MINIREVIEW

Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking

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Abstract

Mixed fermentations using controlled inoculation of *Saccharomyces cerevisiae* starter cultures and non-*Saccharomyces* yeasts represent a feasible way towards improving the complexity and enhancing the particular and specific characteristics of wines. The profusion of selected starter cultures has allowed the more widespread use of inoculated fermentations, with consequent improvements to the control of the fermentation process, and the use of new biotechnological processes in winemaking. Over the last few years, as a consequence of the re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking, there have been several studies that have evaluated the use of controlled mixed fermentations using *Saccharomyces* and different non-*Saccharomyces* yeast species from the wine environment. The combined use of different species often results in unpredictable compounds and/or different levels of fermentation products being produced, which can affect both the chemical and the aromatic composition of wines. Moreover, possible synergistic interactions between different yeasts might provide a tool for the implementation of new fermentation technologies. Thus, knowledge of the *Saccharomyces* and non-*Saccharomyces* wine yeast interactions during wine fermentation needs to be improved. To reach this goal, further investigations into the genetic and physiological background of such non-*Saccharomyces* wine yeasts are needed, so as to apply '-omics' approaches to mixed culture fermentations.

Introduction

Grape juice fermentation is a complex biochemical process in which wine yeasts play fundamental roles during their transformation of grape sugars into ethanol, carbon dioxide and hundreds of other secondary products. The quality of a wine is conditioned by several factors, including viticultural practices, winemaking techniques and the yeast strains used. At the same time, microorganisms can influence the quality of the grapes before the harvest, during the fermentation and during the ageing and/or conservation of the wine.

Over the last few decades, major advances have occurred in our understanding of the ecology, physiology, biochemistry and molecular biology of the yeasts involved in the fermentation process. Also, studies have been carried out to determine the impact of these yeasts on the composition, sensory properties and final flavours of the wine (Swiegers *et al.*, 2005; Domizio *et al.*, 2007; Renouf *et al.*, 2007; Fleet, 2008). At present, it is known that the yeast ecology of the fermentation process is more complex than previously thought, and that non-*Saccharomyces* yeast species play relevant roles in the metabolic impact and aroma complexity of the final product.

In recent years, there has been a growing demand for new and improved wine-yeast strains that are adapted to different types and styles of wines (Pretorius, 2000). In this context, to improve the chemical composition and sensory properties of wine, the inclusion of non-*Saccharomyces* wine yeasts, together with *Saccharomyces* strains as part of mixed and multistarter fermentations, has been proposed as a tool to take advantage of spontaneous fermentation, while avoiding the risks of stuck fermentations (Bisson & Kunkee, 1993; Heard, 1999; Rojas *et al.*, 2003; Romano *et al.*, 2003a; Ciani *et al.*, 2006; Jolly *et al.*, 2006).

Spontaneous and inoculated fermentations

Grape must is a nonsterile substrate that contains several types of microorganisms, and in particular, there may be growth of various yeasts that can ferment the substrate. As a consequence, natural fermentation is carried out through a sequence of different yeast species. Indeed, a series of microbiological analyses of the yeast flora associated with natural fermentation of grape juice revealed that in most enological areas, there is a sequential use of the substrate: initially, apiculate yeasts (*Hanseniaspora*/*Kloeckera*) are the most abundant, although after 3–4 days, they are replaced by *Saccharomyces cerevisiae* (Martini, 1993; Pretorius, 2000). In addition, during the various stages of fermentation, it is possible to isolate other yeast genera, such as *Candida*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Kluyveromyces* and *Metschnikowia* (Fleet *et al.*, 1984; Heard & Fleet, 1985, 1986; Pardo *et al.*, 1989).

The growth of non-*Saccharomyces* species belonging to the genera *Kloeckera*/*Hanseniaspora* and *Candida* is generally limited to the first few days of fermentation, because of their weak ethanol tolerance. However, quantitative studies on grape juice fermentation have shown that *Kloeckera apiculata* and *Candida stellata* can survive at significant levels (up to 10^6–10^7 CFU mL^{-1}) during fermentation, and for longer periods than thought previously (Fleet *et al.*, 1984; Heard & Fleet, 1985; Pardo *et al.*, 1989).

