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Non-*Saccharomyces* yeasts for reduction of ethanol content in wine

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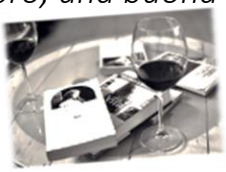
Cycle XXXIII

“Grande è la fortuna di colui che possiede
una buona bottiglia di vino,
un buon libro, una buona amica.”



Molière

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Publications

Fermentazioni miste controllate per migliorare il profilo analitico e sensoriale dei vini

Saccharomyces cerevisiae and *Hanseniaspora uvarum* mixed starter cultures: Influence of microbial/physical interactions on wine characteristics

Exploitation of technological variability among wild non-*Saccharomyces* yeasts to select mixed starter for the production of low alccohol wine

Thanksgivings

Summary

Over recent years, the average ethanol concentration of wine has increased as consequence of increased grape maturity. In fact, climate change has deeply influenced the vine phenology and grape composition, resulting in rising of grapes sugar concentration and, consequently, high alcohol content in wine. This increased ethanol content can have negative consequences on the wine characteristics, affecting the sensory properties of the wines, as high alcohol content reduces the perception of flavour and aroma complexity. Furthermore, wine containing high levels of alcohol gives rise problems to the human health and also to the economic aspects as it determines an increase of taxes. Among the several solutions currently under study, biotechnological approach based on the use of non-*Saccharomyces* yeasts during alcoholic fermentation holds good promise for contributing to reduction ethanol and contemporary to improve wine characteristics. Non-*Saccharomyces* wine yeast species comprise a high number of species, characterized by high physiological diversity. The oenological interest of these microorganisms was initially triggered by their potential positive contribution to the sensorial complexity of wines, through the production of aroma and other sensory-active compounds which are not produced by single fermentation with *Saccharomyces cerevisiae*. The current interest toward these yeasts is addressed also to the ethanol yield on sugar, one of the most invariant metabolic traits of *S. cerevisiae*. In fact, non-*Saccharomyces* yeast can divert carbon away from ethanol production affecting ethanol yield, fermentation efficiency, production of biomass and final by-products. One of these alternative pathways would be sugar respiration under suitable fermentation conditions, especially for Crabtree-negative yeast species, through partial and controlled aeration of the grape juice. However, the oxygenation levels required for yeast respiration could promote, as a side effect, the oxidation of key components for the sensory quality of wines, such as phenolics and aromatic compounds. As a consequence, a strict control of fermentative conditions during oxygen addition is necessary in order to avoid undesirable effects on aromatic quality of wine, such as too high levels of volatile acidity.

The main aim of this research was testing different non-*Saccharomyces* strains in mixed fermentation with *S. cerevisiae* to select the most promising strain combination to be used

as a tool for reducing the ethanol content in wines. In the first step, 33 non-*Saccharomyces* wild strains, belonging to *Debaryomyces polymorphus*, *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Zygosaccharomyces bailii* species, were tested for parameters of technological interest, such as production level of hydrogen sulphide (H_2S), resistance to ethanol, SO_2 and copper, screening for killer-sensitive pattern, evaluation of enzymatic activities (ester-hydrolase, β-glucosidase and β-xylosidase activities). Some non-*Saccharomyces* strains, selected on the basis of results obtained by technological screening, were tested during mixed fermentations at laboratory scale, testing different inoculation protocols, such as simultaneous and sequential inoculum. After this step, one mixed starter culture, characterized by good oenological aptitude and highest ability to reduce ethanol content in wine, was tested in different process conditions, such as fermentation with oxygen addition and use of immobilized cells, in order to evaluate the influence of this parameters on starter behaviour. In the last step, the performance of selected mixed starter culture was validated at pilot scale in order to individuate the mixed starter culture and fermentation conditions to be proposed to winemakers for production of wine with reduced alcohol content and increased aromatic complexity.

Chapter 1

Non-*Saccharomyces* yeasts and their impact in wine production

Abstract

The alcoholic fermentation, that is conversion of fermentable sugars into alcohol by yeasts, is a key process in the production of all alcoholic beverages. However, microbial activity during fermentation is more complex than merely producing ethanol, usually involving the action of a great diversity of yeasts and bacteria and the production of metabolites that affect the organoleptic properties of fermented beverages. Inoculated fermentations using selected starter cultures of *Saccharomyces* is widely believed one of the most important advances in the improvement of product and in the control of the fermentation process. 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 with *Saccharomyces* and different non-*Saccharomyces* yeast species from the wine environment. Non-*Saccharomyces* yeasts, which are naturally present in uninoculated spontaneous fermentations, can provide a tool for increasing aroma and flavour diversity in fermented beverages. The combined use of non-*Saccharomyces* and *Saccharomyces* yeasts, represents a biotechnological tool to ensure the fermentation performance and, at the same time, to modify both the chemical and the aromatic composition of wines. In this chapter, it is reported a review of the main characteristics of non-*Saccharomyces* species that might play a positive role in final wine.

1.1| Biology of yeasts during spontaneous fermentations

Winemaking is one of the oldest biotechnological processes and yeast is the main actor of fermentative process. Historically, these fermentative processes developed from

unknown, uncontrolled, and spontaneous reactions due to complex mixture of microbes present in the diverse natural niches. In wine production, yeast species play an important role, not only for the transformation of sugar to ethanol, but also for the production of specific secondary metabolites, which contribute to wine flavor characteristics. Spontaneous fermentation of grape must in wine is an ecologically complex process, and it is well established that the yeast population change as the fermentation proceeds (Ciani et al., 2010; Fleet, 2008). In fact, grape must is a non-sterile rich substrate that allows growth and fermentative activity of various yeasts. The main microorganism involved in this transformation process belongs to the *Saccharomyces* genus. Other wine yeasts, the so-called non-*Saccharomyces* species, can also develop during the process, and can influence analytical and aromatic wine composition (Wang et al., 2015). Yeast are found throughout nature typically forming communities within specific habitats.

In winemaking environment, grape berry surfaces and cellar equipment surfaces can be considered specialized niches where wine yeasts form communities. These communities change slightly when they enter in contact with the cellar environment where they join with resident microbiota. The main source of this microbial population is grape. The grapes have populations of native or indigenous yeasts that range between 10^4 and 10^6 cells/g of grapes, represented mainly by non-*Saccharomyces* yeasts (Jolly et al., 2014; Mas et al., 2016).

The great quantitative and qualitative variability among non-*Saccharomyces* species found in the early stages of fermentation can be explained by the large number of factors influencing the grape microbiota, such as climatic conditions, localization, cultivar, agronomic practices, stage of ripening, health of the grapes, harvesting procedures and the specific weather conditions in each vintage year. Fermentative species of *Saccharomyces* occur at extremely low populations on grapes and are rarely isolated from intact berries and vineyard soils. In contrast, *S. cerevisiae* is abundant on grape juice and must-coated surfaces of winery equipment, forming an important component of a so-called “residential” or “winery” yeast flora.

Non-*Saccharomyces* yeasts found in the grape must and during fermentation can be divided into three groups:

i. yeasts that are largely aerobic, for example, *Debaryomyces* spp., *Candida* spp., *Pichia* spp., *Rhodotorula* spp., and *Cryptococcus albidus*;
ii. apiculate yeasts with low fermentative activity, for example, *Hanseniaspora spp.* (*Kloeckera* anamorph form): *H. uvarum, H. guilliermondii, H. osmophila, H. vineae*;
iii. yeasts with fermentative metabolism, for example, *Metschnikowia pulcherrima*, *Kluyveromyces marxianus*, *Torulaspora delbrue*ckii and *Zygosaccharomyces bailii*.

During spontaneous grape juice fermentation, a sequential succession of yeasts is observed. Initially, species of *Hanseniaspora*, *Starmerella*, *Issatchenkia*, *Pichia*, *Zygosaccharomyces*, *Torulaspora*, *Schizosaccharomyces*, *Candida*, *Metschnikowia*, *Debaryomyces* and *Cryptococcus* are found in fresh must. Among these, one of the most common yeast species, present in grape must at highest numbers, is *H. uvarum/guilliermondii*. These yeasts were found at high cell densities, up to 10^6-10^8 cells/mL, during the first 3-4 days of fermentation, followed by various *Candida* species. Subsequently, *S. cerevisiae* becomes the dominating microorganism, completing the wine fermentation. Despite the high presence of certain non-*Saccharomyces* yeasts, the majority of them disappear during the early stages of fermentation. This might be due to their slow growth and inhibition by the combined effects of SO_2, low pH, high ethanol and oxygen deficiency (Jolly et al., 2014; Mas et al., 2016; Varela, 2016).

Spontaneous fermentations can give high quality wines with unique and distinct regional characteristics. These wines are generally regarded as having improved characteristics, such as higher flavour complexity, compared to wines from inoculated fermentations. However, the main limits of spontaneous fermentation are represented by unpredictability of the process, responsible of stuck or slow fermentations, and inconsistencies in wine quality. As a consequence, the applied research and industrial production utilize specific starter cultures, composed by *S. cerevisiae* strains. The use of starter culture, in contrast, offers the advantages of a more predictable and rapid process, giving wines with constant quality (Fleet, 2008; Jolly et al., 2014).

1.2| *Saccharomyces cerevisiae* yeast

In winemaking the use of commercial *S. cerevisiae* strains is becoming a common practice, due to the advantages of assuring process performances and product quality. Strains of this species have unique physiological properties that are not found in other yeasts. The most important trait is the ability to ferment sugars vigorously to produce alcohol under both aerobic and anaerobic conditions. The role of *S. cerevisiae* yeasts is not only related to perform the alcoholic fermentation, but also to the influence of this yeast on wine quality (Jolly et al., 2014).

The selection of commercially available starters has been based on different criteria; actually, on the market it is possible to find starters able to ferment musts with high sugar concentration, to resist to high or low fermentation temperatures, to survive in wines with high ethanol content, to perform secondary fermentation (for instance for sparkling wine production), to increase aromatic characteristics, among other properties. Nevertheless, the

main characteristic of these commercial starter cultures is that they are good fermenters, able to complete the alcoholic fermentation. As a consequence, the use of commercial *S. cerevisiae* strains produces very uniform wines, characterized by similar content of analytical compounds and organoleptic profiles, and thus, limiting the variability and complexity that define the typicality of a wine. Wine typicality could be defined as the characteristics that allow the identification of a wine with the territory where it has been produced. The safeguarding of this typicality can be obtained by the selection of native or indigenous yeasts to be used as starters contributing to the sensory characteristics of final product (Bokulich et al., 2014; Mas et al., 2016).

1.3| The contribution by non-*Saccharomyces* yeasts

In the past, the contribution of non-*Saccharomyces* yeasts in winemaking has always been considered negative for their limited oenological attitude and the ability to produce undesirable compounds.

Most of the non-*Saccharomyces* species have limited fermentation potential, such as low fermentation power and rates, as well as low SO_2 resistance. Moreover, the production of acetic acid, ethyl acetate, acetaldehyde, and acetoin at high concentrations generally prevents the use of these strains as starter cultures in wine industry. Currently, one of the main trends in the industry of starter cultures for oenology relies on the survey of the microbial resources associated with spontaneous fermentation in order to design products able to maximize wine quality (Petruzzi et al., 2017; Romano & Capece, 2017). This new trend contributed to an increased interest on the use of non-*Saccharomyces* yeasts in winemaking (Benito et al., 2019a; Padilla et al., 2016), although the endless debate between researchers and oenologists regarding the use of non-*Saccharomyces* in winemaking. In fact, the growth of non-*Saccharomyces* yeasts can still be seen as an uncontrollable risk or as an opportunity of improving wine quality (Padilla et al., 2016; Petruzzi et al., 2017). The sensory profile of wines that are produced after a fermentation process in which, in a greater or lesser extent, various species of non-*Saccharomyces* yeasts have played a role in the winemaking process and, therefore, have contributed to the final result, differ substantially from wines produced using *S. cerevisiae* starter cultures. In this context, the inclusion of selected non-*Saccharomyces* wine yeasts as part of mixed starters together *S. cerevisiae* was suggested as a way of taking advantage of spontaneous fermentations without the risks of stuck fermentations or wine spoilage (Benito et al., 2019a; Padilla et al., 2016). However, the contribution of non-*Saccharomyces* metabolites to wine flavour depends on how they are active during the initial phases of fermentation, and this depends on how they can cope with

the high osmotic pressure, equimolar mixture of glucose and fructose, high sulphite concentration, suboptimal growth temperature, decreasing nutrients as well as increased alcohol concentrations and anaerobic conditions (Goold et al., 2017).

The non-conventional yeasts contribute to the enhancement of flavor and aroma complexity of wine directly by their metabolic activity (production of alcohols and esters) or by the release of extracellular enzymes which transform metabolites produced by *S. cerevisiae* (Petruzzi et al., 2017). The metabolic products resulting from non-*Saccharomyces* growth include terpenoids, esters, higher alcohols, glycerol, acetaldehyde, acetic acid and succinic acid.

Different non-*Saccharomyces* yeasts produce different levels of higher alcohols, such as *n*-propanol, isobutanol, isoamyl alcohol, active amyl alcohol, which at high concentrations are not desired, whereas values comprised in desirable ranges can increase wine complexity. Generally, non-*Saccharomyces* yeasts form lower levels of these alcohols than *S. cerevisiae*, but great strain variability was found (Jolly et al., 2014).

Glycerol, the major yeast metabolite produced during wine fermentation after ethanol, is important because it contributes to smoothness (mouth-feel), sweetness and complexity of wines, but the grape variety and wine style will determine the extent to which glycerol impacts on these properties. Different non-*Saccharomyces* yeasts, particularly *Lachancea thermotolerans* and *Starmerella bacillaris*, can produce high glycerol concentrations during wine fermentation (Comitini et al., 2011).

Some non-*Saccharomyces* yeasts are able to form succinic acid, which could positively influence the analytical profile of wines. However, succinic acid has a "salt-bitter-acid" taste and excessive levels influence negatively wine quality.

Other compounds that are known to play a role in the sensory quality of wine include volatile fatty acids, carbonyl and sulphur compounds. Volatile thiols greatly contribute to the varietal character of some grape varieties. *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *L. thermotolerans* are able to release important quantities of the volatile thiols 3-mercaptohexan-1-ol (3MH).

Non-*Saccharomyces* yeasts possess enzymatic activities, which can catalyze the release of volatile aroma compounds from non-volatile bound precursors. Several flavour and aroma compounds are present in grapes as glycosylated flavourless precursors. These compounds may be hydrolysed by the enzyme β-glucosidase to form free volatiles that can improve the flavour and aroma of wine, but this enzyme is not encoded by the *S. cerevisiae* genome. In contrast, non-*Saccharomyces* yeasts possess β–glucosidase activity and can play a role in releasing volatile compounds from non-volatile precursors.

Other non-*Saccharomyces* extracellular enzymatic activities, such as proteolytic and pectinolytic enzymes, might also be beneficial for winemaking. For example, proteolytic activity of some non-*Saccharomyces* yeasts could lead to a reduction in protein levels, increasing protein stability of the final product (Jolly et al., 2014).

1.4| Non-*Saccharomyces* yeasts: principal genera and characteristics.

Non-*Saccharomyces* yeast is a term used in winemaking sector to indicate many different yeast species. These yeasts are either ascomycetous or basidiomycetous, characterized by vegetative state, with reproduction predominantly by budding or fission, without sexual states with in or on a fruiting body (Jolly et al., 2014). Current taxonomy recognizes 150 yeast genera, comprising nearly 1500 species; of these, more than 40 species have been isolated from grape must in spontaneous fermentations (Goold et al., 2017; Gschaedler, 2017)

In Figure 1.1 microscopic images of the most frequent non-*Saccharomyces* yeasts found in winemaking are reported.

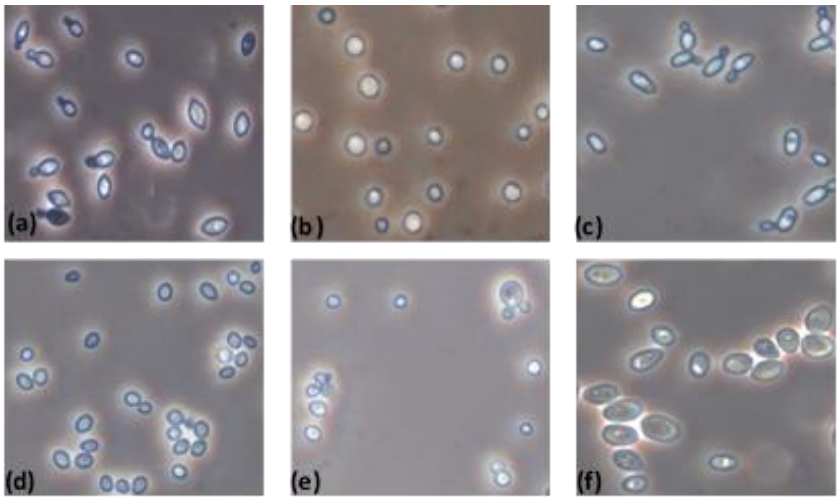

Figure 1.1| Microscopic observations of some non-*Saccharomyces* yeasts: (a) *Hanseniaspora uvarum:* (b) *Torulaspora delbrueckii;* (c) *Starmerella bacillaris,* (d) *Metschnikowia pulcherrima;* (e) *Debaryomyces polymorphus;* (f) *Zygosaccharomyces bailii.*

1.4.1| *Hanseniaspora*/*Kloeckera* species

The apiculate yeasts *Hanseniaspora* (anamorph *Kloeckera*) are one of the non-*Saccharomyces* yeasts present a significant proportion among the normal biota of grape berries, and found in the highest numbers in grape must (Jolly et al., 2014). Therefore, they should be in the best position to make a significant role in alcoholic fermentation and in wine flavour profile (Varela & Borneman, 2017). Species from the *Hanseniaspora* genus possess a characteristic apiculate shape (Figure 1.1a). Eleven species belong to the genus

Hanseniaspora, such as *H. clermontiae*, *H. guilliermondii*, *H. lachancei*, *H. meyeri*, *H. occidentalis*, *H. opuntiae*, *H. osmophila*, *H. pseudoguilliermondii*, *H. uvarum*, *H. valbyensis* and *H. vinae*. Among these, four species are of oenological interest: *H. guilliermondii*, *H. osmophila*, *H. vinae* and *H. uvarum*.

As most of the yeasts present at the beginning of spontaneous fermentation belong to this genus, in spontaneous fermentation the *Hanseniaspora* genus most likely influences alcoholic fermentations during the first phase (Benito et al., 2019a), until alcohol levels of about 4% are reached. At these levels, most *Hanseniaspora* strains cannot survive due to their low tolerance to ethanol. The *Hanseniaspora* species, in particular *H. guilliermondii*, *H. uvarum*, and *H. vinae*, are the most interesting source of enzymes for winemaking, such as β-glucosidase, β-xylosidase, glycolytic, and protease, in particular for application at industrial scale. The aroma improvements correlated to enzymatic activities result in production of higher concentrations of 2-phenylethyl acetate, acetate esters (such as isoamyl acetate), medium-chain fatty acid ethyl esters, and terpenes, and reductions in the final concentration of higher alcohols (Benito et al., 2019a; Jolly et al., 2014).

However apiculate yeasts may be associated with the production of undesirable flavour compounds, such as high levels of volatile acidity, sulphur compounds, etc. However, a wide biodiversity at strain level was found and the use of selected strains of apiculate yeasts might have a positive influence on the flavour profile of certain style wines (Jolly et al., 2014).

1.4.2| *Torulaspora* species

Torulaspora delbrueckii (anamorph *C. colliculosa*) (Figure 1.1b) is one of the most studied non-*Saccharomyces* species in winemaking, and it was one of the first commercial non-*Saccharomyces* yeast to be released (Benito et al., 2019a; Jolly et al., 2014). *T. delbrueckii* (formerly classified as *Saccharomyces rosei*) was previously suggested for vinification of musts containing low levels of sugar and acidity. *T. delbrueckii* is characterized by high purity fermentation, low production of glycerol, acetaldehyde, acetic acid, and ethyl acetate. One of the first advantages attributed to this species, when used in mixed fermentations with *S. cerevisiae*, was the reduction of the volatile acidity in wines (Benito et al., 2019a; Petruzzi et al., 2017). In addition, this species produces high levels of extracellular enzymes of oenological relevance, such as β-glucosidase. In fact, it was shown that the strong level of this enzymatic activity exhibited by this species enhanced wine sensory profile, by modulating the levels of nor-isoprenoids, terpenols, and lactones, in consequence of the hydrolysis from their respective precursors (Petruzzi et al., 2017; Renault et al., 2015). In wines obtained with mixed cultures of *T. delbrueckii* and *S. cerevisiae*, it

was found an increase in acetate esters and medium chain fatty acids, various thiols, α-terpineol and linalool. Most of potential biotechnological applications of *T. delbrueckii* rely on its fermentation capacity (Varela & Borneman, 2017). The management of *T. delbrueckii* is relatively easy compared to other non-*Saccharomyces* species due to its relatively high fermentative power, and the ability to tolerate ethanol concentration up to 9-10% (v/v). Due to the high ethanol resistance, this species can influence the characteristics of final wine during almost all the alcoholic fermentation, although *S. cerevisiae* is required to properly complete the alcoholic fermentation.

This species can improve the intensity and quality of wine aroma, increasing the overall impression and the varietal and fruity characters. Furthermore, *T. delbrueckii* is able to reduce the concentrations of higher alcohols when it is used in sequential fermentations with *S. cerevisiae*, contributing to increase the perception of the varietal character. Several authors have reported the production of wines containing high concentrations of fruity esters. The use of *T. delbrueckii* can decrease the final ethanol concentration in wines of about 1% (v/v), while increasing the glycerol concentration (Benito et al., 2019a; Petruzzi et al., 2017).

1.4.3| *Candida*/*Starmerella* species

The genus *Candida* is composed by more than 314 different species, several of which have been associated with winemaking. *Candida stellata* and *C. zemplinina* are species frequently isolated during the grape must fermentation.

Candida stellata was originally isolated from an overripe grape must in Germany; this yeast is characterized by spherical/ovoid cells, usually found as single cells. This yeast ferments glucose, sucrose, and raffinose and uses lysine as only source of ammonium. It is also able to grow at high pH values and it is not sensitive to ethanol. These features make it a good candidate for the use in co-inoculation with *S. cerevisiae*. This yeast is also frequently associated with musts obtained from botrytized grapes. An interesting feature of this species is the ability to produce high levels of succinic acid, which is also associated with high levels of glycerol. Succinic acid could positively influence the sensory/mouthfeel profile of wines with low levels of acidity.

Both *C. stellata* and *C. zemplinina* possess a strong fructophilic character, that is the preferential consumption of fructose rather than glucose (Varela & Borneman, 2017; Petruzzi et al., 2017), contrary to *S. cerevisiae*, which utilizes preferentially glucose (Jolly et al., 2014). For a long time, *C. stellata* was confused with *C. zemplinina*, mainly in consequence of their similarities, including their ecological habitat, particularly grape and wine environments, but the two species were differentiated on the basis of the analysis of

the ribosomal RNA sequence (Englezos et al., 2015; Sipiczki, 2004). *C. zemplinina* (synonym *Starmerella bacillaris*) is a non-*Saccharomyces* yeast, isolated for the first time in Napa Valley (California, United States) in 2002 (Englezos et al., 2017; Morata et al., 2020). *St. bacillaris* is organized in ellipsoid to elongated cells, single or pairs (Figure 1.1c). This yeast differs from other non-*Saccharomyces* yeasts as it is able to persist up to the middle-end phase of the fermentation process, due to its ability to tolerate high concentrations of ethanol (Englezos et al., 2017). In addition, *St. bacillaris* showed some interesting characteristics, when it is used in combination with *S. cerevisiae*, such as production of high glycerol levels, reduced ethanol yield and reduction of the acetic acid production. Additionally, the ability of this species to produce a broad spectrum of extracellular hydrolytic enzymes of oenological interest, determines an increase of concentrations of terpenes, lactones and malic acid degradation, with consequent increase of aroma complexity (Morata et al., 2020).

1.4.4| *Metschnikovwia* species

Metschnikowia pulcherrima (anamorph *C. pulcherrima*, Figure 1.1d) is another yeast commercially available, commonly associated with grapes and wine. The species more frequently isolated in grape must fermentation are *M. pulcherrima* and *M. fructicola*, although the main member of this genus associated to winemaking is *M. pulcherrima* (Jolly et al., 2014).

Recently, it was demonstrated the effect of this species on sensory profile of wine (Varela & Borneman, 2017). This species is a high producer of β-glucosidase, and its presence in mixed cultures can decrease the volatile acidity and increase the production of some compounds in final wine, such as medium-chain fatty acids, higher alcohol, esters, terpenols and glycerol. Another recent application of this species is the use in mixed fermentation with *S. cerevisiae* for reduction of ethanol concentration of wine, obtaining an ethanol decrease of about 1% (v/v) (Benito et al., 2019b; Varela & Borneman, 2017).

Strains of this non-*Saccharomyces* species produce an extra-cellular α-arabinofuranosidase, that impacts on the concentration of varietal aromas, such as terpenes and volatile thiols. *M. pulcherrima* has been reported to increase the levels of methyl butyl-, methyl propyl-, and phenethyl esters (Jolly et al., 2014; Petruzzi et al., 2017). The most relevant influence on wine quality related to the use of *M. pulcherrima* is the ability of the cystathionine-β-lyase activity produced from some strains to release varietal thiols, such as 4-methyl-4-sulfanylpentan-2-one, in concentrations higher than those produced by *S. cerevisiae* (Benito et al., 2019a). In addition, it has also been observed that *M. pulcherrima* might have an

antagonistic effect toward several yeasts, including *S. cerevisiae* which determines delays in fermentation. This phenomenon was due to a killer effect, although different from *S. cerevisiae* killer phenomenon, and it was linked to the production of pulcherrimin pigment by *M. pulcherrima*. (Petruzzi et al., 2017).

1.4.5| *Pichia*

The genus *Pichia* included 20 different identified species, most of them have been associated with winemaking, such as *P. fermentans*, *P. kluyveri*, *P. membranifaciens* and *P. occidentalis*.

Although different species are related to winemaking, *P. kluyveri* received most attention from wine researchers. *P. kluyveri* has been shown to release flavour precursors from grape juice, with a potential enhancement of wine aroma and flavour. Mixed fermentation with *P. kluyveri* has been reported to lead to higher levels of varietal thiols, especially 3-mercaptohexyl acetate (3MHA), 2-phenylethyl acetate and ethyl octanoate. It was shown that also the total terpene concentration increased, enhancing the grape variety typicity (Benito et al., 2019a; Jolly et al., 2014).

It has been reported that the use of *P. fermentans* in mixed fermentation with *S. cerevisiae* produces wines with increased concentrations of acetaldehyde, ethyl acetate, 1-propanol, *n*-butanol, 1-hexanol, ethyl caprilate, 2,3-butanediol and glycerol. Furthermore, the inoculum of *P. fermentans* increased concentration of ester and mannoproteins in wine (Varela & Borneman, 2017, Petruzzi et al., 2017).

1.4.6| *Rhodotorula* species

Rhodotorula species are orange-red pigmented, saprophytic yeasts that can be isolated from many environmental sources. Although these species are commonly found on grape berries and during wine fermentation, their use in winemaking is very limited (Varela & Borneman, 2017).

1.4.7| *Schizosaccharomyces* species

Three species belong to genus *Schizosaccharomyces*, *Schiz. japonic*us, *Schiz. octosporus* and *Schiz. pombe*, and all of them have been associated with winemaking, although only *Schiz. pombe* species has oenological relevance. *Schizosaccharomyces pombe* was initially considered as a spoilage yeast in consequence of the production of undesirable metabolites with a negative sensorial impact. However, this species is recommended for deacidification of wines produced in cold areas, characterized by too high level of acidity.

In this respect, *Schiz. pombe* is able to metabolize malic acid into ethanol and CO_2, reducing the total wine acidity (Petruzzi et al., 2017). In addition, *Schiz. pombe* specie is characterized by production level of higher alcohols and esters significantly lower than *S. cerevisiae* and other yeast species. This characteristic is very interesting for wine in which the preservation of the varietal aroma of grapes is desired more than the fermentative aroma (Benito et al., 2019a).

The use of *Schiz. pombe* in combined inoculums with *S. cerevisiae* allows the achievement of microbiological stability; in this way, the wine can be bottled without the risk of bottle refermentation. However, one of the main problems of using *Schiz. pombe* is the risk of production of high levels of acetic acid. Different strategies have been tested to reduce this undesirable effect, such as the mixed fermentation with *S. cerevisiae* or *T. delbrueckii* or the use of cells immobilized in alginate. Another undesirable effect of the use of *Schiz. pombe* is an increase in the ethanol concentration, as the degradation of malic acid produces additional ethanol (Benito et al., 2019a; Jolly at al., 2014; Petruzzi et al., 2017).

1.4.8| *Debaryomyces* species

The main species of this genus associated with wine are *Debaryomyces hansenii* and *D. polymorphus* (Figure 1.1e). *D. hansenii* species is an osmotolerant and halotolerant yeast. It was reported that this species produces β-glucosidases, with production of free monoterpenes from several grape-derived non-volatile precursors (Varela & Borneman, 2017). As a consequence, the use of mixed fermentation with *D. polymorphus* and *S. cerevisiae* resulted in an increased concentration of the terpenols, such as citronellol, nerol and geraniol (Petruzzi et al., 2017).

1.4.9| *Zygosaccharomyces* species

Among the species included in the genus *Zygosaccharomyces*, *Z. bailii* and *Z. rouxii* are frequently found during wine production. *Zygosaccharomyces* species was considered to be winery contaminants as this genus is characterized by production of high quantities of acetic acid, although different studies have highlighted the positive contribution of these species to wine fermentation. *Zygosaccharomyces* yeasts are characterized by high resistance to several stresses, such as low pH, high osmolality, high salinity. These yeasts also have been shown to enhance positive attributes in wine, including the improvement of flavour and aroma, in consequence of production of high levels of ethyl esters and mannoproteins (Domizio et al., 2011; Varela & Borneman, 2017). Some researchers found strains of *Z. florentinus* (actually classified as *Zygotorulaspora florentina*) characterized by production of low levels of acetic

acid, H_2S and SO_2 and high fermentation vigour, whereas others found a *Z. bailii* strain able to degrade malic acid and produce low amounts of H_2S. In addition, strains of both species flocculated, a positive characteristic for wine submitted to re-fermentation. Wines produced by mixed fermentation with combinations of *Z. bailii/S. cerevisiae* and *Z. florentinus/S. cerevisiae* contained high level of polysaccharides, which can have a positive influence on wine taste (Jolly et al., 2014). *Z. bailii* is fructophilic, metabolizing fructose more easily than glucose, a positive trait for grape musts from over-ripened grapes (Petruzzi et al., 2017). Furthermore, this species was used for production of low ethanol wine (Varela & Borneman, 2017). The Figure 1.1f shows a microscopic observation of *Z. bailii* cells.

1.4.10| *Lachancea*/*Kluyveromyces* species

Although three *Lachancea* species, *L. thermotolerans*, *L. kluyveri* and *L. lanzarotensis*, have been associated with grapes or wine, most research has been focused on *L. thermotolerans* (formerly *Kluyveromyces thermotolerans*).

Lachancea thermotolerans is the most recommended among non-*Saccharomyces* species to acidify grape juices that suffer from a lack of acidity due to its unique ability, among yeasts, to produce lactic acid during fermentative metabolism. Some strains of this species, characterized by low level of acetic acid production, can be used to reduce the final concentration of volatile acidity in wine. Additionally, it was found that the addition of oxygen during fermentations with *L. thermotolerans* increases the production of glycerol, while reducing the production of ethanol (Varela & Borneman, 2017). This species is characterized by moderate ethanol tolerance, in fact, it is considered a fermentative species able to ferment wines up to levels slightly higher than 10% (v/v) in ethanol, but it must be combined with *S. cerevisiae* to ensure completion of alcoholic fermentation. Additionally, some studies have observed that mixed fermentations between *L. thermotolerans* and *S. cerevisiae* reduce ethanol concentrations from 0.2% to 0.4% (v/v) (Benito et al., 2019a). It was reported that the use of *L. thermotolerans* in mixed fermentations produces wines with increased concentrations of lactic acid, glycerol and 2-phenylethanol (Jolly et al., 2014).

1.5| Application of non-*Saccharomyces* yeasts

The use of non-conventional yeasts, in controlled multistarter fermentation with *S. cerevisiae*, has been proposed and applied to take advantages of some their specific fermentative features. In this regard, numerous studies have found a wide intraspecific variability for oenological characters and a different behaviour when used in co-culture, due to interactions with *S. cerevisiae*. All these aspects have highlighted a significant role of

these non-conventional yeasts in determining the analytical and sensory profile of wine (Ciani et al., 2010; Mas et al., 2016).

The main applications of mixed cultures with non-conventional yeasts are the following: ethanol reduction, control of spoilage microflora and enhancement of wine quality.

1.5.1| Ethanol reduction

Nowadays, the increase of alcohol levels in wine is one of the main challenges affecting the winemaking sector, due to global climate change which determined an increase of grape maturity. In this context, the interest for reduction of ethanol content in wine was increased and among the available tools addressed to this aim, the microbiological approach appears a promising way. In particular, researchers' interest was addressed to investigate the wide variability in ethanol yield among non-*Saccharomyces* yeasts, that could be a potential tool for the reduction of alcohol content in wine (Contreras et al., 2014; Gobbi et al., 2013; Contreras et al., 2015a, b). Low ethanol yield was found in some strains of *St. bacillaris*, and *M. pulcherrima*, and in strains belong to *Hanseniaspora*, and *Zygosaccharomyces* genera (Gobbi et al., 2013). Ethanol yield, like other fermentation features, is a species-related trait but, similarly to other fermentation parameters, a pronounced intraspecies variability was also evident (Comitini et al., 2011; Domizio et al., 2011).

