Chapter 3
Application of Non-*Saccharomyces* Yeasts in Wine Production

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Abstract In the past, *Saccharomyces* spp. yeasts were almost the only option for use in modern winemaking due to their unparalleled ability to metabolize all grape juice sugar into ethanol. For that reason, until some years ago, all commercial dry yeasts were *Saccharomyces* spp. For several years, non-*Saccharomyces* were forgotten at industrial level, and even some of them were considered as spoilage microorganisms. Non-*Saccharomyces* only played a significant role in limited productions that perform spontaneous fermentations following organic polities. However, during the last decade, several researchers have proved numerous non-*Saccharomyces* to be able to improve wine quality and to solve some modern enology challenges. Some of the factors that can improve are acidity, aromatic complexity, glycerol content, ethanol reduction, mannoproteins, anthocyanins, and polysaccharide concentrations. They can also decrease the concentrations of unwanted compounds that affect food safety, such as ochratoxin A, ethyl carbamate, and biogenic amines. Due to all those scientific advances, the main manufacturers have just started to commercialize dry non-*Saccharomyces* such as *Torulaspora delbrueckii*, *Schizosaccharomyces pombe*, *Metschnikowia pulcherrima*, *Lachancea thermotolerans*, and *Pichia kluyveri*. Other non-*Saccharomyces* species with special enology abilities such as *Candida zemplinina*, *Kloeckera apiculata*, *Hanseniaspora vineae*,

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Hanseniaspora uvarum, *C. stellata*, *Kazachstania aerobia*, or *Schizosaccharomyces japonicus* could follow a similar progress. The aim of the chapter is to show which are the main abilities and advantages of these non-*Saccharomyces* in modern winemaking.

Keywords Non-*Saccharomyces* · Winemaking · *Torulaspora delbrueckii* · *Schizosaccharomyces pombe* · *Schizosaccharomyces japonicus* · *Metschnikowia pulcherrima* · *Lachancea thermotolerans* · *Pichia kluyveri* · *Pichia guilliermondii* · *Hanseniaspora* spp.

3.1 Introduction

Comprehensive studies gave attention to *Saccharomyces* and non-*Saccharomyces* yeasts, the main microorganisms involved in spontaneous wine fermentation (Fleet 1993; Fleet and Heard 1993; Lonvaud-Funel 1996). A wide variety of yeast genera and species belong to the indigenous yeasts occurring in grape musts. In spontaneous fermentations a sequential activity of the various non-*Saccharomyces* yeasts is coming up with abundance of apiculate yeasts until autochthonous *Saccharomyces cerevisiae* strains dominate (Varela et al. 2009; Jolly et al. 2014). Consequently, many yeast species and strains participate in the fermentation and the overall quality of the resulting wines (Padilla et al. 2016; Varela 2016). Indigenous yeasts originate from the vineyard, the grapes, the surfaces, and the equipment of the winery (Schütz and Gafner 1993; Fleet 2008). The composition of this yeast genera, species, and strain community differs every year and, therefore, a persistence of certain yeast strains in the winery environment cannot be expected (Rosini 1984; Lonvaud-Funel 1996; Pretorius 2000). In addition, most of the non-*Saccharomyces* yeasts have weak fermenting capacities. Hence, spontaneous fermentations have to be seen as an uncontrollable risk that can lead to sluggish or stuck fermentations and spoiled wines. Consequently, some of the non-*Saccharomyces* yeasts have been considered as spoilage microorganisms (Padilla et al. 2016; Varela 2016). The intensified use of pure dry yeast cultures of the species *S. cerevisiae* since the 1980s offered the winemakers the opportunity for controlled alcoholic fermentations and the production of predictable wines with established and consistent quality criteria (Varela 2016). In contrast it is discussed that wines fermented with pure mono *S. cerevisiae* cultures can lack the diversity and complexity of flavor caused by specific autochthonous yeasts (Lambrechts and Pretorius 2000; Romano et al. 2003; Padilla et al. 2016). On the other hand, intensive research demonstrated that certain non-conventional and selected strains can positively impact on wine quality (Ciani and Maccarelli 1998; Jolly et al. 2006; Fleet 2008; Jolly et al. 2014) due to their ability to enhance the aroma profile, to increase flavor diversity, and/or to contribute to other metabolites that influence the wine character and style (e.g., content of ethanol, acidity, glycerol, mannoproteins, and color stability) or affect wine safety (e.g., decrease of

ochratoxin A, ethyl carbamate, and/or biogenic amines) (Quirós et al. 2014; Benito et al. 2015b; Ciani et al. 2016; Mylona et al. 2016). Furthermore, non-*Saccharomyces* yeasts are discussed to produce enzymes and metabolites of enological importance (Belda et al. 2016; Padilla et al. 2016).