The presence and permanence of these non-*Saccharomyces* yeasts throughout fermentation is influenced by several physico-chemical and microbiological factors. Gao & Fleet (1988) showed that *K. apiculata* and *C. stellata* have increased tolerance to ethanol at lower temperatures (10–15 °C). This behaviour has also been confirmed in mixed cultures using *K. apiculata* and *S. cerevisiae* (Erten, 2002). Such increases in the ethanol tolerance of non-*Saccharomyces* yeasts at low temperatures appear to be the major factor that affects their stronger contribution to low-temperature fermentations.

More recent studies have highlighted the important role of oxygen concentration in the survival of some non-*Saccharomyces* yeasts during fermentation, such as *Torulaspora delbrueckii* and *Klyveromyces thermotolerans* (Hansen *et al.*, 2001). Moreover, it has been shown that cell–cell interactions are involved in inhibition of these two non-*Saccharomyces* species. Thus, in the presence of high concentrations of viable cells of *S. cerevisiae*, the growth of *T. delbrueckii* and *K. thermotolerans* is inhibited (Nissen & Arneborg, 2003; Nissen *et al.*, 2003).

The production of toxic compounds from *S. cerevisiae* has also been hypothesized to be a cause of the early death of *Hanseniaspora guilliermondii* in mixed fermentations (Pérez-Nevado *et al.*, 2006). Indeed, several compounds produced by yeasts during must fermentation may become inhibitory to other yeast species or strains. In addition to ethanol, acetic acid, medium-chain fatty acids, acetaldehyde and the synergistic action of their combinations could play an important role in the inhibitory mechanism that can occur in wine fermentation (Edwards *et al.*, 1990; Bisson, 1999; Ludovico *et al.*, 2001; Fleet, 2003).

The use of selected starter cultures of *S. cerevisiae* can thus play an important role in the suppression of wild yeasts. Inoculated cultures of *Saccharomyces* are expected to suppress either indigenous non-*Saccharomyces* species and *Saccharomyces* strains or to dominate the fermentation. Moreover, the use of antiseptic agents, such as SO_2, to which most of the non-*Saccharomyces* yeasts are scarcely resistant, should guarantee the dominance of the inoculated strains.

However, several studies carried out over the last 25 years or so have shown that the growth of *K. apiculata* and *C. stellata* is not suppressed in inoculated fermentations with selected cultures of *S. cerevisiae* (Heard & Fleet, 1985; Martinez *et al.*, 1989; Mora *et al.*, 1990), while other studies have revealed their quantitatively significant presence even during the various stages of fermentation of inoculated *S. cerevisiae* strains (Bouix *et al.*, 1981; Martinez *et al.*, 1989; Ciani & Rosini, 1993; Mannazzu *et al.*, 2007). The dominance of inoculated strains is thus not always assured, and will depend on the specific conditions of the vinification, such as: (1) the amount and viability of the inoculum, and its correct use; (2) the physiological and metabolic characteristic of the selected yeast culture(s) of the inoculum; and (3) the technology used in the winemaking (e.g. clarification procedures, temperature of fermentation and SO_2 addition) (Amerine & Cruess, 1960; Benda, 1982; Reed & Nagodawithana, 1988; Ciani & Rosini, 1993).

With the commercial availability of active dry cultures of *S. cerevisiae*, the inoculation of grape must has become attractive and convenient (Kraus *et al.*, 1983; Barre & Vezinhet, 1984). As such, at present, the use of selected yeast cultures is widespread in both the newer wine-producing countries, such as the United States, South Africa and Australia, and in the more traditional wine-producing countries, such as Italy, Germany and France (Reed & Nagodawithana, 1988; Fleet & Heard, 1993). In this context, extensive use of starter cultures in all winemaking areas around the world represents an important advance in wine biotechnology. Nevertheless, the generalized use of selected starter cultures is a simplification of microbial fermentation communities that promotes the standardization of the analytical and sensory properties of wines.

The role of non-*Saccharomyces* yeasts in must fermentation

As a nonsterile environment, grape must contains several microorganisms that can grow and convert the initial sugar

content into ethanol, CO_2 and other byproducts, although it is well known that the most important agent for alcoholic fermentation is *S. cerevisiae*. Earlier studies considered non-*Saccharomyces* yeasts as 'wild' yeasts or 'spoilage' yeasts (Castelli, 1954; Amerine & Cruess, 1960; Ribèreau-Gayon & Peynaud, 1960), because they were often isolated from stuck or sluggish fermentations, or from wines with anomalous analytical and sensorial profiles.

