main differences between the two cryotolerant species and *S. cerevisiae* were in carbohydrate metabolism, mainly fructose. However, these two species have developed different strategies for cold resistance. *S. uvarum* presented elevated shikimate pathway activity, while *S. kudriavzevii* displayed increased NAD^+ synthesis (López-Malo et al., 2013). García-Rios et al (2016) analyzing the proteome profiling of these species and comparing with *S. cerevisiae* observed that amino acid biosynthetic pathways can also be mechanisms that better explain biomass yield in cryotolerant strains and also can justify the differences in the aroma synthesis. However, at low temperature, *S. cerevisiae* showed higher concentrations of glycolytic and alcoholic fermentation enzymes.

Contribution to the wine aroma

Wine fermentation aromas result from a complex mixture of chemical compounds produced by yeast secondary metabolism (Lilly et al., 2000; Swiegers and Pretorius, 2005). Higher alcohols (fusel, marzipan and floral aromas) as well as acetate and ethyl esters (fruity and floral aromas) are the main scents in young wines. Higher alcohols are generated from sugar metabolism intermediates, through anabolic reactions, or from branched-chain amino acids, through the Ehrlich pathway, a multistep catabolic reaction (Boulton et al., 1996; Dickinson et al., 1997; Eden et al., 2001; Dickinson et al., 2003). Ethyl ester compounds are produced by condensation of alcohols and coenzyme-A-activated acids (Swiegers and Pretorius, 2005), while acetate esters result from the

306 combination of acetyl-CoA with an alcohol, by the action of alcohol acetyl transferases
307 (Lilly et al., 2000). The amount and nature of these aroma compounds are influenced by
308 many factors, such as must nitrogen content, fermentation temperature and the yeast
309 strain (Lilly et al., 2000; Swiegers and Pretorius, 2005). Several studies have
310 characterized the impact of *S. uvarum* and *S. kudriavzevii* on the fermentation processes
311 and the by-product formation and aroma profile (Gonzalez et al., 2007; Henschke et al.,
312 2000; Gamero et al., 2011a; Gamero et al., 2011b; Gamero et al., 2012; Gamero et al.,
313 2013). These studies showed that *S. uvarum* and *S. kudriavzevii* strains present different
314 aroma compound accumulation patterns during wine fermentation compared to those of
315 *S. cerevisiae* strains (Figure 1). Hence, the utilization of *S. uvarum* and *S. kudriavzevii*
316 strains in winemaking can contribute to a new aromatic composition in the final wines,
317 by increasing the higher alcohol and ester amounts. Specifically, *S. uvarum* generates
318 larger amounts of 2-phenylethanol, 2-phenylethyl acetate and ethyl lactate (Henschke et
319 al., 2000; Masneuf-Pomarede et al., 2010; Gamero et al., 2011a; Gamero et al., 2012)
320 and *S. kudriavzevii* produce larger amounts of higher alcohols and 2-phenylethanol at
321 12ºC (Henschke et al., 2000; Gamero et al., 2011a; Gamero et al., 2013; Stribny et al.,
322 2015). These differences in aroma production have been correlated with differences in
323 gene sequences and in gene regulations. For example, *S. kudriavzevii* strains exhibited
324 an up-regulation in acyltranferase *EHT1* and a down-regulation in acyltranferase *EEB1*
325 (the genes involved in ethyl esters formation) during fermentation at 28ºC (Gamero et
326 al., 2014). The low ethyl ester production in *S. kudriavzevii* also suggests that
327 acyltransferase *EEB1* is more important in the production of these aromatic compounds
328 than *EHT1*, as previously described (Saerens et al., 2008). Also it is interesting to note
329 that the higher level of geraniol uptake, which is then bioconverted into linalool and
330 alpha-terpineol, is one of the most significant abilities of *S. kudriavzevii* among others

(Gamero et al., 2011b). By comparative genomic analysis, among the available genome sequences of *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii,* of genes involved in aroma synthesis, Stribny et al (2015) observed important differences at nucleotide level in the genes *ARO10* (encoding a phenylpyruvate decarboxylase), *ATF1* and *ATF2* (coding for alcohol acetyltransferases 1 and 2, respectively). The heterologous expression of these genes from *S. kudriavzevii* and *S. uvarum* in 3 deletant *S. cerevisiae* T73 strains (Δ*ATF1*, Δ*ATF2*, and Δ*ARO10* strains) confirmed the higher production of several aromatic compounds by *S. kudriavzevii* and *S. uvarum* such as isoamyl alcohol, isobutanol and their esters (Stribny et al., 2016a and 2016b). As occurred with Gpd1p (Oliverira et al., 2014), it has also been observed interesting differences in enzymatic activities between these proteins in the cases of *S. kudriavzevii* and *S. uvarum*. The enzymatic activity of SkAro10p with phenylpyruvate as a substrate was half that ScAro10p whereas the activities of SkAro10p for the other tested substrates were more than three times higher. Differences in the enzymatic properties of ScAtf1p, SkAtf1p and SuAtf1p were also detected (Stribny et al., 2016a). Hence, the utilization of *S. uvarum*, *S. kudriavzevii* or their hybrids strains in winemaking can contribute to a new aromatic composition in the final wines.

THE ROLE OF *S. CEREVISIAE* HYBRIDS IN WINEMAKING

In the case of *Saccharomyces* genus, one of the most interesting mechanisms observed in the adaptation of these yeasts to industrial process is the formation of interspecific hybrids. Allopolyploidy and introgression by interspecific hybridization are the main mechanisms of lateral gene transfer in eukaryotes (de Barros Lopes et al., 2002; Barrio et al., 2006). Interspecific hybridization generates new gene combinations of potential

355 adaptive value conferring, under fluctuating or intermediate environmental conditions,
356 selective advantages to the hybrids with respect to their parental species (Masneuf et al.,
357 1998). Hybrids between *S. cerevisiae* and other *Saccharomyces* species such as the
358 cryotolerant *S. uvarum* (Naumov et al., 2000; Greig et al., 2002; Le Jeune et al., 2007)
359 and *S. kudriavzevii* (Bradburi et al., 2006; González et al 2006; Lopandic et al., 2007;
360 Erny et al., 2012; Peris et al., 2012a) have been isolated from wine, cider and brewing
361 fermentations, and other sources.

362

363 **On the origin of *S. kudriavzevii* wine hybrids**

364 In recent years, hybrids between *S. cerevisiae* and *S. kudriavzevii* have been isolated not
365 only from wine, but also from cider and brewing fermentations, as well as other sources
366 (Bradburi et al., 2006; González et al 2006; Lopandic et al., 2007; Erny et al., 2012;
367 Peris et al., 2012a) suggesting interesting human interactions in their origin and
368 propagation (Peris et al., 2012b). Researchers have looked for *S. kudriavzevii* wine
369 hybrids worldwide but they were only isolated in specific locations in Europe (Figure
370 2). By combining the phylogenetic analysis of gene sequences with all the available
371 information on genetic and genomic characterization of *S. cerevisiae* x S. *kudriavzevii*
372 hybrids a total of seven potential hybridization events were predicted as the *S.*
373 *kudriavzevii* wine hybrids origins (Peris et al., 2012b). Most hybrids seem to have been
374 generated by rare-mating events involving a diploid *S. cerevisiae* strain and a haploid
375 strain of *S. kudriavzevii* generating different chimerical genomes with ploidy values
376 close to 3n (Table 1) (Peris et al., 2012a; Peris et al., 2012b). Two of these hybridization
377 events generated the most frequent wine hybrid types (Figure 2), one was found in
378 Wädenswill, Switzerland, and is phylogenetically related to Trappist brewing hybrids.