The regulatory respiro-fermentative metabolism in yeasts might be used as strategy to reduce the ethanol concentration in wine. In addition to a low ethanol yield, among non-*Saccharomyces* wine yeasts, some strains/species showed a sugar consumption by respiration (Crabtree negative). Both these approaches can limit ethanol production. Since most non-*Saccharomyces* yeasts are unable of completing alcoholic fermentation, *S. cerevisiae* strains should be added in simultaneous or sequentially inoculum modality. In this regard, different combinations of selected non-conventional yeasts, such as *M. pulcherrima*, *St. bacillaris*, and *T. delbrueckii*, have been proposed.

These yeast species are able to divert the carbon flux toward other metabolites rather than ethanol (Englezos et al., 2015; Varela, 2016).The different respiro-fermentative regulatory mechanisms of some non-conventional yeasts, compared to *S. cerevisiae,* was evaluated to reduce the ethanol content through partial and controlled aeration of the grape juice in simultaneous and sequential fermentation (Contreras et al., 2015a, b; Quirós et al., 2014). However, in simultaneous fermentation aeration condition showed consistent increase of volatile acidity, since in this condition *S. cerevisiae* usually produce large amount of acetic acid (Morales et al., 2015). Contrary, non-*Saccharomyces* yeasts produce very low amounts of volatile acidity, also during oxygenation. For these reasons, sequential fermentation,

inoculating before non-conventional yeasts with moderate aeration, followed by inoculum of *S. cerevisiae* in strict anaerobiosis could be a suitable strategy to avoid increase in acetic acid content and obtain, at the same time, the reduction of ethanol content. The results obtained, in terms of ethanol reduction in final wine, was promising (Quirós et al., 2014).

1.5.2| Antimicrobial activity of non-*Saccharomyces* yeasts

Another useful application of non-*Saccharomyces* yeasts in winemaking regards their use for the control of undesired microorganisms. During winemaking, the control of potential spoilage microorganisms is necessary to assure the final quality of wine as the growth of undesirable microorganisms can determine undesired organoleptic features of the final product. In this context, killer yeasts and toxins secreted by these microorganisms represent an interesting solution as antimicrobial agents, for the partial or complete substitution of the use of synthetic compounds. In fact, one of the topical subjects in winemaking is the reduction in the use of SO_2 and its partial or complete substitution with natural antimicrobials. Killer toxins are proteins or glycoproteins, naturally produced by yeasts, that kill cells of sensitive microorganisms; some of these toxins were purified and characterized. The mode of action of most of the killer toxins were well studied, even if the killing modalities towards the sensitive cells in some of the newly discovered killer toxins are still unknown (Liu et al., 2015).

Kluyveromyces phaffii (reclassified as *Tetrapisispora phaffii*) produces a killer toxin able to control the proliferation of apiculate yeasts during the pre-fermentation phase (Comitini & Ciani, 2010). Some researchers identified killer yeasts showing a potential antimicrobial effect against *Dekkera*/*Brettanomyces* in wine. These spoilage yeasts are responsible for formation of undesired odours in wine and actually are considered the major concern for winemakers, since an effective method to control their growth has not yet been developed. Belda et al. (2017) have identified and characterized two killer toxins from *P. membranifaciens* (PMKT1 and PMKT2), which are able to inhibit *B. bruxellensis*. Moreover, other two killer toxins (Kwkt and Pikt), produced by *Kluyveromyces wickerhamii* and *Wickerhamomyces anomalus*, respectively, showed an antimicrobial activity against *Brettanomyces*/*Dekkera* wine-spoilage yeasts (Oro et al., 2016).

1.5.3| Enhancement of wine quality

The use of non-conventional yeasts in controlled mixed fermentation has been proposed and applied also to take advantage of some their specific fermentative features. For example, *Schizosaccharomyce pombe* and/or *Schiz. japonicus* has been proposed as agent

for biological deacidification (Domizio et al., 2017). Another positive trait related to the involvement of non-*Saccharomyces* yeasts is the reduction of volatile acidity. The amount of volatile acidity produced by *S. cerevisiae* is usually low, but it may increase during fermentation of high-sugar media, as *S. cerevisiae* produce acetic acid as response to osmotic stress in consequence of upregulation of genes encoding for aldehyde dehydrogenases. Some non-*Saccharomyces* species do not respond in the same way to osmotic stress.

In this regard, *T. delbrueckii* in mixed fermentation with *S. cerevisiae* showed a consistent reduction of volatile acidity in high sugar fermentation. Similar behaviour was found for *C. stellata* (now reclassified as *St. bombicola*) in mixed culture with *S. cerevisiae*. Polysaccharides production is another relevant character that could be improved with the use of non-*Saccharomyces* yeasts. *S. cerevisiae* releases low amounts of polysaccharides, generally ranging from 50 to 150 mg/L, whereas non-*Saccharomyces* wine yeasts are generally characterized by the capacity to release a high quantity of polysaccharides (Comitini et al., 2011; Domizio et al., 2011; Gobbi et al., 2013). The possibility to increase naturally the content of mannoproteins by the use of these yeasts could represent a valuable possibility to enhance the overall quality of wines. In this regard, *M. pulcherrima, Saccharomycodes ludwigii, L. thermotolerans, Schiz. pombe, Schiz. japonicus* showed high polysaccharides production and could be used in mixed fermentation (Domizio et al., 2017). Polysaccharides can positively influence wine taste and mouth-feel by increasing the perception of wine "viscosity" and "fullness" on the palate (Jolly et al., 2014).

1.6| Concluding remarks

Although winemaking is one of the oldest biotechnological process, many years were necessary to understand the mechanisms behind the fermentation process, mainly regarding the microbiological aspects.

Starting from Pasteur's findings, it was established that alcoholic fermentation is a biotransformation process and *S. cerevisiae* is the primary microorganism involved. Nevertheless, a wide variety of microbial species may participate to alcoholic fermentation and contribute to the properties of final products. However, spontaneous fermentation is a hazardous and uncontrolled process, which was replaced with the practice of pure fermentation by inoculating selected starter cultures of *S. cerevisiae*. The use of antiseptic agents, such as SO_2, to which most of the non-*Saccharomyces* yeasts are scarcely resistant, guarantee the dominance of the inoculated strains. Although the extensive use of starter cultures is an important advance in wine biotechnology, the generalized use of selected cultures represents a simplification of microbial fermentation communities, promoting a

standardization of the analytical and sensory properties of final wines. Recently, in winemaking the request for wines with peculiar and distinctive characteristics was increased. This trend reinforced and encouraged the selection and the use of different yeast genera, with pronounced impacts on aroma and flavour. In fact, if *S. cerevisiae* remains the main manager of alcoholic fermentation, non-*Saccharomyces* species may have a complementary role on aroma, ethanol production, and microbial control in wine. In this context, the involvement of non-*Saccharomyces* yeasts in mixed fermentation with *S. cerevisiae* starter cultures could be a practical way to improve the complexity and to enhance particular characteristics of wine. Furthermore, the current interest on non-*Saccharomyces* yeasts was addressed toward the use of these non-conventional yeasts for ethanol reduction in wine, an emergent request in wine sector. The possibility of using non-*Saccharomyces* yeasts at industrial level for reducing alcohol levels will require an improved understanding of the metabolism of these alternative yeast species, as well as the interactions with different yeast starters during the fermentation of grape must.

History taught us that the best outcome for both winemaker and consumer is achieved when the wine industry harnesses what nature, human ingenuity and cutting-edge science offer in harmony with the unique "artistic" nature of wine.

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Chapter 2

Biotechnological strategies to reduce alcohol content in wines

Abstract

Over recent years, the average ethanol concentration of wine has increased as consequence of increased grape maturity. In fact, climate change has deeply influenced the vine phenology and grape composition, resulting in rising of grapes sugar concentration and, consequently, high alcohol content in wine. This increased ethanol content can have negative consequences on the wine characteristics, affecting the sensory properties of the wines, as high alcohol content reduces the perception of flavour and aroma complexity. Furthermore, wine containing high levels of alcohol gives rise problems to the human health and also to the economic aspects as it determines an increase of taxes. Among the several solutions currently under study, biotechnological approach based on the use of non-*Saccharomyces* yeasts during alcoholic fermentation is very promising for ethanol reduction and contemporary to improve wine characteristics. Non-*Saccharomyces* yeast species comprise a high number of species, characterized by high physiological diversity. The oenological interest of these microorganisms was initially triggered by their potential positive contribution to the sensorial complexity of wines, through the production of aroma and other sensory-active compounds which are not produced by single fermentation with *Saccharomyces cerevisiae*. The current interest toward these yeasts is addressed also to the ethanol yield on sugar, one of the most invariant metabolic traits of *S. cerevisiae*.

2.1| Introduction

Over the past years, it was increased consumers' request for well-structured and high aromatic wines. In order to satisfy these requests, grape and wine producers have extended

harvest times to increase phenolic maturity of the berries and to enhance the content of fruit flavours and colour intensity (Ciani et al., 2016; Goold et al., 2017; Maturano et al., 2019). However, high degree of grape maturity results in increased grape sugar concentration, which in turn results in wines with excessive alcohol levels, more than 15% (v/v). On average, the alcohol strength of red wines from many warm wine producing regions globally rose by about 2% (v/v) during last years (Goold et al., 2017; Mestre Furlani et al., 2017). Although many of these "full-bodied, fruit-forward" wines are well balanced and requested by some consumers, a significant consumer market segment prefers light wines, with less ethanol-derived hotness on the palate.

High levels of alcohol can have several adverse effects. For example, it can alter the sensorial quality of wines in consequence of an increase in the perception of bitterness, astringency and hotness and it can mask certain volatile aromatic compounds (Mestre Furlani et al., 2017; Goold et al., 2017; Varela & Varela, 2019). High ethanol content can lead also to stuck and sluggish fermentations as too high alcohol levels can inhibit some yeast strains, also belonging to *S. cerevisiae*. Furthermore, it is well known that beverages with high alcohol content can have negative psychological effects on human health. Lastly, wines with high ethanol levels can raise economic issues because some countries impose taxes, which can considerably increase the final price (Contreras et al., 2014; Mestre Furlani et al., 2017).

In conclusions, current consumer preferences, health concerns related to alcohol consumption, government policies and environmental conditions (growing global climate warming), have focused research attention on reducing alcohol concentration in wine. In fact, production of quality wines with decreased alcohol concentration, nowadays, is one of the major challenges facing wine producers (Varela et al., 2016). In this context, consumer-focused wine producers are developing and implementing several methods to reducing alcohol concentration of wine, including viticultural and pre-fermentation practices, microbiological strategies, and post-fermentation practices.

The biotechnological strategies initially explored to face this problem were based on genetic engineering of *S. cerevisiae*, a rational choice considering the preponderant role of this species in alcoholic fermentation. However, the use of these recombinant strategies encountered different obstacles, correlated both to yeast metabolism and the regulatory aspects of genetically modified organisms. Furthermore, this strategy can be expensive and can have negative effects on the organoleptic quality of the final product (Ciani et al., 2016; Mestre Furlani et al., 2017).

The most promising biotechnological strategy, currently under study, is the use of selected non-conventional yeasts during alcoholic fermentation (Ciani et al., 2016; Varela & Varela,

2019). Non-*Saccharomyces* wine yeast species were characterized by a wider physiological diversity than *S. cerevisiae*. This diversity also involves ethanol yield on sugar, one of the most invariant metabolic traits of *S. cerevisiae*. Current knowledge indicates that ethanol yield on sugar is not only species-specific, but often strain-specific.

Since most non-*Saccharomyces* wine yeasts are sensitive to high ethanol concentrations, in order to avoid stuck or sluggish fermentation, the use of *S. cerevisiae* starters (Ciani et al., 2016) is still required.

In this context, recent studies have reported a reduction in ethanol concentration using non-*Saccharomyces* yeasts in co-cultures with *S. cerevisiae*, compared to the ethanol concentration obtained with *S. cerevisiae* as single inoculum. The proper mixed starter management during fermentation will allow winemakers to tailor wines to the changing demands of consumers (Ciani et al., 2010; Contreras et al. 2015; Englezos et al., 2016; Maturano et al., 2019; Mestre Furlani et al., 2017; Varela, 2016).

Therefore, with the aim of presenting new evidence on the potentiality of non-*Saccharomyces* yeasts, research was mainly focused on non-*Saccharomyces* yeast selection, and on design of mixed starters, directed to produce wine with a reduced ethanol concentration. Particular consideration is given to the impact of low ethanol-producing yeasts on the volatile composition and sensory profile wine.

2.2| New trends in wine sector

Wine's history parallels that of the civilization of human kind, and for decades, humans have exploited the fermentation activity of yeast as a means of preservation of grape juice.

After the knowledge of fundamental role of yeast metabolism on wine characteristics, together to development of modern vineyard practices, winemaking equipment and ever-changing consumer preferences, placed the global wine industry on a never-ending journey of today's innovation is tomorrow's tradition (Goold et al., 2017; Padilla et al., 2016).

One consumer driven innovation that has become a tradition is the extension of the time for grape harvesting in warm wine producing regions. Late harvests are indeed required to meet actual consumer's preferences toward well structured, full body wines, and optimal phenolic maturity of grapes. These later harvested grapes produced wines not only with enhanced ripe fruit flavours and wine colour intensity, but also reduced undesirable unripe, vegetal wine flavours. However, this practice results in a noticeable increase in the sugar content of the berries, which results in higher alcohol concentration in the final wine. Rich, ripe fruit flavours and more intense colour, but higher alcohol is a double-edged sword of this new style of wine category.

On the other hand, global climate change has deeply influenced the vine phenology and the grape composition, resulting in grapes with lower acidity, altered phenolic maturation and tannin content, and increasing sugar concentration. These changes further contribute to rising alcohol content in wines, in addition to modifying other wine sensory attributes as well as wine microbiology (Ciani et al., 2016; Contreras et al., 2014; Englezos et al., 2016; Goold et al., 2017). In fact, in sunny, warmer regions, the average alcohol content has risen by approximately 2% (v/v) over the past years. While in the past it was rare to encounter wines with alcohol concentrations more than 14% (v/v), now it is not uncommon to find wines with an alcohol concentration higher than 16% (v/v). As a consequence, the interest of the global wine industry in the lowering the alcohol content in wines is increased for different interconnected reasons.

Firstly, it is increased the consumers' attention for health aspects of food and beverages; in the actual health and safety conscious society, wines with high alcohol concentration attract constant negative remarks from health professionals. The harmful effects of alcohol abuse have been widely reported, although the health benefits demonstrated by moderate wine consumption. Additionally, high alcohol concentration can negatively affect the sensory properties of a wine. In fact, the high ethanol content in wine can lead to stuck or sluggish fermentations, and to the production of unbalanced wines that are unpleasant for consumers. Although many wines with higher alcohol concentration are full-bodied and rich in ripe fruit flavours, in some cases and depending on wine style, too high ethanol concentration could increase hotness and bitterness perceptions, while it decreases acidity sensations and masks the perception of some important aroma compounds, such as higher alcohols, esters and monoterpenes. In fact, balance between alcohol strength, tannin, acidity, sweetness and fruit flavour intensity is extremely important for overall quality of the wine (Ciani et al 2016; Contreras et al., 2014; Goold et al., 2017).

Finally, economic issues have to be considered as some countries apply additional taxes on high alcohol wines. In order to overcome these issues, the market focus is looking for reduction of ethanol content in wines. Consequently, the general interest for researchers and winemakers is focused on developing practices aiming to reduce the alcohol concentration in wine by about 1-2% v/v, in order to compensate the impact of global warming and to obtain better-balanced wines. The winemaking industry is addressing this challenge by targeting almost all the different steps of the production cycle, and it is focusing on four main strategies, which are grape-growing and viticultural practices; pre-fermentation and winemaking practices; microbiological practices and strain development programmes; and post-fermentation practices (Ciani et al., 2016; Englezos et al., 2016; Goold et al., 2017;

Gschaedler, 2017). The viticultural practices to reduce ethanol content in wine act to manage grapes sugar content through different approaches, such as reducing leaf area, optimizing the harvest date by not harvesting overripe grapes with excessive sugar concentration. As regards the pre-fermentative stage, the reduction of sugar concentration in must could be achieved through blending of early harvested grapes (containing low sugars) with well ripened, flavour intense grapes, by dilution of grape must with water, depending of country regulation, or using nanofiltration technologies. Another pre-fermentative strategy to remove sugar from grape must could be by addition of enzymes, such as glucose oxidase.

The post-fermentation technologies that could be used for ethanol reduction, include the blending of low and high alcohol wines, physical removal of alcohol by using membrane systems (such as reverse osmosis or pervaporation), osmotic distillation and extraction with supercritical carbon dioxide (Ciani et al., 2016; Goold et al., 2017; Liguori et al., 2018).

Despite their effectiveness of removing the required amount of alcohol, the majority of techniques currently available in the market usually require the application of intense practices on wine, which could be detrimental for wine quality, with high risk of losing some flavour compounds (Iris et al., 2020).

2.3| Microbiological approaches to reduce alcohol content in wine

The microbiological techniques for reducing alcohol content in the wine are mainly focused on the development of yeast strains with decreased efficiencies of ethanol production, for example, strains that produce higher concentrations of glycerol instead of ethanol. However, development and application of yeast strains showing alcohol production below usual level has been a recurrent objective for wine biotechnology for different years, before increasing ethanol content in wines was widely perceived as a problem (Ciani et al., 2016; Goold et al., 2017). Microbial approaches to reduce the ethanol production during wine fermentation include the isolation of new strains of *Sacchromyces* and non-*Saccharomyces* yeasts, characterized by low ethanol production and with optimal oenological properties, or the application of genetic modification techniques able to change the direction of sugar flux away from ethanol and toward other compounds.

2.3.1| Genetic engineering of *Saccharomyces cerevisiae*

In consequence of principal role of *S. cerevisiae* in alcoholic fermentation during winemaking, this is the species of choice for all researches addressed to reduce ethanol yield (Contreras et al., 2014; Padilla et al., 2016; Petruzzi et al., 2017; Valera, 2016). Although high variability was found among wild isolates of *S. cerevisiae*, different strains of this

species produce similar ethanol yield, resulting in comparable alcohol concentrations when different strains ferment the same must. Research efforts have therefore been directed to developing *S. cerevisiae* strains that produce wines with lower alcohol concentrations. In wine yeasts, gene modification technologies have been used to partially divert carbon metabolism away from ethanol formation, by redirecting carbon to other final products, and attempting to maintain wine quality. Researchers have designed several approaches based on genetic engineering in order to partially redirect *S. cerevisiae* normal carbon flux (Ciani et al., 2016; Contreras et al., 2014; Valera, 2016). A schematic representation of genetic modification strategies, with the aim of creating yeasts able to reduce the production of ethanol by redirecting the carbon flux toward the production of other metabolites interesting for wine, such as glycerol, organic acids, and esters, is reported in Figure 2.1 (Goold et al., 2017; Varela & Varela, 2019).

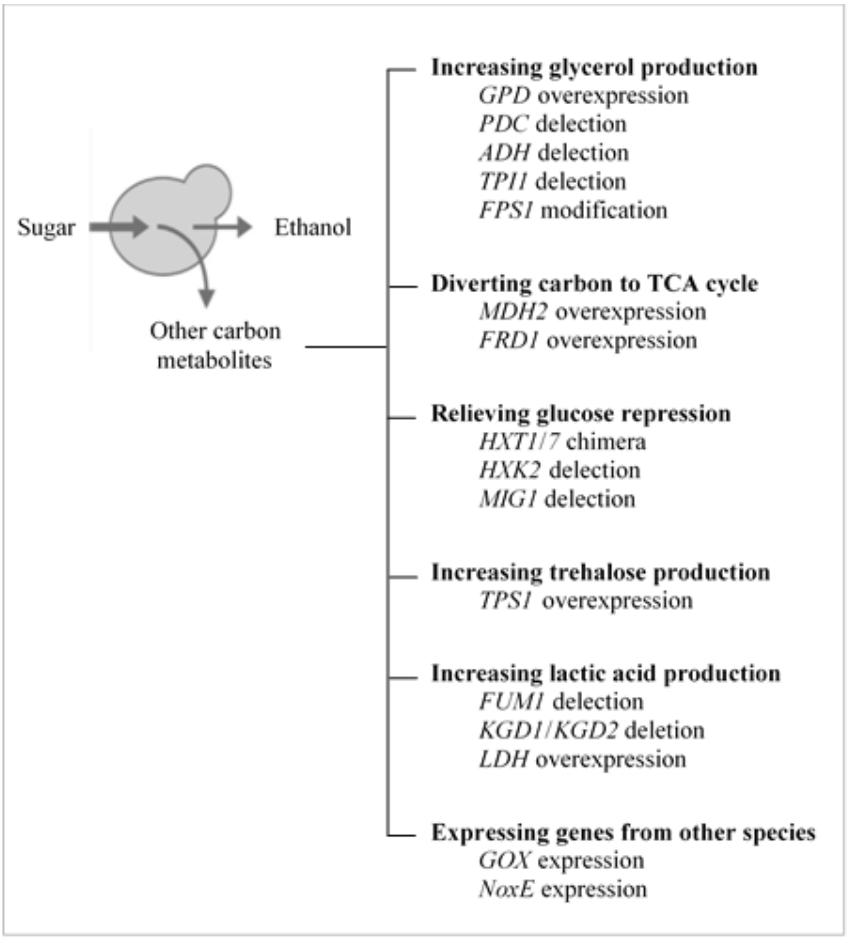

Figure 2.1| Different genetic modification to divert the metabolism of wine yeast in *S. cerevisiae* away from ethanol formation by redirecting carbon to other metabolites (Varela & Varela, 2019).

The most relevant genetic modification strategies to decrease ethanol production in *S. cerevisiae* include: increasing glycerol production, diverting carbon to the tricarboxylic acids

(TCA) cycle, reducing glucose repression, increasing trehalose production, increasing lactic acid production and expressing genes from other species.

Some of the strategies to divert the metabolism of wine yeasts away from ethanol formation, by redirecting carbon toward an increasing glycerol production, are the following:

- the overexpression of the yeast *GPD1* and/or *GPD2* genes, which encode glycerol-3-phosphate dehydrogenase isozymes;
- the modification of the glycerol transporter encoded by *FPS1*;
- the deletion of the *PDC2* gene encoding pyruvate decarboxylase;
- the impairment of alcohol dehydrogenases encoded by *ADH1*, *ADH3*, *ADH4* and *ADH5*;
- the deletion of *TPI1*, which encodes triose phosphate isomerase.

However, among all the genetic modifications, the overexpression of *GPD1* was the most efficient strategy for lowering alcohol yield. The choice of *GPD* genes was additionally driven by glycerol contribution to sweetness, smoothness and wine body. Genetic modifications approaches resulted very effective for reducing ethanol concentration in wine, with some engineered strains producing 1.5-2.5 % v/v less ethanol than the corresponding parental strains (Ciani et al., 2016; Goold et al., 2017; Varela & Varela, 2019).

However, the genetic engineering approach has some limitations. Despite positive results, it is still not legally permitted in many countries. Furthermore, it has to be considered also the negative public perception toward the use of genetically modified organisms in food and beverage production. As a consequence, it is not possible to completely assess the success of these engineered strains.

2.3.2| Use of non-*Saccharomyces* yeasts

Another microbiological alternative to produce wines with a reduced alcohol content and, at the same time, to improve their sensory quality, would be through the selection of non-*Saccharomyces* yeasts. These yeasts are naturally able to direct the sugar consumption to the production of other metabolites with positive sensory impact instead of synthesizing ethanol (Iris et al., 2020, Varela, 2016; Varela & Varela, 2019). Non-*Saccharomyces* wine yeasts were usually different from *S. cerevisiae* in metabolic flux distribution during fermentation and, consequently, in ethanol production, biomass synthesis, and by-product formation (Ciani et al., 2016). In consequence of numerous reports describing the potential of non-conventional yeasts to enhance sensorial complexity and aroma profile of wines (Fleet, 2008; Ciani et al., 2010), their role in the production of fermented beverages has been revised (Ciani et al., 2016; Comitini et al., 2011; Goold et al., 2017; Jolly et al., 2014; Varela

& Varela, 2019), generating a growing interest in isolating and characterizing non-*Saccharomyces* yeasts for development of new starter cultures.

The development of non-*Saccharomyces* commercial starters is related to the increasing consumer demand for wines showing improved sensorial properties and distinctive flavor, in contrast to the limited complexity attributed to wines fermented with *S. cerevisiae* starter strains.

In this context, the intense research activity around non-*Saccharomyces* wine yeasts, increasing awareness about the metabolic diversity of these yeasts, and the availability on the market of non-*Saccharomyces* starters opened new opportunities to exploit metabolism of these yeasts with the aim of also reducing ethanol content of wines. Contrary to *S. cerevisiae*, non-*Saccharomyces* yeasts show, generally, lower ethanol production and ethanol resistance. Current knowledge indicates that, similarly to other metabolic traits, ethanol yield on sugar is not only species-specific, but often strain-specific. Therefore, some non-*Saccharomyces* yeast species can show ethanol yields similar than *S. cerevisiae*, but many of them show reduced ethanol yields in consequence of conversion of some grape sugars to metabolites different from ethanol (Ciani et al., 2016; Gobbi et al., 2014; Padilla et al., 2016; Varela, 2016).

Some of the mechanisms responsible for reduced ethanol yields include altered biomass synthesis, by-product formation and/or alternative regulation of respiration (Goold et al., 2017; Varela, 2016). Several non-*Saccharomyces* species have shown potential for producing reduced alcohol wines when used as single or mixed starter with *S. cerevisiae*.

The species tested until now in mixed starters with *S. cerevisiae* are *Candida zemplinina* (*Starmerella bacillaris*), *Debaryomyces pseudopolymorphus*, *Hanseniaspora guilliermondii, H. uvarum, H. vineae*, *Issatchenkia orientalis*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia fermentans*, *P. kluyveri*, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, and *Zygosaccharomyces bailii* (Gschaedler, 2017).

However, the concept of mixed fermentation is not new to the wine industry. Commercial mixtures of *T. delbrueckii* or *Kluyveromyces* (current *Lachancea*) *thermotolerans* in conjunction with *S. cerevisiae* are already used to produce wines with richer and rounder flavours (Jolly et al., 2014).

Although these commercialized yeast blends were not primarily developed to reduce the concentration of ethanol in wine, selected strains of non-*Saccharomyces* yeasts could be developed as co-cultures for the reduction of alcohol concentration in wine. The choice and compatibility of non-*Saccharomyces* and *S. cerevisiae* strains will be crucial and dependent on wine type (Goold et al., 2017).

Some important parameters should be taken in account for this purpose, such as the inoculation level and the timing between the first and second inoculation, nutrient consumption and sulphite content. High inoculation level of non-*Saccharomyces* yeast improves the competitiveness toward *S. cerevisiae* and other wild yeasts, while the interval between the first and the second inoculation affects the duration of this metabolic activity, which will quickly decline upon inoculation of *S. cerevisiae* (Ciani et al, 2016; Goold et al., 2017). When using non-*Saccharomyces* yeasts in mixed starters, generally two practices of inoculation were used. The first, known as simultaneous inoculation, involves the inoculation of the selected non-*Saccharomyces* yeasts at high cell concentration together with *S. cerevisiae*, while the second, sequential inoculation, implies that the selected non-*Saccharomyces* yeasts are first inoculated at high levels and allowed to ferment for a given amount of time, after that *S. cerevisiae* is added to take over the fermentation. Both practices are feasible, but the potential interactions between yeasts could determine which inoculation strategy is more appropriate (Padilla et al., 2016).

The sequential modality allows to take advantage of the metabolism of the first inoculated non-*Saccharomyces* yeast without the influence of the *Saccharomyces* starter culture. In this way, the reduction in ethanol content will depend on the metabolic characteristics of the non-*Saccharomyces* strain used, which can leave its metabolic footprint before *S. cerevisiae* takes over (Ciani et al., 2016).

Literature data reported that the use of non-*Saccharomyces* species allows to reduce the initial ethanol content by about 1-2% (v/v), depending on the yeast species and fermentation conditions (Benito et al., 2019; Ciani et al., 2016). Some studies have reported moderately reduced ethanol yields when using non-*Saccharomyces* and *S. cerevisiae* strains in co-inoculation or sequential inoculation, with the decreases in the ethanol concentration ranging from 0.2 to 0.7% (v/v), compared to the ethanol concentration achieved with a single *S. cerevisiae* inoculum (Contreras et al., 2014). Iris et al. (2020) reported that non-*Saccharomyces* species have shown potential to produce wines with reduced ethanol concentration when used in mixed fermentations at lab scale with *S. cerevisiae*, obtaining a reduction between 0.5 and 1% v/v.

A sequential inoculation of *M. pulcherrima* followed by *S. cerevisiae* wine strain produced a wine with an ethanol concentration lower that achieved with *S. cerevisiae*. In fact, lab-scale wines showed about 0.9-1.6% (v/v) less ethanol than control wines produced with only *S. cerevisiae*. Similarly, strains of the species *H. uvarum*, *Z. bailii* and *Z. bisporus* were identified as useful candidates to produce wines with reduced ethanol concentration when used as mixed starters (Ciani et al., 2014; Contreras et al., 2014; Gobbi et al., 2014).

Similar results were found by Goold et al. (2017) with *H. opuntiae* and *H. uvarum*. In the sequential fermentations using *L. thermotolerans* as starter culture and inoculating *S. cerevisiae* 48 h after the beginning of fermentation, an average reduction in the alcohol content of approximately 1.5% v/v was achieved, with respect to *S. cerevisiae* pure fermentation (Iris et al., 2020). In other trials, performed at industrial scale, a mixed starter composed by *L. thermotolerans* and *S. cerevisiae* (inoculated after two days) allowed to obtain an ethanol reduction of 0.7% (v/v) (Ciani et al., 2016; Iris et al., 2020).

Among, the non-*Saccharomyces* species of oenological interest, *St. bacillaris* is considered as one of the most promising species for reducing ethanol content in wine (Tristezza et al., 2013). The ability of this species to produce low ethanol from sugar consumed supports the potential use of this wine yeast, in combination with *S. cerevisiae* either in co-inoculated or sequential fermentations (Englezos et al., 2016). Mixed fermentation with *St. bacillaris* and *S. cerevisiae* using a sequential inoculation were tested in different studies. The reduction in ethanol concentration in these trials varied from 0.5% (v/v), for pilot scale fermentations, to 0.70-0.90% (v/v), during laboratory scale fermentation using natural must. The use of *St. bacillaris* in mixed fermentation with *S. cerevisiae* reduced the ethanol content in wine of about 0.32% (v/v), with contemporary increase of glycerol content (Ciani et al 2016; Englezos et al., 2017; Goold 2017).

The species *P. fermentans* has also demonstrated its ability to reduce the alcoholic concentration between 0.9 and 1.6% v/v, when used in sequential fermentation with *S. cerevisiae*. Table 2.1 lists recent studies reporting the use of non-*Saccharomyces* yeast species in mixed fermentation for reduction of the ethanol content in wine, indicating also their impact on wine composition.

Some important characters of non-*Saccharomyces* yeasts are involved in ethanol reduction, such as the oxidative metabolism observed in some non-*Saccharomyces* species. Therefore, oxygenated fermentation, used to stimulate yeast respiration, introduce a new challenge for managing mixed fermentations. *S. cerevisiae* is the Crabtree-positive yeast species; this metabolic feature strongly favours fermentative metabolism over respiratory. Only under conditions of very low sugar availability, respiration is the main energetic metabolic pathway in this species. Unlike *S. cerevisiae* yeasts, many non-*Saccharomyces* yeasts have different respiro-fermentative regulatory mechanisms. They can divert consumption of carbon sources toward products different from ethanol. Under aerobic conditions during the first stages of winemaking, different yeasts are able to consume sugar and therefore, “burn off” carbon, that would otherwise go to ethanol formation, by respiratory metabolism (negative Crabtree Effect), while *S. cerevisiae* is able to ferment sugar despite oxygen availability

(positive Crabtree Effect). However, aeration can enhance the growth and the persistence of non-*Saccharomyces* yeasts during wine fermentation, but it could also negatively impact wine sensory profile increasing the concentration of some off-flavours (Ciani et al., 2016; Iris et al., 2020; Varela, 2016; Varela & Varela, 2019).

Table 2.1| Recent studies evaluating the use of non-*Saccharomyces* yeast to reduction of ethanol content in wine and their impact on wine aroma composition (Varela & Varela, 2019).

Non-*Saccharomyces* yeast (simultaneous or sequential inoculation)	**Ethanol reduction**	**Impact on chemical and sensory composition**	**References**
St. bombicola/ S. cerevisiae	0.7% v/v (Lab-scale)	Increased glycerol concentration, decreased floral and fruity, increased ethyl acetate sensory descriptors	Ciani et al., 2016
St. bombicola/ S. cerevisiae	0.6% v/v (Pilot-scale immobilized cells)	Increased glycerol and succinic acid content, decreased esters and higher alcohols	Varela & Varela, 2019
H. uvarum/ S. cerevisiae	0.8% v/v (Lab-scale)	Increased hazelnut, coffee, caramel, cherry and acetone sensory descriptors	Rossouw & Bauer, 2016
L. thermotolerans/ S. cerevisiae	1.2% v/v (Lab-scale)	Reduced aromatic intensity and aromatic quality, increased herbaceous character	Del Fresno et al., 2017
M. pulcherrima/ S. cerevisiae	0.9-1.6% v/v (Lab-scale)	Increased concentration of esters and higher alcohols, decreased volatile acids content	Contreras et al., 2014
M. pulcherrima/ S. cerevisiae	1.4% v/v (Lab-scale immobilized cells)	Increased geraniol and acetaldehyde content	Canonico et al 2016; Ciani et al 2016; Contreras et al 2014
St. bacillaris/ S. cerevisiae	0.7% v/v (Lab-scale)	Increased glycerol content	Canonico et al 2016; Englezos et al., 2016
St. bacillaris/ S. cerevisiae	1.6% v/v (Lab-scale immobilized cells)	Increased ethyl acetate and isoamyl acetate concentration	Canonico et al., 2016
T. delbrueckii/ S. cerevisiae	1.6% v/v (Pilot-scale)	Increased aromatic intensity and fruity sensory attributes	Tronchoni et al., 2018

Aeration is a practice used during wine production and performed through macro-oxygenation techniques. Some authors studied the possibility of using Crabtree-negative non-*Saccharomyces* yeast strains, in a pre-fermentative aerobic stage, to reduce the levels of sugar in the grape must by sugar respiration and thus limit the potential alcohol content of final wine. Lab-scale trials have reported the use of strains of *M. pulcherrima* and *Kluyveromyces lactis*, able to respire when oxygen was introduced into must, determining a

reduction of ethanol concentration. Other researches showed that, when oxygen was supplied to grape must, *M. pulcherrima/S. cerevisiae* mixed cultures were able to produce lab-scale wines with 2.2 % (v/v) lower alcohol concentration than wines obtained by pure cultures of *S. cerevisiae*. Similar results were obtained by using *T. delbrueckii* and *Z. bailii*; when aeration was provided in sequential inoculation, these strains reduced ethanol concentration of 1.5 and 2.0 % (v/v), respectively, compared to levels detected in *S. cerevisiae* control wines (Morales et al., 2015; Varela, 2016).