Pure culture fermentations with non-*Saccharomyces* wine yeasts have shown several negative metabolite and fermentation characteristic that generally exclude their use as starter cultures. The most important spoilage metabolites produced by non-*Saccharomyces* wine yeasts are acetic acid, acetaldehyde, acetoin and ethyl acetate, along with off-odours, such as the vinyl and ethyl phenols that are linked to the development of *Brettanomyces/Dekkera* spp. (Chatonnet *et al.*, 1995). Moreover, most of the non-*Saccharomyces* wine-related species show limited fermentation aptitudes, such as low fermentation power (the maximum amount of ethanol in the presence of an excess of sugar) and rate, and a low SO_2 resistance. However, in mixed fermentations such as natural fermentations, some negative enological characteristic of non-*Saccharomyces* yeasts may not be expressed or be modified by *S. cerevisiae* cultures. In this context, following the investigations of the last decades on the quantitative presence and persistence of non-*Saccharomyces* wine yeasts during fermentation, several studies have been carried out to determine their oenological properties and their possible roles in winemaking (Romano *et al.*, 1992, 1997; Ciani & Picciotti, 1995; Lema *et al.*, 1996; Ciani & Maccarelli, 1998; Egli *et al.*, 1998; Henick-Kling *et al.*, 1998; Rojas *et al.*, 2001; Zohre & Erten, 2002; Fleet, 2003; Jolly *et al.*, 2003; Farkas *et al.*, 2005; Hermle *et al.*, 2005; Domizio *et al.*, 2007; Kim *et al.*, 2008; Viana *et al.*, 2008). Experimental evidence has highlighted the positive role of non-*Saccharomyces* yeasts in the analytical composition of wine (Cabrera *et al.*, 1988; Herraiz *et al.*, 1990; Moreno *et al.*, 1991; Lema *et al.*, 1996). Some non-*Saccharomyces* yeast species can improve the fermentation behaviour of yeast starter cultures and the analytical composition of wine, or lead to a more complex aroma. Consequently, during recent years, there has been a re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking (Fleet & Heard, 1993; Ciani, 1997; Esteve-Zarzoso *et al.*, 1998; Heard, 1999; Fleet, 2008) and today more attention is being paid to the ecology of fermenting yeasts, to better understand the impact of non-*Saccharomyces* strains on the chemistry and sensory properties of wine (Pretorius, 2000; Romano *et al.*, 2003a; Swiegers *et al.*, 2005).

In this context, the enzymatic activities of non-*Saccharomyces* wine yeasts can influence the wine profile. Investigations of poly-galacturonase and β-D-xylosidase production by non-*Saccharomyces* yeasts involved in winemaking showed that these activities are widely dispersed in these yeasts and can be used to enhance wine quality (Manzanares *et al.*, 1999; Fernandez *et al.*, 2000; Strauss *et al.*, 2001). Another biocatalytic activity widely associated with non-*Saccharomyces* wine yeasts is β-glucosidase activity. β-Glucosidase hydrolyses terpenyl-glycosides, and can enhance the wine aroma. In contrast to grape glucosidase, β-glucosidase produced by yeast is not inhibited by glucose, and it is involved in the release of terpenols during fermentation. This β-glucosidase activity has been found in several yeast species associated with winemaking, especially among the non-*Saccharomyces* species (Vasserot *et al.*, 1989; Günata *et al.*, 1990; Rosi *et al.*, 1994; Manzanares *et al.*, 1999; Ferreira *et al.*, 2001; Rodriguez *et al.*, 2004; Fia *et al.*, 2005; González-Pombo *et al.*, 2008). The diffusion of this activity among non-*Saccharomyces* wine yeasts has confirmed the role of these yeasts in enhancing wine aroma (Manzanares *et al.*, 1999; Fernandez *et al.*, 2000; Ferreira *et al.*, 2001; Strauss *et al.*, 2001; González-Pombo *et al.*, 2008).