The other type is widely distributed from the Rhine valley (Alsace and Germany) to the Danube valley (Austria, Croatia and Hungary) wine regions.

After ~6,000 years of adaptation to wine fermentation conditions in warm climates of the Mediterranean and Fertile Crescent regions, wine *S. cerevisiae* yeasts were taken by the Romans, together with the vines and winemaking tools, to the limits of their empire, the Rhine and Danube Rivers. In these regions of Oceanic and Continental climates (Figure 2), these *S. cerevisiae* wine strains could have problems to perform wine fermentations at low temperatures at which other *Saccharomyces* species are better adapted. In such climate conditions, however, hybrids have clear advantages over the parental species.

These *S. cerevisiae* x *S. kudriavzevii* hybrids likely originated several times but they probably spread during the Middle Age when Christian monks spread the viticulture and enology practices all over Europe (Figure 2) (Burton and Kerr, 2011). For example, in Central Europe Cistercian monks extended the Burgundian family of grape varieties, mainly Chardonnay and Pinots, as well as German varieties, and with them also the main lineage of hybrid yeasts responsible of wine fermentation (Erny et al., 2012; Peris et al., 2012a; Peris et al., 2012b).

Molecular characterization of enological *Saccharomyces* hybrids

At the moment, very few information about the physiological and molecular part of *S. uvarum* hybrids are available. However, some more studies have been done on the *S. kudriavzevii* hybrids. Oenological characterization of *S. cerevisiae* and *S. uvarum* and S. *cerevisiae x S. kudriavzevii* hybrid strains has demonstrated that the hybrids are well adapted to ferment at low and intermediate temperatures, producing moderate or higher

403 levels of glycerol and less acetic acid and more aromas (higher alcohols and esters) with
404 regard to reference strains of *S. cerevisiae* and *S. kudriavzevii* (Masneuf et al., 1998;
405 Gamero et al., 2013; González et al., 2007).

406 Natural hybrids between *S. cerevisiae* and *S. kudriavzevii* conducting wine
407 fermentations have been characterized by genetic approaches (Belloch et al., 2008; Peris
408 et al., 2012a; Peris et al., 2012b, Borneman et al., 2013). It is interesting to note that
409 some of the advantages of this hybrids (fermenting at low temperature and produce
410 more glycerol) can be correlated with the genome composition (Figure 2 and Table 1).
411 Thus, it has been observed that most natural hybrids present as less, one *S. kudriavzevii*
412 allele for the genes related with the cold stress, ergosterol and glycerol metabolism
413 (Table 1). But also have been correlated some of these differences with gene expression
414 (Combina et al., 2012; Gamero et al., 2015). The commercial hybrid W27 induced eight
415 genes related to these functional groups compared with *S. cerevisiae* reference species.
416 When expression of these W27 hybrid genes was compared with *S. kudriavzevii*,
417 significant differences were observed in genes *PGI1* and *TIP1* involved in glycerol
418 biosynthesis; in genes mainly belonged to the *PAU*, *DAN/TIR* families, linked to cold
419 shock adaptation, while one gene (*ARE1*) is involved in sterol metabolic processes and
420 in aroma synthesis (Figure 2). Besides, the quantitative contribution to the overall gene
421 expression of the alleles coming from one parental strain or the other was clearly
422 determined by the fermentation temperature for some genes as has been demonstrated
423 for *ARO1* and *ATF2* genes where *S. kudriavzevii* allele was more expressed than that of
424 *S. cerevisiae* particularly at 12ºC (Gamero et al., 2015).

425 At present, the only contribution of *S. kudriavzevii* to winemaking is through its
426 interspecific hybrids with other *Saccharomyces species,* most of them with *S.*
427 *cerevisiae,* that are present, and very frequently predominant, in wine fermentations of

European regions of Continental and Oceanic climates (Figure 2). Several wine hybrids were physiologically and genetically characterized (Figure 2), indicating that these hybrids take advantage of their chimerical genomes (Table 1), coming from both parental species, to adapt to changes in their environments (González et al., 2007; Gangl et al., 2009; Belloch et al., 2008; Arroyo-López et al., 2009), especially to grow at lower temperatures, a trait acquired from the *S. kudriavzevii* parent (González et al., 2006, González et al., 2007; Gangl et al., 2009; Salvadó et al., 2011; Tronchoni et al., 2012). The enological characterization of some of those *S. cerevisiae* x *S. kudriavzevii* hybrids have shown that *S. cerevisiae* provide ethanol tolerance and high fermentative capacity (Arroyo-López et al., 2010; Le Jeune et al., 2007; Bradbury et al., 2006) whereas *S. kudriavzevii* genome contributes to their adaptation to low temperatures (Belloch et al., 2008; Arroyo-López et al., 2009). In fact, glycerol production in the hybrids seems intermediate between both parentals in must fermentations, which suggest that the adaptation to low temperatures by increased glycerol production has been partially inherited from *S. kudriavzevii* (González et al., 2007, Gangl et al., 2009). Regarding ethanol, hybrids usually produce similar or higher levels compared to *S. cerevisiae* (Gangl et al., 2009). On the other hand, both species showed a differentiated pattern of aroma compounds accumulation in winemaking which can generate a diverse aroma profile in *S. cerevisiae* x *S. kudriavzevii* hybrids. Aroma production by some hybrids (W27 and HA1841) showed that aroma production profile of these hybrids was similar to that of *S. kudriavzevii* at low fermentation temperature, whereas at moderate or high fermentation temperatures, they showed higher similarities with *S. cerevisiae* (González et al., 2007; Gangl et al., 2009). Other studies have shown differences between aroma profiles of hybrids and parental species except for higher alcohols production that were

452 comparable to those of *S. cerevisiae* at 28°C and *S. kudriavzevii* at 12°C (Bellon et al.,
453 2011; Cus and Jenko, 2013; Gamero et al., 2013).

454

455 **GENERATION OF ARTIFICIAL HYBRIDS**

456 Genomic blind approaches, as artificial hybridization, are the most adequate
457 methodologies to generate of new industrial strains (Giudici et al., 2005). The most
458 promising examples of wine yeast strains improvement involved generation of
459 interspecies hybrids within the species of the *Saccharomyces* genus (Bellon et al., 2011)
460 or further inbreed program of them (Bizaj et al., 2012). The final destination of the
461 generated hybrid should be considered to select the hybridization method since the use
462 of genetically modified organisms (GMOs) in food is limited by current legislations in
463 different countries, as well as by public concern (Schilter and Constable, 2002;
464 Pretorius and Hoj, 2005; Cebollero et al., 2007). According to Directive 2001/18/EC of
465 the European Parliament and the Council of the European Union, hybrids generated by
466 mating of spores and rare-mating—based on the natural rare event of mating type
467 switching in industrial yeasts—must not be considered as GMOs. Recent studies, have
468 focused on optimize and develop programs of generation of artificial hybrids (Bellon et
469 al., 2011, Pérez-Través et al., 2012; Pérez-Través et al., 2014; da Silva et al., 2015;
470 Solieri et al., 2015; Lopandic et al., 2016). It has been described that hybrids obtained
471 by rare-mating are easily obtained and contain a complete set of chromosomes of both
472 parents (Pérez-Través et al., 2012; Pérez-Través et al., 2014). These artificial hybrids
473 have a more complete subset of genetic material inherited from each parental strain just
474 before they are generated. Consequently, they possess an extremely high genetic
475 plasticity which could render a potentially better adaption to the environment. Due to

476 the fact that a loss of genetic material occurs during hybrids generation and genetic
477 stabilization, hybrids possessing a high amount of DNA became a better resource to
478 obtain the best suitable hybrid strain for industrial purposes. However, to guarantee the
479 genetic invariability of recently generated hybrids during future industrial utilization,
480 the genetic stabilization is a last crucial aspect to be considered in every hybridization
481 program (Pérez-Través et al., 2012; Pérez-Través et al., 2014). After generating the
482 hybrid, 30-50 generations were enough to obtain genetically stable interspecific *S.*
483 *cerevisiae*-*S. kudriavzevii* and *S. cerevisiae* intraspecific hybrids, respectively (Pérez-
484 Través et al., 2012). Indeed, Pérez-Través et al. (2012) presented evidences of the
485 existence of extensive genetic rearrangements among genetically similar genomes
486 during hybrid genetic stabilization.