More recently, the use of sequential fermentation with immobilized non-*Saccharomyces* wine yeasts (*St. bombicola, M. pulcherrima, H. uvarum* and *H. osmophila*) was proposed as strategy to reduce the ethanol concentration in wine. Wines produced with these starters showed reduced ethanol concentration, in a range between 1.65 and 1.00% (v/v), in comparison to *S. cerevisiae* wines. In particular, by using a sequential fermentation of 72 hours, it was obtained an ethanol reduction of 1.64% (v/v) for *St. bombicola*, 1.46% (v/v) for *M. pulcherrima*, 1.21% (v/v) for *H. uvarum* and 1.00% (v/v) for *H. osmophila*.

Wines produced by co-inoculation and sequential fermentation with *St. bombicola*, immobilized in beads, and *S. cerevisiae* were characterized by an ethanol content lower than 0.9% (v/v) (co-inoculation) and 1.6% v/v (sequential fermentation) in comparison to wine obtained with *S. cerevisiae* in pure fermentation. Furthermore, the obtained wines did not contain negative fermentation products, but rather an increase of some desirable compounds (Canonico et al., 2016; Ciani et al., 2016; Iris et al., 2020; Varela, 2016).

2.4| Concluding remarks

In conclusion, numerous scientific evidences showed that strategies based on the use of specific yeast starters, in particular selected mixed starters, can be effective in producing wine with reduced ethanol concentration. The benefits of mixed cultures should be tested in different grape musts since different nutritional characteristics of grape must might modify the impact of the individual components of the starter on the final wine. Moreover, mixed cultures should be tested at industrial or semi-industrial scales because it has been reported that the production of different metabolites can vary depending on the fermentation volume and the oxygen conditions. Thus, rational design of mixed cultures should take into account not only results from screening at laboratory scale, that allow exploiting positive features of non-*Saccharomyces* yeasts, but also trial at pilot scale levels, in order also to explore the potential interactions among microorganisms. Currently, only a limited number of studies describing fermentation trials at industrial scale is available. Although these trials are often expensive and require an industrial partner, it is necessary to test selected starters at industrial

scale in order to evaluate their real contribution to the sensorial characteristics of wine and their ability to reduce alcoholic concentration of the final wine.

If the 20th century was the time of *S. cerevisiae*, the 21st is the time of non-*Saccharomyces* yeasts. The interest toward the applications and commercialization of these species is constantly increasing for formulation of new style wines, characterized by reduced ethanol concentration, and modified wine aroma, colour and structure (Morata et al., 2020).

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Chapter 3

Screening on oenological properties of non-*Saccharomyces* yeasts

Abstract

The current researches in winemaking has highlighted the beneficial contribution of non-*Saccharomyces* yeasts to wine quality, in terms of complexity and organoleptic richness. By considering that the contribution of non-*Saccharomyces* yeasts during the fermentation process cannot be ignored, an useful tool to enhance the wine quality is represented by the use of selected autochthonous non-*Saccharomyces* strains. At this aim, the study of physiology of these yeasts is necessary for selection of yeast strains that have specific influences on process efficiency and wine quality. The research activity of this work started with first screening among 33 non-*Saccharomyces* strains, belonging to four specie (*Debaryomyces polymorphus, Hanseniaspora uvarum, Starmerella bacillaris and Zygosaccharomyces bailii*). The strains were evaluated for genetic and some technological traits.

The genetic diversity of 33 strains was investigated, using MSP-PCR with the primer $(GTG)_5$. All the strains were characterized for resistance to antimicrobial compounds (such as ethanol, sulfur dioxide and copper) and presence of enzymatic activities of oenological interest. Some of the tested strains showed interesting oenological traits, representing suitable candidate to be used as mixed starter cultures with *S. cerevisiae* for modulation of wine flavour and aroma.

3.1| Introduction

It's well known that the inoculation of selected starter cultures of *S. cerevisiae* is one of the most common practice used in the wineries worldwide in order to assure a more

controlled process with better predicted outcomes, avoiding the risks of contamination and inconsistent quality due to vintage variation (Binati et al., 2019). However, despite all these advantages of using selected starters of *S. cerevisiae*, in more recent years a strong debate among winemakers and scientists about the "standardization" of the wines due to the use of the same cultures was diffused among winemakers and scientists. The homogeneity of fermentations is one of the goals of the inoculation, but at the same time the extensive use of this approach determines the loss of wine aromatic complexity and distinct characteristics (Fleet, 2008; Mateus et al., 2020; Padilla et al., 2016), which could presumably be achieved with the indigenous microbiota associated with spontaneous fermentations (Binati et al., 2019). In fact, the inoculum of starters at high concentrations rapidly dominate over the indigenous population, limiting their involvement in the process. Many genera of yeasts belonging to the group called non-*Saccharomyces* participate in the winemaking process in the first steps of spontaneous alcoholic fermentation, although they are usually suppressed by *S. cerevisiae* due to their limited tolerance to ethanol (Comitini et al., 2011).

Several environmental parameters can affect the number and presence of different species, such as grape variety, geographical location, climate, vineyard treatments, technological practices, processing stage and season (pre-harvest, harvest, post-harvest) (Varela & Borneman, 2017).

In the past three decades, the interest toward the beneficial role of non-*Saccharomyces* yeasts in wine biotechnology was increased. These yeasts increase the sensory complexity and quality of wines (Berbegal et al., 2017), mainly in consequence of presence of enzymatic activities, such as esterases, β-glucosidases, lipases, and proteases (Belda et al., 2016; Tofalo et al., 2016).

As reported for *S. cerevisiae*, also among non-*Saccharomyces* yeasts a wide biodiversity at strain level for genetic and technological characteristics is reported (Padilla et al., 2016). In consequence of this, before the use in winemaking, a strong selection process is necessary in order to individuate the non-*Saccharomyces* strain most suitable to be used as mixed starter in combination with *S. cerevisiae*.

The goal of this step was to screen different non-*Saccharomyces* strains in order to investigate the potential of these strains to be employed in alcoholic fermentations. For this purpose, a collection of yeasts of different origins was subjected to molecular and physiological characterizations, with emphasis on parameters of enological interest. Non-*Saccharomyces* yeasts, belonging to the species *D. polymorphus*, *H. uvarum*, *St. bacillaris* and *Z. bailii*, were studied in order to individuate new strains with potentially positive oenological traits. The 33 strains were submitted to a preliminary screening, based on

evaluation of genetic variability, by using MSP-PCR with the primer $(GTG)_5$, and for the main characteristics of oenological interest, such as the production level of undesirable compounds (hydrogen sulphide) or resistance to antimicrobial compounds potentially present during winemaking, such as ethanol, sulfur dioxide and copper.

3.2| MSP-PCR Fingerprinting

Monitoring the contribution of each species or population, both in industrial microbiology or yeast diversity studies, involves the isolation and analysis of a large number of isolates. Yeast identification is currently based on sequencing of domains 1 and 2 (D1/D2) of the rRNA gene and/or the ITS1-5.8S-ITS2 region, proposed as a universal barcode (Kurtzman et al., 2014).

The evaluation of biodiversity among high numbers of strains can be performed by using RAPD and micro/minisatellite PCR techniques, which, although characterized by low levels of reproducibility, are widely used for strain biotyping. Among them, the Microsatellite/Minisatellite Primed (MSP)-PCR Fingerprinting techniques using the primers $(GAC)_5$, $(GACA)_4$, $(GTG)_5$ and M13 has been widely applied in the literature (Ramirez-Castrillon et al., 2014). M13 core sequence, widely used in the study on ascomycetous yeasts, was useful to evaluate the variability among autochthonous yeast population during spontaneous fermentations of grape musts. PCR with this primer was found to result in characteristic band patterns when different wine yeast species were compared (Lopandic et al., 2008; Pfliegler et al., 2014). This primer was also used (Tofalo et al. 2009) to characterize *St. bacillaris* isolates.

The primer $(GTG)_5$ was frequently used to discriminate species of the genus *Saccharomyces* and to characterize strains of non-*Saccharomyces* species (Caruso et al., 2002; Ramirez-Castrillon et al., 2014). Rantsiou et al. (2012) used both these micro/minisatellite primers to successfully differentiate between pathogenic *Candida* spp, although a low intraspecific variation was described.

3.3| Non-*Saccharomyces* yeasts selection criteria

The primary goal of yeast is to rapidly and efficiently convert simple sugars into ethanol without developing unpleasant flavors. Several factors affect the yeast ability to grow in the fermentative media related with the type and style of beverage produced; therefore, the ability to adapt and to cope with this hostile environment is considered the main feature to select yeast. Yeast cells are exposed to several stress conditions from the beginning to the end of the fermentation process, resulting in the reduction of their growth and survival rate,

which can determine a decrease in fermentation efficiency. Yeasts able of overcoming these conditions with low loss of viability are the best candidate for fermentation process. In this section, some of the most relevant inhibiting conditions present in winemaking are summarized.

Carbohydrates

Carbohydrates are the most important nutrient since they are metabolized to form biomass, ethanol, and different by-products such as volatile compounds, glycerol, and others that will affect the sensorial characteristics. However, they are also responsible of stress due to osmotic pressure in the cells after their inoculation. Therefore, tolerance to high sugar concentration is one of the main criteria for yeast selection. Yeast cells have developed mechanisms to adjust to high external osmolarity and maintain or re-establish an inside-directed driving force for water. Adaption to this stress usually takes several hours in which yeast cells accumulate glycerol and trehalose and change their cell wall composition to counter the loss of water by the osmotic pressure. The level of stress depends on the type and concentration of sugars present in the growth medium (Castilleja et al., 2017).

Ethanol

Ethanol produced during fermentation is known to inhibit yeast growth, resulting in a primary factor affecting yeast efficiency; the viability of yeast cells in the presence of ethanol constitutes a key feature on strain selection for fermentative purposes (Lam et al., 2014). Ethanol affects many aspects of yeast survival, as the fluidity of the plasmatic membrane, the vacuole morphology, the activity of crucial glycolytic enzymes, and the mitochondrial DNA. Ethanol also causes the denaturation of hydrophilic and hydrophobic proteins, affecting various transport systems, such as the general amino acid permease and glucose uptake processes. These factors determine adaptation of strains to the hostile environment present during alcoholic fermentation (Castilleja et al., 2017).

Sulfur dioxide (SO_2)

The addition of sulfur dioxide (SO_2) is a common practice aimed to inhibit native microbiota (mainly non-*Saccharomyces* yeasts and bacteria) present in the grape must and to favor the growth of native or inoculated *Saccharomyces* strains. Strains of *Saccharomyces* also produces SO_2 during metabolism of sulfate ions (Castilleja et al, 2017, Wells & Osborne, 2011). If the amount of SO_2 added and produced by *Saccharomyces* strain are high and remain until the end the process, safety concerns can arise as this compound can causes

health problems in sensitivity consumers. As a consequence, the selection of yeast strains resistant to SO_2 and low SO_2 producers is desirable (Castilleja et al., 2017).

Interaction with other microorganisms

Different microorganisms interact during wine production. The variety of these interactions and their impacts on efficiency and product quality should be individually determined, as they will depend on the associated microbiota. In this context, it has to be considered the "killer phenotype," which refers to yeasts able to secrete polypeptide toxins which kill sensitive cells. Killer toxins differ between species and strains, all killer toxins are usually active and stable at pH 4-5 and 20-25°C; nevertheless, each toxin has an optimum pH and temperature at which it manifests its killer character more effectively.

Killer toxins are proteinaceous antimicrobial compounds secreted by yeasts, potentially able to inhibit sensitive yeasts. Killer toxins were first discovered in *S. cerevisiae* strains in 1963 by Makower and Bevan and in non-*Saccharomyces* yeast genera by Phillis kirk and Young (1975). Killer toxins produced by *S. cerevisiae* and their relevance in winemaking have been thoroughly investigated in literature. Although non-*Saccharomyces* killer toxins have been investigated to a lesser extent than those of *S. cerevisiae*, they generally exhibit broader spectra of activity than the latter (Mehlomakulu et al., 2014). Over 11 different killer toxins have been described, and they are produced by strains belonging to the *Hanseniaspora*, *Pichia*, *Saccharomyces*, *Torulaspora*, genera. Killer yeasts are widespread in nature where they can be found in higher percentages than laboratory strains, indicating the existence of a competitive advantage. One of the mechanisms responsible of advantage and described in some killer yeast is related to the presence of dsRNA viruses. These mycoviruses encode killer toxins that provide benefits to the producing cells by killing sensitive yeast cells. Killer toxin production has been related in *S. cerevisiae* with the presence of two dsRNA viruses: L-A, the helper virus, and the M killer virus, that encodes a killer toxin that determines its phenotype (K1, K2, K28 or Klus). The potential use of killer yeasts and their toxins has been proposed for production of different alcoholic beverages (brewery, winery, and distillery) (Belda et al., 2017; Pérez-Nevado et al., 2006).

3.4| Enzymes with oenological interest

Undoubtedly, aroma is one of most important characteristics that contribute to the quality of wine. Wine aroma is composed by 100s of different compounds, and the balance and interaction of all of them determine the wine aromatic quality. Wine aroma can be subdivided into three groups: the varietal or primary aroma, determined by the grape variety;

the fermentative or secondary aroma; and tertiary aroma, resulting from the transformation of aromas during aging. Non-*Saccharomyces* yeasts can influence the aroma through the production of enzymes and metabolites (Padilla et al., 2016).

The aroma-contributing volatile compounds are mainly monoterpenols, C13-norisoprenoids, and aliphatic and aromatic alcohols (Swiegers & Pretorius, 2005). The monoterpenols, such as linalool, geraniol, nerol, citronellol and α-terpineol, plays a fundamental role for wine character as these compounds provide floral notes and have low odor thresholds.

C13-norisoprenoids derive from carotenoids and β-ionone and β-damascenone are considered important volatiles of non-floral grapes (Ristic et al., 2010; Yuan & Qian, 2016). In grapes, these aroma compounds are present in free/volatile/odorous and mono- or disaccharide glucosidic/nonvolatile/odorless forms. The latter are not odorant compounds which can be submitted to during fermentation through the action of wine yeasts. The principal compounds are aroma precursors linked to sugar molecules, mainly terpenol and C13-norisoprenoid glycosides, and the non-volatile precursor of volatile thiols, conjugated to cysteine or glutathione. Contrary to *Saccharomyces* species, the non-*Saccharomyces* yeasts produce and secrete several enzymes that may convert neutral compounds of grapes into aromatic compounds, enhancing the sensory attributes of wines (García et al., 2016; Hu et al., 2016). The production of extracellular enzymes by yeasts has been widely studied. *Saccharomyces cerevisiae* is not recognized as a good producer of extracellular enzymes, whereas non-*Saccharomyces* wine yeasts (including genera such as *Candida*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*) have been described as potential sources for the production of enzymes, such as proteases, esterases, pectinases, lipases and glycosidases (Manzanares et al., 1999, 2000; Palmeri & Spagna, 2007).

The main enzymes produced by yeast and involved in the release of aroma compounds from odorless grape precursors are glycosidases, that hydrolyze the non-volatile glycosidic precursors, and carbon-sulfur lyases, that release volatile thiols from aroma-inactive cysteine-bound conjugates. Glycosidases, including β-D-apiosidase, α-L-arabinofuranosidase, β-D-glucosidase, α-L-rhamnosidase and β-D-xylosidase activities, have been described as being involved in flavour releasing processes (Manzanares et al., 1999).

β-Glucosidases

Since the demonstration that the aromatic components are present in the grape berry both in free form and bound to sugars as glycosides, a continuous research to find

glycosidases able to release varietal aromas from precursors was performed. Glycosidically-bound volatiles are highly complex and diverse, especially regarding the aglycone moiety. The sugar parts consist of β-d-glucopyranosides and different diglycosides: 6-O-α-l-arabinofuranosyl-β-d-glucopyranosides, 6-O-α-l-arabinopyranosyl-β-d-glucopyranosides (vicianosides), 6-O-α-l-rhamnopyranosyl-β-d-glucopyranoside (rutinosides), 6-O-β-d-apiofuranosyl-β-d-glucopyranosides, 6-O-β-d-glucopyranosyl-β-d-glucopyranosides, and 6-O-β-d-xilopyranosyl-β-d-glucopyranosides (primeverosides). The aglycon part is often formed with terpenols, but other flavor precursors can occur, such as linear or cyclic alcohols, C-13 norisoprenoids, phenolic acids, and probably volatile phenols such as vanillin. Terpene glycosides can be hydrolysed by an enzymatic activity (Maicas & Mateo, 2005, Padilla et al., 2016) to enrich wine flavor by releasing free aromatic compounds from natural glycoside precursors. Enzymatic hydrolysis of glycosides is carried out with various enzymes which act sequentially according to two stages. In the first stage, α-l-rhamnosidase, α-l-arabinosidase, or β-d-apiosidase make the cleavage of the terminal sugar and rhamnose, arabinose, or apiose and the corresponding monoterpenyl β-d-glucosides are released. In the second stage, the liberation of monoterpenols takes place after the action of a β-d-glucosidase on the previous monoterpenyl β-d-glucosides (Figure 3.1).

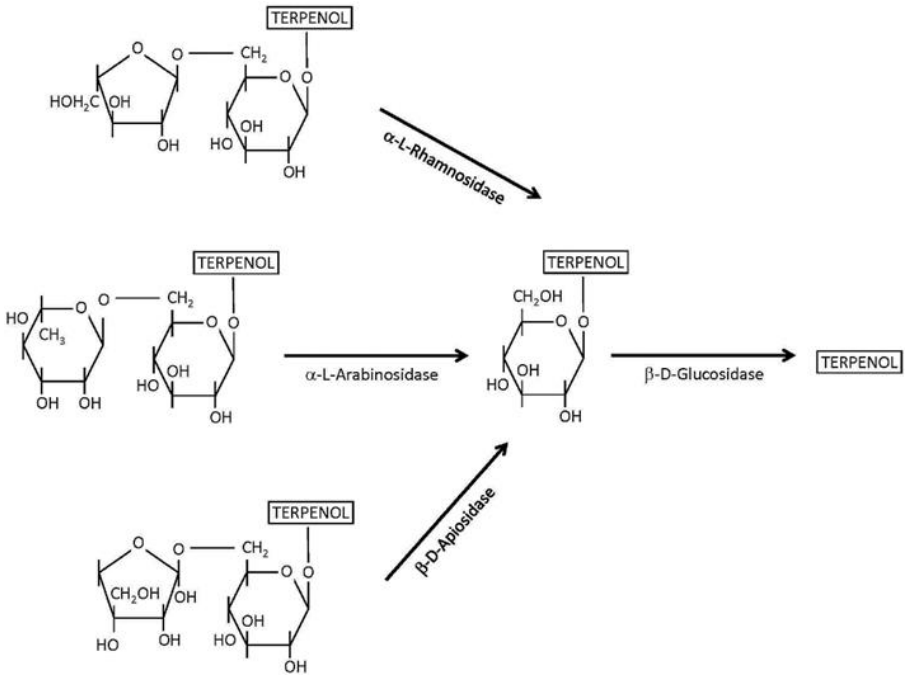

Figure 3.1| Sequential enzymatic hydrolysis of dissacharidic flavor precursors (Mateo & Maicas, 2016).

The selectivity of β-d-glucosidase is mainly for the sugar and it is related to the presence of glucose, and it was found to be dependent on the structure of the aglycon. In fact, the β-d-glucosidase is more selective towards tertiary alcohols than to primary alcohols (steric

hindrance), and to the origin of the enzyme. The aglycon moieties of glycosides include monoterpenes, and also C13-norisoprenoids, benzene derivatives, and aliphatic alcohols, the sugar moiety is represented by glucose or disaccharides. This glycosidically bound aroma fraction, upon hydrolysis, can give rise to odourous volatiles or to volatiles able to generate odour-active compounds during wine storage by acid–base mechanism (Mateo & Maicas, 2016). β-glucosidase activity was detected in strains on the basis of its hydrolytic activity on p-nitrophenyl-β-d-glucoside (pNPG) and terpene glucosides of Muscat juice. This enzymatic activity is induced by the presence of bound β-glucose as carbon source in the medium and seems to be a characteristic of the yeast strain. This β-glucosidase is associated with the yeast cell wall, is quite glucose independent but may be inhibited by acidic wine conditions or high ethanol concentrations.

Non-*Saccharomyces* yeasts belonging to the genera *Debaryomyces*, *Hansenula*, *Candida*, *Pichia*, and *Hanseniaspora* possess various degrees of β-glucosidase activity and can play a role in releasing volatile compounds from non-volatile precursors. For example, co-fermentation of grape juice with *D. polymorphus* and *S. cerevisiae* resulted in an increased concentration of the terpenols citronellol, nerol, and geraniol in wine (Otero et al., 2003). Equally, fermentation of grape juice with mixed cultures of *C. zemplinina*/*S. cerevisiae* and *T. delbrueckii*/*S. cerevisiae* produced wines with high concentrations of terpenols compared to wines fermented with only *S. cerevisiae* (Mateo & Maicas, 2016). The exploitation of indigenous yeast biodiversity for the presence of strains possessing specific β-glucosidases represents an useful tool for production of wines with more varietal aromatic characteristics.

β-Xylosidase

β-Xylosidase enzyme is industrially relevant in consequence of its involvement in the degradation of hemicellulose by hydrolysis of the main heteroglycan (xylan). Xylan presents a d-xylopyranose skeleton linked by β-d-(1-4) bonds, which may contain residues of l-arabinofuranose, 4-O-methyl-d-glucuronic, acetate groups and other substituents. Xylan hydrolysis is carried out in 2 consecutive steps: in the first step, the activity of endoxylanases hydrolize β-(1-4) internal bonds, releasing xylooligosaccharides. Subsequently β-xylosidase breaks the terminal bonds, releasing xylose monomers (López et al., 2015; Manzanares et al., 1999; Mateo & Maicas, 2016).

Esterasi

The accumulation of esters in wine is known to be a result of the balance between the yeast's ester-synthesizing enzymes and hydrolysis reactions involving esterases (responsible

for cleavage and, in some cases, formation of ester bonds) (García et al., 2016). Esterases are a diverse group of enzymes that catalyze the hydrolysis of ester bonds in triacylglycerides to glycerol and fatty acids.

Ethyl acetate is the most abundant ester in wine; this compound can have favorable effects on wine aroma at concentrations below 80 mg/L, whereas at high concentration it is responsible for the altered sensory properties known as acescency.

Proteolytic and pectinolytic enzymes

Proteolytic and pectinolytic (polygalacturonase) enzymes are other extracellular enzymatic activities produced by non-*Saccharomyces* yeasts which may also be beneficial for winemaking. For example, proteolytic activity of some non-*Saccharomyces* yeasts reduces the protein concentration of the grape juice by approximately one-third, determining an increase in protein stability of the final product. Protein precipitation in bottled wines, especially in white and red with low amounts of polyphenols, causes protein haze as a consequence of protein coagulation with unfavorable storage conditions. These denatured proteins can either flocculate into a hazy suspension or form sediments in bottle. Species found to produce the greatest number of extracellular enzymes are *C. stellata*, *H. uvarum*, and *M. pulcherrima* (Mateo & Maicas et al., 2016).

3.5| Materials and methods

3.5.1| Yeast strains and growth conditions

In this study, non-*Saccharomyces* yeast strains, deposited in the UNIBAS Yeast Collection (UBYC) of the SAFE (School of Agriculture, Forestry, Food and Environmental Sciences; University of Basilicata, Italy), was used. In particular, a total of the thirty-three non-*Saccharomyces* yeast strains belonging to the following species: *Debaryomyces polymorphus* (Db), *Hanseniaspora uvarum* (Ha), *Starmerella bacillaris* (Sb) and *Zygosaccharomyces bailii* (Zb), was used. The strains were previously isolated from spontaneous fermentation of grapes of different varieties, coming from different geographical areas of Italy, and prickly pear fruit, collected in 2017 and 2018 vintages. The used strains are listed in Table 3.1.

All the yeasts has been previously identified by means of Restriction Fragment Length Polymorphism (RFLP) analysis of the 5.8S ITS rDNA region (Esteve-Zarzoso et al., 1999; Granchi et al., 1999) by using the restriction enzymes *Hae*III, *Hinf*I, *Dde*I and *Dra*I

(Promega, Milano, Italy); the restriction enzyme *Dra*I was used to distinguish *St. bacillaris* from *Candida stellata* (Nisiotou & Nychas, 2007).

Table 3.1| Origin of the strains used in the study.

Species	Strain code	Source of isolation	Number of strains
Debaryomyces polymorphus	Db1, Db2, Db3	Grape must	3
Hanseniaspora uvarum	Ha1, Ha2, Ha3, Ha4, Ha5, Ha6, Ha7, Ha8, Ha9, Ha10, Ha11, Ha12, Ha13, Ha14, Ha15, Ha16, Ha17	Grape must	17
Starmerella bacillaris	St1, St2, St3, St4, St5, St6, St7	Grape must	7
Starmerella bacillaris	St8, St9, St10, St11	Fruit (prickly pear)	4
Zygosaccharomyces bailii	Zb1, Zb2	Grape must	2
Saccharomyces cerevisiae	EC1118	Commercial starter (Lallemand)	1

Strains were grown on YPD (1% yeast extract, 2% bacteriological peptone, 2% glucose, 2% agar, w/v) slants for medium-term conservation at 4°C, while for log-term storage, each strain was cryopreserved at -80°C in glycerol 30% (v/v).

In addition, the *Saccharomyces cerevisiae* commercial strain Lallemand EC1118 (purchase as active dry yeast by Lallemand Inc., Toulouse, France) was used.

3.5.2| Molecular characterization of non-*Saccharomyces* yeast strains

3.5.2.1| DNA extraction

Genomic DNA was extracted from pure cultures of each strain grown on YPD medium at 26 °C for 24 h, by using a synthetic resin (Instagene Bio-Rad Matrix), as previously reported by Capece and Romano (2009), with some modifications. A single yeast colony was suspended in 1 mL of STE buffer, containing 10 mM Tris-HCl, 1 mM EDTA, 0,1 M NaCl (Sigma), 1 mM EDTA (Sigma). Afterwards, the mixture was vortexed for 1 min and centrifuged at maximum speed for 5 min.

The upper phase was removed and the pellet was wash and suspend by the addition the 1 mL of distilled water, after that it was vortexed for 1 min and centrifuged at maximum speed for 5 min. In the following step, the supernatant was removed, and the pellet was re-suspended in 0.2 mL of Insta Gene matrix (Sigma) and incubated at 56 °C for 30 min. After

that, it was vortexed for 1 min and boiled at 100°C for 8 min. The supernatant was transferred in a new 1.5 mL Eppendorf tube, and the extracted DNA was stored at -20 °C until analysis. Yield, purity and integrity of DNA samples were determined using a SPECTROstar Nano (UV-Vis spectrometer, BMG LaBTECH) (Figure 3.2).

Figure 3.2| UV-Vis spectrometer SPECTROstar Nano quantification of DNA, RNA and protein samples.

3.5.2.2| Genotypic characterization

The thirty-three non-*Saccharomyces* yeast strains were submitted to genetic characterization by microsatellite-primed PCR (MSP-PCR) with synthetic oligonucleotide $(GTG)_5$, by following the protocol described by Guaragnella et al. (2020), modified in some steps.

The amplification reaction was carried out in a final volume of 50 µL containing 10 µL of *Taq* Polymerase 5X Buffer (Promega), 4.0 µL of the 25 mM $MgCl_2$ (Promega, Milano, Italy), 5 µL of 5 µM primer $(GTG)_5$ (5'-GTGGTGGTGGTGGTGT-3'), 1 µL of 10 mM dNTP (Promega), 0.25 µL (5 U/µL) of *Taq* DNA polymerase (Promega), and 5 µL of the extracted DNA, with sterile water, added upon to final volume. The PCR reactions were carried out in Thermal Cycler System (Bio-Rad, Milano, Italy), using the following amplification conditions: initial denaturation at 95°C for 5 min, 35 cycles at 94°C for 1 min for denaturing, 1 min at 52°C, 2 min at 72°C for extension, and a final step at 72°C for 5 min.

PCR-products were analyzed by electrophoresis in 2% (w/v) agarose gel, prepared in 1X TBE buffer (Tris-HCl, Boric acid, 0.5 M EDTA, pH = 8.0). The gels were run at 100 V for 90 min, stained with SYBR® Safe (Invitrogen, United states) and captured by the Gel Doc™ XR+ system (Bio-Rad). As size marker, 100 bp DNA ladder marker (Biolabs) was used (Figure 3.3).

Gels containing MSP-PCR profiles of the yeast strains were analyzed using FQuest 4.5 software (BIo-Rad).

Dendrogram obtained by hierarchical clustering of the band fingerprints were generated using the Pearson's correlation coefficient and the unweighted pair group method with arithmetic averages (UPGMA) algorithm (tolerance 1%, optimization 0.50%).

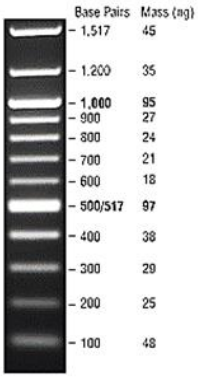

Figure 3.3| Marker "100bp DNA ladder" (Biolabs).

3.5.3| Screening for physiological parameters

3.5.3.1| Technological characterization of yeast strains

All the strains belonging to the different non-*Saccharomyces* species were evaluated for characteristics potentially useful in winemaking. In particular, the 33 strains were tested for resistance to antimicrobial compounds usually present during winemaking, such as sulphur dioxide (SO_2), copper sulphate ($CuSO_4$) and ethanol (EtOH), and for production of hydrogen sulphide (H_2S).

The abilities of the strains to grow in the presence of different concentrations of sulphur dioxide, copper and ethanol were tested directly by plating yeast strains on agarized medium. The yeast cells (refreshed from 24 h) were suspended in sterile physiological solution, and diluted until to an absorbance value ranging between 0.2 and 0.4 optical density, measured at 600nm (OD_{600}) by using the UV-Vis spectrometer SPECTROstar Nano, and corresponding approximately to 10^4 cells/mL. Five µl of this cell suspension were inoculated by spotting onto agar plates, added with the different antimicrobial compounds.

Sulphur dioxide resistance was tested in agarized grape must, buffered at pH 3.6 (with citrate-phosphate buffer), added with increasing amounts of SO_2 (in the form of potassium metabisulphite), in order to obtain doses from 25 mg/L up to 200 mg/L, with increments of 25 mg/L.

Copper resistance was evaluated as strain ability to grow on synthetic complete medium containing different amounts of $CuSO_4$ (100, 200, 300, 400 and 500 µmol/L). The composition of the synthetic medium was the following: agar 20 g/L, YNB (Yeast Nitrogen Base without amino acids and sulphate, Difco) 6.7 g/L, glucose 20 g/L.

Ethanol tolerance of the strains was evaluated in agarized grape must (pH 3.6), added with concentration ranging from 2 until 16 % (% vol/vol) of ethanol.

The strain resistance to the three compounds (SO_2, $CuSO_4$, EtOH) was evaluated on the basis of positive growth after 48 h at 26°C, in comparison with a control, represented by strain growth on medium without addition of the antimicrobial compound. The degree of resistance of each strain was reported as minimal dose which allowed its growth.

The production level of hydrogen sulphide (H_2S) was tested using a qualitative method performed on the medium Bismuth Sulphite Glucose Glycerin Yeast (BiGGY) Agar. The medium was spot-inoculated with fresh yeast cells and the plates were incubated at 26°C for 24 h. On this medium, yeast cells develop colonies with different browning degree in function of amounts of hydrogen sulphide produced, ranging from brown/dark brown (high production level of H_2S), hazelnut (medium amounts level of H_2S), until white/cream (no or low production level of H_2S) (Alberico et al., 2019; Capece et al., 2010, 2019; Domizio et al., 2011; Mauriello et al., 2009).

3.5.3.2| Killer activity

Yeast strains used in this study were tested to verify the presence/absence of killer phenotype. All isolates were grown in YPD medium at 26°C for 24 h. The test was carried out on medium composed of Malt extract broth (2%), agar (2%), and methylene blue (0.003%), and buffered at pH 4.6 with 0.1 M of citric acid phosphate buffer. As sensitive reference strain, *S. cerevisiae* DBVPG 6500 (NCYC 1006; National Collection of Yeast Cultures, Norwich, England) was used, by spreading a concentration of approximately 10^9 UFC/mL (final concentration) on the surface of solidified agar plate. The non-*Saccharomyces* strains were inoculated by a spot on the plates, which were incubated at 26°C for three days. The *S. cerevisiae* killer strain DBVPG 6567 was used as positive control.

The tested yeast strains were designated as killer strains when the colony was surrounded by a clear zone in which no growth of the inoculated sensitive strain (*S. cerevisiae* DBVPG 6500) has occurred.

The level of killing activity was measured as the diameter of the clear zone of inhibition around the colony after incubation. Furthermore, the strains were tested to verify the neutral/sensitive character. In this case, the reference killer strain (*S. cerevisiae* DBVPG 6567) was suspended in sterile physiological solution and inoculated on the surface of medium at a concentration of about 10^9 UFC/mL, as previously described. The strains in study and sensitive reference strain (*S. cerevisiae* DBVPG 6400) were spotted on the plates, inoculated with reference killer strains, and the plates were incubated at 26°C for three days. The strain was classified as sensitive in case of formation of blue colony, whereas strains forming cream colony were indicated as neutral (Capece et al., 2013; Ramírez et al., 2015).