In addition to the enzymatic activities of non-*Saccharomyces* wine yeasts, other specific properties of winemaking interest have been evaluated to improve our knowledge of the metabolic characteristics, and to test the intraspecific variability of these wine yeasts. Non-*Saccharomyces* strains can be selected on the basis of their ability to produce favourable metabolites that contribute to the definition of the final bouquet of a wine. Viana *et al.* (2008) screened 38 yeast strains belonging to the *Candida*, *Hanseniaspora*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* genera for acetate ester formation. Here, they identified *Hanseniaspora osmophila* as a good candidate for mixed cultures, due to its glucophilic nature, the ability to produce acetaldehyde within a range compatible for wine and acetate ester production, in particular of 2-phenylethyl acetate. A rapid method to evaluate wine-yeast performance based on the ability of a yeast species to produce levels of metabolites that contribute towards improving wine quality has been proposed (Romano *et al.*, 2003b). In particular, through determination of 2,3-butanediol and acetoin stereoisomers, these have been demonstrated to be characteristics for the *S. cerevisiae* and *K. apiculata* yeast species (Romano *et al.*, 2003b), confirming that *S. cerevisiae* is a higher producer of 2,3-butanediol in comparison with *K. apiculata*. Moreira *et al.* (2008) investigated the role of *H. guilliermondii* and *Hanseniaspora uvarum* in pure and mixed starter cultures with *S. cerevisiae*, for the production of heavy sulphur compounds and esters. Their results highlight that these apiculate yeasts enhance the production of desirable compounds, such as esters, without increasing the undesirable heavy sulphur compounds.

Multistarter fermentation in winemaking

There has been controversy over the use of spontaneous and inoculated fermentations using selected yeast strains,

particularly with respect to the organoleptic quality of the final wine. Thus, on the basis of sensory wine testing, some authors have claimed the advantages of either spontaneous or inoculated fermentations. In the case of spontaneous fermentation, the impact of the different kinds of yeasts on the wine aroma and flavour may lack consistency, as spontaneous fermentation is an uncontrolled process. On the other hand, the total suppression of indigenous non-*Saccharomyces* species can reduce the aroma complexity of the final wines. Indeed, an inoculum of a selected *S. cerevisiae* strain can not only result in the inhibition of potential spoilage yeasts but also of other yeast species whose presence in the fermentation process in defined amounts and for defined durations can positively contribute to the wine aroma. However, natural multistarter cultures remain an uncontrolled process, and multistarter cultures need to be used under better defined conditions. Similarly, the combined or the sequential use of different yeast starter species in the development of new fermentation technologies needs to be monitored.

Some non-*Saccharomyces* species associated with winemaking have been suggested as starter cultures for a long time, due to their specific metabolic characteristics. It is possible, therefore, to promote the activity of non-*Saccharomyces* yeasts in winemaking by limiting or delaying the use of selected *S. cerevisiae* starter cultures.

The use of a selected multistarter (controlled mixed cultures) was proposed several years ago. In the middle of the last century, to reduce the acetic acid content of wine, Castelli (1955, 1969) encouraged the sequential use of *T. delbrueckii* (formerly known as *Saccharomyces rosei*) and *S. cerevisiae*. Later on, other studies investigated the use of controlled mixed cultures to reduce the volatile acidity and enhance the organoleptic profiles of wines (Moreno *et al.*, 1991). Recently, the impact of mixed and sequential *T. delbrueckii*–*S. cerevisiae* cultures in high sugar fermentation was evaluated to determine whether it can improve the quality of wines and reduce the acetic acid content (Bely *et al.*, 2008). Mixed *T. delbrueckii*–*S. cerevisiae* cultures at a 20 : 1 ratio produced 53% and 60% reductions in the volatile acidity and acetaldehyde, respectively, while sequential cultures showed lower effects on the reduction of these metabolites.

One of the most investigated uses of mixed cultures in winemaking relates to the biological deacidification of must and/or wine. For some time, the use of *Schizosaccharomyces pombe* to reduce malic acid in grape juice and/or wine was suggested (Peinaud & Sudrad, 1962; Rankine, 1966; Munyon & Nagel, 1977). Snow & Gallender (1979) proposed the sequential inoculation of *S. pombe* and *S. cerevisiae* to improve the competition between the yeasts and to reduce or eliminate the negative sensorial characteristics due to *S. pombe*. The deacidification of wines under commercial winemaking conditions using a mutant of *Schizosaccharomyces malidevorans* was also evaluated (Thornton & Rodriguez, 1996). A more controlled biological deacidification process was obtained using *S. cerevisiae* and immobilized *S. pombe* cells (Magyar & Panyik, 1989; Yokotsuka *et al.*, 1993; Ciani, 1995). In this process, *S. cerevisiae* carried out the fermentation using almost all of the sugar available, while the immobilized *S. pombe* cells used malic acid. Under these conditions, the undesirable effects of *S. pombe* on the wine quality were limited or eliminated. Recently, dry immobilized cells of *S. pombe* for malic acid consumption in winemaking have been proposed (Silva *et al.*, 2003), and a commercial yeast strain from *S. pombe* is now available in an immobilized form to reduce the malic acid content in wine (ProMalic®; Proenol, http://www.proenol.pt/files/products/ProMalic_09_2008.pdf). In addition to *S. pombe*, a strain of *Issatchenkia orientalis* can degrade malic acid rapidly (Seo *et al.*, 2007), and has been proposed to reduce the malic acid content in wine as mixed cultures with *S. cerevisiae* (Kim *et al.*, 2008).