The application of mixed and selected non-*Saccharomyces* yeasts with *S. cerevisiae* strains is intensively investigated within the last decade to combine the advantages and to improve wine quality. Thus, it is possible to intensify the opportunity of great specific and distinct wine types and styles, with a wide range of organoleptic characteristics and with lower risks of sluggish and stuck fermentations and spoilage (Romano et al. 2003; Ciani et al. 2006; Padilla et al. 2016).

The following chapter gives brief review on the latest research related to the use of non-*Saccharomyces* yeasts as single or mixed starter cultures for wine production and presents already existing applications.

3.2 Non-*Saccharomyces* Species

3.2.1 Torulaspora delbrueckii

Among the non-*Saccharomyces* species studied for their application to face the modern challenges of winemaking, *Torulaspora delbrueckii* is the best studied and more utilized yeast at industrial level (Benito 2018a; Benito et al. 2018). Unlike most of the non-*Saccharomyces* yeasts, the fermentative capacity of *T. delbrueckii* (Quirós et al. 2014) allows its implantation during the first phases of the alcoholic fermentation. Even though *T. delbrueckii* shows limited ethanol resistance compared with *S. cerevisiae*, it seems to be able to tolerate over 9% (v/v) (Bely et al. 2008). Because of that, this species can have an important role during the fermentation process, contributing positivity to several wine quality parameters. In addition, *T. delbrueckii* can properly complete the entire fermentation in beer or sparkling base wine production, due to the lower alcohol level of these beverages. In the case of wine fermentations, sequential fermentation with *S. cerevisiae*, with 2 to 5 days of delay, must be carried out to achieve the effects that *T. delbrueckii* might impact on wines. *T. delbrueckii* primarily has influence on the acetic acid content of wine. Fermentations in which *T. delbrueckii* is involved typically showed low levels of acetic acid (Bely et al. 2008). Acetic acid decreases between 0.13 and 0.27 g/L have been observed in *T. delbrueckii* sequential fermentation (Benito 2018a). Also, this yeast is able to lower the ethanol concentration of wines, over 1% (v/v) (Contreras et al. 2014). This impact is important in warmer viticultural regions (most affected by the climatic change) where musts reach higher concentrations of sugars and, therefore, higher concentration of ethanol after fermentation. Increases of glycerol content, which can have great influence on wine sensory properties, have been reported in a range of 0.1 to 1 g/L (González-Royo et al. 2015; Belda et al. 2017; Medina-Trujillo et al. 2017; Puertas et al. 2017).

T. delbrueckii is reported to consume between 20% (Belda et al. 2015) and 25% (Chen et al. 2018) of the initial malic acid concentration in must. Regarding succinic acid production, Puertas et al. (2017) reported an increase of 0.46 g/L, compared to pure *S. cerevisiae* fermentation. Higher amounts of mannoproteins and polysaccharides are also produced by *T. delbrueckii*, impacting the mouthfeel properties of wines (Belda et al. 2015). Reduction on acetaldehyde concentration in wine has been observed in most of research works using *T. delbrueckii*, in comparison to *S. cerevisiae* fermentations. Decrease from 20 to 40 mg/L has been reported (Belda et al. 2017; Puertas et al. 2017).

In relation to wine color quality, lower concentration of some anthocyanins was shown by *T. delbrueckii* and *S. cerevisiae* sequential fermentation than by single *S. cerevisiae* fermentation (Belda et al. 2015). Opposite results were observed regarding other anthocyanins (Bañuelos et al. 2016).