487

488

489 **CONCLUSIONS**

490 Yeasts starters are essential for the winemaking industry in order to obtain high quality
491 standards. *S. cerevisiae* strains has been classically used but wineries require new
492 strains with different physiological characteristics to face new consumer demands and
493 other problems as the ones derived from climate change. Several studies have shown
494 that wine *Saccharomyces* non-*cerevisiae* as *S. kudriavzevii* and *S. uvarum* are suitable
495 for winemaking due to their cryophilic character, based on increased glycerol
496 production, adapted membrane composition and enhanced translation efficiency, and
497 the ability to produce valuable aromatic compounds. These species have great potential
498 to solve some of these problems either as a part of natural or artificial *S. cerevisiae* x *S.*
499 *kudriavzevii* hybrids, in co-cultures or directly as a new starter.

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REFERENCES

1. Aguilera, J., Randez-Gil, F., and Prieto, J. A. (2007). Cold response in *Saccharomyces cerevisiae*: new functions for old mechanisms. FEMS Microbiol. Rev. **31**:327-341.
2. Ale, C. E., Bru, E., Strasser de Saad, A. M., and Pasteris, S.E. (2014). Effect of physicochemical factors on glycerol production by simultaneous cultures of wine micro-organisms using the response surface method. J. Appl. Microbiol. **117**:1336-47.
3. Ansell, R., Granath, K., Hohmann, S., Thevelein, J. M., and Adler, L. (1997). The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J. **16**:2179-2187.
4. Arroyo-López, F. N., Querol, A., and Barrio, E. (2009). Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae, S. kudriavzevii* and their interspecific hybrid. Int. J. Food Microbiol. **131**:120-7.
5. Arroyo-López, F. N., Pérez-Torrado, R., Querol, A., and Barrio, E. (2010). Modulation of the glycerol and ethanol syntheses in the yeast *Saccharomyces*

kudriavzevii differs from that exhibited by *Saccharomyces cerevisiae* and their hybrid. Food Microbiol. **27**:628-37.

6. Arroyo-López, F.N., Pérez-Través, L., Querol, A., and Barrio, E. (2011). Exclusion of *Saccharomyces kudriavzevii* from a wine model system mediated by *Saccharomyces cerevisiae*. Yeast. **28**:423-35.

7. Barrio, E. González, S.S., Arias, A., Belloch, C., and Querol, A. (2006). *Molecular mechanisms involved in the adaptive evolution of industrial yeasts*. In: Querol, A., Fleet, G.H. eds, *Yeasts in Food and Beverages*, Ed 1st Vol 2. Springer-Verlag, 153-174.

8. Belda, I., Navascués, E., Marquina, D., Santos, A., Calderón F. (2016). Outlining the influence of non- conventional yeasts in wine ageing over lees. Yeast 33: 329–338

9. Belloch, C. Orlic, S., Barrio, E., and Querol, A. (2008). Fermentative stress adaptation of hybrids within the *Saccharomyces* sensu stricto complex. Int. J. Food Microbiol. **122**:188–195.

10. Bellon, J. R., Eglinton, J. M., Siebert, T., Pollnitz, A. P., Rose, L., De Barros Lopes, M., and Chambers, P. J. (2011). Newly generated interspecific wine yeast hybrids introduce flavour and aroma diversity to wines. Appl. Microbiol. Biotechnol. **91**:603–612.

11. Beltran, G., Novo, M., Leberre, V., Sokol, S., Labourdette, D., Guillamón, J. M., Mas, A., François, J., and Rozès, N. (2006). Integration of transcriptomic and metabolic analyses for understanding the global responses of low-temperature winemaking fermentations. FEMS Yeast Res. **6**:1167–118.

12. Bisson, L. F. (1999). Stuck and Sluggish Fermentations. Am. J. Enol. Vitic. **50**:107–119.

549 13. Bizaj, E. Cordente, A. G., Bellon, J. R., Raspor, P., Curtin, C. D., and Pretorius,
550 I. S. (2012). A breeding strategy to harness flavor diversity of Saccharomyces
551 interspecific hybrids and minimize hydrogen sulfide production. FEMS Yeast
552 Res. **12**:456-65.
553 14. Borneman, A. R., Schmidt, S. A., and Pretorius, I. S. (2013), At the cutting-edge
554 of grape and wine biotechnology. Trends Genet. **29**:263-71.
555 15. Boulton, R. B., Singleton, V. L., and Bisson, L. F. (1996). Principle and
556 Practices of Winemaking. Chapman & Hall.

557 16. Bradbury, J.E., Richards, K. D., Niederer, H. A., Lee, S. A., Rod Dunbar, P., and
558 Gardner, R. C. (2006). A homozygous diploid subset of commercial wine yeast
559 strains Antonie Van Leeuwenhoek. **89**:27-37.
560 17. Burton, J., and Kerr, J. (2011) *The Cistercians in the Middle Ages*. The Boydell
561 Press.
562 18. Canonico, L., Comitini, F., Oro, L., and Ciani, M. (2016). Sequential
563 fermentation with selected immobilized non-*Saccharomyces* yeast for reduction
564 of ethanol content in wine. Front. Microbiol. **7**:278.

565 19. Cebollero, E. D., González-Ramos, L., Tabera, R., and González (2007).
566 González Transgenic wine yeast technology comes of age: is it time for
567 transgenic wine? Biotechnol. Lett. **29**:191–200.
568 20. Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G., Romano, P.
569 (2016). Yeast Interactions in Inoculated Wine Fermentation. Front Microbiol.
570 22;7:555.
571 21. Ciani, M., Morales, P., Comitini, F., Tronchoni, J., Canonico, L., Curiel, JA.,
572 Oro, L., Rodrigues, AJ., Gonzalez, R. (2016). Non-conventional Yeast Species
573 for Lowering Ethanol Content of Wines. Front Microbiol. 4;7:642.