Because *K. thermotolerans* shows positive oenological characteristics, such as low production of volatile acidity and high production of fixed acidity [L(+) lactic acid form], Mora *et al.* (1990) explored its use in wine fermentation, to improve the analytical and sensorial characteristics of wines. With the aim of obtaining biological acidification of wine, a mixed culture of *K. thermotolerans* and *S. cerevisiae* was investigated, which provided up to a 70% increase in titratable acidity and consequently a reduction of 0.3 pH units (Kapsopoulou *et al.*, 2005, 2007).

The use of a multistarter fermentation process has also been proposed to simulate natural must fermentation, to confer greater complexity to a wine. Herraiz *et al.* (1990) analysed the influence of pure, mixed and sequential cultures of *K. apiculata*, *T. delbrueckii* and *S. cerevisiae* on the volatile composition of the resulting wines, showing evident differences in the metabolism by *S. cerevisiae* in pure and mixed cultures. The evaluation of the volatile metabolites produced by mixed and sequential cultures of apiculate yeasts and *S. cerevisiae* confirmed these results (Zironi *et al.*, 1993). Multistarter fermentations (mixed and sequential) of *T. delbrueckii* and *K. thermotolerans* together with *S. cerevisiae* were also investigated, to optimize mixed wine fermentations using these non-*Saccharomyces* yeast species (Ciani *et al.*, 2006). In this context, blends of active dried yeasts of *S. cerevisiae*/*K. thermotolerans*/*T. delbrueckii* denominated Vinflora® Harmony.nsac (Christian Hansen) and single non-*Saccharomyces* (*Zygosaccharomyces bailii*) have become commercially available.

Recently, several studies have investigated multistarter fermentations using apiculate yeasts and *S. cerevisiae*. The influence of temperature and SO_2 on the growth and metabolism of a mixed fermentation of *K. apiculata* and *S. cerevisiae* was investigated, showing increased viability in

K. apiculata in mixed fermentations (Mendoza *et al*., 2007). Studies on the influence of *H. uvarum* and *H. guilliermondii* on sulphur compounds, higher alcohols and ester production in mixed fermentations with *S. cerevisiae* reported an enhancement in the production of desirable compounds (Moreira, 2005; Moreira *et al*., 2008). In particular, in mixed fermentation, *H. uvarum* increased the isoamyl acetate content in wine, whereas *H. guilliermondii* resulted in an enhancement of 2-phenylethyl acetate (Moreira *et al*., 2008).

The combined use of *S. cerevisiae* and non-*Saccharomyces* wine yeasts has also been proposed to enhance the glycerol content in wine (Ciani & Ferraro, 1996). In this fermentation process, a strain of *C. stellata*, which was recently reclassified as *Starmerella bombicola* (Sipiczki *et al*., 2005), was used as a biocatalyst in an immobilized form. Grape must fermentation carried out by the combination of immobilized *C. stellata* cells and *S. cerevisiae* has improved the analytical composition of the resulting wine (Ciani & Ferraro, 1998).

These results were also confirmed in a pilot-scale winemaking process (Ferraro *et al*., 2000). Mixed fermentations using a *C. stellata* strain in combination with *S. cerevisiae* were assayed in the production of Chardonnay wines (Soden *et al*., 2000). Coinoculation and sequential inoculation were compared with monoculture of the two yeasts, focusing attention on the sensory analyses. The results here indicated differences among the different wines with increases in some of the positive and negative characteristic shown by multistarter fermentations, in comparison with the monoculture.

Candida cantarellii, another fermenting species of the wine environment, has also been proposed in multistarter fermentations, to enhance glycerol and to develop wines with particular characteristics (Toro & Vazquez, 2002).