3.5.3.3| Qualitative tests for Enzymatic activities

All the 33 non-*Saccharomyces* strains were submitted to qualitative screening for three enzymatic activities relevant for wine quality, such as β-glucosidase, β-xylosidase and esterase activities. In all the tests, before the inoculation on substrate specific for each enzymatic activity, the yeast strains were cultured on YPD agar at 26°C for 24 h.

β-*glucosidase activity*. To evaluate the β-glucosidase activity, the method described by Englezos et al. (2015) and Domizio et al. (2014) with some modifications, was applied. The β-glucosidase activity was evaluated by streaking yeast strains on agar plates containing arbutin as substrate. The composition of basal medium was the following: 5 g/L arbutin (hydroquinone β-d-glucopyranoside (Sigma), 6.7 g/L Yeast Nitrogen Base (YNB) without amino acids (Difco) and 20 g/L of agar. The pH of the medium was adjusted to 5.0 before for 15 minutes at 121°C. Four millilitres of 1% ferric ammonium citrate solution (sterilized by filtration) were added to 100 mL of medium before pouring the medium in the plates. The plates were incubated at 30°C for 5 days. The presence of the β-glucosidase activity was indicated by a change in colour medium around the colonies. In fact, strains with this activity hydrolyze the substrate, developing dark brown colour in the agar. The strains of *S. cerevisiae* EC1118 and *H. uvarum* were used as negative and positive controls, respectively.

β-*xylosidase activity*. The presence of β-xylosidase activity among yeast strains was verified according to the method described by Manzanares et al. (1999), modified in some steps. β-xylosidase screening was carried out on agar plates containing a medium composed by 6.7 g YNB, without amino acids and ammonium sulphate, 5 g ammonium sulphate, 5 g xylose and 20 g agar per litre of medium; the pH was adjusted to 5.5.
4-Methylumbelliferyl-β-D-xyloside (MUX, Sigma) was spread onto the surface of the agar plates. The yeasts were inoculated by spotting on the agar surface and the plates incubated at 26°C for 24 to 48 h. The hydrolysis of MUX by the action of β-D-xylosidase activity resulted in the release of 4-methylumbelliferone (MU), which was visualised under UV illumination as fluorescent halos surrounding yeast colonies. *S. cerevisiae* EC 1118 was used as negative control.

Esterase activity. The ability of the yeasts to hydrolyze esters was evaluated on a medium, containing 10 g/L bacteriological peptone, 5 g/L NaCl, 0.1 g/L $CaCl_2$ and 15 g/L agar. The medium was autoclaved 121°C for 15 minutes, after that 5 mL/L of sterile Tween 80 (the oleic acid monoester of polyoxyethylene sorbitan) was added to the cooled medium (about 50°C). The plates were spot inoculated with the strains, incubated at 30°C for 48 h and were daily observed through 10 days. The presence of esterase activity was indicated by a visible opaque halo around the colony (Binati et al., 2019; Buzzini & Martini, 2002,

Englezos et al., 2015; Slifkin, 2000). *S. cerevisiae* DBVPG 6597 (NCYC 1006; National Collection of Yeast Cultures, Norwich, England) strain was used as positive control.

3.5.3.4| Quantitative evaluation of β-glucosidase and β-xylosidase activities

Strains positive to the qualitative screening for enzymatic activities were submitted to quantitative screening of the extracellular β-glucosidase and β-xylosidase activities, using the protocol described by López et al. (2015, 2016) and Ferreira et al. (2001), with some modifications. The assay for β-glucosidase and β-xylosidase activities was performed by quantifying the *p*-nitrophenol released from the chromogenic substrates, specific for each test. Overnight yeast cultures were inoculated in YPD broth and incubated at 28°C for 48 h with agitation; after that, $1x10^6$ cells/mL were inoculated in induction medium, composed by YNB without amino acids (0.67%), supplemented with 20 g/L glucose (2%) and 0.4 mL of ferric ammonium citrate solution (1% w/v). The flasks were incubated overnight at 28°C in an orbital shaker at 150 rpm. Extracellular activities were determined in 1 mL of supernatant, recovered by centrifugation at 3000 rpm for 10 min.

The enzymatic activities were evaluated by determining the amount of *p*-nitrophenol (pNP) released from the *p*-nitrophenyl-β-D-glycoside (pNPG) and *p*-nitrophenyl-β-d-xyloside (p-NPX) for β-glucosidase and β-xylosidase activity respectively, by adding 0.2 mL of pNPG and pNPX solution (5 mmol/L) in citrate-phosphate buffer (citric acid 0.1 M, Na_2HPO_4 0.2 M, pH 5) to 0.2 mL of each supernatant fluid and incubating at 30°C for 1 h.

The reaction was stopped by adding 1.2 mL of Na_2CO_3 solution (0.2 M). The amount of pNP released was determined spectrophotometrically at 400 nm. The enzymatic activity was quantified using a standard curve of pNP ranging between 10 and 300 nmol/mL. Results were expressed as nmol of pNP released for mL for hours. For each strain two biological replicates were analyzed.

The enzymatic activities were subjected to statistical analysis by One-Way Analysis Of Variance (ANOVA); the statistical significance was set at $p < 0.05$. Tukey's test was used to compare the mean values of enzymatic activity among the strains.

3.5.3.5| Statistical elaboration of data obtained by screening for physiological parameters

All the data obtained by technological characterization were standardized and used as variables for cluster analysis. Results related to H_2S production and resistance to antimicrobial compounds were converted into adimensional values, assigning the values reported in Table 3.2.

Table 3.2| Adimensional values assigned to technological parameters.

Technological characteristics	**Adimensional values**			
	0	**1**	**2**	**3**
H_2S Production	white/cream	hazelnut	brown	dark brown
SO_2 resistance (mg/L)	50	100-125	175	200
Cu resistance (µmol/L)	< 100	100	200	300
EtOH resistance (% v/v)	6	8-10	12	14

The results correlated to quantitative analysis of β-glucosidase and β-xylosidase activities were converted into adimensional values on the basis of mean and Standard Deviation (SD), by assigning the following values:

- class 0 is represented by strains possessing these characteristics at very low level (< mean – SD);
- class 1 includes strains possessing these traits at low level (mean – SD ≤ levels ≤ mean);
- class 2 contains strains possessing these traits at medium/high level (mean < levels ≤ mean + SD);
- class 3 is represented by strains possessing these traits at very high level (> mean + SD).

The matrix obtained by these values was submitted to cluster analysis and the dendrogram was generated using Ward's method with Euclidean distance by using statistical software RStudio.

3.5.4| Growth in high concentration of ethanol and SO_2

After the first genetic and technological screening, strains, representative of the different species and possessing different genetic/technological traits, were selected for further characterization. The selected strains were analyzed for ability to grow in high ethanol and sulphur dioxide concentrations.

The tests in ethanol and SO_2 were performed in 96-well microtiter plates, following the protocol described by Englezos et al. (2015) and Tofalo et al. (2012), with slight modifications. Briefly, yeast cells were prepared by inoculating one colony (fresh cells) in 5 mL of YPD medium and incubated overnight at 26°C without shaking. Then, the cells were harvested by centrifugation at 9000 rpm for 10 min and the pellet was washed twice with sterile physiological solution (8.5 g/L NaCl) and then re-suspended in the same solution to obtain a concentration of about 10^6 UFC/mL. The growth under the two stress conditions

were tested in YNB medium, with amino acids (6.7 g/L), pH 5.5, and supplemented with 20 g/L of glucose, sterilized by filtration with a 0.2 μm membrane filter. This medium was supplemented with different amounts of ethanol until to reach final concentrations of 10, 12, 14 and 16% (v/v), and different amounts of SO_2, added as potassium metabisulfite ($K_2S_2O_5$) to reach final concentrations of 50, 100, 150 and 200 mg/L of total SO_2. Medium without addition of ethanol or SO_2 was used as control.

Twenty μL of cell suspension were inoculated in 180 μL of the respective medium in 96-well microtiter plates. The microplates were incubated at 26°C and the optical density (OD) was measured at 630nm using a microtiter plate reader every 24 h for 2 days, after an orbital shaking of 30 s, in order to re-suspend the cells in the medium before the measurement. The cell growth was determined by the ratio between the growth of the isolates in broth with and without ethanol or SO_2 at the specific incubation times. Strains showing a percentage of growth ratio < 10% were considered not resistant. *S. cerevisiae* EC1118 strain was used as positive control. These tests were carried out in duplicate, and the results are represented as the average and standard deviation (± SD). One-way Analysis of Variance (ANOVA) was applied to the results and the data were considered significant if the associated P was < 0.05 by using Tukey tests. The statistical analyses were performed with software PAST3 version 3.20 (Hammer et al., 2018).

3.5.5| Evaluation of sulphur dioxide resistance in microfermentation

The resistance to SO_2 of selected strains was evaluated also by determining the influence of this compound on fermentative activity of the 19 non-*Saccharomyces* strains. At this aim, each strain was inoculated in 10 mL of natural red grape must (Aglianico variety), thermally treated at 80°C for 20 minutes, supplemented with 30 mg/L of total SO_2. As control, grape must without SO_2 addition was used. The main characteristics of the used grape must are summarised in Table 3.3. The sugar content was measured with a bench-top refractometer, while the other parameters were determined by a Fourier Transfer Infrared WineScan instrument (OenoFoss, Hillerød, Denmark).

The non-*Saccharomyces* strains were grown overnight in YPD broth at 28°C with agitation, and a volume of biomass containing about $2x10^7$ cells/mL, detected by measuring optical density at 600nm, was used to inoculate the grape must.

Each microfermentation experiment was carried out in duplicate under static conditions at 26°C (semi-anaerobic conditions), for about 20 days. The commercial strain EC1118 (*S. cerevisiae*) was used as positive control. The evolution of the fermentations was evaluated by measuring daily weight loss, caused by carbon dioxide (CO_2) release during the process,

and sugar concentration. Fermentations were considered to be finished when the weight loss of the samples was constant for 2-3 consecutive days. At the end of the process, the experimental wines were refrigerated for 3 days at 4°C to allow clarification, racked and stored at -20°C until required for chemical analysis.

Table 3.3| Main oenological characteristics of the grape must used for microfermentations trials.

Parameters	Values
°Brix	24.6
pH	3.77
Total acidity (g/L)	4.1
Assimilable nitrogen (mg/L)	234.49
Alpha Amino (mg/L)	120.6
Ammonia (mg/L)	113.9
Gluconic acid (g/L)	0.94
Malic acid (g/L)	0.03

The SO_2-resistance was expressed as ratio between strain fermentative vigour in SO_2-added fermentations and the fermentative vigour without SO_2 addition. The fermentative vigour was measured as the amount of CO_2 produced at the second day of fermentation.

Analysis of variance (ANOVA) was used to evaluate differences for sulphur dioxide resistance among the strains. The significant differences were determined using Tukey tests, and the data were considered significant if the associated P was < 0.05. The software PAST3 version 3.20 (Hammer et al., 2018) was used for the statistical analysis.

3.6| Results and Discussions

3.6.1| Molecular characterization of non-*Saccharomyces* strains

By considering the increasing interest of winemakers toward the role of non-*Saccharomyces* yeasts on wine characteristics, the first part of this research activity was addressed to the selection of non-*Saccharomyces* strains to be used in mixed fermentation. In this context, a total of 33 non-*Saccharomyces* yeasts, belonging to the species *D. polymorphus* (3 strains), *H. uvarum* (17 strains), *St. bacillaris* (11 strains) and *Z. bailii* (2 strains), were chosen (Figure 3.4).

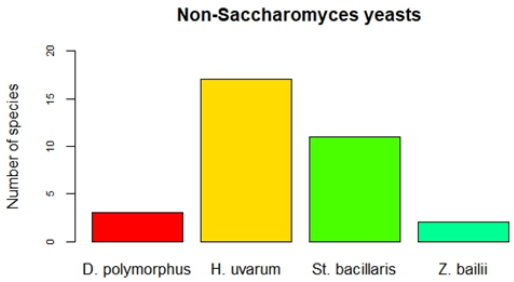

Figure 3.4| Thirty-three non-*Saccharomyces* yeast strains.

The first step of screening among non-*Saccharomyces* yeasts was addressed to evaluate the genetic variability, by using MSP-PCR analysis as described in Materials and Methods. This method has proved to be suitable for studying variability among a wide number of yeast strains, according to the PCR patterns amplified with specific oligonucleotides. In this study, for strain typing it was chosen the oligonucleotide $(GTG)_5$, a primer widely used for assessment of yeast communities (Guaragnella et al., 2020), and description of new genus, species or genotypes within species. The molecular profiles obtained by MSP-PCR are reported in Figure 3.5 (a, b).

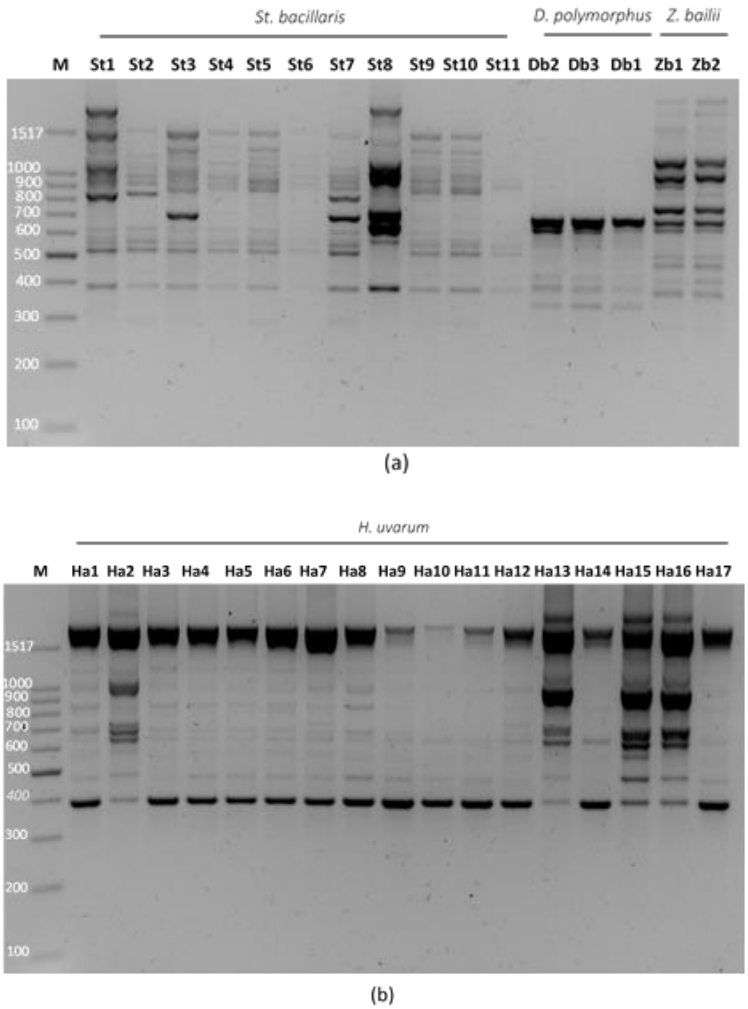

Figure 3.5| Representative profiles of selected *St. bacillaris*, *D. polymorphus*, *Z. bailii* (Fig.3.3a), and *H. uvarum* (Fig. 3.4b) strains, generated by MSP-PCR analysis with the oligonucleotide $(GTG)_5$.

This technique gave fingerprints containing between 7 and 20 bands, with sizes ranging from approximately 300 to 2000 base pairs. The fingerprinting profiles were analyzed based only on the number and size of well reproducible bands, whereas faint and badly reproducible bands were not considered during analysis. Furthermore, in some fingerprints differences in band intensity occurred, but the band intensity was not used as a variable for grouping the isolates.

Bands with molecular weight lower than 500 bp were consistently present in almost all the profiles of analyzed yeasts, in particular among *H. uvarum* and *St. bacillaris* strains, whereas the presence of bands with molecular weight higher than 500 bp was more variable. Molecular profiles obtained by PCR fingerprinting with the $(GTG)_5$ primer were clustered using the UPGMA algorithm with Dice correlation-based distance measures, obtaining the dendrogram reported in Figure 3.6.

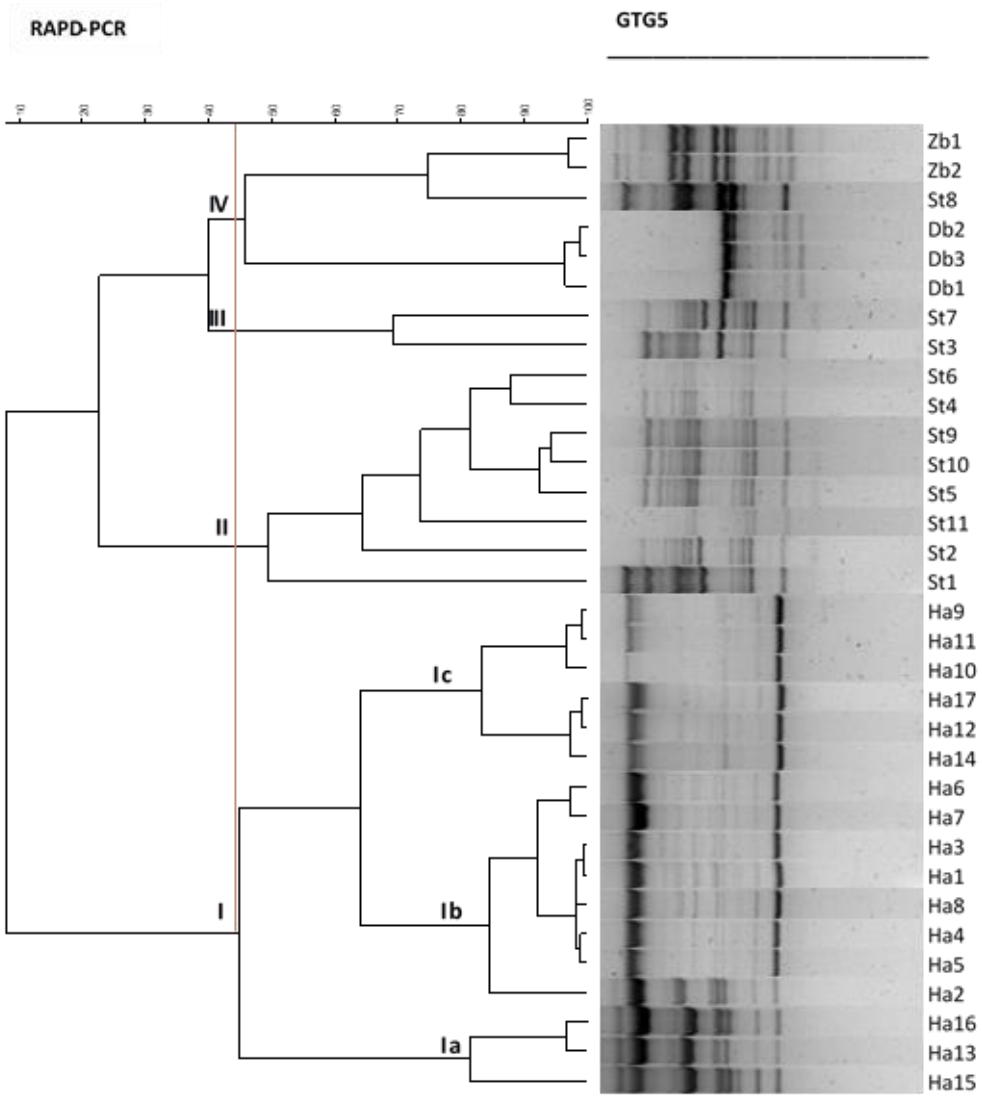

Figure 3.6| Cluster analysis of 33 non-*Saccharomyces* strains isolated from spontaneously fermented. Dendrogram was produced by hierarchical clustering of the electrophoretic patterns obtained by MSP-PCR with the oligonucleotide $(GTG)_5$, using the Dice similarity coefficient with UPGMA algorithm. The orange line indicates the similarity value (44 %) for separation of biotypes.

By considering a similarity level of 44%, the strains were distributed into four well-defined clusters (I, II, III, IV). All clusters were composed by strains belonging to the same species, except the cluster IV, including Zb1, Zb2 and St8 strains, belonging to the species *Z. bailii* (Zb1 and Zb2) and *St. bacillaris* (St8). *H. uvarum* strains clustered in group I, which can be subdivided into 3 sub-groups (Ia, Ib and Ic), characterized by very similar patterns, with low percentage of variability. *St. bacillaris* strains are grouped in clusters II and III, whereas only one strain (St8) is included in group IV. Contrary to *H. uvarum*, *St. bacillaris* strains showed molecular profiles quite different among them, and consequently low percentage of similarity was found among strains grouped in clusters II and III of the dendrogram.

As previously reported, the group 4 is composed by three *D. polymorphus* isolates, showing very similar molecular profiles among them, and two *Z. bailii* isolates, characterized by a highly similar PCR fingerpintings, other than St8 strain.

In conclusion, this typing method was able to discriminate the isolates in function of species, but it was also useful to detect the variability among strains belonging to the same non-*Saccharomyces* species, as previously reported (Englezos et al., 2015; Lemos Junior et al., 2016; Pfliegler et al., 2014), in particular for *St. bacillaris* strains.

3.6.2| Phenotypic characterization

3.6.2.1 | Technological characterization of yeast strains

The initial screening of 33 non-*Saccharomyces* yeasts belonging to 4 different species (*D. polymorphus*, *H. uvarum*, *St. bacillaris* and *Z. bailii*) was based on characterization for the main characteristics of oenological interest, enzymatic and killer activities. As it's well known that not all non-*Saccharomyces* isolates have the technological characteristics relevant for wine production, the screening for the main oenological criteria represents a fundamental step in selecting yeast isolates for potential use as starter cultures. The technological characteristics evaluated in this step were the resistance to antimicrobial compounds potentially present in winemaking, such as sulphur dioxide, copper, and ethanol, and hydrogen sulphide production. These characteristics were evaluated on agar-based media (Figure 3.7) and the obtained results are summarized in Table 3.4. The non-*Saccharomyces* strains showed marked differences for the characteristics analysed.

As regards SO_2 tolerance, great variability was observed within the *H. uvarum* species, with some strains showing moderate resistance (175 mg/L), whereas only two (Ha16 and Ha17) were very sensitive to this compound, tolerating not more 50 mg/L of SO_2. The yeasts belonging to the specie *D. polymorphus*, *St. bacillaris* and *Z. bailii* showed resistance to SO_2

from 175 to 200 mg/L, with the exception of Db1 strain, which grew until 125 mg/L of SO_2. Considering that SO_2 is the most used antimicrobial compound added to grape must, the high variability found among tested strains underlines the importance of strain selection for this parameter.

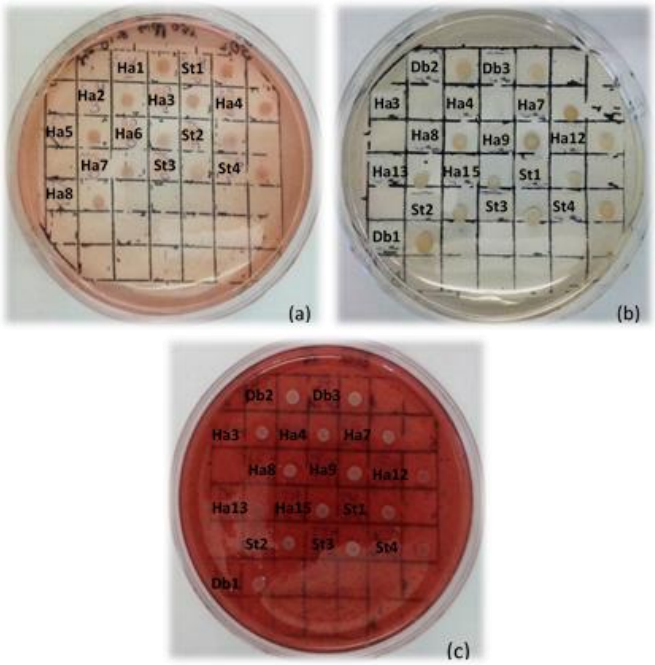

Figure 3.7| Evaluation of resistance to antimicrobial compounds on the basis of growth on plates containing several concentrations of sulphur dioxide (a), copper (b) and ethanol (c).

Also for copper sulphate tolerance, a variability among the strains was found, in this case the resistance level was correlated to species (Table 3.4). In fact, all *D. polymorphus* strains were tolerant to the highest tested concentration (300 μmol/L), as well as some *St. bacillaris* strains, whereas medium-low level of tolerance was found for *H. uvarum*, with tolerance level ranging between 100 and 200 μmol/L. The two *Z. bailii* showed a low resistance to the copper sulphate, with the maximum doses tolerated of 100 μmol/L.

Copper formulates are effective against a high number of crop pests and this compound is considered a traditional fungicide as it has been used against powdery mildew since the 1880s. Furthermore, the use of copper sulphate is allowed also in organic viticulture. It was reported that the acquisition of resistance to copper sulphate could be associated with the ancient use of this fungicide in vineyards.

The wide use of this compound in vine treatment can determine the potential presence of copper sulphate residues in grape musts and, therefore, the use of non-*Saccharomyces* strains possessing high copper resistance represents an additional advantage to assure successful fermentative process (Capece et al, 2019).

Table 3.4| Results obtained for technological characterization of the 33 non-*Saccharomyces* yeasts used in this study.

Strains code	SO_2 (mg/L)	Cu (μmol/L)	EtOH % (v/v)	H_2S Production	Other characteristics	
					Killer activity[1]	Neutral/ Sensitive[2]
Db1	125	300	12	cream	K^+	-
Db2	200	300	14	cream	K^+	-
Db3	200	300	14	cream	K^+	-
Ha1	175	200	14	hazelnut	K^-	S
Ha2	175	200	14	hazelnut	K^-	S
Ha3	175	200	14	hazelnut	K^-	S
Ha4	175	100	14	hazelnut	K^-	S
Ha5	175	100	14	hazelnut	K^-	S
Ha6	175	100	14	hazelnut	K^-	S
Ha7	175	200	14	hazelnut	K^-	S
Ha8	175	200	14	hazelnut	K^-	S
Ha9	100	200	12	hazelnut	K^-	S
Ha10	100	100	12	hazelnut	K^-	S
Ha11	100	200	12	hazelnut	K^-	S
Ha12	100	200	12	hazelnut	K^-	N
Ha13	100	200	12	hazelnut	K^-	S
Ha14	100	100	12	hazelnut	K^-	S
Ha15	125	200	12	hazelnut	K^-	S
Ha16	50	100	14	hazelnut	K^-	S
Ha17	50	100	14	hazelnut	K^-	S
St1	175	200	14	hazelnut	K^-	N
St2	200	300	14	dark brown	K^-	S
St3	175	300	14	dark brown	K^-	N
St4	175	300	14	hazelnut	K^-	S
St5	200	300	14	hazelnut	K^-	S
St6	200	300	14	hazelnut	K^-	S
St7	200	200	14	hazelnut	K^-	S
St8	200	200	14	hazelnut	K^-	S
St9	200	200	14	hazelnut	K^-	S
St10	200	200	14	hazelnut	K^-	N
St11	200	200	14	hazelnut	K^-	S
Zb1	200	100	14	hazelnut	K^-	N
Zb2	200	100	14	hazelnut	K^-	N

[1]Killer phenotype: K^+ killer activity; K^- no killer activity
[2]Neutral/sensitive phenotype: "N" Neutral; "S" Sensitive

The effect of increasing ethanol concentration on growth of non-*Saccharomyces* was further evaluated. Ethanol stress represents toxicity specially to yeast cell membranes, as ethanol alters membrane organisation and permeability, thus affecting glucose uptake and fermentation rates under oenological conditions (Navarro-Tapia et al., 2018; Tofalo et al., 2009). Since non-*Saccharomyces* yeasts have a lower tolerance to ethanol compared to *S. cerevisiae*, it is expected that the indigenous isolates would not be able to grow in the

presence of high ethanol concentrations. Almost all the strains exhibited medium tolerance to ethanol, growing on plates containing 14% (v/v) of EtOH, except some *H. uvarum* strains, which didn't grow at concentration higher than 12%, such as one *D. polymorphus* strain (Db1).

All *S. bacillaris* and *Z. bailii* strains proved to be more resistant to this stress, contrarily to results obtained by Aponte and Blaiotta (2016), which, analyzing different non-*Saccharomyces* species, found that isolates of *S. bacillaris* tolerated ethanol concentrations not higher than 10%.

In general, the screening of non-*Saccharomyces* isolates for parameters of technological interest revealed a different behaviour among all the strains analysed. The majority of strains resulted low-tolerant to SO_2, with 37% of isolates exhibiting tolerance to the highest dose of SO_2 tested (200 mg/L), whereas for $CuSO_4$ and ethanol higher level of resistance was found. In fact, 49% of isolates tolerated 300 µmol/L $CuSO_4$, whereas 76% of isolates grew in presence of 14% v/v ethanol (Figure 3.8).

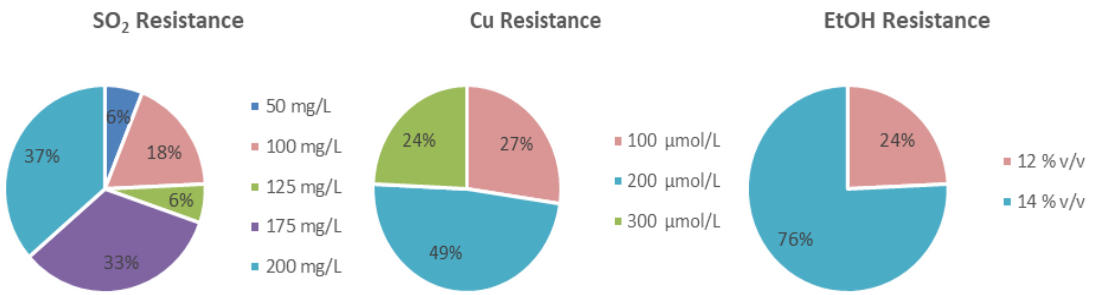

Figure 3.8| Distribution of non-*Saccharomyces* strains for tolerance to antimicrobial compounds.

As regards hydrogen sulphide, this compound has a detrimental effect on wine organoleptic properties, due to its characteristic unpleasant aroma. The production level of H_2S among the non-*Saccharomyces* strains was assessed on BiGGY agar, by comparing the colour of colonies formed by each strain. The colony colours ranged from black and dark brown, hazelnut and cream, as a function of the increasing amounts of hydrogen sulphide produced (Figure 3.9).

Only three *D. polymorphus* strains resulted no-producers of H_2S as they developed cream colonies on BIGGY agar, whereas most of the strains developed colonies characterized by hazelnut colour, resulting medium producers of this undesirable compound. A very high production level of H_2S was exhibited by two *St. bacillaris* strains (St2 and St3), which developed dark brown colonies (Table 3.4, Figure 3.10).

Figure 3.9| Colours of colonies developed on BIGGY agar medium as function of production level of H_2S**.**

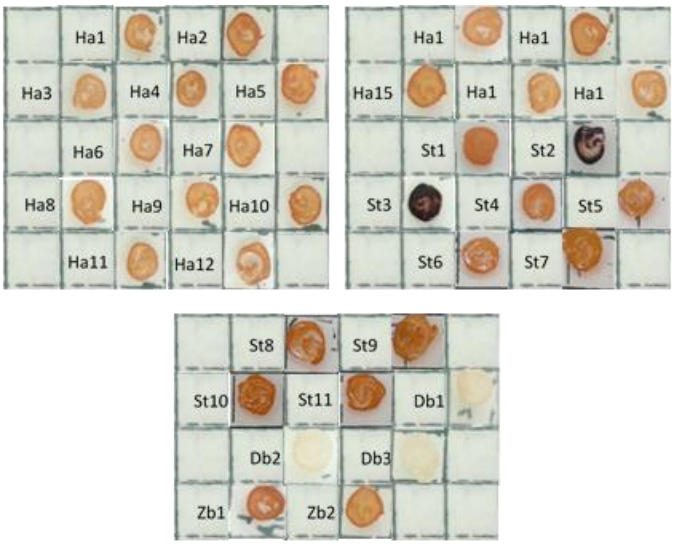

Figure 3.10| Evaluation of H_2S production among 33 non-*Saccharomyces* strains as function of colony colour developed on BIGGY agar medium.

The results obtained in our research regarding the production level of H_2S are in agreement with previous studies on non-*Saccharomyces* (Domizio et al. 2011; González-Arenzana et al. 2017).

Subsequently, the isolates were subjected to screening for evaluation of killer, neutral or sensitive phenotype, that could be of interest in oenology (Figure 3.11) as this character might be related to strain dominance during wine fermentation. In fact, the use of starter possessing killer activity may potentially favour the strain dominance during winemaking. The killer yeast strain *S. cerevisiae* DBVPG 6565 was used as control, since its killer activity has been previously reported.

The killer assay revealed that most of the studied non-*Saccharomyces* yeasts showed sensitive phenotype (S), whereas neutral (N) and killer (K^+) phenotypes were less frequent (18 and 9%, respectively). In particular, all the three *D. polymorphus* strains exhibited the killer activity since these strains inhibited the growth of the killer-sensitive reference strain *S. cerevisiae* DBVPG 6500, whereas the neutral phenotype was found only among 1 *H. uvarum* (Ha12), 3 *St. bacillaris* (St1, St3 and St10) and the two *Z. bailii* strains. Previous studies reported that the most non-*Saccharomyces* yeasts presented neutral or sensitive phenotype (González-Arenzana et al. 2017; Rodríguez et al. 2004).