574 22. Combina, M., Pérez-Torrado, R., Tronchoni, J., Belloch, C., and Querol, A.
575 (2012). Genome-wide gene expression of a natural hybrid between
576 *Saccharomyces cerevisiae* and *S. kudriavzevii* under enological conditions. Int.
577 J. Food Microbiol. **157**:340-5.
578 23. Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I.
579 and Ciani, M. (2011). Selected non-*Saccharomyces* wine yeasts in controlled
580 multistarter fermentations with Saccharomyces cerevisiae. Food Microbiol. 2011
581 **28(5):**873-82.
582 24. Contreras, A. C., Hidalgo, P. A., Henschke, P. J., Chambers, C., Curtin, C., and
583 Varela, C. (2015). Evaluation of non-Saccharomyces yeasts for the reduction of
584 alcohol content in wine. Appl. Environ. Microbiol. **80**:1670–1678.
585 25. Cossins, A. R., and Prosser, C. L. (1978). Evolutionary adaptation of membranes
586 to temperature. Proc. Natl. Acad. Sci. U S A. **75**:2040–2043.
587 26. Cus, F., and Jenko, M. (2013). The Influence of Yeast Strains on the Composition and
588 Sensory Quality of Gewürztraminer Wine. Food Technol. Biotechnol. **51**:547–553.
589 27. da Silva, T., Albertin, W., Dillmann, C., Bely, M., la Guerche, S., Giraud, C.,
590 Huet, S., Sicard, D., Masneuf-Pomarede, I., de Vienne, D., Marullo, P. (2015).
591 Hybridization within *Saccharomyces* Genus Results in Homoeostasis and
592 Phenotypic Novelty in Winemaking Conditions PLoS One. 6;10(5): 1-24
593 28. de Barros Lopes, M., Bellon, J. R., Shirly, N. J., and Ganter, P. F. (2002).
594 Evidence for multiple interspecific hybridization in *Saccharomyces* sensu stricto
595 species. FEMS Yeast Res. **1**:323-331.
596 29. Dickinson, J. R., Lanterman, M. M., Danner, D. J., Pearson, B. M., Sanz, P.,
597 Harrison, S. J., and Hewlins, M. J. (1997). 13C nuclear magnetic resonance

598 investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces*
599 *cerevisiae*. J. Biol. Chem. **272**:26871-8.
600 30. Dickinson, J. R., Eshantha, L., Salgado, J., and Hewlins, M. J. E. (2003). The
601 catabolism of amino acids to long chain and complex alcohols in *Saccharomyces*
602 *cerevisiae*. J. Biol. Chem. **278**:8028-34.
603 31. Eden, A., Van Nedervelde, L., Drukker, M., Benvenisty, N., and Debourg, A.
604 (2001). Involvement of branched-chain amino acid aminotransferases in the
605 production of fusel alcohols during fermentation in yeast. Appl. Microbiol.
606 Biotechnol. **55**:296-300.
607 32. Erny, C., Raoult. P., Alais, A., Butterlin, G., Delobel, P., Matei-Radoi, F.,
608 Casaregola, S., and Legras, J. L. (2012). Ecological success of a group of
609 *Saccharomyces cerevisiae/Saccharomyces kudriavzevii* hybrids in the Northern
610 European wine making environment. Appl. Environ. Microbiol. **78**:3256-65.
611 33. Eustace, R., and Thornton R. J. (1987). Selective hybridization of wine yeasts
612 for higher yields of glycerol. Can. J. Microbiol. **33**:112-117.
613 34. Fortner, D. M., Troy, R. G., and Brow, D. A. (1994). A stem/loop in U6 RNA
614 defines a conformational switch required for pre-mRNA splicing. Genes Dev.
615 **8**:221–233.
616 35. Gamero, A., Hernández-Orte, P., Querol, A., and Ferreira, V. (2011a). Effect of
617 aromatic precursor addition to wine fermentations carried out with different
618 *Saccharomyces species* and their hybrids. Int. J. Food Microbiol. **147**:33-44.
619 36. Gamero, A., Manzanares. P., Querol, A., and Belloch, C. (2011b). Monoterpene
620 alcohols release and bioconversion by *Saccharomyces* species and hybrids. Int.
621 J. Food Microbiol. **145**:92-7.
622 37. Gamero, A., Tronchoni, J., Querol, A., and Belloch, C. (2013a). Production of

623 aroma compounds by cryotolerant *Saccharomyces* species and hybrids at low
624 and moderate fermentation temperatures. J. Appl. Microbiol. **114**:1405-14.
625 38. Gamero, A., Wesselink, W., and de Jong, C. (2013b). Comparison of the
626 sensitivity of different aroma extraction techniques in combination with gas
627 chromatography-mass spectrometry to detect minor aroma compounds in wine.
628 J. Chromatogr. A. **1272**:1–7.
629 39. Gamero, A., Belloch C., Ibáñez C., and Querol A. (2014). Molecular analysis of
630 the genes involved in aroma synthesis in the species *S. cerevisiae, S.*
631 *kudriavzevii* and *S. bayanus var. uvarum* in winemaking conditions. PLoS One.
632 **9**:e97626.

633 40. Gamero, A., Belloch, C., and Querol, A. (2015). Genomic and transcriptomic
634 analysis of aroma synthesis in two hybrids between *Saccharomyces cerevisiae*
635 and *S. kudriavzevii* in winemaking conditions. Microb. Cell Fact. **14**:1-10.
636 41. Gangl, H., Batusic, M., Tscheik, G., Tiefenbrunner, W., Hack, C., and Lopandic,
637 K. (2009). Exceptional fermentation characteristics of natural hybrids from
638 *Saccharomyces cerevisiae* and *S. kudriavzevii*. N. Biotechnol. **25**:244-51.
639 42. García-Ríos, E., Querol A, Guillamón, JM. (2016). iTRAQ-based proteome
640 profiling of Saccharomyces cerevisiae and cryotolerant species *Saccharomyces*
641 *uvarum* and *Saccharomyces kudriavzevii* during low-temperature wine
642 fermentation. J. Proteomics. **146**, 70–79
643 43. Giudici, P., Solieri, A., Pulvirenti, M., and Cassanelli S. (2005). Strategies and
644 perspectives for genetic improvement of wine yeasts. Appl. Microbiol.
645 Biotechnol. **66**:622–628.
646 44. González, S. S., Barrio, E., Gafner, J., and Querol, A. (2006). Natural hybrids
647 from *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces*

kudriavzevii in wine fermentations. FEMS Yeast Res. **6**:1221-34.

45. González, S. S., Barrio, E., and Querol A (2007). Enological characterization of natural hybrids from *Saccharomyces cerevisiae* and *S. kudriavzevii*. Int. J. Food Microbiol. **116**:11-8.
46. Greig, D., Louis, E. J., Borts, R. H., and Travisano, M. (2002). Hybrid speciation in experimental populations of yeast. Science. **298**:1773-1775.
47. Hayashi, M., and Maeda, T. (2006). Activation of the HOG pathway upon cold stress in *Saccharomyces cerevisiae*. J. Biochem. **139**:797-803.
48. Henschke, P.A., McWilliam, S., Fogarty, M., Francis, L., Kwiatkowsli, M., Høj, P., and Henschke, P. (2000). The effect of Saccharomyces bayanus var. uvarum-mediated fermentation on the chemical composition and aroma profile of Chardonnay wine. Aust. J. Grape Wine Res. **6**:190–196.
49. Henschke, P.A., and Rose A. H. (1991). *Plasma membrane* In: Rose, A.H. Harrison, J.S. (Eds.) *The yeasts, Yeast Organelles, IV*, Academic Press Limited, 297–345.
50. Hilliker, A. K., Mefford, M. A., and Staley, J. P. (2007). U2 toggles iteratively between the stem IIa and stem IIc conformations to promote pre-mRNA splicing. Genes Dev. **21**:821–834.
51. Hubmann, G., Guillouet, S., and Nevoigt, E. (2011). Gpd1 and Gpd2 fine-tuning for sustainable reduction of glycerol formation in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **77**: 5857-67.
52. Ishikawa, T., and Noble, A. C. (1995). Temporal perception of astringency and sweetness in red wine. Food Qual. Pref. **6**:27–33.
53. Izawa, S., Sato, M., Yokoigawa, K., and Inoue, Y. (2004). Intracellular glycerol influences resistance to freeze stress in *Saccharomyces cerevisiae*: analysis of a

673 quadruple mutant in glycerol dehydrogenase genes and glycerol-enriched cells.
674 Appl. Microbiol. Biotechnol. **66**:108-114.