Mixed fermentations have also been proposed to enhance specific volatile compounds to improve the wine aroma. Garcia *et al*. (2002) proposed the use of a mixed culture of *Debaryomyces vanriji* and *S. cerevisiae* to increase volatile compounds (particularly geraniol) in Muscat wine. More recently, cofermentation with *S. cerevisiae* and *Pichia kluyveri* was proposed to increase varietal thiol concentrations in Sauvignon Blanc (Anfang *et al*., 2009). This study showed that a 1 : 9 starting ratio of *S. cerevisiae*: *P. kluyveri* enhanced the 3-mercaptohexyl acetate concentrations in Sauvignon Blanc. *Pichia fermentans*, another wine-related yeast, has been proposed for multistarter fermentation with *S. cerevisiae* (Clemente-Jimenez *et al*., 2005); here, the positive influence of the non-*Saccharomyces* yeast on several volatiles and byproducts was shown only in sequential culture, with the inoculation of *S. cerevisiae* after 2 days. In another investigation, some non-*Saccharomyces* yeasts were studied for possible use in the overlees ageing of red wines (Palomero *et al*., 2009).

Therefore, non-*Saccharomyces* wine yeasts have some specific oenological characteristic that are not present in *S. cerevisiae* species and that can have additive effects on the wines. Controlled mixed cultures of *S. cerevisiae* and non-*Saccharomyces* wine yeasts can improve the analytical and aromatic profile of wines through metabolic interactions between the yeast species (Languet *et al*., 2005; Salmon *et al*., 2007). In this context, the use of the immobilization technique in mixed cultures allows the careful control of the multistarter process, and several studies have proposed its use. The fermentation processes relative to the use of mixed cultures are summarized in Table 1.

Yeast interactions in mixed fermentations

Besides paving the way towards the implementation of new strategies for the management of fermentation processes, investigations into multistarter fermentations require the elucidation of both the physiological and metabolic interactions between *S. cerevisiae* and non-*Saccharomyces* wine strains. Indeed, preliminary evidence has shown that when some yeasts develop together under fermentation conditions, they do not passively coexist, but rather they interact and produce unpredictable compounds and/or different levels of fermentation products, which can affect the chemical and aromatic composition of wines (Howell *et al*., 2006; Anfang *et al*., 2009).

Possible synergistic interactions between different yeasts might represent a tool for new fermentation technologies. Mendoza *et al*. (2007) showed that during mixed fermentations, the production of biomass of *Saccharomyces* and non-*Saccharomyces* yeasts is lower than that produced by the two strains in pure cultures. However, the presence of both *Saccharomyces* and non-*Saccharomyces* yeasts promotes an increase in the persistence of non-*Saccharomyces* yeasts during the fermentation process (Ciani *et al*., 2006; Mendoza *et al*., 2007). Indeed, on the basis of these studies and other experimental evidence, interactions between *Saccharomyces* and non-*Saccharomyces* wine yeasts have effects not only on the persistence of the non-*Saccharomyces* yeasts but also on the behaviour of the *S. cerevisiae* wine strains. This can be seen as variations in the degree of flocculation in mixed cultures of *K. apiculata* and *S. cerevisiae*. In mixed fermentation, the flocculent strain of *K. apiculata* interacts with a nonflocculent strain of *S. cerevisiae*, inducing coflocculation of these two yeasts (Sosa *et al*., 2008). The influence of mixed fermentation on the growth and death rates of *S. cerevisiae* and non-*Saccharomyces* and on the possible interactions is currently under study. Investigations carried out in mixed cultures to evaluate the influence of cell-to-cell contact with *T. delbrueckii*, and *K. thermotolerans* and *S. cerevisiae* indicate a lesser ability of these non-*Saccharomyces* to compete for space in comparison with *S. cerevisiae*. The causes of this behaviour are still not clear (Nissen & Arneborg, 2003; Nissen *et al*., 2003).

Table 1. Mixed fermentation processes that have been proposed in winemaking, using *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts