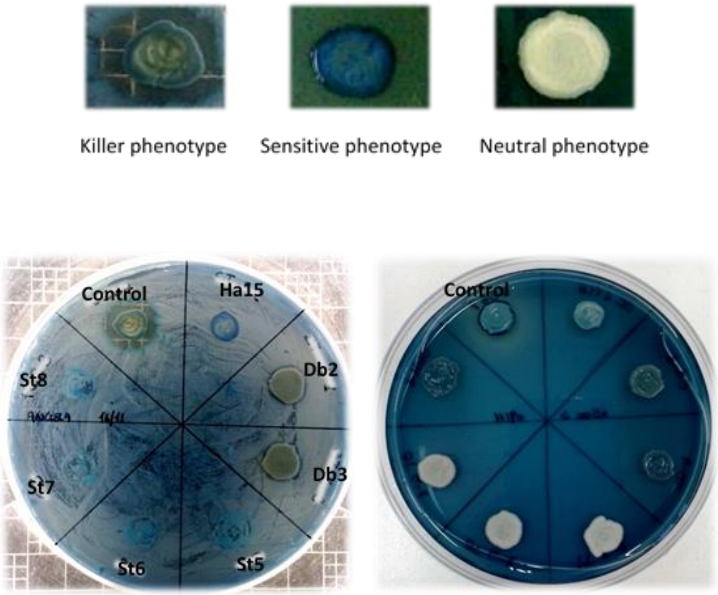

Figure 3.11| Screening for killer activity and neutral/sensitive phenotype among non-*Saccharomyces* strains.

3.6.2.2| Qualitative and Quantitative assays of enzymatic activities

In the second stage, the 33 non-*Saccharomyces* strains were screened for evaluation of enzymatic activities of oenological relevance as these activities can play an important role in modifying wine organoleptic characteristics.

The enzymatic activities analyzed were β-glucosidase, β-xylosidase and esterase, tested in plates containing specific substrates, obtaining the results reported in Table 3.5, in which the different activities were subdivided in the following classes: no activity (−), weak (+), moderate (++) and strong (+++). For evaluation of β-glucosidase activity, the non-*Saccharomyces* strains were tested in a medium containing a β-glucosidic substrate, in particular arbutin (β-D-glucoside) was used as the sole carbon source. Inclusion of ferric ammonium citrate in the YNB-arbutin plates produced a browning of the medium around β-glucosidase positive colonies (see Figure 3.12a).

As reported in Table 3.5, the majority of strains (73%) showed β-glucosidase activity, whereas only about 27% of them did not show activity (belonging to *St. bacillaris*). All *H. uvarum*, *D. polymorphus* and *Z. bailii* strains showed β-glucosidase activity, although at different level among the different strains, with the highest level of activity (+++) exhibited by two (of three) *D. polymorphus* and some *H. uvarum* strains. The two *Z. bailii* strains showed weak/moderate activity, whereas only two *St. bacillaris* strains (St1 and St2) showed β-glucosidase activity at weak level. Our results are in agreement with literature data, reporting that β-glucosidase activity is a widespread trait among non-*Saccharomyces* yeasts (Padilla et al., 2016).

The data obtained indicated the ability of the selected non-*Saccharomyces* strains to cut the β-glycosidic bond, enhancing the flavour of aromatic grape varieties (González Flores et al. 2017) and resulting very useful for industrial application.

Table 3.5| Qualitative screening of enzymatic activities in non-*Saccharomyces* strains.

Strain code	Qualitative enzymatic activities[1]		
	β-glucosidase	β-D-xylosidase	Esterase
Db1	+++	+++	-
Db2	+++	+++	-
Db3	+++	++	-
Ha1	+	-	-
Ha2	++	-	-
Ha3	++	+++	-
Ha4	++	++	-
Ha5	+	-	-
Ha6	+	-	-
Ha7	+	-	-
Ha8	++	-	-
Ha9	+++	++	-
Ha10	++	-	-
Ha11	+	++	-
Ha12	++	-	-
Ha13	++	-	-
Ha14	++	-	-
Ha15	+++	-	-
Ha16	+++	-	-
Ha17	++	+++	-
St1	+	-	-
St2	+	-	-
St3	-	-	-
St4	-	-	-
St5	-	-	-
St6	-	-	-
St7	-	-	-
St8	-	-	-
St9	-	-	-
St10	-	-	-
St11	-	-	-
Zb1	++	++	-
Zb2	+	-	-

[1]Presence of each activity: +++ = strong activity; ++ = moderate activity; + = weak activity; - = no activity.

The β-D-xylosidase activity was detected after 24 h of incubation and it was evaluated in function of the formation of UV fluorescent halos surrounding yeast colonies, resulting from the liberation of 4-methylumbelliferone from MUX (Figure 3.12c). The β-xylosidase activity was less diffused than β-glucosidase activity among the tested strains. Only few *H. uvarum*

strains (Ha3, Ha4, Ha9, Ha11 and Ha17), one *Z. bailii* strain (Zb1) and all the three *D. polymorphus* strains exhibited β-xylosidase activity, although only *D. polymorphus* strains showed high level of this enzymatic activity. None of *St bacillaris* strains produced β-xylosidase.

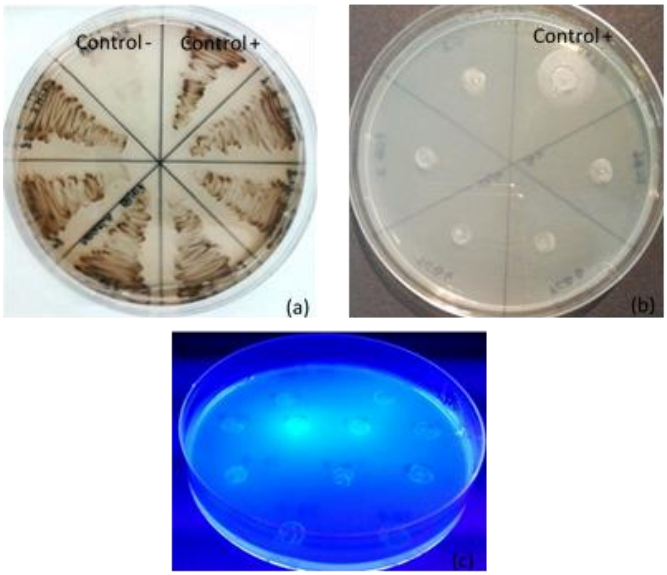

Figure 3.12| Evaluation of enzymatic activities on screening plates. (a) β-glucosidase activity on arbutin as substrate; (b) Esterase activity on Tween 80 medium; (c) β-xylosidase activity on MUX as substrate.

However, among all the 33 non-*Saccharomyces* strains, only 9 strains (Db1, Db2, Db3, Ha3, Ha4, Ha9, Ha11, Ha17 and Zb1) gave positive results for both the enzymatic activities tested. The non-*Saccharomyces* strains were evaluated also for the production of esterase enzyme, by using an agarized medium containing Tween 80 (Figure 3.12b). The presence of a halo around inoculated colony indicates a positive test, with production of esterase. None of the tested strains resulted able to produce esterase enzyme (Table 3.5), although the halo around the colony was found for the positive control (*S. cerevisiae*).

In summary, regarding the enzymatic activities evaluated in this study all *D. polymorphus* strains show glucosidase and β-xylosidase activities; also *H. uvarum* strains produced the two activities but with lower percentages of positive strains for β-xylosidase than for glucosidase (20 and 100%, respectively). As regards *St. bacillaris*, one only activity was found (β-glucosidase), showed by only two strains (St1 and St2), whereas for *Z. bailii*, one strain (Zb1) showed both the activities and Zb2 only β-glucosidase.

The yeast strains showing β-glucosidase and β-xylosidase activities on plates were selected for quantitative determination of these enzymes.

The β-glucosidase and β-xylosidase activities were evaluated by detecting the hydrolysis of the substrate *p*-nitrophenyl-β-d-glucopyranoside (p-NPG) and *p*-nitrophenyl-β-D-

xylopyranoside (p-NPX), respectively, which yields *p*-nitrophenol, a substance which was spectrophotometrically measured at 400nm.

The concentration of p-NPG and p-nPNX was measured by using a linear calibration curve (R2 > 0.996, Figure 3.13) of 4-nitrophenol (Sigma) solution, with a concentration of 100.000 nmol/L. The results were expressed as the amount of enzyme able to liberate 1 nmol of p-nitrophenol (p-NP) for minute.

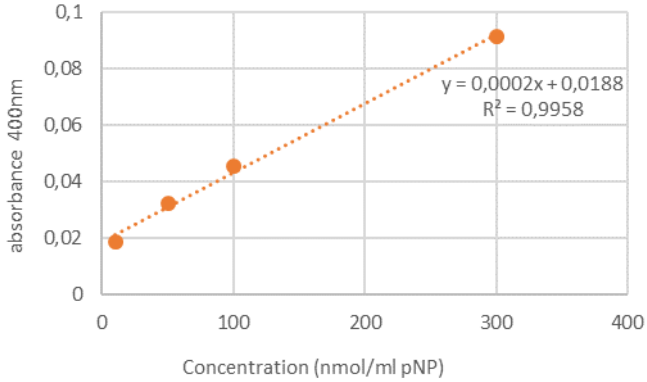

Figure 3.13| Linear calibration curve for enzymatic activities.

All enzymatic measurements were done in duplicate. The results are reported in Table 3.6, in which data presented are the mean ± standard deviation. Quantitative screenings for extracellular β-glucosidase and β-xylosidase activity revealed high level of variability among the non-*Saccharomyces* strains. All *H. uvarum* strains exhibit levels medium/high of β-glucosidase activity, in particular, a very high level of this activity was exhibited by Ha3, Ha9, Ha12, Ha13, Ha14, Ha15, ha16 and Ha17 (between 102.060 and 117.570 nmol pNP/ mL/ h). The three *D. polymorphus* strains (Db1, Db2 and Db3) exhibited a high level of activity (higher than 120 nmol pNP/mL/h). Almost all *St. bacillaris* strains did not exhibit levels of β-glucosidase activity, only in two strains (St1 and St2) a weak enzymatic activity was observed (8.182 and 26.550 nmol pNP/ mL/h), whereas *Z. bailii* strains showed a medium level of β-glucosidase. Interestingly, some of these strains (Db1, Db2, Db3, Ha3, Ha9, Ha14, Ha15 and Ha16) showed an enzymatic activity higher than the *H. uvarum* reference strain H2 (114.305 nmol pNP/mL/h). From a biotechnological point of view, high levels of β-glucosidase activity have been previously associated with increased hydrolysis of bound monoterpenes, which can enhance the fruity character of the wines (Guaragnella et al., 2020; Jolly et al., 2014).

As regards the quantitative screening for extracellular β-xylosidase activity, only 9 non-*Saccharomyces* strains exhibited a level of activity. As regards strains belonging to *D. polymosphus* species, two strains showed high levels of enzymatic activity, whereas only Db3 strain exhibited low level of β-xylosidase activity. Two *H. uvarum* strains (H3 and

Ha17) showed high enzymatic activity (106.14 and 93.896 nmol pNP/mL/h), whereas no remarkable level was detected in the *Z. bailii* strain (22.468 nmol pNP/mL/h). All *St. bacillaris* strains did not exhibit detectable levels of β-xylosidase activity.

Table 3.6|Quantitative enzymatic activities of the selected non-*Saccharomyces* strains.

Strain code	Quantitative enzymatic activities[1]	
	β-glucosidase	β-D-xylosidase
Db1	128.590 ± 8.658^{ab}	155.121 ± 17.317^{a}
Db2	157.162 ± 25.975^{ab}	108.182 ± 20.203^{b}
Db3	151.039 ± 28.862^{ab}	48.998 ± 5.772^{c}
Ha1	48.998 ± 5.772^{c}	nd
Ha2	87.774 ± 8.658^{ac}	nd
Ha3	116.345 ± 14.431^{a}	106.141± 5.772^{b}
Ha4	95.937 ± 20.203^{ac}	57.162 ± 5.772^{cd}
Ha5	46.958 ± 8.658^{c}	nd
Ha6	44.917 ± 5.772^{c}	nd
Ha7	51.039 ± 2.886^{c}	nd
Ha8	83.692 ± 20.203^{ac}	nd
Ha9	177.570 ± 14.431^{bd}	51.039 ± 2.886^{cd}
Ha10	81.652 ± 11.545^{ac}	nd
Ha11	55.121 ± 8.658^{c}	28.590 ± 5.772^{c}
Ha12	110.223 ± 5.772^{a}	nd
Ha13	102.060 ± 5.772^{ac}	nd
Ha14	120.427 ± 8.658^{a}	nd
Ha15	116.345 ± 8.658^{a}	nd
Ha16	120.427± 2.886^{a}	nd
Ha17	100.019 ± 14.431^{ac}	93.896 ± 11.545^{bd}
St1	26.550 ± 8.658^{ce}	nd
St2	8.182 ± 5.772^{ce}	nd
Zb1	83.692 ± 8.658^{ac}	22.468 ± 8.658^{c}
Zb2	59.203 ± 14.431^{c}	nd

[1]The activities was measured as the amount of β-glucosidase that released nmol of p-nitrophenol (p-NP) for mL for hours (nmol pNP/mL/h). H2 and EC1118 were used as positive and negative, respectively, reference strains. Data are means ± standard deviations of two replicas. The different lowercase letters in superscript within the same column indicate significant differences between strains (Tukey's test $P \leq 0.05$).
nd = not tested as negative to qualitative test

3.6.2.3| Statistical analysis of data obtained by preliminary screening

The data obtained by preliminary screening of the 33 non-*Saccharomyces* strains aimed to evaluate technological traits useful for winemaking were converted into adimensional values (Table 3.7), following the criteria previously reported (Table 3.2), and the obtained matrix was submitted to cluster analysis.

Table 3.7| Adimensional values assigned to all data obtained from technological screening of 33 non-*Saccharomyces* strains.

Strains	H_2S Production	SO_2 Mg/L	Cu (µmol/L)	EtOH %(v/v)	β-glucosidase[1]	β-D-xylosidase[1]
Db1	0 (cream)	1 (125)	3 (300)	2(12)	3 (128.590)	3 (155.121)
Db2	0 (cream)	3 (200)	3 (300)	3 (14)	3 (157.162)	3 (108.182)
Db3	0 (cream)	3 (200)	3 (300)	3 (14)	3 (151.039)	2 (48.998)
Ha1	1 (hazelnut)	2 (175)	2 (200)	3 (14)	1 (48.998)	0 (0)
Ha2	1 (hazelnut)	2 (175)	2 (200)	3 (14)	2 (87.774)	0 (0)
Ha3	1 (hazelnut)	2 (175)	2 (200)	3 (14)	2 (116.345)	3 (106.141)
Ha4	1 (hazelnut)	2 (175)	1 (100)	3 (14)	2 (95.937)	2 (57.162)
Ha5	1 (hazelnut)	2 (175)	1 (100)	3 (14)	1 (46.958)	0 (0)
Ha6	1 (hazelnut)	2 (175)	1 (100)	3 (14)	1 (44.917)	0 (0)
Ha7	1 (hazelnut)	2 (175)	2 (200)	3 (14)	1 (51.039)	0 (0)
Ha8	1 (hazelnut)	2 (175)	2 (200)	3 (14)	2 (83.692)	0 (0)
Ha9	1 (hazelnut)	1 (100)	2 (200)	2 (12)	3 (177.570)	2 (51.039)
Ha10	1 (hazelnut)	1 (100)	1 (100)	2 (12)	2 (81.652)	0 (0)
Ha11	1 (hazelnut)	1 (100)	2 (200)	2 (12)	1 (55.121)	2 (28.509)
Ha12	1 (hazelnut)	1 (100)	2 (200)	2 (12)	2 (110.223)	0 (0)
Ha13	1 (hazelnut)	1 (100)	2 (200)	2 (12)	2 (102.060)	0 (0)
Ha14	1 (hazelnut)	1 (100)	1 (100)	2 (12)	3 (120.427)	0 (0)
Ha15	1 (hazelnut)	1 (125)	2 (200)	2 (12)	2 (116.345)	0 (0)
Ha16	1 (hazelnut)	0 (50)	1 (100)	3 (14)	3 (120.427)	0 (0)
Ha17	1 (hazelnut)	0 (50)	1 (100)	3 (14)	2 (100.019)	3 (93.896)
St1	1 (hazelnut)	2 (175)	2 (200)	3 (14)	1 (26.550)	0 (0)
St2	3 (dark brown)	3 (200)	3 (300)	3 (14)	0 (8.182)	0 (0)
St3	3 (dark brown)	2 (175)	3 (300)	3 (14)	0 (0)	0 (0)
St4	1 (hazelnut)	2 (175)	3 (300)	3 (14)	0 (0)	0 (0)
St5	1 (hazelnut)	3 (200)	3 (300)	3 (14)	0 (0)	0 (0)
St6	1 (hazelnut)	3 (200)	3 (300)	3 (14)	0 (0)	0 (0)
St7	1 (hazelnut)	3 (200)	2 (200)	3 (14)	0 (0)	0 (0)
St8	1 (hazelnut)	3 (200)	2 (200)	3 (14)	0 (0)	0 (0)
St9	1 (hazelnut)	3 (200)	2 (200)	3 (14)	0 (0)	0 (0)
St10	1 (hazelnut)	3 (200)	2 (200)	3 (14)	0 (0)	0 (0)
St11	1 (hazelnut)	3 (200)	2 (200)	3 (14)	0 (0)	0 (0)
Zb1	1 (hazelnut)	3 (200)	1 (100)	3 (14)	2 (83.692)	2 (22.468)
Zb2	1 (hazelnut)	3 (200)	1 (100)	3 (14)	1 (59.203)	0 (0)

[1]Enzymatic activities reported as nmol pNP/mL/h

The obtained dendrogram, reported in Figure 3.14, showed that the strains were distributed in six main groups, named A, B, C, D, E and F. The technological characteristics of strains composing these groups are reported in the Table 3.8. The strain grouping is correlated to the species, i.e. strains belonging to the same species were included in the same group, except clusters D and F. These clusters are composed mainly by *H. uvarum* strains, except Zb1 (*Z. bailii*) and Db1 (*D. polymorphus*), for group D, and Zb2 (*Z. bailii*) and St1 (*St. bacillaris*),

for cluster F. All the other *St. bacillaris* strains were included in A and B groups, whereas the other *H. uvarum* strains grouped in cluster E and the other two *D. polymorphus* strains composed the group C. As regards the technological characteristics of strains included in the different groups, clusters A and B include strains exhibiting high level of tolerance to antimicrobial compounds and the lowest level of β-glucosidase and β-xylosidase activities. The characteristic mainly differentiating strains included in group A from those included in cluster B is the production level of H_2S, which was very high in A and low in B.

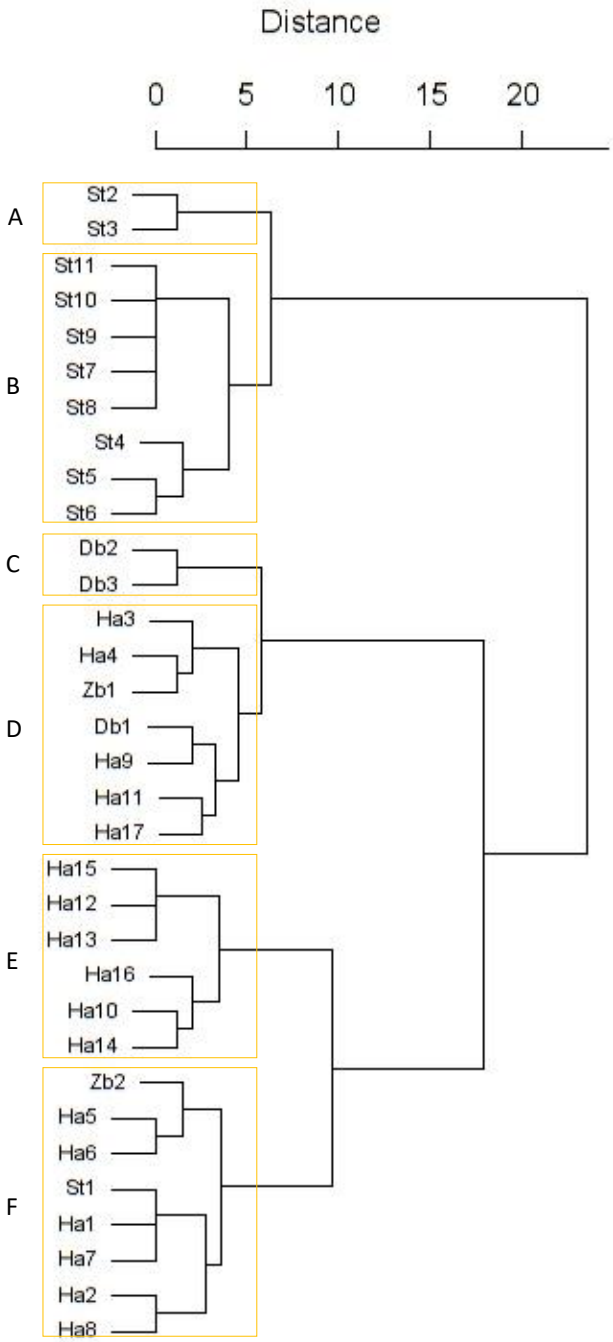

Figure 3.14| Dendrogram obtained after cluster analysis performed on technological characteristics (production level of H_2S, resistance to antimicrobial compounds and qualitative enzymatic activities) used for preliminary screening of 33 non-*Saccharomyces* strains.

Table 3.8| Characteristics of non-*Saccharomyces* strains included in groups obtained by cluster analysis of data obtained by preliminary screening (Figure 3.14).

Group	SO_2 mg/L	Cu µmol/L	EtOH % (v/v)	β-glucosidase[1]	β-xylosidase[1]	H_2S[2] Production
A	175-200	300	14	0-8.182	0	3
	(2-3)	**(3)**	**(3)**	**(0)**	**(0)**	
B	175-200	200-300	14	0	0	1
	(2-3)	**(2-3)**	**(3)**	**(0)**	**(0)**	
C	200	300	14	151.039-157.162	48.998-108.182	0
	(2)	**(3)**	**(3)**	**(3)**	**(2-3)**	
D	50-200	100-300	12-14	55.121-177.570	22.468-155.121	0-1
	(0-3)	**(1-3)**	**(2-3)**	**(1-3)**	**(2-3)**	
E	50-125	100-200	12-14	81.652-120.427	0	1
	(0-1)	**(1-2)**	**(2-3)**	**(2-3)**	(0)	
F	175-200	100-200	14	26.550-87.774	0	1
	(2-3)	**(1-2)**	**(3)**	**(1-2)**	**(0)**	

[1]Enzymatic activities, no activity = 0.
[2]Qualitative production of H_2S on BiGGY Agar (0 = cream colony; 1 = hazelnut; 2 = brown colony; 3 = dark brown colony)

The two strains of *D. polymorphus* (Db2 and Db3) composing the cluster C showing the best combination of the technological parameters tested. In fact, these strains were characterized by high ability to tolerate high concentration of sulphur dioxide, copper and ethanol, high levels of both the enzymatic activities tested and lowest level of H_2S production.

As a consequence, these strains can be considered excellent strains as potential candidates for winemaking in mixed fermentation with *Saccharomyces cerevisiae*. The cluster D is composed by six strains belonging to different species, which showed a high variability for tolerance level to the antimicrobial compounds and for β-glucosidase activity (ranging from low to high), whereas these strains were characterized by medium-high level of β-xylosidase activity. The cluster E includes strains belonging to the *H. uvarum* species, which are characterized by the following common characteristics: a low level of H_2S production, tolerance level to sulphur dioxide (very sensitive), medium/high β-glycosidase activity and medium level of copper tolerance. The strains grouped in F are characterized by medium/high level of resistance to sulphur dioxide, medium/low tolerance to copper and medium level of β-glucosidase activity, whereas both strains grouped in E and F resulted not producers of β-xylosidase.

On the basis of these technological characteristic, nineteen strains, representative of the different clusters and characterized by high level of tolerance to antimicrobial compounds and/or enzymatic activities, were selected and submitted to further characterization (Table 3.9). In particular, two *D. polymorphus* strains, eleven *H. uvarum* strains, five *St. bacillaris*

and one to *Z. bailii* strains were chosen. The selected strains were evaluated for ability to grow in high ethanol and sulphur dioxide concentrations and sulphur dioxide resistance in microfermentation.

Table 3.9| Main technological characteristics of selected strains.

Strains	**SO_2** mg/L	**Cu** µmol/L	**β-glucosidase** nmol pNP/mL/h	**β-D-xylosidase** nmol pNP/mL/h
Db1	125	300	128.590 ± 8.658	155.121 ± 17.317
Db2	200	300	157.162 ± 25.975	108.182 ± 20.203
Ha2	175	200	87.774 ± 8.658	nd
Ha3	175	200	116.345 ± 14.431	106.141± 5.772
Ha5	175	100	46.958 ± 8.658	nd
Ha7	175	200	51.039 ± 2.886	nd
Ha9	100	200	177.570 ± 14.431	51.039 ± 2.886
Ha10	100	100	81.652 ± 11.545	nd
Ha12	100	200	110.223 ± 5.772	nd
Ha14	100	100	120.427 ± 8.658	nd
Ha15	125	200	116.345 ± 8.658	nd
Ha16	50	100	120.427 ± 8.658	nd
Ha17	50	100	100.019 ± 14.431	93.896 ± 11.545
St1	175	200	26.550 ± 8.658	nd
St2	200	300	8.182 ± 5.772	nd
St4	175	300	nd	nd
St5	200	300	nd	nd
St8	200	200	nd	nd
Zb1	200	100	83.692 ± 8.658	22.468 ± 8.658

nd = not tested as negative to qualitative test

3.6.3| Growth in high concentration of ethanol and SO_2

The selected strains were further evaluated for resistance to stress factors present during winemaking, such as high concentration of ethanol and SO_2, that represent the strongest stress factors for non-*Saccharomyces* strains. The evaluation of strain resistance to these stress conditions was performed by measuring the influence of high concentration of ethanol and SO_2 on strain growth. In particular, the 19 strains were inoculated in liquid medium, added with increasing doses of ethanol (14-16% v/v) and SO_2 (150-200 mg/L), and incubated at 26°C for 24 hours. The strain response was reported as relative cell growth, determined by comparing the growth with and without the stress factor.

Ethanol tolerance varied among strains, and with increasing ethanol concentration, leading to reduced growth for all the tested strains (Figure 3.15).

The most ethanol-tolerant strain was Zb1 (*Z. bailii*), followed by the two strains of *D. polymorphus* (Db1 and Db2), whereas *H. uvarum* strains were the most sensitive to the high concentration of ethanol.

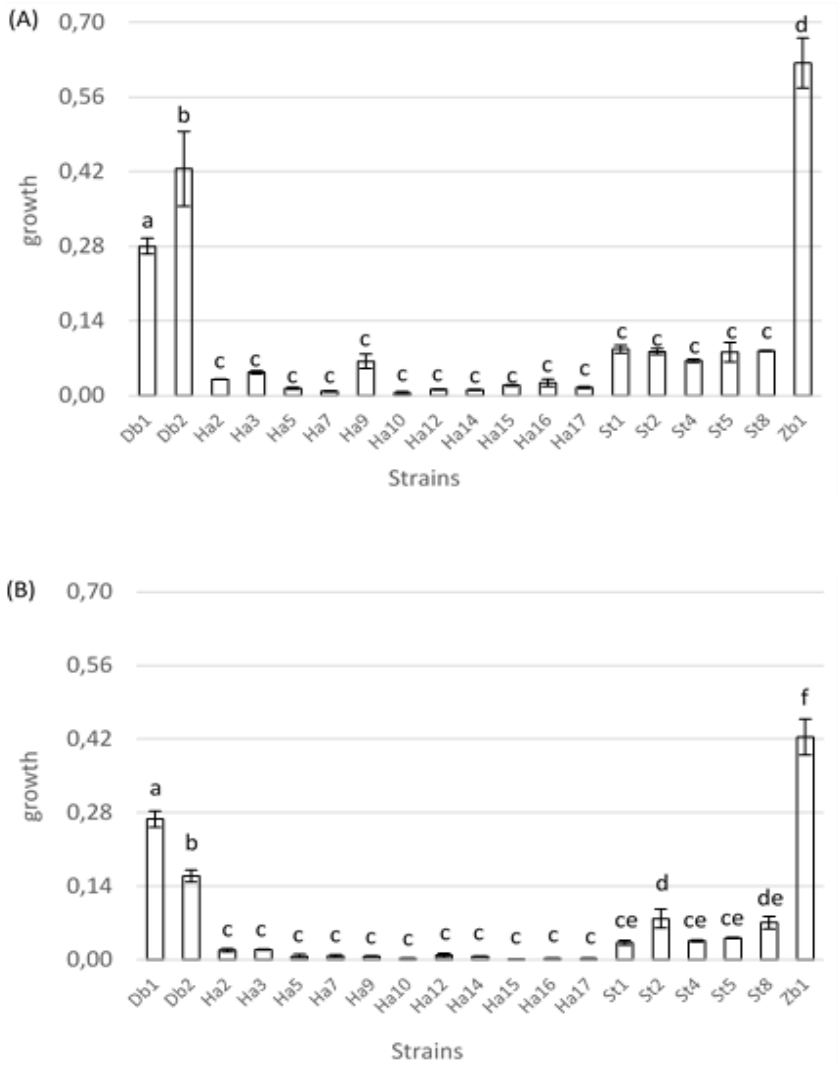

Figure 3.15| Strain growth in presence of 14% (A) and 16% of ethanol (B), expressed as the ratio between the growth in broth with and without ethanol. All the data are reported as mean and standard deviation. Different superscript letters indicate significant differences among the strains (Tukey's test $p < 0.05$).

As regards the growth in presence of high concentration of SO_2 (Figure 3.16), all the strains were inhibited by the presence of high doses of this compound (150 and 200 mg/L). The highest level of tolerance was found among the two *D. polymorphus* strains, as already reported for ethanol tolerance, whereas Zb1 strain was very sensitive to the presence of high concentration of sulphur dioxide.

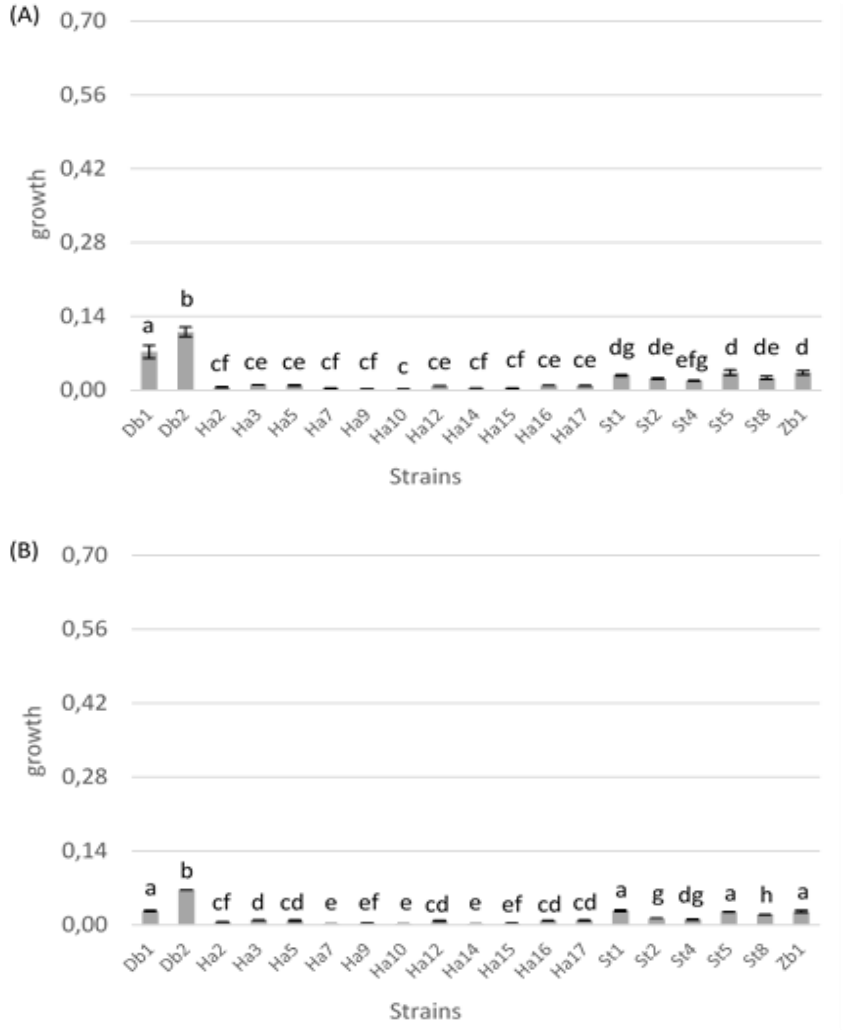

Figure 3.16| Strain growth in presence of 150 (A) and 200 (B) mg/L of SO_2, expressed as the ratio between the growth in broth with and without SO_2. All data are expressed as mean and standard deviation. Different superscript letters indicate significant differences among the strains (Tukey's test $p < 0.05$).

3.6.4| Evaluation of sulphur dioxide resistance in microfermentation

The 19 strains were also evaluated for the influence of SO_2 on the fermentative activity, during the early stages of fermentation, as this compound is normally added to winemaking. The resistance of the strains to SO_2 was evaluated by measuring strain fermentative vigour in presence of 30 mg/L of SO_2, calculated on the basis of weight loss after 48 hours of fermentation. For each strain, the ratio between fermentative vigour with SO_2 and the fermentative vigour without SO_2 addition was used to express the SO_2 resistance level (Figure 3.17).

The SO_2 addition determined a reduction of fermentative vigour of the strains, indicating strain sensitivity to this compound.

As reported in Figure 3.17, several *H. uvarum* strains showed a reduction of the fermentative vigour after SO_2 addition, with values ranging between 0.60 and 0.68, whereas three strains (Ha3, Ha14 and Ha17) were less affected by SO_2 addition, in which the ratio between

fermentative vigour with and without SO_2 showed values higher than 0.70. Similar behaviour was observed among the other strains (two *D. polymorphus*, one *Z. bailii* and four *St. bacillaris* strains) and the most resistant strain was one *St. bacillaris* strain (St8), which showed the highest ratio between fermentative vigour with and with SO_2 (0.92), higher also than the value observed for the *S. cerevisiae* strain EC1118.