675 54. Jones, G. V., White, M. A., Cooper, O. R., and Storchmann, K. (2005). Climate
676 change and global wine quality. Climate Change. **73**:319-343.

677 55. Kurata, S., Shen, B., Liu, J. O., Takeuchi, N., Kaji, A., and Kaji, H. (2013).
678 Possible steps of complete disassembly of post-termination complex by yeast
679 eEF3 deduced from inhibition by translocation inhibitors. Nucleic Acids Res.
680 **41**:264–276.

681 56. Lencioni, L., Romani, C., Gobbi, M., Comitini, F., Ciani, M., Domizio, P.
682 (2016). Controlled mixed fermentation at winery scale using *Zygotorulaspora*
683 *florentina* and Saccharomyces cerevisiae. Int J Food Microbiol. 2016 **3;234**:36-
684 44.

685 57. Le Jeune, C., Lollier, M., Demuyter, C., Erny, C., Legras, J. L., Aigle, M., and
686 Masneuf-Pomarède, I. (2007). Characterization of natural hybrids of
687 Saccharomyces cerevisiae and *Saccharomyces bayanus* var. *uvarum*. FEMS
688 Yeast Res. **7**:540–549

689 58. Li, Z., and Brow, D.A. (1996). A spontaneous duplication in U6 spliceosomal
690 RNA uncouples the early and late functions of the ACAGA element in vivo.
691 RNA. **2**:879–894.

692 59. Lilly, M., Lambrechts, M. G., and Pretorius I. S. (2000). Effect of increased
693 yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates.
694 Appl. Environ. Microbiol. **66**:744-53.

695 60. Llaudy M. del C., Canals, R., González-Manzano, S., Canals, J. M., Santos-
696 Buelga, C., and Zamora, F. (2006). Influence of micro-oxygenation treatment

697 before oak aging on phenolic compounds composition, astringency, and color of
698 red wine. Agric. Food Chem. **54**:4246-52.
699 61. Lopandic, K., Gangl, H., Wallner, E., Tscheik, G., Leitner, G., Querol, A.,
700 Borth, N., Breitenbach, M., Prillinger, H., and Tiefenbrunner, W. (2007).
701 Genetically different wine yeasts isolated from Austrian vine-growing regions
702 influence wine aroma differently and contain putative hybrids between
703 *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii*. FEMS Yeast Res.
704 **7**:953-65.
705 62. Lopandic, K., Pfliegler, WP., Tiefenbrunner, W., Gangl, H., Sipiczki, M.,
706 Sterflinger, K. (2016). Genotypic and phenotypic evolution of yeast interspecies
707 hybrids during high-sugar fermentation. Appl Microbiol Biotechnol.
708 100(14):6331-43.
709 63. López-Malo, M., Querol, A., and Guillamon, J. M. (2013). Metabolomic
710 comparison of *Saccharomyces cerevisiae* and the cryotolerant species *S.*
711 *bayanus var. uvarum* and *S. kudriavzevii* during wine fermentation at low
712 temperature. PLoS One. **8**:e60135.
713 64. Masneuf, I., Hansen, J., Groth, C., Piskur, J., and Dubourdieu, D. (1998). New
714 hybrids between Saccharomyces sensu stricto yeast species found among wine
715 and cider production strains. Appl. Environ. Microbiol. **64**:3887-3892.
716 65. Masneuf-Pomarede, I., Bely, M., Marullo, P., Lonvaud-Funel, A., and
717 Dubourdieu, D. (2010). Reassessment of phenotypic traits for *Saccharomyces*
718 *bayanus* var *uvarum* wine yeast strains. Int. J. Food Microbiol. **139**:79-86.
719 66. Milanovic, V., Maurizio, C., Oro, L., and Comitini F. (2012). Starmerella
720 bombicola influences the metabolism of Saccharomyces cerevisiae at pyruvate

721 decarboxylase and alcohol dehydrogenase level during mixed wine
722 fermentation. Microb. Cell Fact. 11:1-18
723 67. Morales, P., Rojas, V., Quirós, M., and Gonzalez, R. (2015). The impact of
724 oxygen on the final alcohol content of wine fermented by a mixed starter culture.
725 Appl. Microbiol. Biotechnol. **99**:3993-4003.

726 68. Naumov, G., James, S. A., Naumova, E. S., Louis, E. J., and Roberts, I. N.
727 (2000). Association of Saccharomyces bayanus var. uvarum with some French
728 wines: genetic analysis of yeast populations. Res. Microbiol. **151**:683–691.
729 69. Naumov, G.I., Naumova, E. S., Aigle, M., Masneuf, I., and Belarbi, A. (2001).
730 Genetic reidentification of the pectinolytic yeast strain SCPP as *Saccharomyces*
731 *bayanus var. uvarum*. Appl. Microbiol. Biotechnol. **55**:108-11.
732 70. Norbeck, J., and Blomberg, A. (1997). Metabolic and regulatory changes
733 associated with growth of *Saccharomyces cerevisiae* in 1.4 M NaCl. Evidence
734 for osmotic induction of glycerol dissimilation via the dihydroxyacetone
735 pathway. J. Biol. Chem. **272**:5544-5554.

736 71. Oliveira, B. M., Barrio, E., Querol, A., and Pérez-Torrado, R. (2014). Enhanced
737 enzymatic activity of glycerol-3-phosphate dehydrogenase from the cryophilic
738 *Saccharomyces kudriavzevii*. PLoS One. **9**:e87290.
739 72. Paget, C. M., Schwartz, J. M., and Delneri, D. (2014). Environmental systems
740 biology of cold-tolerant phenotype in *Saccharomyces* species adapted to grow at
741 different temperatures. Mol Ecol. **23**:5241-57.
742 73. Pahlman, A. K. Granath, K., Ansell, R., Hohmann, S., and Adler, L. (2001). The
743 yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol
744 biosynthesis and differentially involved in the cellular responses to osmotic,
745 anaerobic, and oxidative stress. J. Biol. Chem. **276**:3555-3563.

746 74. Panadero, J., Pallotti, C., Rodriguez-Vargas, S., Randez-Gil, F., and Prieto, J. A.
747 (2006). A downshift in temperature activates the high osmolarity glycerol HOG
748 pathway, which determines freeze tolerance in *Saccharomyces cerevisiae*. J.
749 Biol. Chem. **281**:4638-4645.

750 75. Pérez-Través, L. Lopes, C. A., Barrio, E., and Querol, A. (2012). Evaluation of
751 different genetic procedures for the generation of artificial hybrids in
752 *Saccharomyces* genus for winemaking. Int. J. Food Microbiol. **156**:102-11.
753 76. Pérez-Través, L. Lopes, C. A., Barrio, E., and Querol A. (2014). Stabilization
754 process in *Saccharomyces* intra and interspecific hybrids in fermentative
755 conditions. Int. Microbiol.**17**:213-24.
756 77. Peris, D., Lopes, C. A., Belloch, C., Querol A., and Barrio E. (2012a).
757 Comparative genomics among *Saccharomyces cerevisiae* x *Saccharomyces*
758 *kudriavzevii* natural hybrid strains isolated from wine and beer reveals different
759 origins. BMC Genomics. **13**:407.
760 78. Peris, D. Lopes, C. A., Arias, A., and Barrio, E. (2012b). Reconstruction of the
761 evolutionary history of *Saccharomyces cerevisiae x S. kudriavzevii* hybrids
762 based on multilocus sequence analysis. PLoS One. **7**, e45527.
763 79. Perriman, R. J., and Ares, M. (2007). Rearrangement of competing U2 RNA
764 helices within the spliceosome promotes multiple steps in splicing. Genes Dev.
765 **21**:811–820.
766 80. Pretorius, I. S., and Hoj, P. B. (2005). Grape and wine biotechnology:
767 challenges, opportunities and potential benefits. Aust. J. Grape Wine Res. Res.
768 **11**:83–108.
769 81. Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel
770 approaches to the ancient art of winemaking. Yeast **16**:675-729.