Species used	Aim	Process	References
S. cerevisiae *T. delbrueckii*	Reduction of acetic acid production	Sequential cultures	Castelli (1969); Herraiz *et al*. (1990); Ciani *et al*. (2006); Salmon *et al*. (2007); Bely *et al*. (2008)
S. cerevisiae *S. pombe*	Malic acid degradation	Sequential cultures Immobilized cells (batch process) Immobilized cells (continuous process)	Snow & Gallender (1979); Magyar & Panyik (1989); Yokotsuka *et al*. (1993), Ciani (1995)
S. cerevisiae *C. stellata*	Enhancement of glycerol content	Immobilized cells (pretreatment or sequential cultures)	Ciani & Ferraro (1996); Ciani & Ferraro (1998); Ferraro *et al*. (2000)
S. cerevisiae *C. cantarellii*	Enhancement of glycerol content	Mixed or sequential cultures	Toro & Vazquez (2002)
S. cerevisiae *C. stellata*	Improve wine aroma profile	Mixed or sequential cultures	Soden *et al*. (2000)
S. cerevisiae *H. uvarum (K. apiculata)*	Simulation of natural fermentation (improvement of aroma complexity)	Mixed or sequential cultures	Herraiz *et al*. (1990); Zironi *et al*. (1993); Moreira (2005); Ciani *et al*. (2006); Moreira *et al*. (2008); Mendoza *et al*. (2007)
S. cerevisiae *K. thermotolerans*	Reduction of acetic acid production Enhancement of titratable acidity	Sequential cultures	Mora *et al*. (1990); Ciani *et al*. (2006); Kapsopoulou *et al*. (2007)
S. cerevisiae *Issatchenkia orientalis*	Reduction of malic acid content	Mixed fermentation	Kim *et al*. (2008)
S. cerevisiae *Pichia fermentans*	Increased and more complex aroma	Sequential cultures	Clemente-Jimenez *et al*. (2005)
S. cerevisiae *Pichia kluyveri*	Increased varietal thiol	Mixed fermentation	Anfang *et al*. (2009)
S. cerevisiae *Candida pulcherrima*	Improve wine aroma profile	Mixed fermentation	Zohre & Erten (2002); Jolly *et al*. (2003)
S. cerevisiae *Debaryomyces vanriji*	Increase in geraniol concentration	Mixed fermentation	Garcia *et al*. (2002)
S. cerevisiae *Schizosaccharomyces* spp. *Saccharomycodes* spp. *Pichia* spp.	Influence on sensorial and physico-chemical properties of wines	Ageing over the lees during wine maturation	Palomero *et al*. (2009)

Metabolic profiles during fermentation show interactions in mixed cultures within the *Saccharomyces* starter cultures (Howell *et al*., 2006), and the multistarter inoculum compared with monoculture fermentation reveals differences. Indeed, blending of monoculture wines to mimic the composition of mixed-culture wines does not account for these differences. More recently, coinoculated fermentations using different *S. cerevisiae* starter cultures have shown differences in chemical and sensory profiles from both pure fermentations and from equal blends of single-strain wines (King *et al*., 2008).

In *Saccharomyces*/non-*Saccharomyces* mixed cultures, interactions due to the wide intergeneric metabolic diversity should be higher. In the case of the interaction between *S. cerevisiae* and *S. bombicola* (formerly *C. stellata* DBVPG 3827) (Sipiczki *et al*., 2005), complementary consumption of glucose and fructose was seen (Ciani & Ferraro, 1998). Using sequential, continuous fermentation and immobilized yeast cells, preliminary evidence has highlighted the exchange of acetaldehyde between these two yeast species. The excess of acetaldehyde production by *S. bombicola*, due to the low activity of alcohol dehydrogenase (Ciani *et al*., 2000), was rapidly metabolized by *S. cerevisiae*, which is a more active alcoholic fermentation species (Ciani & Ferraro, 1998). In this context, an investigation into the acetaldehyde movement between *S. cerevisiae* and *Saccharomyces bayanus* has been reported (Cheraiti *et al*., 2005). These interactions in acetaldehyde production (reduction) were also detected in mixed fermentations using *S. cerevisiae* and *T. delbrueckii* (Ciani *et al*., 2006; Bely *et al*., 2008) and *K. thermotolerans* (Ciani *et al*., 2006).

Another compound involved in interactions between two yeast species in mixed fermentation is acetoin; this is largely produced by *S. bombicola* in a pure culture, and completely

Table 2. Main interactions described in mixed fermentation of wines

Species used	Compound or behaviour	Interactions	References
S. cerevisiae *H. uvarum*	Growth and viability	Persistence of non-*Saccharomyces*	Ciani *et al*. (2006); Mendoza *et al*. (2007)
S. cerevisiae *T. delbrueckii*	Cell-to-cell contact	Increase in death rate of non-*Saccharomyces*	Nissen & Arneborg (2003); Nissen *et al*. (2003)
S. cerevisiae *C. stellata*	Acetaldehyde, acetoin, glucose and fructose	Complementary consumption	Ciani & Ferraro (1998)
S. cerevisiae *H. uvarum/guillermondii*	Ethyl acetate Esters	Reduction Increase	Moreira *et al*. (2008)
S. cerevisiae *P. anomala*	Isoamyl acetate (EAHase)	Increase in production by *S. cerevisiae*	Kurita (2008)
S. cerevisiae *P. kluyveri*	3-Mercaptohexyl acetate	Increase in thiols	Anfang *et al*. (2009)
Mixed 'wild' yeasts	Volatile compounds	Increased and more complex aroma	Garde-Cerdán & Ancín-Azpilicueta (2006); Varela *et al*. (2009)

EAHase, ethyl acetate-hydrolysing esterase.

metabolized by *S. cerevisiae* in mixed fermentation (Ciani & Ferraro, 1998).