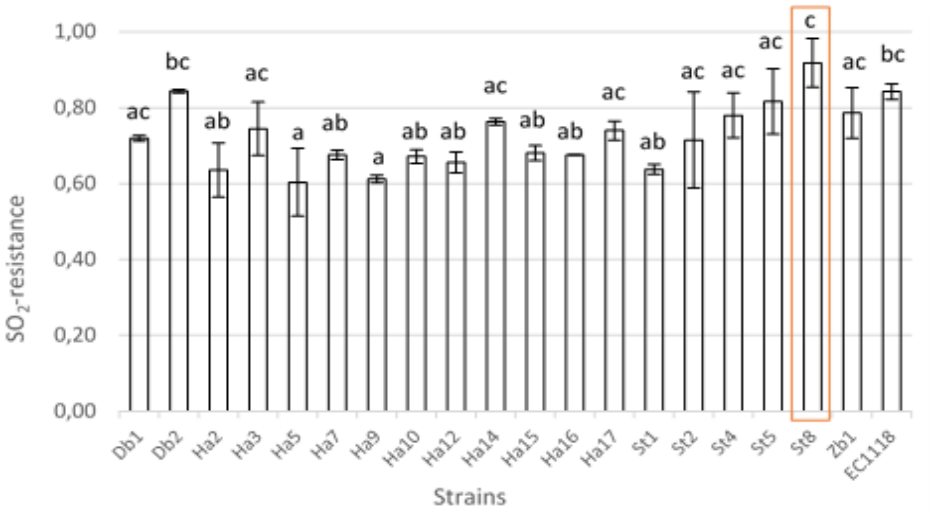

Figure 3.17| Evaluation of strain resistance to SO_2 during microfermentation. The SO_2 resistance level was expressed as the ratio between the fermentative vigour in presence of 30 mg/L of SO_2 and without SO_2 addition. The different letters indicate differences statistically significant (Tukey's test, $p < 0.05$) among strains.

On the basis of obtained results, eight non-*Saccharomyces* strains (Db2, Ha3, Ha9 St1, St2, St5, St8 and Zb1), characterized by the highest resistance level to EtOH and SO_2 and high levels of enzymatic activities, were selected and submitted to further characterization.

3.7| Conclusions

This step of the research has been focused on preliminary screening among non-conventional yeasts to individuate strains, characterized by traits of oenological interest, to be used as potential candidates in mixed starter cultures for the production of low alcohol wine with peculiar aromatic traits.

A total of 33 non-*Saccharomyces* strains, belonging to *D. polymorphus*, *H. uvarum*, *St. bacillaris* and *Z. bailii* species were studied. Although these yeast species were described in literature for the use as mixed starter cultures, it was necessary to exploit the technological characteristics of the chosen strains as it's well known that not all the strains within a species show the same desirable characteristics.

Technologically important properties useful for winemaking applications of non-conventional yeasts are high tolerance to antimicrobial compounds present in winemaking, as ethanol, sulphite and copper, killer properties, limited production of H_2S. Furthermore,

also the presence of high levels of enzymes with of oenological interest is a required characteristic as the presence of these activities promotes the liberation of grape terpenoids. In fact, the aromatic quality of wine is the result of a strict interaction between grape must composition and yeast strains performing the fermentation; as a consequence, yeast abilities to release volatile compounds from grape precursors and to synthesize de novo volatile compounds are very important traits for wine starter cultures.

The first screening showed a wide intraspecific difference within the yeast species investigated, indicating that strain selection is of great importance, as not all strains within a species can necessarily show the same desirable or undesirable characteristics. These results underline the fundamental role of the screening program followed in the laboratory to select the most promising non-*Saccharomyces* strains able to efficiently ferment musts.

As regards the enzymatic activities, high variability was found for β-glucosidase and the highest level of these activity was exhibited by the strains Db1, Db2, Db3, Ha9, Ha14 and Ha16. In particular, *D. polymorphus* and *Z. bailii* strains exhibited interesting and desirable properties to improve wine sensory profile, such as the highest β-glucosidase and β-xylosidase activities, and the good resistance to antimicrobial compounds, a characteristic allowing them to survive during grape must fermentation.

Also *H. uvarum* strains showed interesting technological traits, but, due to their sensibility to high concentrations of ethanol, copper and sulphur dioxide, it could be used only in mixed culture with *S. cerevisiae* in order to complete the fermentation process. Moreover, all *H. uvarum* strains exhibited a high β-glucosidase activity.

All *St. bacillaris* strains exhibited a good resistance to antimicrobial compounds, and only two strains St1 and St2 showed presence of β-glucosidase activity.

Non-*Saccharomyces* strains able to tolerate stress conditions of fermentative process and exhibiting enzymatic activities are suitable to be used in the first stages of fermentation process to improve the quality of wine.

The goal of an efficient selection program is to find the best strains within determined species that are able to maximize the advantages and minimize the disadvantages. The obtained results highlight the importance to characterize a large number of strains for the selection of starters to be integrated in innovative mixed fermentation strategies, with the final goal of enhancing the quality of wine. Further research will be needed to fully evaluate the technological suitability and aromatic contribution of the selected strains in combination with *S. cerevisiae*.

Mixed starter cultures with non-*Saccharomyces* strains, carefully selected in function of wine characteristics and market trends, can be considered an innovative biotechnological

tool not only to improve wine complexity, but also to satisfy the current challenge of wine industry addressed to "alcohol reduction in wines".

From an initial amount of 33 non-*Saccharomyces* yeasts, we have selected 8 strains characterized interesting enological traits, as presence of β-glucosidase and β-xylosidase activities, and tolerance to fermentative stress.

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Chapter 4

Mixed fermentation at laboratory scale with selected non-*Saccharomyces* strains

Abstract

Non-*Saccharomyces* yeasts play a substantial role in the early stages of wine fermentation. With the increase in alcohol concentration, indigenous or commercial strains of *S. cerevisiae* take over and complete the transformation of the grape must sugars into ethanol, CO_2, and other secondary metabolites. Although the activity of non-*Saccharomyces* yeasts is limited to the first fermentation days, their presence has an impact on the wine composition, and consequently, their contribution during the fermentation process cannot be ignored. The use of these yeasts can be useful to meet the new challenges of current consumer preferences, addressed to well-structured, full-bodied wines with a rich flavor.

The aim of this step was to assess the oenological potential of some selected strains, belonging to *D. polymorphus* (Db2), *H. uvarum* (Ha3 and Ha9), *St. bacillaris* (St1, St2, St5 and St8) and *Z. bailii* (Zb1), tested in simultaneous (SiF) and sequential fermentation (SeF), with commercial *S. cerevisiae* EC 1118 strain.

The different combinations of strains tested influenced the growth dynamics, the fermentation behavior and wine composition. Moreover, both proposed strategies yielded wines containing ethanol levels lower than control wine (*S. cerevisiae* pure culture). On the basis of results related to fermentation kinetics, secondary compounds formation, and ethanol reduction, four strains were further selected (Ha3, St1, St8 and Zb1) and tested in co-fermentation with the *S. cerevisiae* EC 1118 in natural grape must. The last experiment demonstrated that co-inoculation was the most useful inoculation procedure for wine quality and ethanol reduction.

4.1| Introduction

Wine fermentation is an ecologically complex process that involves diverse yeast species at different stages. These species interact with each other and show a population succession, which is mostly characterized by large populations of non-*Saccharomyces* species at early stage and the dominance of *Saccharomyces* in the final step (Chen et al., 2018; Englezos et al., 2018; Fleet et al., 2008; Pietrafesa et al., 2020; Padilla et al., 2016; Tristezza et al., 2016; Wang et al., 2016). Yeast species play important role in the wine-making process: transforming sugars to ethanol and CO_2, producing specific secondary metabolites, and finally, contributing to wine flavor characteristic (Fleet, 2003; Romano et al., 2003; Seguinot et al., 2020; Jolly et al., 2006) (Figure 4.1).

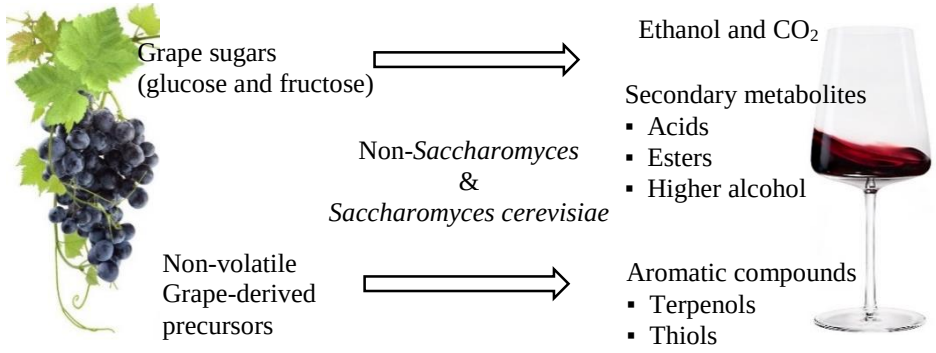

Figure 4.1| Main compounds produced during the wine-making process.

In recent years, increasing interest in the beneficial role of non-*Saccharomyces* yeasts in wine biotechnology was diffused (Aplin et al., 2019; Sadoudi et al., 2012; Su et al., 2020; Wang et al., 2016), resulting in several studies addressed to the use of controlled fermentations using *Saccharomyces* and non-*Saccharomyces* yeast species in winemaking (Ciani et al., 2010; Fleet, 2008; Pietrafesa, 2020). An emerging application of mixed fermentation is the use of this starters as a biotechnological tool to enhance specific characteristics of the wine, improving their complexity (Hu et al., 2018). It has been shown that some of the metabolites produced by these yeasts may be beneficial and contribute to the complexity of the wine when they are used in mixed fermentations with *S. cerevisiae* cultures (Padilla, 2016; Varela, 2016; Rodriguez-Gomez et al., 2010; Ruiz et al., 2018).

It was showed that, when pure non-*Saccharomyces* yeasts are cultivated with *S. cerevisiae* strains, their negative metabolic activities may not be expressed or could be modified by the metabolic activities of the *S. cerevisiae* strains. Numerous aspects support the use of multi-starter fermentation in winemaking, such as modification of specific analytical compounds,

increase of glycerol content, enhancement of total acidity or reduction of acetic acid content of the wine; enhancement of the analytical profile of the wine (esters, volatile thiols); reduction of the ethanol content of the wine; control of the spoilage microflora in the wine; and improvements to the overall quality and complexity of the wine (Contreras et al., 2014; Domizio et al., 2014; Gobbi et al., 2013; Hu et al., 2018; Jolly et al., 2006; Maturano et al., 2019; ; Quirós et al., 2014; Varela, 2016).

The aim of this step was to evaluate the fermentation performance and influence on organoleptic quality of wine of mixed starter cultures, composed by 8 non-*Saccharomyces* strains, selected after the previous step, and *S. cerevisiae* EC1118 during laboratory scale fermentations. In particular, the strains Db2 (D. *polymorphus*), Ha3, Ha9 (*H. uvarum*), St1, St2, St5, St8 (*St. bacillaris*) and Zb1 (*Z. bailii*) were tested in mixed fermentation by using two different inoculum modalities, simultaneous (SiF) and sequential fermentation (SeF), both in synthetic and natural grape must. Furthermore, in order to study the interaction mechanisms based on cell-to cell contact between strains composing mixed starters, it was investigated the interaction between *one S. cerevisiae* wild strain and one *H. uvarum* strain in mixed fermentation, by using the double-compartment fermentor pointed out by Renault et al. (2013).

4.2| Influence of non-*Saccharomyces* yeasts on wine aroma profile

As already reported, the first use of mixed starters including selected non-*Saccharomyces* yeasts was addressed to the production of wines with unique aromatic characteristics. The aroma is one of most important characteristics that contribute to the quality of wine, and is composed by 100s of different compounds. Three different types of aroma can be distinguished: the varietal or primary aromas that are grape-variety-specific, secondary aroma (produced by yeast metabolism during alcoholic fermentation), and tertiary aroma, resulting from the transformation of aromas during aging. Non-*Saccharomyces* yeasts can influence both the primary and secondary aroma through the production of enzymes and metabolites, respectively (Padilla et al., 2016).

Influence on Primary Aroma

Primary or varietal aroma is formed during the ripening of grapes and its contribution to the final wine aroma is considered an appreciated feature. The production of active compounds of primary wine odour takes place in the exocarp of the grape berry and its final concentration in wine is mainly influenced by the vine variety, but also by the state of ripeness and agronomic and oenological practices. Compounds forming primary aroma

belong to a limited number of chemical families, including methoxypyrazines, C13-norisoprenoids, volatile sulfur compounds, and terpenes. Methoxypyrazines are products of amino acid metabolism, and they have been associated to vegetal, while C13-norisoprenoids derive from carotenoids and, particularly β-ionone and β-damascenone, are considered impact volatiles of non-floral grapes. Moreover, in grape berries and corresponding wines, approximately seventy terpenoid compounds have been identified, among them, five monoterpenoid alcohols (i.e. linalool, geraniol, nerol, citronellol, and α-terpineol) are the most abundant and the strongest contributors to wine aroma. These compounds provide floral notes and have low odor thresholds (Padilla et al., 2016). Most of the primary aroma compounds occur in their linked forms, which makes them non-volatile and hence odorless. However, the linked aromas can be liberated during fermentation through the action of wine yeasts, mainly belonging to non-*Saccharomyces* species (Dutraive et al., 2019).

Particularly important are aroma precursors linked to sugar molecules, mainly terpenol and C13-norisoprenoid glycosides, and the non-volatile precursor forms of volatile thiols, conjugated to cysteine or glutathione (Padilla et al., 2016).

Influence on Secondary Aroma

Most of the compounds determining wine aroma arise from the fermentation process, and their concentrations are mainly dependent on the dominant yeasts and the fermentation conditions. Although ethanol, glycerol, and CO_2 are quantitatively the most abundant compounds, their contribution to the secondary aroma is relatively limited. Volatile fatty acids, higher alcohols, esters, and, to a lesser extent, aldehydes, have a greater contribution to secondary aroma (Figure 4.2).

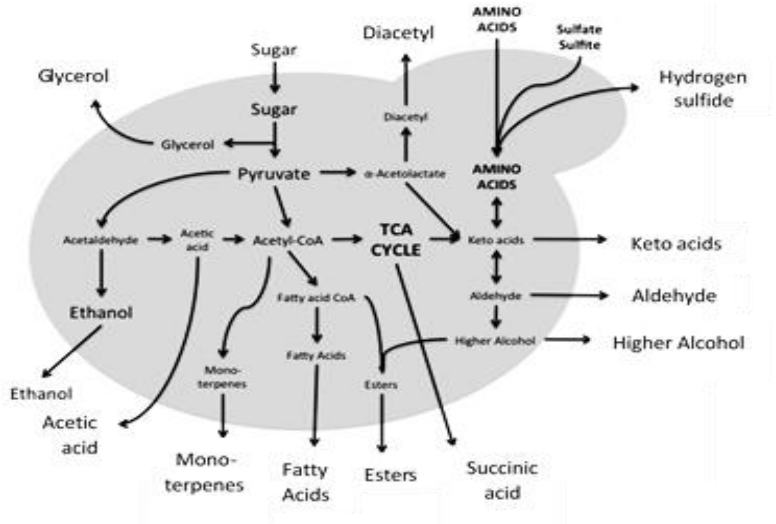

Figure 4.2| Main metabolites synthesized during the alcoholic fermentation through different pathways of yeasts metabolism.

The biosynthesis of these compounds is species- and strain-dependent, and those compounds of yeast metabolism can have a positive or negative impact on wine aroma and quality, in function of the concentration present in the wine.

Volatile Fatty Acids. Acetic acid is responsible for 90% of the volatile acidity of wines, while the remaining fatty acids, such as propanoic and butanoic acid, are present in small quantities. This compound becomes unpleasant at concentrations near its flavor threshold of 0.7-1.1 g/L and usually values between 0.2 and 0.7 g/L are considered optimal (Tristezza et al., 2016). The studies on acetic acid production by non-*Saccharomyces* yeasts have generated variable results. For example, some non-*Saccharomyces* genera such as *Hanseniaspora* and *Zygosaccharomyces* have been traditionally described as producers of excessive amounts of acetic acid (Romano et al., 2003), such as the species *Schizosaccharomyces* pombe is commonly associated with high levels of acetic acid. Levels of acetic acid ranging from about 0.6 g/L to more than 3.4 g/L have been described for *H. uvarum* strains (Romano et al., 2003; Benito et al., 2014). The *T. delbrueckii* strains are characterized by production low levels of volatile acidity, when compared to *S. cerevisiae* (Comitini et al., 2011). Similar behavior was found in *St. bacillaris* yeast (Englezos et al., 2015).

Higher Alcohols. These are the largest group of aromatic compounds and contribute to the aromatic complexity of wine at concentrations below 300 mg/L, whereas a concentrations exceed 400 mg/L, higher alcohols are considered to have a negative effect on aroma. These compounds are synthesized by the yeast as an intermediate in amino acids metabolism (Mestre et al., 2019). *Hanseniaspora* and *Zygosaccharomyces* species have been described as producers of low amounts of higher alcohols. Regarding specific alcohols, increased production of 2-phenylethyl alcohol, compound associated with pleasant aromas, has been described as a characteristic of *M. pulcherrima*, *L. thermotolerans*, *St. bacillaris* (Andorrà et al., 2010) and *T. delbrueckii* (Tristezza et al., 2016).

Esters. Esters are the most abundant compounds found in wine, with around 160 identified compounds to date. They are the result of pathways that appear during the alcoholic fermentation. Their proportion in wines is strain-specific, depends from the fermentation conditions (temperature and pH), and evolves, positively or negatively, during wine aging (Dutraive et al., 2019). These compounds have a pleasant impact in wine aroma, with ethyl acetate associated to fruity notes, ethyl hexanoate and ethyl octanoate to an apple flavor, and

ethyl decanoate to floral and fruity aromas. Although various esters can be formed during fermentation, the most abundant are those derived from acetic acid (ethyl, isoamyl, isobutyl, and 2-phenylethyl acetate) and ethyl esters of saturated fatty acids (ethyl butanoate, ethyl caproate, ethyl caprylate, and ethyl caprate). The main ester in wine is ethyl acetate, and it can impart spoilage character at levels above 150-200 mg/L. Species belonging to the genera *Candida*, *Hansenula*, and *Pichia* were described as having a greater capacity to produce ethyl acetate than wine strains of *S. cerevisiae*. Among *Hanseniaspora* species, *H. uvarum* is reported to be a good producer of esters, in general, whereas *H. guilliermondii* and *H. osmophila* are strong producers of 2-phenylethyl acetate (Tristezza et al., 2016). *Z. bailii* species was characterized as producer of several esters when inoculated together with *S. cerevisiae*, and increased aromatic complexity in wine (Garavaglia et al., 2014).

Aldehydes. These compounds with apple-like odors are important to the aroma and bouquet of wine due to their low sensory threshold values. Among aldehydes, acetaldehyde constitutes more than 90% of the total content of wines, and its amount can vary from 10 up to 300 mg/L. *S. cerevisiae* strains usually produce higher acetaldehyde levels (5-120 mg/L) than non-*Saccharomyces* species (up to 40 mg/L). An average acetaldehyde concentration of around 25 mg/L was described for *H. uvarum* strains, although significant differences in production among strains were observed (Romano et al., 2003; Tristezza et al., 2016).

4.3| Yeast interactions in mixed fermentations

Wine fermentation is a complex microbial process, where various microorganisms coexist and interact influencing the persistence of fermenting yeasts and the analytical profiles of wine. As consequence of the re-evaluation of the role of non-*Saccharomyces* yeasts, there is an increasing interest on the use of different species in mixed inoculated fermentation where the yeast interactions play a fundamental role (Ciani et al., 2015). The management of mixed fermentation strongly influences the dominance and persistence of yeast species. The investigations into multistarter fermentations require the elucidation of both the physiological and metabolic interactions between *S. cerevisiae* and non-*Saccharomyces* wine strains. 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 (Anfang, 2009; Ciani et al., 2010). Although the physiological and biochemical basis for the interactions among wine yeasts are unclear, environmental factors, production of yeast metabolites or

yeast–yeast interaction could be involved. In this context, the management of mixed fermentations, such as cell concentration and inoculation modalities, require more knowledge on environmental factors and metabolic activities influencing the yeast interactions. During wine fermentation, yeast species can be involved in several interactions through the production of toxic compounds, or as a result of competition for nutrients (Ciani et al, 2015) (Figure 4.3).

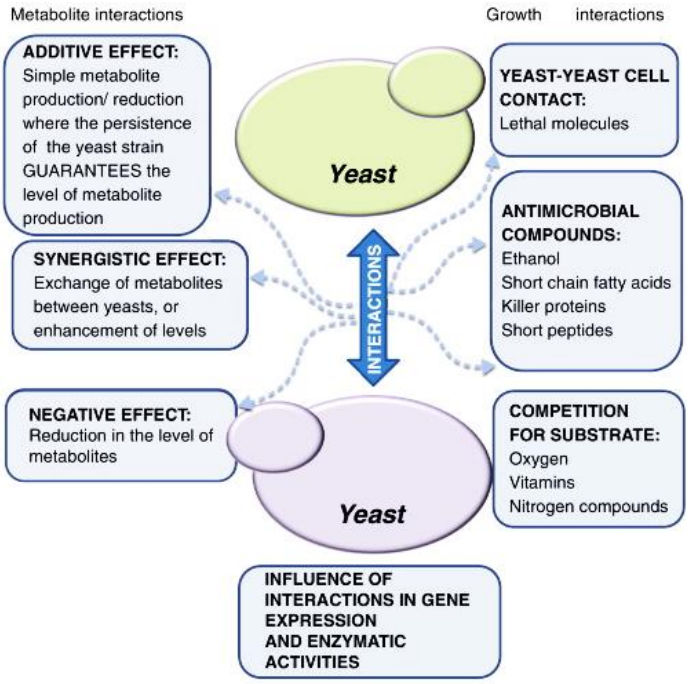

Figure 4.3| Factors affecting yeast interactions in mixed wine fermentations (Ciani et al., 2015)

In terms of inhibitory interactions mediated by metabolites with toxic effects, the most evident example is the production of ethanol during the fermentation process, by *S. cerevisiae* yeasts toward non-*Saccharomyces* yeasts (Ciani et al., 2016). The selective pression exerted by high levels of alcohols has been defined as the main factor responsible for the dominance of *S. cerevisiae* towards other non-*Saccharomyces* yeasts. Together with ethanol, other factors can have strong selective pressure in mixed wine fermentation, such as the production of medium-chain fatty acids and high amounts of acetic acid, can negatively affect the growth of co-fermenting yeasts. Another mechanism that regulates the presence of yeast species during wine fermentation is the oxygen. Oxygen availability plays an important role as selective factor in mixed cultures, affecting growth and fermentation performance of wine yeasts (Jolly et al., 2014). *Saccharomyces cerevisiae* and non-*Saccharomyces* wine yeasts exhibit a different behavior in presence of a low oxygen content; in anaerobic conditions, *S. cerevisiae* is able to grow quickly, while non-*Saccharomyces*

yeasts grow poorly under the same conditions (Ciani et al., 2016). Another factor, that could influence the behaviour and the dominance of yeast strains in mixed fermentation, is the availability of nitrogen source and vitamins (Liu et al., 2015). When non-*Saccharomyces* species grow fast in fermentation process, these yeasts can consume amino acids and vitamins, limiting the subsequent growth of the *S. cerevisiae* strain (Fleet, 2003). In addition, the presence of more yeast species might improve the uptake, and the consequent consumption, of some amino acids by *S. cerevisiae* strains, resulting in a synergistic mechanism of nitrogen use. Preliminary findings in this topic indicate that in multi-starter fermentation of *S. cerevisiae* and *H. uvarum*, less nitrogen is used than pure culture fermentations, which suggests that there is no competition for assimilable nitrogen compounds between *S. cerevisiae* and apiculate yeasts (Ciani et al., 2015).

Other metabolic activities that influence the controlled multistarter fermentations could be grouped in production of antimicrobial molecules and cell-to-cell contact mechanisms. Several studies investigated on the nature of the toxic compounds produced by *S. cerevisiae* responsible of the early death of non-*Saccharomyces* strains during mixed fermentations, founding that the killing effect was due to proteins compounds, such as killer toxins (Albergaria et al., 2010). The killer phenomenon has been widely described in winemaking and among wine-yeast species. The *S. cerevisiae* killer yeasts show a narrow spectrum of action, while non-*Saccharomyces* killer yeasts show wide inter-generic killer actions. Together with antimicrobial compounds, medium fatty acids, produced during alcoholic fermentation above a given threshold, could exhibit inhibitory actions toward *S. cerevisiae* and/or other species (Ciani et al., 2015).

Cell-cell contact has been described as another mechanism playing a fundamental role in interaction between wine yeasts. Some authors (Nissen et al., 2003; Nissen & Arneborg, 2003) suggested this mechanism was involved in interaction between *S. cerevisiae* and *T. delbrueckii*; these authors found that the presence of viable *S. cerevisiae* cells at high density may inhibit growth and induce the early death of *T. delbrueckii* species. Similar results were found by other authors (Renault et al., 2013), which confirmed that *S. cerevisiae* induced the death of *T. delbrueckii* in a mixed culture, underlying the involvement of physical cell-cell contact or close proximity, since *T. delbrueckii* cells remained viable and metabolically active when physically separated from *S. cerevisiae*.

It is not known whether contact with *S. cerevisiae* induced death, thus stopping *T. delbrueckii* metabolic activity, or whether *S. cerevisiae* contact inhibited metabolic activity, thus inducing cell death. Otherwise, *S. cerevisiae* growth and viability remained unchanged compared to the pure *S. cerevisiae* culture. As regards the influence of yeast interaction on

analytical composition of wine, two different metabolic mechanisms shown by yeast in mixed cultures can be distinguished, simple additive effects or specific metabolic interactions. In some cases, the aromatic profile of the wine is influenced by the simple addition of metabolites produced by each yeast from partial consumption of carbon or nitrogen sources, or by a specific metabolic activity. Moreover, the persistence of the specific yeast in the mixed fermentation determines the level of metabolite production or the metabolic activity. For example, the enhancement of glycerol levels and total acidity shown by mixed fermentation with non-*Saccharomyces*/*S. cerevisiae*, are strictly related to the persistence and competitiveness of the non-*Saccharomyces* strains (Ciani et al., 2015).

The fructophilic yeast *St. bacillaris* in mixed fermentations for sweet wine production determined an acetic acid production by *S. cerevisiae*. The high concentration of the sugars, which are responsible for the up-regulation of the genes encoding the aldehyde dehydrogenases, results in the high production of acetic acid in *S. cerevisiae*. The consumption of fructose by *St. bacillaris*, and the consequent osmotic pressure reduction, promotes a reduction in acetic acid production by the *S. cerevisiae* strain.

4.4| Materials and methods

4.4.1| Mixed fermentation in synthetic grape juice

4.4.1.1| Yeast strains

In this step, the yeast strains tested were the following:

- one *D. polymorphus* strain, Db2;
- two *H. uvarum* strains, H3 and H9;
- four *St. bacillaris* strains, St1, St2, St5 and St8;
- one *Z. bailii* strain, Zb1;
- one *S. cerevisiae* strain, EC1118 (Lallemand).

The eight non-*Saccharomyces* strains were previously selected on the basis of oenological characteristics, such as their phenotypic features, high β-glucosidase and β-xylosidase activities, resistance to high concentration of ethanol and SO_2, and high fermentative vigour. Table 4.1 shows the characteristics of selected yeast strains.

The yeast strains were maintained on yeast extract-peptone-dextrose (YPD) medium (10 g/L yeast extract; 20 g/L peptone; 20 g/L glucose; 20 g/L agar) at 4°C.

Table 4.1 | Main technological characteristics of the selected strains.

Strain code	Quantitative enzymatic activities		EtOH[(b)]	SO_2[(b)]
	β-glucosidase[(a)]	β-xylosidase[(a)]		
Db2	157.162 ± 25.98	108.182 ± 20.20	0.43 ± 0.07	0.11 ± 0.01
Ha3	116.345 ± 14.43	106.141± 5.77	0.04 ± 0.00	0.01 ± 0.00
Ha9	177.570 ± 14.43	51.039 ± 2.89	0.06 ± 0.01	0.01 ± 0.00
St1	26.550 ± 8.66	0.00 ± 0.00	0.09 ± 0.01	0.03 ± 0.00
St2	8.182 ± 5.77	0.00 ± 0.00	0.08 ± 0.01	0.02 ± 0.00
St5	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.02	0.03 ± 0.01
St8	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.00	0.02 ± 0.00
Zb1	83.692 ± 8.66	22.468 ± 8.66	0.62 ± 0.05	0.03 ± 0.00

[a]Enzymatic activities reported as nmol pNP/mL/h
[b]Strain tolerance to ethanol and SO_2, expressed as the ratio between the growth in broth with (14% v/v ethanol and 150 mg/L SO_2) and without the stress factor .

4.4.1.2| Mixed fermentations

The selected strains were tested in laboratory scale fermentations using synthetic grape juice medium. The composition of medium used, reported in Table 4.2, was described by Liu et al. (1995), Lonvaud-Funel et al (1988) and Weiller and Radler (1976) (as reported in Wang et al., 2003), but with some modifications. The final concentration of sugars was 180 g/L (90 g/L of glucose and 90 g/L of fructose), pH adjusted to 3.5.

Table 4.2| Composition of synthetic grape juice medium.

Components		Amounts
Carbon		
	D-Glucose (g/L)	115.0
	D-Fructose (g/L)	115.0
Acids		
	KH Tartrate (g/L)	5.0
	L-Malic acid (g/L)	3.0
	Citric acid (g/L)	0.20
Salts		
	K_2HPO_4 (g/L)	1.14
	$MgSO_4{\cdot}7H_2O$ (g/L)	1.23
	$CaCl_2{\cdot}2H_2O$ (g/L)	0.44
Nitrogen source		
Amino acid/Ammonia (25 x stock)		
	L-Alanine (mg/L)	26.0
	L-Arginine (mg/L)	188.0
	L-Asparagine (mg/L)	39.0
	L-Aspartic acid (mg/L)	89.0
	L-Glutamic acid (mg/L)	126.0
	L-Glutamine (mg/L)	51.0
	Glycine (mg/L)	14.0
	L-Histidine·HCL·H_2O (mg/L)	39.0

Table 4.2| Composition of synthetic grape juice medium (continue).

Components	Amounts
Nitrogen source	
Amino acid/Ammonia (25 x stock)	
L-Isoleucine (mg/L)	51.0
L-Leucine (mg/L)	76.0
L-Lysine·HCL (mg/L)	63.0
L-Methionine (mg/L)	39.0
L-Phenylalanine (mg/L)	39.0
L-Proline (mg/L)	126.0
L-Serine (mg/L)	101.0
L-Threonine (mg/L)	89.0
L-Tryptophan (mg/L)	26.0
L-Tyrosine (mg/L)	6.0
L-Valine (mg/L)	51.0
$(NH_4)_2HPO_4$ (mg/L)	100.0
Tace minerals (100 x stock)	
$MnCl_2·4H_2O$ (µg/L)	198.2
$ZnCl_2$ (µg/L)	135.5
$CuCl_2$ (µg/L)	13.6
$FeCl_2$ (µg/L)	32.0
H_3BO_3 (µg/L)	5.7
$Co(NO_3)_2·6H_2O$ (µg/L)	29.1
$NaMoO_4·2H_2O$ (µg/L)	24.2
KIO_3 (µg/L)	10.8
Vitamins (100 x stock)	
Myo-inositol (mg/L)	20.0
Pyridoxine·HCl (mg/L)	0.40
Nicotinic acid (mg/L)	0.40
Ca pantothenate (mg/L)	0.20
Thiamin·HCl (mg/L)	0.10
Riboflavin (mg/L)	0.04
Biotin (mg/L)	0.03
Folic acid (mg/L)	0.04
Other components	
Ergosterol (mg/L)	10.0
Tween 80 (mg/L)	0.5
pH	3.2 – 3.5

After preparation, the medium was filtered through a 0.22-µm-pore-size Millipore nitrocellulose membrane. All the stock solutions, such as vitamins, amino acid and trace minerals, were filtered through 0.22-µm-pore-size Millipore nitrocellulose membrane separately, and added aseptically to the medium. Finally, Ergosterol and Tween 80 stock solutions were added to the medium.

Fermentation tests were carried out in sterile 130 mL Erlenmeyer flask filled with 100 mL of synthetic must, equipped with stoppers and kept under static conditions at 26°C. The

flasks were inoculated with 48-h pre-cultures grown in YPD broth at 26°C with shaking. After incubation, the cells were harvested by centrifugation (3000 rpm for 5 min), and re-suspended in grape must.

The non-*Saccharomyces* strains were inoculated at 10^7 cells/mL, while the *S. cerevisiae* strain EC1118 was inoculated, at two different concentrations: 10^7 and 10^3 cells/mL. Each non-*Saccharomyces* strain was inoculated in combination with the *S. cerevisiae* strain in two different modalities, simultaneous (SiF) and sequential (SeF) inoculum. In the fermentation trial SeF, the inoculum ratio was 1:1, that is non-*Saccharomyces* strain 10^7 cells/mL and *S. cerevisiae* strain 10^7 cells/mL; in these trials, firstly, it was inoculated the non-*Saccharomyces* strain, whereas the *S. cerevisiae* strain was inoculated when the alcohol content reach about 5% (v/v). In the fermentation trials SiF, the inoculum ratio was 10.000:1, that is non-*Saccharomyces* strain 10^7 cells/mL and *Saccharomyces* 10^3 cells/mL; in this case, the two yeast strains were simultaneously inoculated.

The positive control was represented by pure culture fermentation, obtained inoculating the *S. cerevisiae* EC1118 at concentration of 10^7 cells/mL, whereas non-inoculated must was used as negative control.

Overall, nineteen fermentation trials were performed and, considering that the each fermentation was performed in duplicate, a total of thirty eight fermentations were followed.