771 82. Querol, A., Fernández-Espinar, M. T., del Olmo, M., and Barrio, E. (2003).
772 Adaptive evolution of wine yeast. Int. J. Food Microbiol. **86**:3-10.
773 83. Redón, M., Guillamón, J. M., Mas, A., and Rozès, N. (2011). Effect of growth
774 temperature on yeast lipid composition and alcoholic fermentation at low
775 temperature. Eur. Food Res. Technol. **232**:517-527.
776 84. Remize, F., Barnavon, L., and Dequin, S. (2001). Glycerol export and glycerol-
777 3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for
778 glycerol production in *Saccharomyces cerevisiae*. Metab. Eng. **3**:301-312.
779 85. Ribereau-Gayon, J., Peynaud, E., Ribereau-Gayon, P., and Sudraud P. (1977).
780 In. Traité D'oenologie. Sciences et Techniques Du Vin, pp. 353-361. Paris:
781 Dunod.
782 86. Saerens, S. M. G., Delvaux, F., Verstrepen, K. J., Van Dijck, P., Thevelein, J.
783 M., and Delvaux, F. R. (2008). Parameters affecting ethyl ester production by
784 *Saccharomyces cerevisiae* during fermentation. Appl. Environ. Microbiol.
785 **74**:454-61.
786 87. Sakamoto, T., and Murata, N. (2002). Regulation of the desaturation of fatty
787 acids and its role in tolerance to cold and salt stress. Curr. Opin. Microbiol.
788 **5**:208-10.
789 88. Salvadó, Z., Arroyo-López, F. N., Guillamón, J. M., Salazar, G., Querol, A., and
790 Barrio, E. (2011). Temperature adaptation markedly determines evolution within
791 the genus *Saccharomyces*. Appl. Environ Microbiol. **77**:2292-302.
792 89. Schilter, B., and Constable A. (2002). Regulatory control of genetically
793 modified (GM) foods: likely developments Toxicol. Lett. **127**:341–349.
794 90. Schuller, D., and Casal, M. (2005). The use of genetically modified
795 *Saccharomyces cerevisiae* strains in the wine industry. Appl. Microbiol.

796 Biotechnol. **68**:292-304.

797 91. Solieri, L., Verspohl, A., Bonciani, T., Caggia, C., and Giudici, P. (2015). Fast
798 method for identifying inter- and intra-species Saccharomyces hybrids in
799 extensive genetic improvement programs based on yeast breeding. J. Appl.
800 Microbiol. **119**:149-61.

801 92. Staley, J. P., and Guthrie, C. (1999). An RNA switch at the 5′ splice site requires
802 ATP and the DEAD box protein Prp28p. Mol. Cell. **3**:55–64.

803 93. Stribny, J., Gamero, A., Pérez-Torrado, R., and Querol, A. (2015).
804 *Saccharomyces kudriavzevii* and *Saccharomyces uvarum* differ from
805 *Saccharomyces cerevisiae* during the production of aroma-active higher alcohols
806 and acetate esters using their amino acidic precursors. Int. J. Food Microbiol.
807 **205**:41-6.

808 94. Stribny, J., Querol, A., and Pérez-Torrado, R. 2016a. Differences in Enzymatic
809 Properties of the *Saccharomyces kudriavzevii* and *Saccharomyces uvarum*
810 Alcohol Acetyltransferases and Their Impact on Aroma-Active Compounds
811 Production. Front. Microbiol. **7**:897.

812 95. Stribny, J., Romagnoli, G., Pérez-Torrado, R., Daran, J. M., and Querol, A.
813 2016b. Characterisation of the broad substrate specificity 2-keto acid
814 decarboxylase Aro10p of *Saccharomyces kudriavzevii* and its implication in
815 aroma development. Microb. Cell Fact. **15**:51.

816 96. Swiegers, J. H., and Pretorius, I. S. (2005). Yeast modulation of wine flavor.
817 Adv. Appl. Microbiol. **57**:131-75.

818 97. This, P., Lacombe, T., and Thomas, M. R. (2006). Historical origins and genetic
819 diversity of wine grapes. Trends Genet. **22**:511–519.

820 98. Torija, M.J., Beltran, G., Novo, M., Poblet, M., Guillamón, J. M., Mas, A., and
821 Rozès, N. (2003). Effects of fermentation temperature and *Saccharomyces*
822 species on the cell fatty acid composition and presence of volatile compounds in
823 wine. Int. J. Food Microbiol. **85**:127-36.
824 99. Tosi, E., Azzolini, M., Guzzo, F., and Zapparoli, G. (2009). Evidence of
825 different fermentation behaviours of two indigenous strains of *Saccharomyces*
826 *cerevisiae* and *Saccharomyces uvarum* isolated from Amarone wine. J. Appl.
827 Microbiol. **107**:210-8.
828 100. Tronchoni, J., Arroyo-Lopez, F. N., Gamero, A., Querol, A., and Barrio,
829 E. (2009). Differences in the glucose and fructose consumption profiles in
830 diverse *Saccharomyces* wine species and their hybrids during grape juice
831 fermentation. Int. J. Food Microbiol. **134**:237-243.
832 101. Tronchoni, J., Rozès, N., Querol, A., and Guillamón, J. M. (2012). Lipid
833 composition of wine strains of *Saccharomyces kudriavzevii* and *Saccharomyces*
834 *cerevisiae* grown at low temperature. Int. J. Food Microbiol. **155**:191–198.
835 102. Tronchoni, J., Medina, V., Guillamón, J. M., Querol, A., and Pérez-
836 Torrado, R. (2014). Transcriptomics of cryophilic *Saccharomyces kudriavzevii*
837 reveals the key role of gene translation efficiency in cold stress adaptations.
838 BMC Genomics. **15**:432.
839 103. Tulha, J., Lima, A., Lucas, C., and Ferreira, C. (2010). *Saccharomyces*
840 *cerevisiae* glycerol/H+ symporter Stl1p is essential for cold/near-freeze and
841 freeze stress adaptation. A simple recipe with high biotechnological potential is
842 given. Microb. Cell Fact. **9**:82.

104. Varela, C. (2016). The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. Appl Microbiol Biotechnol. 2016 **100(23):**9861-9874.

105. Wang, C., Mas, A., Esteve-Zarzoso, B. (2016). The Interaction between *Saccharomyces cerevisiae* and Non-*Saccharomyces* Yeast during Alcoholic Fermentation Is Species and Strain Specific. Front Microbiol.

106. White, M. A., Diffenbaugh, N. S., Jones, G. V., Pal, J. S., and Giorgi F. (2006). Extreme heat reduces and shifts United States premium wine production in the 21st century. Proc. Natl. Acad. Sci. U S A. **103**:11217-22.