Aroma profiles of wines are also positively affected by *T. delbrueckii*, due to higher production of desirable volatile compounds, especially on aroma complexity and fruity character. Higher concentrations of fruity esters have been reported in sequential fermentation with *T. delbrueckii* (Renault et al. 2015; Belda et al. 2017), although a decrease in ester amount has also been observed (Azzolini et al. 2015; Puertas et al. 2017). As most of non-*Saccharomyces* yeasts, *T. delbrueckii* can reduce the concentration of higher alcohols in sequential fermentations (Milanovic et al. 2012; Belda et al. 2015). This is important because of the improvement of varietal compounds perception in final wine flavor. Nevertheless, increase of higher alcohol production has also been reported (Azzolini et al. 2015).

Concerning varietal compounds, significant increases of terpene compounds have been observed in *T. delbrueckii* sequential fermentation (Cus and Jenko, 2013; Whitener et al. 2017). Among non-*Saccharomyces* species, *T. delbrueckii* stands out because of its capability to release varietal thiols from their odorless precursors in musts. Renault et al. (2016) observed increase on 3-sulfanylhexan-1-ol (3SH) thiol release, but not on 4-methyl-4-sulfanylpentan-2-one (4MSP) release, in *T. delbrueckii* fermentations. Nevertheless, important increases on 4MSP productions have been reported with another *T. delbrueckii* strain (Belda et al. 2017).

In spite these impacts on wine quality of *T. delbrueckii*, high strain-dependent effects have been reported for *T. delbrueckii* (Azzolini et al. 2015; Escribano et al. 2018). Regarding commercialization of *T. delbrueckii* in wine industry, five strains are available for winemaking: PRELUDE™ (Chr. Hansen Holding A/S, Hoersholm, Denmark), BIODIVA™ (Lallemand Inc., Rexdale, Canada), ZYMAFLORE® ALPHA (LAFFORT®, Bordeaux, France), Viniferm NS-TD® (Agrovin S.A., AlcÁzar de San Juan, Spain) and PRIMAFLORA® VB BIO (Sud et Bio, Lattes, France). All of them are recommended to improve wine quality by decreasing volatile acidity and improving flavor complexity.

3.2.2 Schizosaccharomyces *spp.*

3.2.2.1 *Schizosaccharomyces pombe*

Schizosaccharomyces pombe is unique among the other non-*Saccharomyces* yeast because of its capacity to reach ethanol concentrations of about 15% (v/v) (depending on the strain), in regular wine fermentations. In addition, this yeast has the exclusive ability to deacidify wines through the conversion of malic acid into small amounts of ethanol and CO_2 (Benito et al. 2014). For that reason, the initial application of this species was focused in decreasing the acidity of wines from northern European cold wine regions, where the low grape maturity supposes high contents in malic acid, over 6 g/L. Under those circumstances, *S. pombe* metabolizes all the malic acid during the alcoholic fermentation generating increases in pH of about 0.5 units, producing smoother wines.

However, collateral effects such as high concentrations of volatile acidity, which occasioned strong vinegar character, are commonly reported when *S. pombe* ferments as a single inoculum. Most *S. pombe* strains tend to produce concentrations in acetic acid over 0.8 g/L, although some strains show moderate productions (Benito et al. 2014; Benito et al. 2016). This undesirable effect was initially minimized by using *S. pombe* strains in combination with selected *S. cerevisiae* strains (Benito et al. 2012). The main problem about selecting proper *S. pombe* strains is its low incidence in nature, lower than 0.5% in grapes and other fruits. Therefore, it is very difficult to isolate strains to accomplish a proper selection process. Nowadays the development of specific selective-differential growth media for *S. pombe* has solved that problem (Benito et al. 2018). During the last years, other industry processes, such as sparkling wine, plum wine, apple fermentation, or bilberry fermentation, which present higher concentration of malic acid than grapes, start to use *S. pombe* during their alcoholic fermentations in order to reduce malic acid content of their wines.

S. pombe has also been used in red wines (Benito et al. 2012) in totally different circumstances than the cool northern European viticultural regions. Warm viticulture areas from the south of Europe present contents of malic acid about 1 g/L in wines, with pH of about 3.9 and probable alcohol levels of about 15% (v/v). Under those circumstances, performing the microbiological stabilization process of malolactic fermentation by lactic acid bacteria is highly risky. The main risks are difficult alcoholic fermentation endings where lactic bacteria consume residual sugars generating high volatile acidity concentrations. In addition, the glycosidase activity and cell absorption of most lactic bacteria usually decrease the color of red wine in about 10 to 20%. If the malolactic fermentation is avoided after alcoholic fermentation, this process usually will take place in the bottle generating turbidity or deterioration of the top cork. Additionally, health problems for human beings such as the production of high concentrations of biogenic amines or ethyl carbamate usually take place when lactic bacteria perform in high pH. In this context, the use of

S. pombe, combined with *Lachancea thermotolerans*, can compensate the low acidity of warmer viticultural regions musts (Benito et al. 2015a).