Hydrogen Sulphide test strips was used for detection of hydrogen sulphide production by yeasts; a high H_2S production level appears as blackening of the lower part of the strip (level of blackening is directly correlated with production level of H_2S), while a no-production of H_2S is showed as no blackening.

4.4.1.3| Fermentation kinetics and yeast enumeration

The fermentation kinetics were monitored daily by measuring the weight loss of the flasks due to the carbon dioxide release. The process was considered completed when a constant weight of the samples was recorded for two-three consecutive days. The fermentation progress was calculated by the difference of the initial weight at day 0 and the weight from the day (Dutraive et al., 2019). Yeast strain kinetic growth was checked by plate counting on two different agar media: Wallerstein Laboratory (WL) Nutrient Agar medium (Sigma-Aldrich) (Pallmann et al., 2001) and Lysine Agar medium (Oxoid Unipath Ltd, Hampshire, UK) with addition of bromocresol green.

Fermenting must samples were taken from each flask at different fermentation steps. Briefly, samples were serially diluted with sterile saline solution and the number of colonies forming units per millilitre (UFC/mL) was determined by plating 100 μL of two appropriately chosen

dilutions on both the media. The plates were incubated at 26°C for 5 days. The two media were chosen in order to differentiate *Saccharomyces* and non-*Saccharomyces* growth. In fact, Lysine Agar medium is a selective medium unable to support the growth of *S. cerevisiae*, and it was used for the viable count of the non-*Saccharomyces* yeasts. WL is a differential medium that allows the putative identification of wine yeasts on the basis of colour and morphology of yeast colonies (Domizio et al., 2011, Pallmann et al., 2001; Polizzotto et al., 2016). Dilution plates containing a statistically representative number of colonies were counted.

4.4.1.4| Analytical Determinations

From each flask, daily samples were taken to monitor sugar concentration by measuring Brix degrees using a bench-top refractometer. Experimental wines obtained from the inoculated fermentation were analyzed for conventional chemical parameters, such as ethanol, total acidity, malic and lactic acid, volatile acidity, residual sugars, glucose, fructose, pH, by a Fourier Transfer Infrared WineScan instrument (OenoFoss™, Hillerød, Denmark). The OenoFoss™ is a dedicated analyser for rapid, routine measurement of key parameters in winemaking (Figure 4.4).

Figure 4.4| OenoFoss™ for rapid analysis of wines.

Furthermore, the content of the main secondary influencing wine aroma, such as acetaldehyde, n-propanol, isobutanol, amyl alcohols, ethyl acetate, acetic acid and acetoin, were determined by direct injection gas chromatography of 1 µl sample into a 180 cm × 2 mm glass column packed with 80/120 Carbopack B/5% Carbowax 20 M (Supelco, Bellefonte, PA). The column was run from 70 to 140 °C, the temperature being ramped up at a rate of 7 °C/min. The carrier gas was helium at a flow rate of 20 ml/min. Levels of the secondary compounds were determined by calibration lines, as described by Capece et al. (2013).

4.4.1.5| Statistical analysis

Analysis of variance (ANOVA) was used to evaluate differences in chemical compounds of the experimental wines obtained by different inoculation modalities, by using Tukey's test to compare the mean values. Principal component analysis (PCA) was carried out on the data of wines produced from mixed starters at laboratory scale. The PAST3 software ver. 3.20 (Hammer, Harper, & Ryan, 2018) was used for the statistical analyses.

4.4.2| Mixed fermentations in natural grape must

4.4.2.1| Yeast strains

On the basis of the results obtained from fermentation in synthetic grape must, 4 non-*Saccharomyces* strains were selected:

- *St. bacillaris* St1 and St8;
- *H. uvarum* Ha3;
- *Z. bailii* Zb1.

The selected strains were tested in mixed fermentations at laboratory scale in natural grape must by using simultaneous inoculation (Figure 4.5).

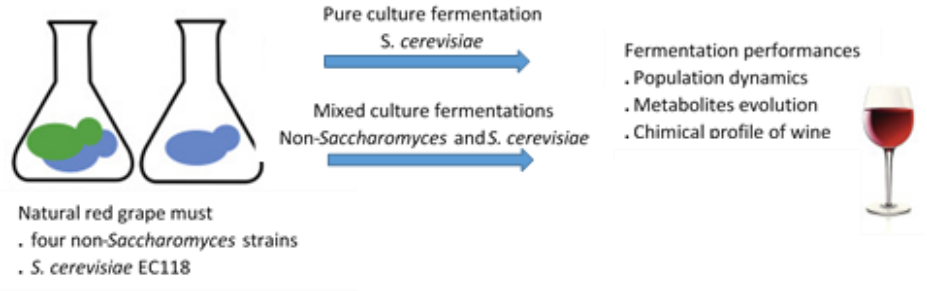

Figure 4.5| Mixed and pure culture fermentation in natural red grape must.

4.4.2.2 | Mixed fermentations

In each fermentation, the *S. cerevisiae* EC1118 strain was co-inoculated with one non-*Saccharomyces* strain, by using different inoculation ratio (10^3 cells/mL for *S. cerevisiae* and 10^7 cells/mL for non-*Saccharomyces*). As control, pure fermentations with the *S. cerevisiae* strains (10^7 cells/mL) were used.

The fermentations were carried out in natural red grape must, Aglianico variety, with the grape skin. The grape must was thermally treated at 80°C for 20 minutes in order to inactivate the yeasts population naturally present, and stored at -20°C until the use. Under sterile conditions, 400 mL of the pasteurized must was transferred in 500 mL sterile glass

bottle closed with a Müller valve filled with sulphuric acid. The grape must composition was the following: 206.6 g/L of sugars, pH 3.53, a total acidity 6.85 g/L, yeast assimilable nitrogen (YAN) composed of 70.60 mg/L of amino acids and 42.30 mg/L of ammonium.
Each fermentation was inoculated with cultures pre-grown in the YPD at 28°C for 48 h. The evolution of the fermentation was evaluated by measuring daily weight loss, caused by carbon dioxide (CO_2) release during the fermentation, and by assessing sugar concentration. The sugar content was measured with a bench-top refractometer, while the other compounds was determined by a Fourier Transfer Infrared WineScan instrument (OenoFoss, Hillerød, Denmark).
Fermentations were considered to be finished when the weight loss of the samples was constant for two-three consecutive days. Fermentation vigour (FV) and fermentation power (FP) of the strains were evaluated. FV was expressed as grams of CO_2 produced in 400 ml of must during the first 48 h of fermentation, while FP was expressed as grams of CO_2 produced at the end of fermentation.
The viable yeast cell population was evaluated by plate counting on WL and Agar Lysine media, following the protocol reported in the paragraph 4.3.1.3. Fermenting must samples were taken from each flask at days 0, 2, 4, 7 and 10 of fermentation. All the fermentations were performed at 26°C under static conditions, and three independent biological replicates were performed. At the end of the fermentation (< 2.0 g/L of residual sugars), wines were analysed for standard chemical parameters and wine samples were stored for analyses of main secondary metabolites.

4.4.2.3| Profiling of wine composition

Ethanol, organic acids production, as well as the glucose and fructose consumption were determined during and at the end of the fermentation by a Fourier Transfer Infrared WineScan instrument (OenoFoss™, Hillerød, Denmark). The glycerol was determined by enzymatic kit, following the manufacturer's instructions (Megazyme, Ireland). Furthermore, at the end of fermentations, concentration of secondary compounds (acetaldehyde, n-propanol, isobutanol, amyl alcohols, ethyl acetate and acetic acid) in wines was determined by direct injection gas chromatography, as previously reported (paragraph 4.3.1.4).

4.4.2.4| Statistical analysis

Each assay was performed in triplicate and the results were reported as the average of the three determinations with the corresponding standard deviation (±SD). Experimental data obtained during fermentations were analyzed by repeated analysis of variance

(ANOVA) measurements. Significant differences were determined by Tukey's test and the results were considered significant if the associated p value was below 0.05. A heat map was calculated with the increase or decrease in the production of volatile compounds for each mixed inoculation in relation to the pure culture of EC 1118, using the RStudio software.

4.4.3| Mixed fermentations in double-compartment fermentor

In order to evaluate the interaction between *S. cerevisiae* and *H. uvarum* and to evaluate the influence of cell-cell contact, *S. cerevisiae* (S) and *H. uvarum* (H) strains were tested in mixed fermentation by using a 2.4-L double-compartment fermentor (Renault et al., 2013) in two experimental conditions. In one condition (nonseparated [NS]), each fermentor compartment was inoculated with both strains (2×10^6 cells/mL of H and 2×10^2 cells/ml of S). In the second condition (separated [S]), the two strains were inoculated separately, by adding H at 2×10^6 cells/mL in the right compartment and S strain (2×10^2 cells/mL) in the left side. In both cases, natural red grape must (Merlot) was used (pH 3.34, sugar concentration 214 g/L, available nitrogen 122 mgN/L); before yeast inoculation, the must was sterilized by filtration (0.45 μm nitrate cellulose membrane). All the fermentations were carried out at 26°C. The fermentation kinetic was monitored by CO_2 release (Renault et al., 2013). The viable yeast cell population was evaluated by plate counting on WL at different fermentation steps. This experiment was performed in duplicate.

Samples of wines were taken from each compartment of the double fermentor at different times. The fermentation course and cell counts of the two species in S and NS modalities were evaluated as described previously in the paragraph 4.3.1.3.

4.4.3.1| Analysis of experimental wines obtained in double-compartment fermentor

The obtained wines were analysed for ethanol concentration (volume %) by infrared refractance (Infra Analyser 450, Technicon, Plaisir, France). Sugar (expressed as gramme per litre) and volatile acidity (expressed in grammes per litre of acetic acid) were determined chemically by colorimetry (460 nm) in continuous flux (Sanimat, Montauban, France), whereas TPC and AP were measured following the protocol previously described. Acetaldehyde, n-propanol, isobutanol, amyl alcohols and ethyl acetate were determined by direct injection gas chromatography (Capece et al., 2013). The wines were analysed also for esters content, both on final wines and at 40% of alcoholic fermentation completion, by solid-phase microextraction followed by gas chromatography mass spectrometry (SPME-GC-MS), following the protocol reported by Renault, Coulon, de Revel, Barbe and Bely (2015). Samples of wines were taken from each compartment of the double fermentor.

4.4.3.2| Statistical analysis

Analysis of variance (ANOVA) was used to evaluate differences in chemical and volatile compounds of the experimental wines obtained, which was done after the verification of variance homogeneity (Levene test, $p < 0.05$). Tukey's test was used to compare the mean values wines from double-compartment fermentor. Principal component analysis (PCA) was carried out on the data of esters detected in wines from double-compartment fermentor (both at 40% of alcoholic fermentation completion and at the end of the process). The PAST software ver. 3.20 (Hammer, Harper, & Ryan, 2018) was used for the statistical analyses.

4.5| Results and Discussions

4.5.1| Mixed fermentation in synthetic grape juice: Simultaneous and Sequential inoculum

Eight wild non-*Saccharomyces* strains (Db2, Ha3, Ha9, St1, St2, St5, St8 and Zb1), were selected in the first step and tested as mixed starters with a commercial *S. cerevisiae* strain in lab-scale microvinification trials (Figure 4.6). In this step, the fermentative behaviour of mixed starter cultures was tested by using two modality of inoculation, simultaneous and sequential inoculum.

Figure 4.6| Laboratory-scale fermentations.

4.5.1.1| Fermentation performance

The fermentation kinetics, represented by CO_2 release, of mixed cultures in micro-vinification trials are showed in Figures 4.7 and 4.8. The trend of CO_2 production was very similar in all the simultaneous fermentations, with an increase after the second fermentation day, except for the mixed fermentation with *St. bacillaris* strains which start already after the first day, such as the control *S. cerevisiae* EC1118 (Sc). All the simultaneous fermentations were completed in 14 days, without significant differences among them,

except for the mixed fermentation with Zb1+Sc starter, which was completed in 16 days. At the end of the process, the maximum CO_2 production was found in the fermentation inoculated with St1+Sc starter (about 13.01 g/100 mL), whereas the lowest amount (about 11.99 g/100 mL) was detected in the fermentation inoculated with Ha9+Sc starter.

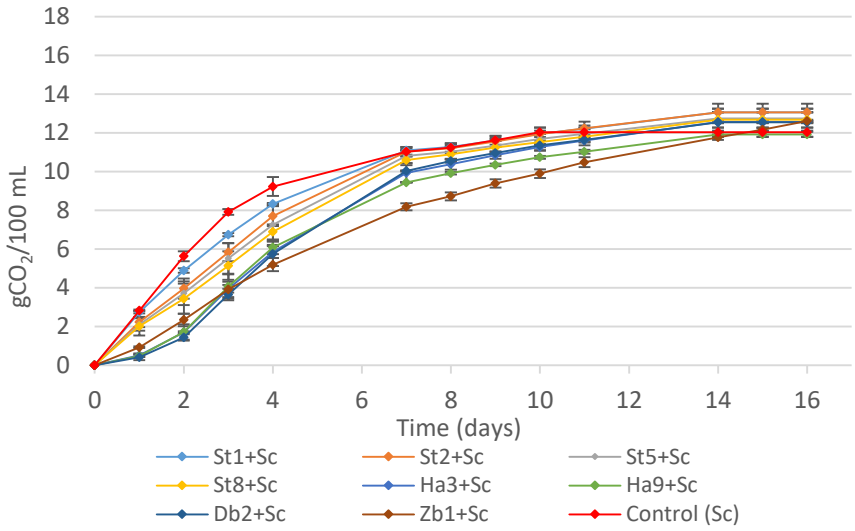

Figure 4.7| Fermentation kinetics of mixed starters cultures of *D. polymorphus* (Db2), *H. uvarum* (Ha3 and Ha9), *St. bacillaris* (St1, St8, St2 and St5) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of two independent experiments.

A similar trend for CO_2 production was observed in sequential fermentations (Figure 4.8), with constant increase in CO_2 production from the first fermentation day, whereas after the fourth fermentation day it was observed a high increase of CO_2 production, most probably as a consequence of the addition of *S. cerevisiae* EC1118.

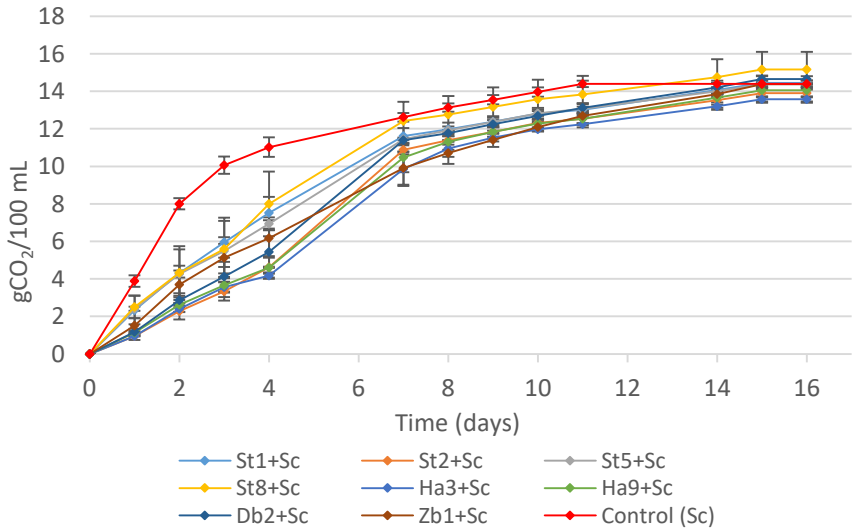

Figure 4.8| Fermentation kinetics of mixed starters cultures of *D. polymorphus* (Db2), *H. uvarum* (Ha3 and Ha9), *St. bacillaris* (St1, St8, St2 and St5) and *Z. bailii* (Zb1) strains sequentially inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of two independent experiments.

The amount of CO_2 produced at the end of fermentation was similar in all the trials, ranging between 13.91 and 15.17 g/100 ml, with highest production found for fermentation inoculated with St8+Sc starter. The duration of fermentation was higher for mixed cultures than for single starter culture; in fact, all the mixed starters completed the process in 15 days, with similar trend all the trials, whereas fermentation inoculated with pure culture of *S. cerevisiae* EC1118 (control fermentation) ends the process.

Analogous results on the time courses of sequential fermentations were recently found by several authors. Englezos et al. (2018) reported that a sequential fermentation with *S. bacillaris* and *S. cerevisiae* in white grape must took 14 days to finish, while 9 days were needed for the single inoculation with *S. cerevisiae*. Although fast and reliable completion of fermentation are of primary importance in the wine industry, the advantages of the use of non-*Saccharomyces* yeasts in mixed fermentations is thought to compensate the slower fermentation with the advantages in wine quality (Hranilovic et al., 2018). Moreover, a slow fermentation kinetics could be considered as positive for a better retention of volatile compounds (Binati et al., 2020).

The Figures 4.9 and 4.10 show the sugar consumption during the mixed fermentations. The evolution of sugar consumption, reported as reduction of Brix degree during the time, reflects the same trend observed for CO_2 production, as expected.

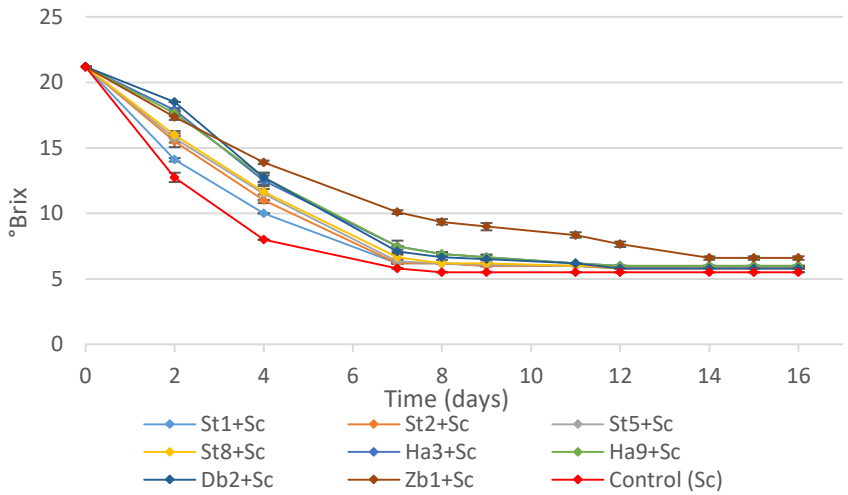

Figure 4.9| Sugar consumption of mixed starters cultures of *D. polymorphus* (Db2), *H. uvarum* (Ha3 and Ha9), *St. bacillaris* (St1, St8, St2 and St5) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of two independent experiments.

Also for this parameter, in both the conditions (simultaneous and sequential inoculum) the mixed starters ended the process later than control fermentation (*S. cerevisiae* EC1118). Furthermore, as already reported for CO_2 evolution (Figure 4.8), in sequential inoculation in the first fermentation days a gradual reduction of Brix degree was observed, whereas after

the fourth days of fermentation, a high decrease of sugar content was observed. This time point corresponds to the addition of the *S. cerevisiae* strain, which probably determined the high increase of fermentation rate and, consequently, the sugar reduction.

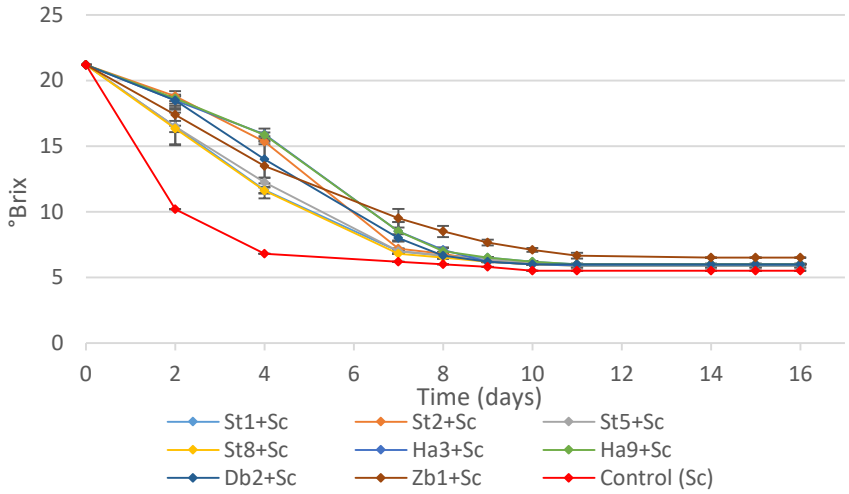

Figure 4.10| Sugar consumption of mixed starters cultures of *D. polymorphus* (Db2), *H. uvarum* (Ha3 and Ha9), *St. bacillaris* (St1, St8, St2 and St5) and *Z. bailii* (Zb1) strains sequentially inoculated with *S. cerevisiae* EC 1118 (Sc). Pure culture of *S. cerevisiae* EC 1118 (Control Sc) was used as a control. Data are means ± standard deviation of two independent experiments.

4.5.1.2| Evaluation of yeast population dynamic

The studies on yeast population dynamics during inoculated fermentation with mixed starter cultures will help in understanding the interactions between yeast strains included in the mixed starter and the final impact of non-*Saccharomyces* strains on wine quality. The microbial population dynamics of the mixed fermentations are shown in Figure 4.11. The evolution of yeast population during fermentative process was monitored by viable count on WL and LYS media. The persistence level of non-*Saccharomyces* strains during the mixed fermentations was variable in function of yeast strain/species and inoculation modality. In fact, in all the fermentations the presence of non-*Saccharomyces* strains at the end of the process was higher in simultaneous inoculum than sequential inoculum, except for fermentations inoculated with *H. uvarum* strains (Figures 4.11B), in which no differences between two modalities of inoculum were found. In mixed fermentations inoculated with *D. polymorphus* strain, in simultaneous modality cells count decreases at 2.5 x 10^5 UFC/mL after 4 days, whereas from the 7^{th} to the 10^{th} day, the viable count of strain Db2 decreased at 1.6 x 10^4 UFC/mL. In the sequential inoculum, Db2 strain reached a maximum of yeast cells (1.6 x 10^8 UFC/mL) in 3 days, after that the viable count decreased and at the 10^{th} day of the fermentation no *D. polymorphus* cells were found. In this case, the number of viable cells of this non-*Saccharomyces* strain decreased when *S. cerevisiae* strain was inoculated in synthetic must fermentation (Figure 4.11A, b).

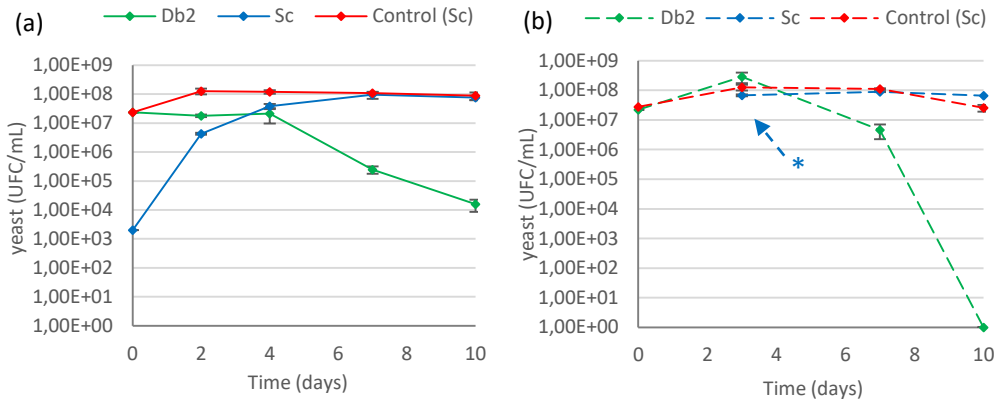

Figure 4.11A| Evolution of yeast populations in mixed fermentations inoculated with *D. polymorphus* (Db2) and *S. cerevisiae* (Sc) in simultaneous (a) and sequential (b) modalities. Values are mean of two independent duplicates. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. * indicate the inoculation time of *S. cerevisiae*.

The evolution of yeast cells of *H. uvarum* strains (Ha3 and Ha9) during the process followed the same trend in both the inoculum modalities, with a decrease of yeast cells after 4 days of fermentation. Furthermore, after 10 days of fermentation, no *H. uvarum* colonies were found on plates (Figure 4.11B).

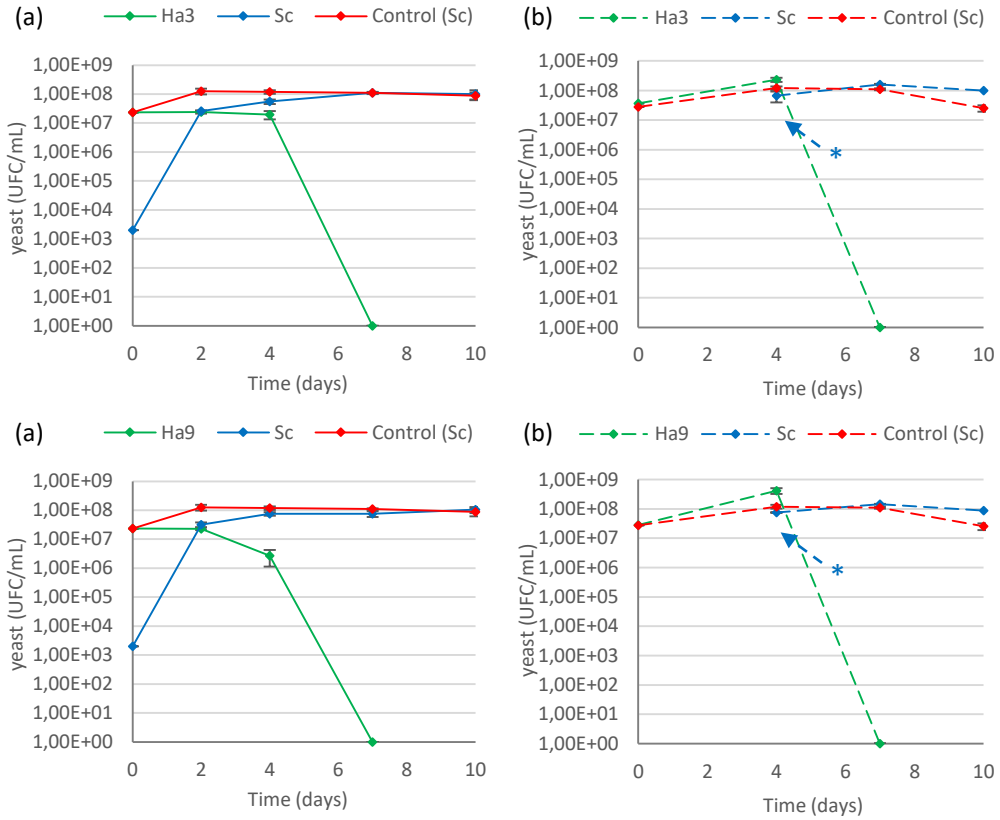

Figure 4.11B| Evolution of yeast populations in mixed fermentations inoculated with *H. uvarum* (Ha3 and Ha9) and *S. cerevisiae* (Sc) in simultaneous (a) and sequential (b) modalities. Values are mean of two independent duplicates. Pure culture of *S. cerevisiae* EC 1118 (Control Sc) was used as a control. * indicate the inoculation time of *S. cerevisiae*.

In mixed fermentations with *St. bacillaris* strains (St1, St2, St5 and St8) (Figure 4.11C), for simultaneous inoculum a slight increase of cell count is observed in the first two days of

fermentations, after that cell count slightly decrease and at the end of the process a number of viable cells ranging between 2 x 10^2 and 3.4 x 10^3 UFC/mL was found.

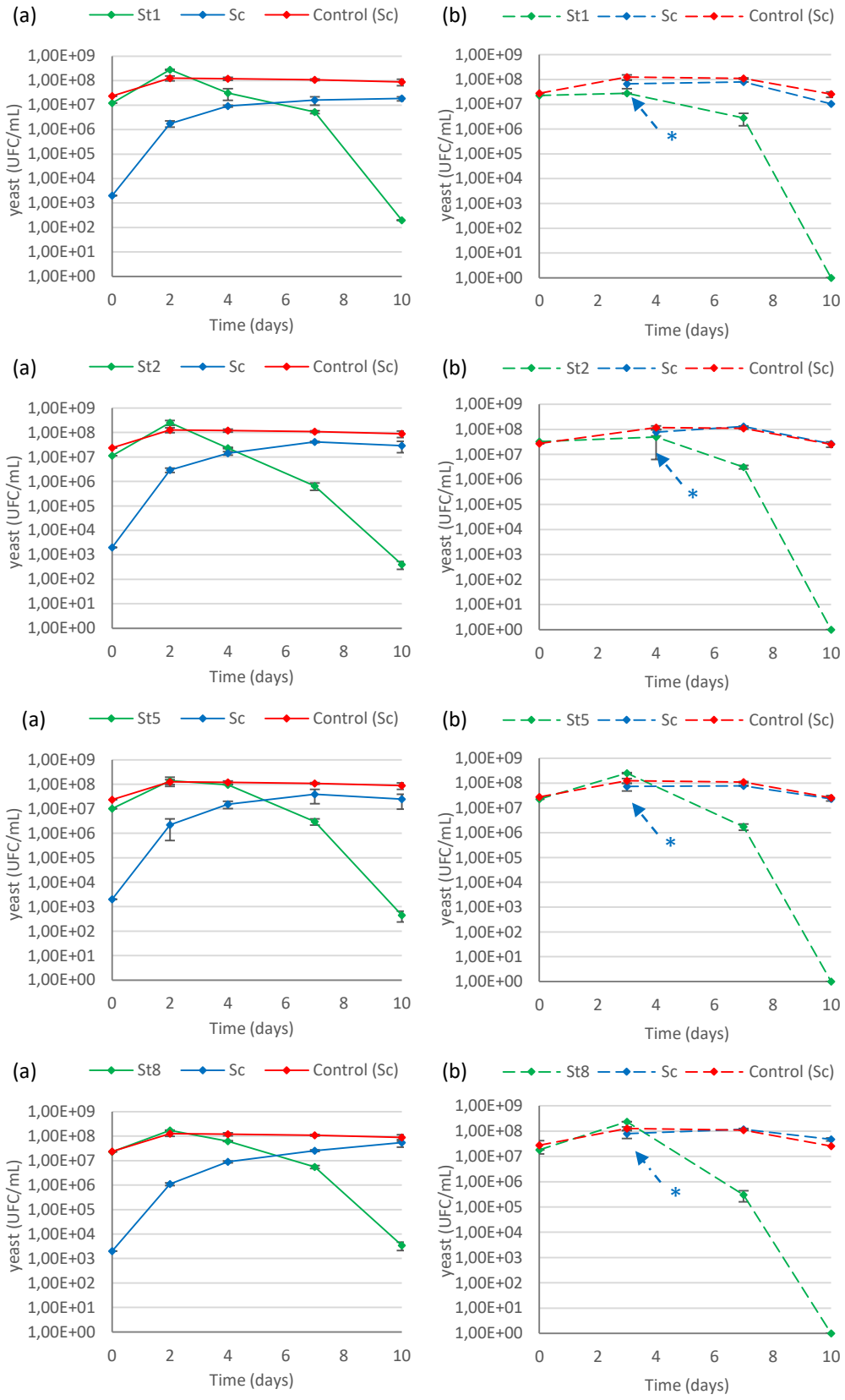

Figure 4.11C| Evolution of yeast populations in mixed fermentations inoculated with *St. bacillaris* (St1, St2, St5, St8) and *S. cerevisiae* (Sc) in simultaneous (a) and sequential (b) modalities. Values are mean of two independent duplicates. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. * indicate the inoculation time of *S. cerevisiae*.

As regards fermentations inoculated in sequential modality (Figure 4.6C, b), for St5 and St8 strains, in the first days of fermentation a trend similar to simultaneous inoculum was found, with an increase of viable cells, whereas after the third fermentation days the number of viable cells decreases and at the end of the process no *St. bacillaris* cells were found. For the other two strains (St2 and St5), the same behaviour was observed at the end of the process, whereas in the first days of the process the number of viable yeast cells increased only slightly.

As regards mixed fermentation with *Z. bailii* (Figure 4.11D), a different trend in the evolution of *Z. bailii* cells was found as function of inoculation modality.

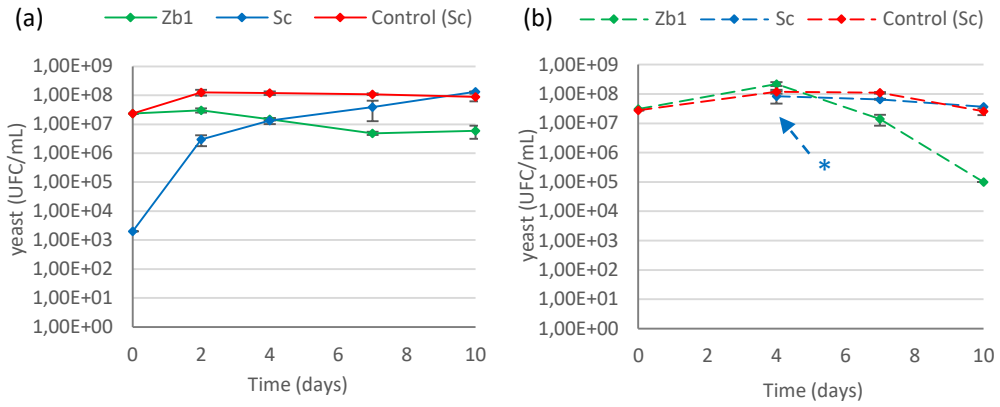

Figure 4.11D | Evolution of yeast populations in mixed fermentations inoculated with *Z. bailii* (Zb1) and *S. cerevisiae* (Sc) in simultaneous (a) and sequential (b) modalities. Values are mean of two independent duplicates. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. * indicate the inoculation time of *S. cerevisiae*.

In simultaneous inoculum, the number of Zb1 cells remains constant in the first two days of the process, after that a reduction in number of viable cells is observed (Figure 4.11D, a), although a number quite high of viable cells was found at the end of the fermentation (6 x 10^6 cells/mL). For sequential inoculum (Figure 4.11D, b), during the first four days, *Z. bailii* cells increase, after that a high reduction of number of viable cells is observed and in the final wine the number of *Z. bailii* cells is about 1 x 10^5 cells/mL.