107. Zavanelli, M., Britton, J., Igel, A., and Ares, M. Jr. (1994). Mutations in an essential U2 small nuclear RNA structure cause cold-sensitive U2 small nuclear ribonucleoprotein function by favoring competing alternative U2 RNA structures. Mol. Cell Biol. **14**:1689.

857 **Figure legends**

858 Figure1. Summary of new challenges for the winemaking industry and the potential
859 benefits of using *S. kudriavzevii* and *S. uvarum* as a starter. The experience acquired
860 from the S. kudriavzevii and S. uvarum wine performances suggest relevant benefits
861 that can potentially solve most of these new problems. such as glycerol accumulation,
862 changes in membrane composition (higher levels of medium chain fatty acids
863 (MCFA)and squalene (SQ), whereas phosphatidic acid (PA) content and the
864 phosphatidylcholine/phosphatidylethanolamine ratio (PC/PE) lowered) or enhanced
865 translation efficiency (ribosome recycling). They may considerably contribute to the
866 aroma profile for S. cerevisiae by increasing the levels of higher alcohols and esters in
867 the final wine. Genes involved overexpressed in these species are depicted.

868

869 Figure 2. Distribution of the *S. cerevisiae* x *S. kudriavzevii* hybrids. The geographic
870 origin and sources from which the different hybrids were isolated are indicated on a
871 climatic map of Europe. These hybrids are linked according to their group assignations,
872 based on previous phylogenetic analyses (Erny et al., 2012; Peris et al., 2012a; Peris et
873 al., 2012b). Within squares numbers indicate the number of strains described until now
874 (up) and the range of *S. kudriavzevii* genome present in % (down).

875

876

877 **Tables**

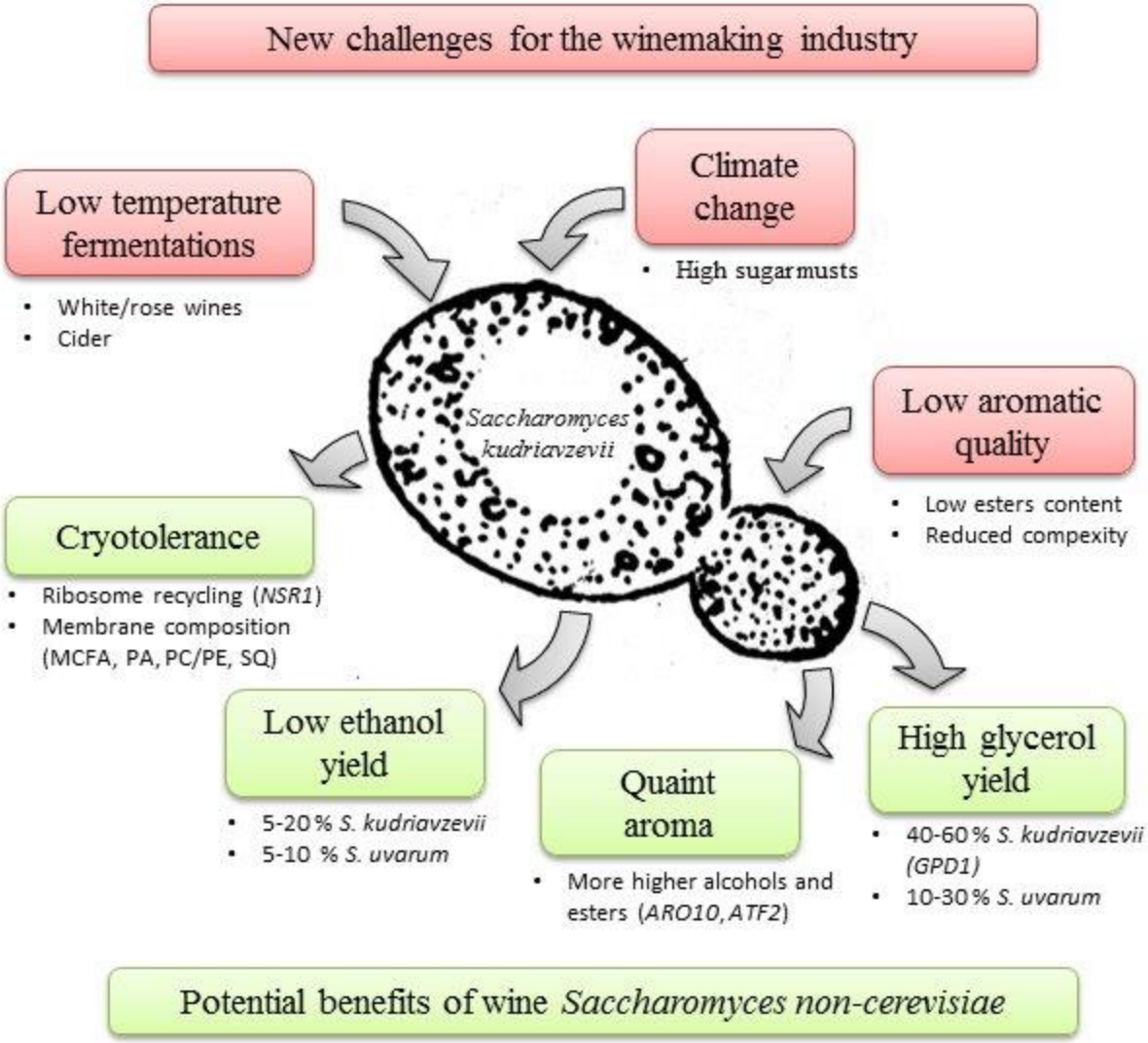
878 Table 1. Genome composition for the natural hybrids between *S. cerevisiae* and *S.*
879 *kudriavzevii* for genes involved in their physiological differences (Peris et al., 2012a).