Positive interactions for volatile compounds between wild yeasts and *S. cerevisiae* starter cultures were reported by Garde-Cerdán & Ancín-Azpilicueta (2006), where an enhancement of ester concentrations in comparison with pure fermentations was shown. More recently, important roles have been established for volatile compounds in the differentiation of wines made with 'wild indigenous and inoculated yeasts' (Varela *et al*., 2009). The chemical basis of the wild-yeast fermentation characteristic was referred to an increase in 2-methylpropanol, 2-methylbutanoic acid, ethyl 2-methylpropanoate, ethyl decanoate and ethyl dodecanoate. Moreira *et al*. (2008) compared pure and mixed fermentations of *H. uvarum*. *Hanseniaspora guilliermondii* and *S. cerevisiae*, confirming the improvement in ester production and the reduction in ethyl acetate in mixed fermentations, in comparison with pure cultures.

To investigate the efficacy of mixed fermentations on analytical profiles, a *Pichia anomala* petit mutant with low levels of ethyl acetate (low activity of ethyl acetate-hydrolysing esterase) was used (Kurita, 2008). In this case, the reduced presence of ethyl acetate determined the interactions between the *P. anomala* petit mutant and *S. cerevisiae*. Ethyl acetate production by this strain of *P. anomala* caused an increase in the acetate ester-hydrolysing esterase activity in *S. cerevisiae*. As a result, the desired amounts of isoamyl acetate are accumulated in the mixed cultures without an excess of ethyl acetate. Positive interactions in mixed fermentation have also been shown for thiol production (Anfang *et al*., 2009). Indeed, mixed fermentation of *P. kluyveri* and *S. cerevisiae* caused an increase in 3-mercaptohexyl acetate in comparison with pure cultures. This interaction appears to be at the strain level, rather than at the species level, but the nature of the interaction remains unknown. The interactions described in mixed fermentations of wines are shown in Table 2.

Future perspectives

Mixed fermentation using controlled *S. cerevisiae* starter cultures and non-*Saccharomyces* yeasts is a practical way to improve the complexity and to enhance the particular characteristic of a wine. However, the interactions among the different starter cultures that appear during the fermentation and the modalities of inoculation need to be further investigated. Indeed, our knowledge of the metabolic interactions between *S. cerevisiae* and non-*Saccharomyces* wine yeasts under winemaking conditions is limited. Moreover, few papers have investigated the organoleptic characteristic of wines, and the evaluation of the sensory profile of controlled-mixed culture fermentation should be carried out.

To study the biochemical, physiological and molecular bases of yeast interactions under winemaking conditions several approaches are needed. To investigate the physiological properties of natural and commercial *S. cerevisiae* yeasts, gene expression approaches (Cavalieri *et al*., 2000; Hauser *et al*., 2001; Rossignol *et al*., 2003; Varela *et al*., 2005; Wu *et al*., 2006) and comparative genome analyses (Borneman *et al*., 2008) have been carried out. The adaptation of yeast cells to wine fermentation conditions has also been investigated at the mRNA and protein levels (Zuzuarregui *et al*., 2006; Rossignol *et al*., 2009). A recent study proposed the use of an ^{1}H-nuclear magnetic resonance-based metabolomic approach for an understanding of the fermentation behaviours of wine yeast strains (Son *et al*., 2009). In addition, a comparative transcriptomic and metabolomic

approach has been utilized to identify the impact of the expression of single genes on the production of volatile aroma compounds in wine yeasts (Rossouw *et al.*, 2008).

In this context, to elucidate the metabolic mechanisms involved in the interactions in mixed culture must fermentations, all -omics approaches (transcriptomic, proteomic and metabolomic) could have a potential impact on the elucidation of these modifications. However, our limited knowledge at present of the genetic background and metabolic regulation of non-*Saccharomyces* wine yeasts provides limited use of molecular tools for investigations into the regulatory mechanisms of yeast interactions during wine fermentation. For these reasons, our knowledge of genetic and metabolic regulation of non-*Saccharomyces* wine yeasts still needs to be improved.

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