Other beneficial ability of *S. pombe* is its production of high pyruvic acid concentration, five times higher than *S. cerevisiae*. This fact allows the production of high-color pigments such as Vitisin A, composed of pyruvic acid and anthocyanin (Benito et al. 2017). The production of moderate levels of acetaldehyde by some specific *S. pombe* strains, below its undesirable perception threshold, also increases the concentration of the stable color pigment Vitisin B.

The *S. pombe* cell structure is rich in α-galactomannose and β-glucans (Domizio et al. 2017; Domizio et al. 2018; Benito et al. 2019). This fact makes *S. pombe* able to release higher concentrations of polysaccharides (from 2.5 to 5 times higher than *S. cerevisiae*). Those polysaccharides and mannoprotein contents usually increase sensory parameters related to the wine structure and reduce the wine astringency.

Another interesting property of *S. pombe* is its urease enzymatic activity that allows removing all the urea from wine (Benito et al. 2015a). Urea is the main precursor of the carcinogenic compound ethyl carbamate. Additionally, the wines fermented by *S. pombe* do not contain nutrients that could be metabolized by lactic acid bacteria such as malic acid. Therefore, the wines are also stable against possible undesirable productions of biogenic amines such as histamine from lactic acid bacteria.

Some specific strains of *S. pombe* can remove gluconic acid from grape juice during the alcoholic fermentation (Peinado et al. 2009). This activity allows to avoid the possible negative effects produced by this compound when it is presented in high concentrations in rotten grapes.

Finally, *S. pombe*, other non-*Saccharomyces* yeasts, produces lower concentrations in higher alcohols, regarding *S. cerevisiae* (Benito et al. 2016), varying from 25 to 50% depending on the strain and the specific higher alcohol. That effect produces in some occasions fruity wines, with strong varietal character not masked by higher alcohols and without lactic notes from malolactic fermentation.

3.2.2.2 *Schizosaccharomyces japonicus*

Although most studies regarding *Schizosaccharomyces* genus focus on *S. pombe* applications in fermentation industries, another species from that genus named *Schizosaccharomyces japonicus* stands out because of its beneficial effects on winemaking (Domizio et al. 2018). *S. japonicus* also shows the ability to degrade malic acid (from 71 to 82%), and it also possesses a high fermentative power, up to 14% (v/v) of ethanol, being slower fermenter than *S. cerevisiae*. Some studies report that *S. japonicus* can produce higher concentrations of glycerol than *S. pombe*. The main undesired effect of the species is that they tend to produce high concentrations of acetic acid, about 0.76 g/L. That problem is nowadays palliated using immobilized cells in alginate beads that allow producing moderate final acetic acid concentrations of about 0.4 g/L. As *S. pombe*, *S. japonicus* produces low concentrations of higher alcohols. Nevertheless, some strains are reported to pro-

duce higher concentrations in 2-phenyl ethanol and isoamyl acetate than the *S. cerevisiae* controls. Finally, it has been reported higher concentrations of polysaccharides and the end of fermentation when *S. japonicus* is used, even higher than *S. pombe* in about 15%.

3.2.3 Metschnikowia pulcherrima

At the moment, there is one strain of *Metschnikowia pulcherrima* commercially available. The manufacturer advertises a competitive advantage based on its α-L-arabinofuranosidase activity. When the *M. pulcherrima* strain FLAVIA™ Mp346 is inoculated in grape juice, it increases the release of varietal compounds such as terpenes and volatile thiols (Lallemand Inc., Rexdale, Canada). However, co-fermentation with *S. cerevisiae* should be carried out to ensure a proper alcoholic fermentation ending. Other authors report that the compatibility between *M. pulcherrima* and *S. cerevisiae* must be considered before inoculation in order to avoid possible delays due to the inhibition effect of killer toxins production (Jolly et al. 2014).