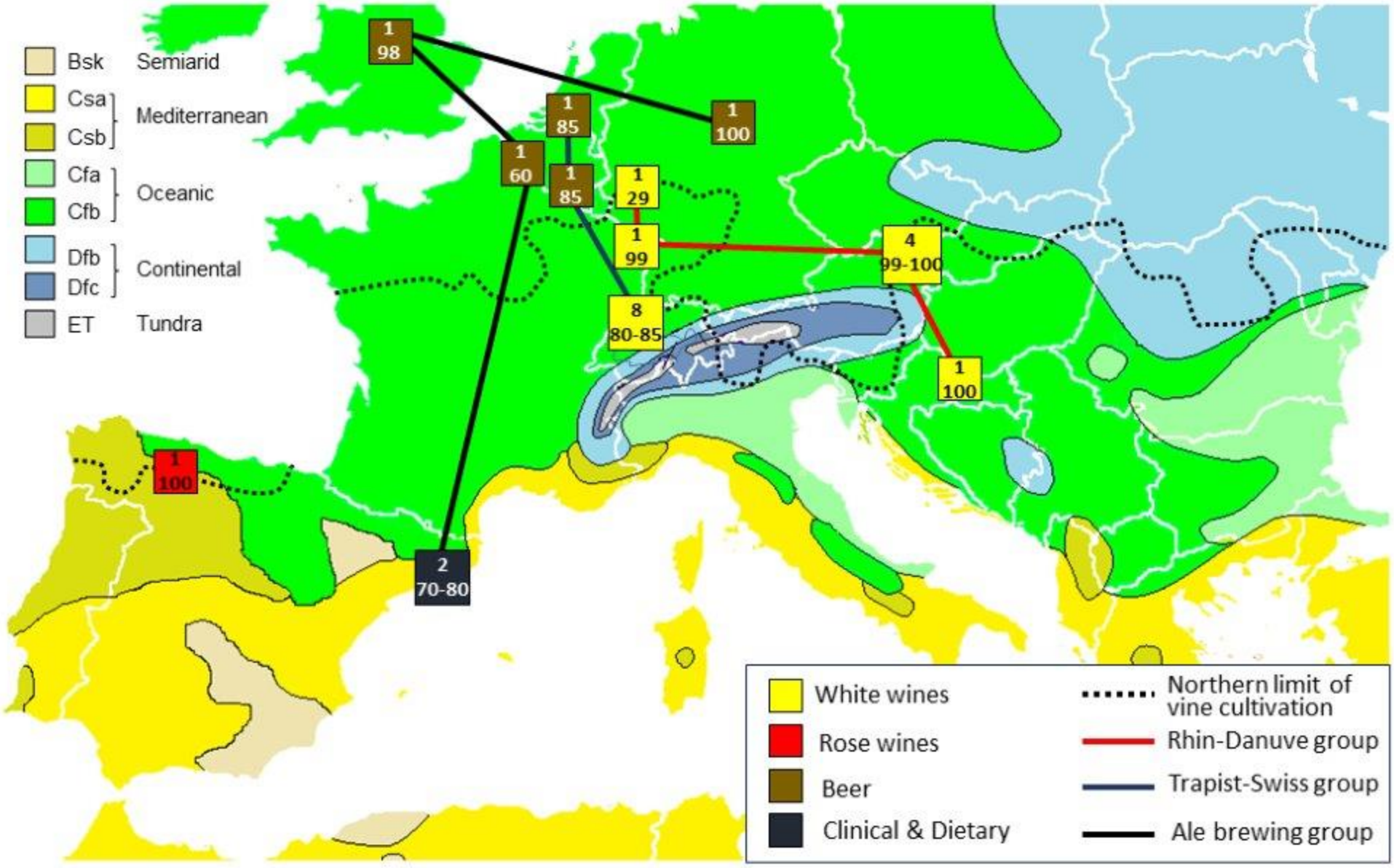
ORF	Gene	AMH	PB7	Vin7	HA*	HA 1841	SOY3	W27	W46	SPG 16-91	441
Cold stress											
YNL112W	*DBP2*	CC	CK	CK	CK	CK	CK	CCK	CCK	CCK	CCK
YJL153C	*INO1*	CC	CK	CK	CK	CK	CK	CK	CK	CK	CK
YJL223C	*PAU1*	CC	CK	CK	CK	CK	CK	CK	CK	CK	CK
YEL049W	*PAU2*	CC	CK	CK	CK	CC	CK	CCK	CCK	CCK	CCK
YCR104W	*PAU3*	CC	CK	CC	CK	CK	CK	CK	CK	CK	CK
YLR461W	*PAU4*	CC	CK	CK	CK	CK	CK	CK	CK	CC	CK
YNR076W	*PAU6*	CC	CK	CK	CK	CK	CK	CC	CCC	CCC	CCC
YAR020C	*PAU7*	C	CK	CK	CK	CK	CK	CK	CK	CK	CC
YBR067C	*TIP1*	CC	CK	CK	CK	CK	CK	CK	CK	CK	CK
YER011W	*TIR1*	CC	CK	CK	CK	CC	CK	CKK	CKK	CKK	CKK
Ergosterol metabolism											

YGR175C	*ERG1*	CC	CK	CC	CCK	CK	CK	CK	CK	CK	CK
YHR007C	*ERG11*	C	CK	CK	CK	CK	CK	CK	CK	CK	CCK
YGR060W	*ERG25*	CK	CK	CK	CCK	CK	CK	CK	CK	CK	CK
YGL001C	*ERG26*	CK	CK	CK	CCK	CK	CK	CK	CK	CK	CK
YLR100W	*ERG27*	CC	CK	CK	CK	CK	CK	CK	CK	CK	CK
YLR056W	*ERG3*	CC	CK	CK	CK	CK	CK	CK	CK	CK	CK
YGL012W	*ERG4*	CK	CK	CK	CCK	CK	CK	CK	CK	CK	CK
YHR072W	*ERG7*	C	CK	CK	CK	CK	CK	CK	CK	CK	CKK
YNR043W	*MVD1*	CC	CK	CK	CK	CK	CK	CKK	CKK	CKK	CKK
YHR042W	*NCP1*	C	CK	CK	CK	CK	CK	CK	CK	CK	CKK
Glycerol metabolism											
YBR196C	*PGI1*	CC	CK	CK	CK	CK	CK	CK	CK	CK	CK
YDR050C	*TPI1*	CC	CK	CK	CK	CK	CC	CC	CC	CC	CC
Osmotic stress											
YGR175C	*ERG1*	CC	CK	CC	CCK	CK	CK	CK	CK	CK	CK
YLR056W	*ERG3*	CC	CK	CK	CK	CK	CK	CK	CK	CK	CK

YDL022W	*GPD1*	CC	CK	CK	CK	CK	CK	CC	CC	CC	CC
YLR362W	*STE11*	CC	CK	CK	CK	CK	CK	CK	CK	CK	CK
Oxidative stress											
YHR183W	*GND1*	C	CK	CK	CK	CK	CK	CK	CK	CK	CCK
YOL151W	*GRE2*	CK	CK	CK	CK	CK	CK	CC	CC	CC	CC
YJL101C	*GSH1*	CC	CK	CK	CK	CK	CK	CK	CK	CK	CK
YGR209C	*TRX2*	CC	CK	CC	CCK	CK	CK	CK	CK	CK	CK
YGR019W	*UGA1*	CK	CK	CK	CCK	CK	CK	CK	CK	CK	CK
Membrane fluidity											
YLR153C	*ACS2*	CC	CK	CK	CK	CK	CK	CK	CK	CK	CK
YER026C	*CHO1*	CC	CK	CK	CK	CC	CK	CKK	CKK	CKK	CKK
YGL055W	*OLE1*	CK	CK	CK	CCK	CK	CK	CK	CK	CK	CK
YNL169C	*PSD1*	CC	CK	CK	CK	CK	CK	CCK	CCK	CCK	CCK

*Include strains HA1835, HA1837, HA1842. Colors indicate absence of *S. kudriavzevii* (K) alleles with one (grey), two (magenta) or three (orange) *S. cerevisiae* (C) gene copies; or two S. kudriavzevii alleles with one copy of *S. cerevisiae* (green); or one *S. kudriavzevii* allele with two copy of *S. cerevisiae* (blue).


New challenges for the winemaking industry
Low temperature fermentations
White/rose wines
Cider
Climate change
High sugar musts
Saccharomyces kudriavzevii
Low aromatic quality
Low esters content
Reduced compexity
Cryotolerance
Ribosome recycling (NSR1)
Membrane composition (MCFA, PA, PC/PE, SQ)
Low ethanol yield
5-20 % S. kudriavzevii
5-10 % S. uvarum
Quaint aroma
More higher alcohols and esters (ARO10, ATF2)
High glycerol yield
40-60 % S. kudriavzevii (GPD1)
10-30 % S. uvarum
Potential benefits of wine Saccharomyces non-cerevisiae


Bsk Semiarid
Csa
Csb
Mediterranean
Cfa
Cfb
Oceanic
Dfb
Dfc
Continental
ET Tundra
1 98
1 60
1 85
1 85
1 100
1 29
1 99
4 99-100
8 80-85
1 100
1 100
2 70-80
White wines
Rose wines
Beer
Clinical & Dietary
Northern limit of vine cultivation
Rhin-Danuve group
Trapist-Swiss group
Ale brewing group