Regarding basic fermentation parameters, sequential fermentations with *M. pulcherrima* and *S. cerevisiae* result in a slight increase of glycerol (0.2 g/L) and reductions of malic acid (0.2 g/L) and acetaldehyde (10 mg/L), while the final acetic acid levels do not increase (Ruiz et al. 2018). Although some strains are reported to be able to reduce the ethanol content during sequential fermentation in about 1% (v/v) (Contreras et al. 2014), most reported benefits about *M. pulcherrima* strains are related to the volatile aroma composition.

Most studies report *M. pulcherrima* as a lower producer of higher alcohols, which can mask varietal aromas such as terpenes or thiols. Ruiz et al. (2018) report a reduction of about 25% respect to the *S. cerevisiae* controls. Some authors mention *M. pulcherrima* as a higher producer of fruity total esters (Jolly et al. 2014), while other scientist did not observe evident differences, based on the *S. cerevisiae* strain (Ruiz et al. 2018). In other studies only the increases of specific esters such as ethyl octanoate, which are related to pleasant aromas like pine apple, were observed (Benito et al. 2015b).

Some studies report no influence on total terpenes or even decreases in their concentration (Benito et al. 2015b); however, great differences are reported in the case of thiols (Ruiz et al. 2018). The release of the polyfunctional thiol 4MSP is the most notorious advantage, observing an increase of seven times higher concentrations than the *S. cerevisiae* control (Ruiz et al. 2018). That effect notably enhances the varietal character of thiolic varieties such as Verdejo or Sauvignon blanc. Nevertheless, this effect is not observed by other researches (Sadoudi et al. 2012) because this impact is strain-dependent. Therefore, *M. pulcherrima* strains must be previously selected regarding this specific trait, mainly due to its cystathionine-β-lyase activity, although other unknown metabolic pathways could be involved.

3.2.4 Lachancea thermotolerans

Yeasts belonging to *Lachancea* genus stand out due to its potential application in biotechnological processes. Currently, this genus comprises 11 species, based on D1/D2 sequence analysis. These species can be isolated from multiple substrates, including both wild and human anthropogenic niches (Porter et al. 2019). *L. thermotolerans*, formerly classified as *Kluyveromyces thermotolerans*, *was selected as the type species. Among Lachancea* spp., *L. thermotolerans*, *L. fermentati*, and, to a lesser extent, *L. lanzarotensis* have been associated with grape must and wine environments, presenting biochemical traits with enological interest (Porter et al. 2019) and, therefore, have potential applicability on winemaking.

Hranilovic et al. 2017a, studied the population structure of *L. thermotolerans*, revealing clusters explained by multiple domestication events. They propose adaptation to different niches. Also, the phenotyping of the various strains revealed a concordance between these clusters and their fermentation capacity and their volatile profile obtained.

Despite *L. thermotolerans* is a moderate fermentative species, during wine production a co-fermentation with more vigorous yeasts as *S. cerevisiae* (Gobbi et al. 2013) or *Schizosaccharomyces pombe* (Benito et al. 2017) is carried out. *L. thermotolerans strains are* already commercialized for its use in winemaking, as a single strain (CONCERTO™) and in a blend with *T. delbrueckii* and *S. cerevisiae* strains (MELODY™, Chr. Hansen Holding A/S, Hoersholm, Denmark).

Due to its ability to produce L-lactic acid during alcoholic fermentation, an uncommon trait among yeasts, *L. thermotolerans* is the most frequently used species in wine industry for improving wine quality by acidification of grape musts. This fact is of great relevance in warmer viticultural regions due to the increase of sugars and the decrease of acidity in grapes caused by global warming. Lactic acid production by *L. thermotolerans* has been reported from 0.3 to 9.6 g/L and an increase of the pH value from 3.3 to 3.5 has been observed (Gobbi et al. 2013). In red wines from warm viticultural regions, the level of lactic acid produced by this yeast can be higher, even if there is a bacterial lactic acid production during malolactic fermentation (Benito 2018b).

Although lactic acid production is the main application of this yeast, the use of *L. thermotolerans* in winemaking can improve other wine quality parameters mentioned below, even in a strain-dependent manner.

L. thermotolerans can be used in the wine industry for the prevention of toxins produced during fermentation. Because of its role as a biocontrol agent against the growth of ochratoxigenic fungi, its inhibition impact on ochratoxin A accumulation has been reported (Ponsone et al. 2016). Some studies about the impact of *L. thermotolerans* on winemaking report malic acid degradation from 8% (Gobbi et al. 2013) to 25% (Kapsopoulou et al. 2005); however this ability is highly strain-dependent. Relating to acetic acid, low concentrations are obtained during fermentations with *L. thermotolerans*. Comitini et al. (2011) observed that different strains

of *L. thermotolerans* have a 50% lower production of volatile acids than *S. cerevisiae* strains, ranging from 0.32 to 0.58 g/L.

Due to their low fermentation power, *L. thermotolerans* strains produce low final levels of ethanol, leaving residual sugars in the final wine. It has been reported that in sequential fermentation with *S. cerevisiae*, an ethanol decrease of 0.20% (v/v) (Benito et al. 2015b) or 0.40% (v/v) (Hranilovic et al. 2017b) can be achieved. In contrast, several studies observed an increase in the formation of glycerol, e.g., from 0.29 to 0.69 g/L in Gobbi et al. (2013) and Benito et al. (2015b). Decreases in acetaldehyde production have been shown in single *L. thermotolerans* fermentations regarding single *S. cerevisiae* fermentations (Ciani et al. 2006). All these reported *L. thermotolerans* effects require in most cases sequential fermentations with *S. cerevisiae* or *S. pombe* strains (Benito 2018b).

Several works have reported the influence of *L. thermotolerans* strains on wine aroma profile. Due to the reported glucosidase activity for some *L. thermotolerans* strains, an increase in the release of terpenes has been observed during single and sequential fermentations with this species (Benito et al. 2015a, b; Comitini et al. 2011). Against that a decrease in the production of higher alcohols, especially in 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol has been noticed during co-fermentation with *L. thermotolerans* and *S. cerevisiae*, in comparison to a single fermentation with *S. cerevisiae* (Gobbi et al. 2013; Benito et al. 2015b; Balikci et al. 2016; Escribano et al. 2018). Opposite results were also observed (Comitini et al. 2011; Chen et al. 2018), demonstrating again the variability between *L. thermotolerans* strains. Similar effects were perceived regarding ester production. Both increase (Benito et al. 2015b; Hranilovic et al. 2017b) and decrease (Escribano et al. 2018) on ester production during wine fermentation have been reported.

Other parameters related to wine quality can also be improved by using *L. thermotolerans*. Several studies have reported increase in color intensity due to the increment of anthocyanin concentration (Benito et al. 2015a; Hranilovic et al. 2017b; Chen et al. 2018). Polysaccharides and mannoproteins have a great influence on mouthfeel of wine. Some works reported increases on polysaccharide concentrations when *L. thermotolerans* participated in the fermentation, compared to pure cultures of *S. cerevisiae* (Gobbi et al. 2013; Domizio et al. 2014). This increasing effect has not been observed in mannoproteins with the use of *L. thermotolerans* in aging over lees (Benito 2018b).

Special mention deserves the combined use of *L. thermotolerans* and *S. pombe* in wine fermentation. *S. pombe* has been used to avoid the negative effects of malolactic fermentation by lactic acid bacteria, e.g., toxin production, loss of color, or increase in acetic acid (Domizio et al. 2017). Nevertheless, *S. pombe* use might reduce acidity of wine. For this reason, combination of *L. thermotolerans* and *S. pombe* is a good solution in grape must with low acidity. Thus, *L. thermotolerans* lactic acid production compensates for the loss of acidity produced by *S. pombe* due to its malic acid metabolism, without malolactic fermentation by lactic acid bacteria (Benito et al. 2015a).

In conclusion, *L. thermotolerans* strains have a great potential use for the winemaking industry in an improvement of wine parameters, during a combined use with higher fermentative yeasts like *S. cerevisiae* or *S. pombe*. Despite some undesirable traits have been reported in this non-*Saccharomyces* species, these disadvantages seem to be strain-dependent. Therefore, a good strain selection procedure allows to select suitable strains for the application in wine quality improvement.

3.2.5 Pichia *spp.*

3.2.5.1 *Pichia kluyveri*

There is one strain of *Pichia kluyveri* commercially available (FROOTZEN®, Chr. Hansen Holding A/S, Horsholm, Denmark). *P. kluyveri* is known to have superior capabilities of releasing 3SH and 3-sulfanylhexyl acetate (3SHA) from precursors found in grape must (Anfang et al. 2009). These compounds impart passion fruit and other tropical aromas in thiolic white grape varieties. Their growth is inhibited by 4 to 5% ethanol. In co-fermentations with *S. cerevisiae*, *P. kluyveri* showed to produce higher levels of specific esters such as 2-phenylethyl acetate and ethyl octanoate in about 23% and 10% more than the *S. cerevisiae* control (Benito et al. 2015b). The same study also observed an increase of about 20% in total terpenes. According to the sensorial analysis, the wines fermented by *P. kluyveri* showed higher Riesling typicity than the control.

3.2.6 Hanseniaspora *spp.*

Despite their medium/low fermentation capacity and the current lack of any commercial strains, *Hanseniaspora* genus yeasts stand out due to their emerging potential in the winemaking industry. This apiculate yeast genus, composed of ten species, presents an extremely high occurrence on grape-associated microflora. In addition, positive enological traits have been reported for these species that can contribute to the chemical composition of wines, in combination with *S. cerevisiae* in sequential fermentation (Martin et al. 2018). Some authors observed that *H. vineae* can contribute to the fruity aroma of wines by increasing the concentration of acetate esters and isoprenoids during wine fermentation, accompanied with a decrease of fatty acid and ethyl esters concentrations compared to *S. cerevisiae* strains (Martín et al. 2019). Giorello et al. (2018) also reported these effects on *H. vineae*, identifying gene duplication and absences compared to *S. cerevisiae* genome that explain this impact on wine flavor. With regard to the release of varietal compounds, *H. uvarum*

and *H. vineae* showed β-glucosidase and β-xylosidase enzymatic activities that can potentially increase the production of terpenes during the fermentation (López et al. 2015). Mendes-Ferreira et al. (2001) also demonstrated the ability of a *H. uvarum* strain to release monoterpenols such as linalool, geraniol, or α-terpineol. Although these apiculate yeasts have been associated with the production of high volatile acidity, this trait seems to be strain-dependent (Martin et al. 2018). Also, *H. uvarum* and *H. guilliermondii* strains have been described as producers of high levels of heavy sulfur-containing aromatics (Moreira et al. 2005). Regarding its contribution to the color quality of red wines, several *Hanseniaspora* genus species have been shown to add to contribute to the polyphenolic composition of sequential fermentation wines (Lleixa et al. 2016). Finally, *Hanseniaspora* spp. yeasts not only can impact on wine parameters directly but indirectly by affecting the genetic expression profile of *S. cerevisiae*. Barbosa et al. (2015) demonstrate effect of *H. guilliermondii* on the transcriptomic response of *S. cerevisiae*, particularly on the expression of flavor-active compound-associated genes.

3.3 Conclusions

The increasing and already numerous research studies indicate the growing interest in the application of non-*Saccharomyces* yeasts as pure single or mixed cultures for controlled mixed fermentations with simultaneous or sequential inoculations to achieve more flavor complexity and stylistic features (Padilla et al. 2016; Varela et al. 2017). In addition, the mixed fermentations with selected non-*Saccharomyces* and *Saccharomyces* yeasts are more and more used to meet the challenges of the climatic change as lowering the alcohol content (González-Royo et al. 2015; Contreras et al. 2015; Morales et al. 2015) and the increase or decrease of acidity (Kapsopoulou et al. 2007; Benito et al. 2015). Further applications like wine color stabilization (Benito et al. 2017); low formation of hydrogen sulfide (H_2S), sulfites (SO_2), acetaldehyde, and other SO_2-bindung compounds; ability to reduce the copper content, biogenic amines, etc. will be of an increasing demand to optimize wine quality and safety (Comitini et al. 2017).

Continuative research is required to get more experience with the application of mixed yeast cultures and single non-*Saccharomyces* fermentations and to optimize the control over mixed culture fermentations. In particular, strain-dependent interactions and cell-to-cell contact in mixed cultures which might affect the entire metabolism and regulation processes have to be studied in detail (Nissen et al. 2003; Kemsawasd et al. 2015; Padilla et al. 2016) for an improved and adequate design of mixed yeast cultures for additional and new applications related to the requirements and demands of the wine industry.

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