As regards the evolution of *S. cerevisiae* EC1118 population in mixed fermentations, similar cell count was observed in both the inoculum modalities and the evolution of *S. cerevisiae* cells in pure culture of *S. cerevisiae* used as (control) reflects the typical growth kinetic, with the presence of high cell number until the end of the fermentations.

The results obtained in this step showed that *D. polymorphus*, *St. bacillaris* and *Z. bailii* strains tested are able to survive during the fermentation, if these non-*Saccharomyces* strains are inoculated simultaneously with *S. cerevisiae*, whereas in sequential inoculation these strains are unable to survive until the end of the process. On the basis of these results, the

viability of non-*Saccharomyces* strains seems to be affected by the inoculum modalities and the contact time with *S. cerevisiae* cells.

Previous studies show similar trends on the decline of non-*Saccharomyces* yeasts during the fermentative process. This phenomenon could have numerous explanations. The loss of viability of the non-*Saccharomyces* in the mixed fermentations can be related to production of yeast metabolites, such as ethanol, medium chain fatty acids and acetaldehyde. Killer toxins produced during the exponential phase by specific strains can also have an inhibitory impact on growth of some yeasts. More recently, it was found that some *S. cerevisiae* strains could secrete peptides inhibiting the growth of non-*Saccharomyces* yeasts (Binati et al., 2020; Dutraive et al., 2019).

4.5.1.3| Analysis of experimental wines

In order to determine the effect of yeast inoculum modalities on the final composition of wine, experimental wines were analysed for oenological parameters and main volatile compounds and the data are shown in Tables 4.3 (simultaneous trials) and 4.4 (sequential trials).

As already reported, all the starter cultures completed the fermentations, with a residual sugar content, both as glucose and fructose, lower than 3 g/L.

As regards co-inoculum (Table 4.3), all the samples from mixed fermentations contained an ethanol concentration lower than control sample (pure culture of EC1118), with values ranging from 11.58 to 12.19 % (v/v), whereas the ethanol content of wine from single fermentation was 12.38 % (v/v).

As regards volatile acidity, the activity of non-*Saccharomyces* strains did not increase volatile acidity; in fact, the volatile acidity of samples inoculated with mixed starters was lower or very similar to level detected in control wine, except for the mixed fermentations inoculated with Db2+Sc and Ha3+Sc starters. However, in all the experimental wines, the volatile acidity ranged from 0.61 to 1.13 mg/L, always being within the acceptable limits. In fact, it has been reported that the optimal concentration of acetic acid in wine is 0.2-0.7 g/L, and the acceptability level of this parameter is comprised between 0.7-1.1 g/L, depending on the style of wine (Capece et al., 2019; Álvarez-Pérez et al., 2014) whereas the OIV states that the maximum acceptable limit for volatile acidity in wines is 1.2 g/L of acetic acid (Gil et al., 2006).

The experimental wines were analysed also for the content of volatile compounds usually present in high quantity in wines, and involved in the wine flavour, such as acetaldehyde, ethyl acetate *n*-propanol, isobutanol, amyl alcohols (Table 4.3).

Table 4.3| Oenological parameters and main volatile compounds of experimental wines obtained from mixed starters cultures of selected non-*Saccharomyces* strains (Db2, Ha3, Ha9, St1, St8, St2, St5 and Zb1) simultaneously inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as control.

Oenological characteristics	**Db2+Sc**	**Ha3+Sc**	**Ha9+Sc**	**St1+Sc**	**St2+Sc**	**St5+Sc**	**St8+Sc**	**Zb1+Sc**	**Control (Sc)**
Ethanol	12.09±0.07^{ab}	11.96±0.01^{ab}	12.19±0.02^{ac}	11.84±0.06^{ab}	11.91±0.23^{ab}	11.93±0.21^{ab}	11.81±0.18^{bc}	11.58±0.20^{b}	12.38±0.05^{a}
Fructose	2.40±0.14^{ab}	2.45±0.21^{b}	2.20±0.14^{ab}	1.40±0.28^{c}	1.45±0.07^{c}	1.40±0.14^{c}	1.40±0.14^{c}	1.80±0.00^{ac}	1.80±0.14^{ac}
Glucose	1.50±0.00^{a}	1.40±0.00^{a}	1.55±0.07^{a}	0.90±0.00^{a}	1.05±0.07^{a}	0.85±0.21^{a}	1.05±0.07^{a}	5.70±0.71^{b}	1.45±0.07^{a}
Total acidity	5.22±0.14^{ac}	5.37±0.00^{a}	5.15±0.16^{ac}	4.89±0.01^{c}	5.00±0.11^{ac}	5.13±0.15^{ac}	5.18±0.08^{ac}	6.00±0.03^{b}	5.02±0.01^{ac}
Volatile acidity	1.13±0.06^{a}	0.88±0.04^{b}	0.71±0.00^{bc}	0.68±0.04^{cd}	0.61±0.03^{c}	0.65±0.02^{c}	0.83±0.02^{bd}	0.68±0.12^{bc}	0.86±0.01^{bd}
Main volatile compounds									
Acetaldehyde	48.22±11.25^{ab}	43.51±14.59^{ab}	65.95±5.28^{a}	29.26±0.30^{b}	30.62±2.76^{bc}	32.76±4.40^{bc}	25.13±4.16^{b}	38.72±7.91^{ab}	59.36±4.16^{ac}
Ethyl acetate	28.95±9.45^{a}	11.84±3.59^{ab}	17.44±5.66^{ab}	7.91±0.31^{b}	8.07±1.42^{b}	9.02±1.85^{b}	7.71±2.80^{b}	9.12±1.07^{b}	7.38±3.39^{ab}
n-Propanol	23.06±4.62	19.67±2.12	22.01±4.29	21.15±2.36	25.64±4.07	29.74±3.34	29.52±5.52	18.91±0.46	19.83±1.44
Isobutanol	19.22±0.42^{ab}	21.32±0.61^{ab}	18.69±2.83^{ab}	25.07±0.04^{ab}	19.91±1.47^{ab}	15.51±1.90^{a}	17.86±2.12^{a}	36.93±12.96^{b}	28.01±2.53^{ab}
D-amilic alcohol	38.52±1.01^{a}	36.80±0.46^{ab}	36.39±1.20^{ab}	25.24±0.71^{ab}	23.74±2.50^{b}	24.3±2.80^{b}	13.04±9.05^{b}	35.14±1.08^{a}	40.12±0.77^{a}
Isoamyl alcohol	96.41±8.22^{a}	91.70±0.98^{a}	95.48±5.62^{a}	53.34±2.26^{b}	51.51±6.21^{b}	47.91±2.28^{b}	49.86±1.85^{b}	87.59±3.54^{ac}	104.04±2.32^{ac}

Data are means ± standard deviation of two independent experiments. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$) between mixed and control fermentation. The oenological parameters are expressed as g/L, with exception of ethanol, expressed as % v/v; volatile compounds are expressed as mg/L.

The content of acetaldehyde, one of the most important carbonyl compounds synthetized during alcoholic fermentation, ranged between 38.72 mg/L (Zb1+Sc) and 65.95 mg/L (Ha9+Sc). This compound at moderate concentrations contributes to fruity flavors, while high levels (>200 mg/L) affects negatively the aroma in wines (Capece et al., 2019). The ethyl acetate was found in concentrations ranging from 7.71 mg/L (St8+Sc) to 28.95 mg/L (Db2+Sc). Ethyl acetate may add pleasurable, fruity aroma to the wine bouquet at low concentrations, whereas it affects negatively the final aroma at a content higher than 150 mg/L (Tristezza et al., 2016).

Generally, mixed starters produced experimental wines characterized by lower amount of alcohols (*n*-propanol, isobutanol, amyl alcohols) than the wine from *S. cerevisiae* EC1118 strain, used as control, except for *n*-propanol. The highest difference between single and mixed starter wines was found for fermentation performed by mixed starter including *St. bacillaris* strains for the content of amyl alcohols. In particular, the samples obtained by these mixed starters (St1+SC, St2+Sc, St5-Sc, St8+Sc) contained a very lower amount of both D-amyl and isoamyl alcohols than experimental wine fermented with EC1118 strain.

Oenological parameters and main volatile compounds detected in the wines obtained by the sequential inoculum are shown in the Table 4.4. Similarly to simultaneous inoculum, all the starters fermented the sugars contained in the synthetic must, leaving in the final samples less than 3 g/L of residual sugars.

Furthermore, the samples obtained by mixed starter sequentially inoculated with EC1118 strain contained lower ethanol than control experimental wine, as already found for co-inoculation. The lowest level of volatile acidity was found in experimental wine obtained by H9+Sc starter (0.69 mg/L), a result very surprising as apiculate yeasts are known to be high producers of acetic acid, whereas in the other samples volatile acidity ranged between 0.91 and 1.18 mg/L. However, also in this case, the values of volatile acidity are included in the acceptability value for this parameter.

As regards acetaldehyde, this compound was detected in the range between 64.38 (Zb1+Sc) and 27.75 mg/L (St1+Sc), whereas the ethyl acetate content ranged from 6.85 mg/L (St1+Sc) to 28.14 mg/L (Db2+Sc). While for acetaldehyde and ethyl acetate, no differences between wines from mixed starters and control wine were found, different results were obtained for higher alcohols. All mixed cultures produced wines containing slightly higher amount of n-propanol, in concentrations ranging from 20.69 mg/L (St8+Sc) to 31.76 mg/L (Ha9+Sc), than the wine from EC1118 strain (20.69 mg/L), used as control.

Table 4.4| Oenological parameters and main volatile compounds of experimental wines obtained from mixed starters cultures of selected non-*Saccharomyces* strains (Db2, Ha3, Ha9, St1, St8, St2, St5 and Zb1) sequentially inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as control.

Oenological characteristics	Db2+Sc	Ha3+Sc	Ha9+Sc	St1+Sc	St2+Sc	St5+Sc	St8+Sc	Zb1+Sc	Control (Sc)
Ethanol	11.46±0.08^{a}	11.61±0.07^{a}	11.66±0.05^{a}	11.37±0.01^{ab}	11.70±0.13^{a}	11.54±0.09^{a}	11.34±0.13^{ab}	11.53±0.00^{a}	11.79±0.20^{a}
Fructose	2.00±0.14^{ab}	1.45±0.07^{a}	1.40±0.00^{a}	1.35±0.07^{a}	1.45±0.21^{a}	1.35±0.07^{a}	1.40±0.00^{a}	1.75±0.07^{a}	2.80±0.71^{b}
Glucose	2.10±0.00^{ad}	1.05±0.07^{bd}	1.15±0.07^{bc}	1.10±0.14^{bd}	1.05±0.35^{bd}	1.00±0.14^{bd}	0.95± 0.07^{bd}	1.80±0.28^{ac}	1.70±0.14^{cd}
Total acidity	5.70±0.06^{ab}	5.88±0.03^{ac}	5.98±0.03^{ac}	5.35±0.16^{bd}	5.73±0.12^{ab}	5.52±0.03^{ad}	5.78±0.25^{ab}	6.30±0.09^{c}	5.18±0.12^{d}
Volatile acidity	1.18±0.01^{a}	1.03±0.04^{ac}	0.69±0.01^{b}	0.88±0.06^{c}	0.97±0.04^{c}	1.17±0.01^{ad}	1.16±0.01^{ad}	0.91±0.10^{c}	0.99±0.05^{cd}
Main volatile compounds									
Acetaldehyde	46.26±3.92^{ab}	48.42±8.63^{ab}	33.11±3.61^{a}	27.75±0.28^{a}	39.46±1.31^{a}	38.32±10.33^{a}	42.96±6.36^{ab}	64.38±2.09^{b}	39.25±2.85^{a}
Ethyl acetate	28.14±6.98^{a}	13.45±0.33^{bc}	17.81±2.33^{c}	6.85±0.20^{b}	6.91±0.06^{b}	8.43±0.44^{bc}	8.88±1.17^{bc}	9.97±0.01^{bc}	10.09±1.29^{bc}
n-Propanol	28.54±0.78^{ab}	28.68±0.37^{ab}	31.76±2.16^{b}	22.73±3.34^{a}	22.33±3.34^{a}	24.74±0.73^{ab}	20.69±2.24^{a}	22.13±2.83^{a}	20.24±0.67^{a}
Isobutanol	29.70±0.51	26.24±1.06	30.91±8.37	33.14±0.07	33.07±8.64	23.46±0.34	24.38±0.32	32.80±4.00	32.15±2.08
D-amilic alcohol	40.78±2.70^{ac}	43.50±0.50^{a}	46.81±4.65^{a}	29.46±5.72^{bc}	27.06±0.50^{b}	33.13±0.53^{bd}	33.39±3.10^{bd}	42.27±2.70^{ad}	48.53±0.39^{a}
Isoamyl alcohol	102.66±0.14^{ab}	94.42±2.55^{ab}	104.50±1.07^{ab}	80.75±12.44^{a}	79.04±21.57^{a}	82.30±6.57^{a}	83.04±9.35^{a}	115.62±7.14^{ab}	127.23±2.59^{b}

Data are means ± standard deviation of two independent experiments. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$) between mixed and control fermentation. The oenological parameters are expressed as g/L, with exception of ethanol, expressed as % v/v; volatile compound are expressed as mg/L.

As regards the levels of the other higher alcohols detected (isobutanol and amyl alcohols), control wine contained higher level of these compounds than samples from mixed starters, confirming the result already described for amyl alcohols in experimental wines obtained by simultaneous inoculums (Table 4.3). Higher alcohols contribute to the aromatic complexity of wine in concentrations below 300 mg/L, while above 400 mg/L they could have a negative effect on aroma (Padilla et al., 2016). Although these compounds represented the most abundant groups in all the analysed samples, all the starters produced an amount of alcohols lower to 300 mg/L.

The analysis of main volatile compounds provides a simply way of measuring the ability of different strains to produce wines with different profiles, since the main difference among wines inoculated with different yeast strains lies in the concentration of aromatic compounds rather than in the type of metabolite produced (Tristezza et al., 2016).

As the aim of this study was the selection of mixed starter culture able to reduce the ethanol content in the wine, it was calculated the ethanol reduction of each mixed starter cultures in both the inoculum modalities. The ability of mixed starter to reduce the ethanol content was calculated as ratio between ethanol produced by *S. cerevisiae* pure culture and ethanol produced from each mixed starter in simultaneous and sequential inoculation (Figure 4.12).

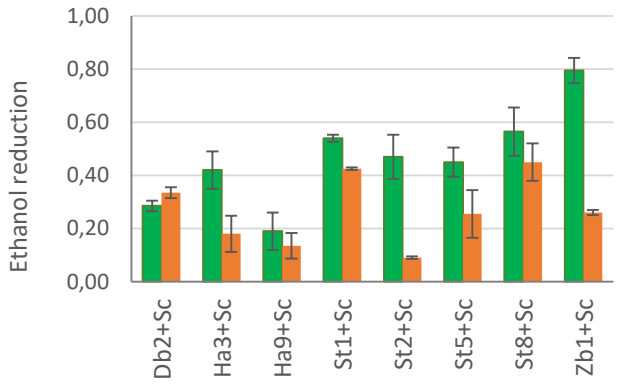

Figure 4.12| Starter ability to reduce the ethanol content in experimental wines obtained by simultaneous (■) and sequential (■) inoculation.

All the mixed starters, in both inoculum modalities, determined an ethanol reduction, and this result was expected as all the wines from mixed starters contained a lower level of ethanol than wine obtained by control fermentation, as already reported (Tables 4.3 and 4.4). The reduction level was higher for simultaneous than for sequential inoculum for all the starters, except Db2+Sc, in which a slightly higher reduction was found in sequential inoculum. In fermentations using simultaneous inoculations, the ethanol reduction ranged between 0.19 (Ha9+Sc) until 0.80 (Zb1+Sc), whereas in sequential inoculation the starter ability to reduce the ethanol content ranged between 0.09 (St2+Sc) and 0.45 (St8+Sc). These

results study indicate that the ability of starter culture to reduce ethanol content is function of both strain combination and inoculum modality. This finding is in agreement with previous observations, in which the inclusion of non-*Saccharomyces* strains in mixed fermentations greatly influences the ethanol production (Englezos et al., 2017).

All the parameters determined in experimental wines obtained by using mixed starter cultures in both the inoculation modalities, simultaneous (samples indicated with SiF code) and sequential (samples indicated with SeF code), and wine obtained by control fermentation (Control (Sc)_SiF and Control (Sc)_SeF, in both the experiments) were submitted to Principal Component Analysis (PCA). The plot of all the experimental wines on the plane defined by the first two components is shown in Figure 4.13A, whereas the loadings of each variable in the first and second PC are reported in the Figure 4.13B and C, respectively.

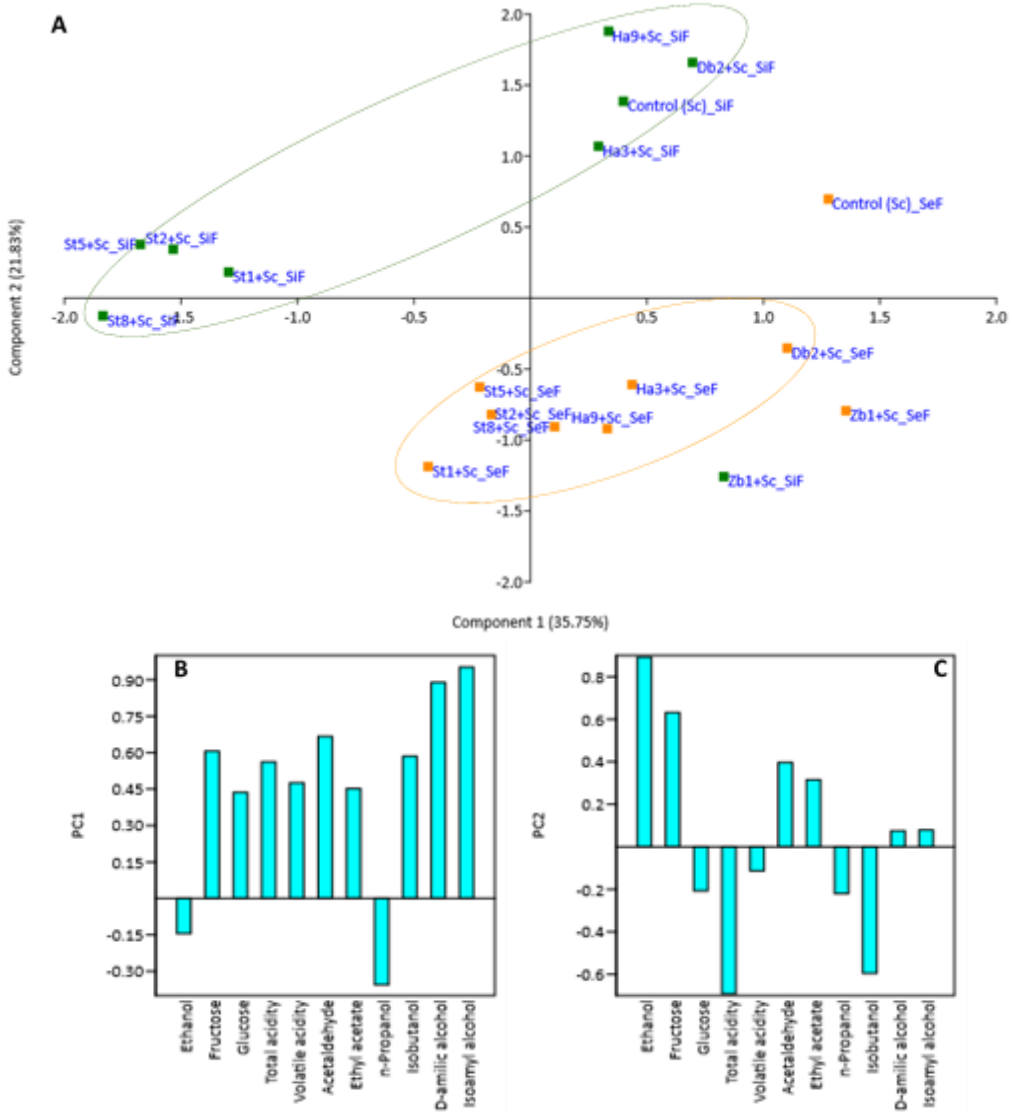

Figure 4.13| Scatter Plot (A) and loading plots of the first (B) and second (C) principal component corresponding to Principal component analysis (PCA) of the oenological parameters and main volatile compounds detected in experimental wines obtained by simultaneous (SiF) (■) and sequential (SeF) (■) inoculum of selected non-Saccharomyces strains. Pure culture of S. cerevisiae EC 1118 (Control Sc) was used as control.

The two principal components, PC1 and PC2, accounted for 56% of the total variance (36 and 22%, respectively). The PC1 was positively correlated mainly with D-amyl and isoamyl alcohols and negatively mainly associated with *n*- propanol (Figure 4.13B), whereas the PC2 was mainly positively related to content of ethanol and fructose residual and negatively with total acidity and isobutanol (Figure 4.13C). This analysis allowed to differentiate the experimental wines in function of inoculation modality; in fact, almost all the samples obtained with non-*Saccharomyces* strains inoculated simultaneously with *S. cerevisiae* strain are located in upper part of the scatterplot (except St8+Sc and Zb1+Sc, Figure 4.13A), whereas all the experimental wines obtained by sequential inoculum are grouped together in the lower part of the scatterplot, with exception of control sample and mixed starter including *Z. bailii* strain. As expected, the control experimental wines (obtained with pure culture of EC1118 strain) are located in the same quadrant of the plot. Similar result was observed for wines obtained with Zb1+Sc starter, that is the wine obtained by sequential inoculum is located in the same quadrant of sample inoculated simultaneously with Zb1 and EC1118. Furthermore, in each fermentation modality, the wines are distributed in function of yeast species used as starter; in fact, all the wines obtained by mixed starters including *St. bacillaris* strains are grouped very near, both in sequential and simultaneous inoculum. The same behaviour was observed for wines from mixed starters including the two *H. uvarum* strains (Ha3 and Ha9).

4.5.1.4| Qualitative production of hydrogen sulphide during fermentation

Hydrogen sulphide (H_2S) test strips was used for qualitative detection of (H_2S) production by starter cultures during fermentation. The qualitative level of H_2S production was directly correlated with browning level of filter paper strips at the end of fermentative process (Figure 4.14).

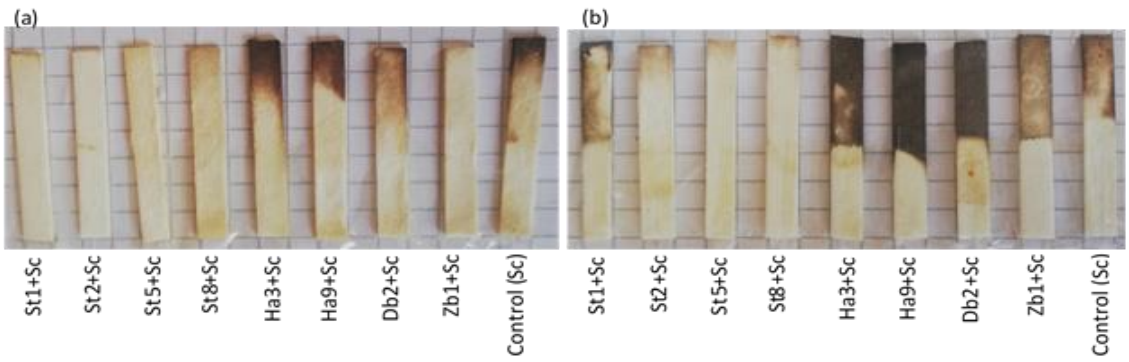

Figure 4.14| Hydrogen sulphide production estimated on Hydrogen Sulphide test strips during (a) simultaneous and (b) sequential inoculum.

Generally, in simultaneous inoculum (Figure 4.14a) the amount of H_2S produced was lower than level detected in sequential inoculum (Figure 4.14b) for all the starters tested. The mixed starters including the strains Ha3, Ha9 and Db2 produced the highest amounts of H_2S in both the inoculation modalities, whereas the lowest amounts of H_2S was produced in mixed starters composed by St1 and St2 strains inoculated simultaneously to EC1118 strain. In the other starter combinations, medium production level of H_2S was observed.

4.5.2| Mixed fermentations in natural grape must

On the basis of the results obtained in the previous step, the mixed starters including the non-*Saccharomyces* strains Ha3 (*H. uvarum*), St1, St8 (*St. bacillaris*), and Zb1 (*Z. bailii*) was selected to study the interaction between non-*Saccharomyces* and *S. cerevisiae* in natural grape must (Figure 4.15).

Figure 4.15| Mixed fermentations in grape must in flasks closed with a Müller valve filled with sulphuric acid.

These mixed starters were chosen on the basis of ethanol reduction and balanced production of aromatic compounds. In details, these 4 mixed starters yielded the highest ethanol reduction (Figure 4.12) and they produced wines characterized by desirable levels of secondary compounds and volatile acidity (Table 4.3). As regards the inoculation modality, it was chosen co-inoculation of non-*Saccharomyces* and EC1118 strain as in this condition the ethanol reduction was higher than reduction observed in sequential inoculation for all the mixed starters tested (Figure 4.12).

During the fermentative process, fermentation kinetic was monitored by measuring the CO_2 evolution, whereas cell growth of both inoculated species was evaluated at different times during the process (Figure 4.16 A-D). The control fermentation, in which pure culture of *S. cerevisiae* EC1118 was inoculated, finished the process before than mixed starters (within 11 days), whereas the mixed starters composed by *S. cerevisiae* (Sc) and *H. uvarum* (Ha3), *St. bacillaris* (St1 and St8), and *Z. bailii* (Zb1) were completed in 17-22 days. The Ha3 strain in association with Sc showed limited persistence during the alcoholic fermentation (Figure

4.16A); in fact, Ha3 persisted at concentrations of about 2 x 10^6 UFC/mL during the first 4 days of fermentation, after that it was showed a dramatic decrease of viable yeast cells, and after sixth fermentation days, no viable *H. uvarum* cells were detected. The growth of Sc strain co-inoculated with Ha3 showed growth kinetic similar to growth kinetic of pure culture. In agreement with this trend, at the end of fermentation, the mixed starter Ha3+Sc and single Sc starter showed a very similar production of CO_2.

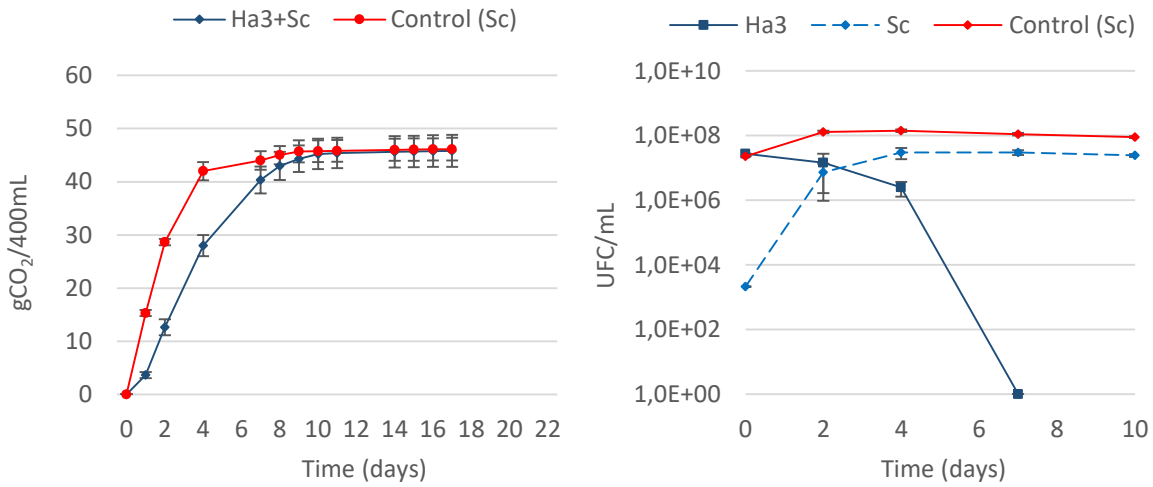

Figure 4.16A| Fermentation kinetics, reported as grams of CO_2 produced during the process, and evolution of yeast populations in mixed fermentations inoculated with *H. uvarum* (Ha3) and *S. cerevisiae* (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of three independent experiments.

As regards *St. bacillaris*, in mixed fermentations both St1 and St8 strains persisted throughout the fermentation process, with a high decrease of viable count after 7 days of fermentation (Figure 4.16B and C).

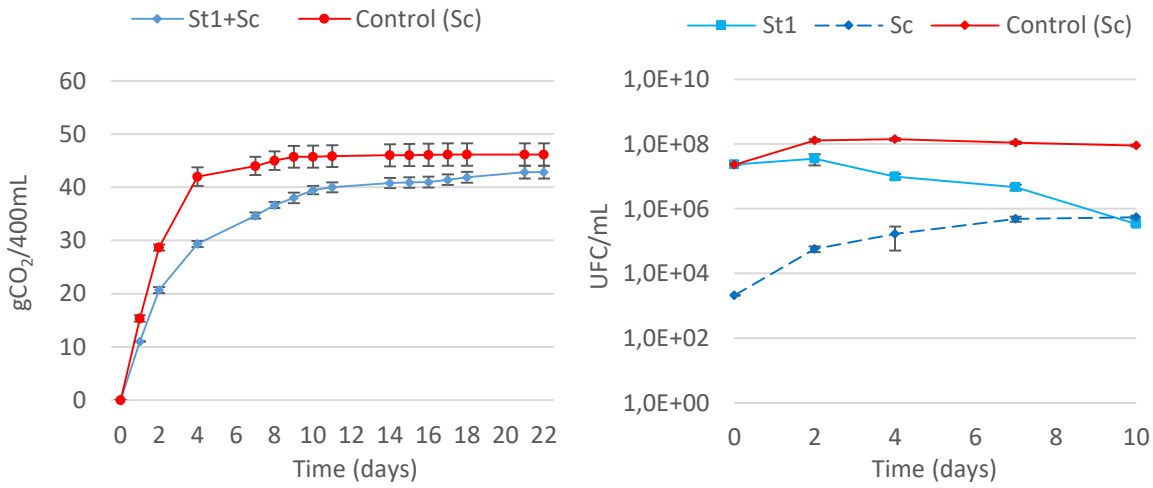

Figure 4.16B| Fermentation kinetics, reported as grams of CO_2 produced during the process, and evolution of yeast populations in mixed fermentations inoculated with *St. bacillaris* (St1) and *S. cerevisiae* (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of three independent experiments.

At the end of the process, the viable counts of St1 and St8 strains is very similar to viable cells of the co-inoculated EC1118 strain. Contrarily to results observed for mixed fermentation with *H. uvarum* strain, in these fermentations viable count of *S. cerevisiae* co-inoculated with the two *St. bacillaris* was lower than viable cells number observed in the control (5 x 10^5 and 9 x 10^7 UFC/mL, respectively). These results can indicate a potential influence of *St. bacillaris* strains on cell viability of *S. cerevisiae* or the existence of competition phenomena, for example for nutritional factors, between the two species during the fermentation.

At the end of fermentation, both the mixed starters including *St. bacillaris* strains showed a CO_2 production similar (about 43 and 41 g/400 mL, for St1+Sc and St8+Sc, respectively) and lower than CO_2 produced by control fermentation (about 46 g/400 mL).

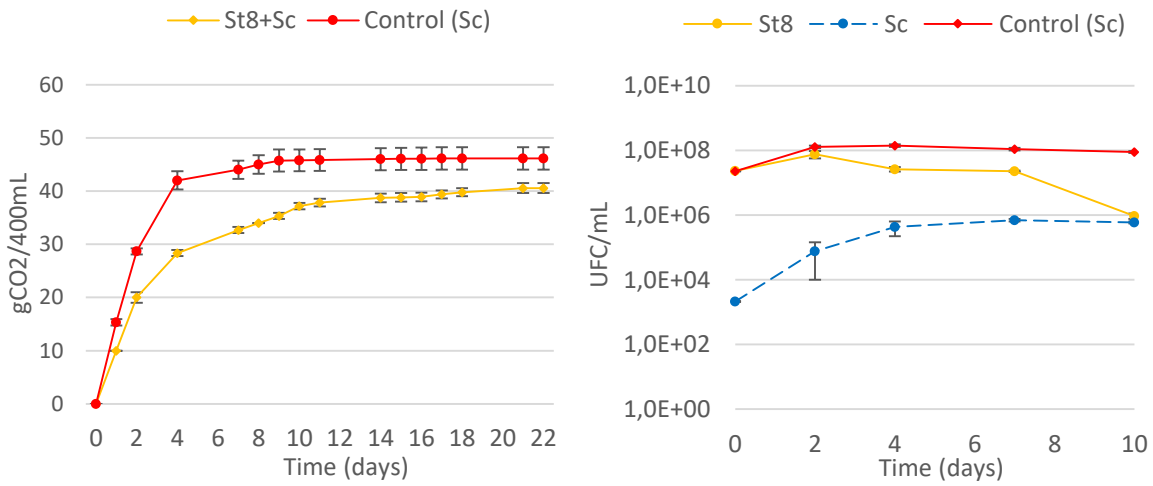

Figure 4.16C| Fermentation kinetics, reported as grams of CO_2 produced during the process, and evolution of yeast populations in mixed fermentations inoculated with *St. bacillaris* (St8) and *S. cerevisiae* (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of three independent experiments.

In the mixed fermentation including *Z. bailii* Zb1, the yeast cells of non-*Saccharomyces* cells remain quite constant during the process and after 10 days the viable count was 4 x 10^6 UFC/mL (Figure 4.16D).

As already reported for *St. bacillaris* mixed fermentations, at the end of the process the viable cell of *S. cerevisiae* inoculated together with Zb1 was lower than *S. cerevisiae* cells of control fermentation. At the end of fermentation, the mixed starter Zb1+Sc showed production of CO_2 of about 46 g/400 mL, comparable to CO_2 produced by single starter, although high differences between the single and mixed fermentation were found for duration of fermentation, which needs more than 20 days to finish for mixed fermentation and less than 10 days for single starter fermentation.

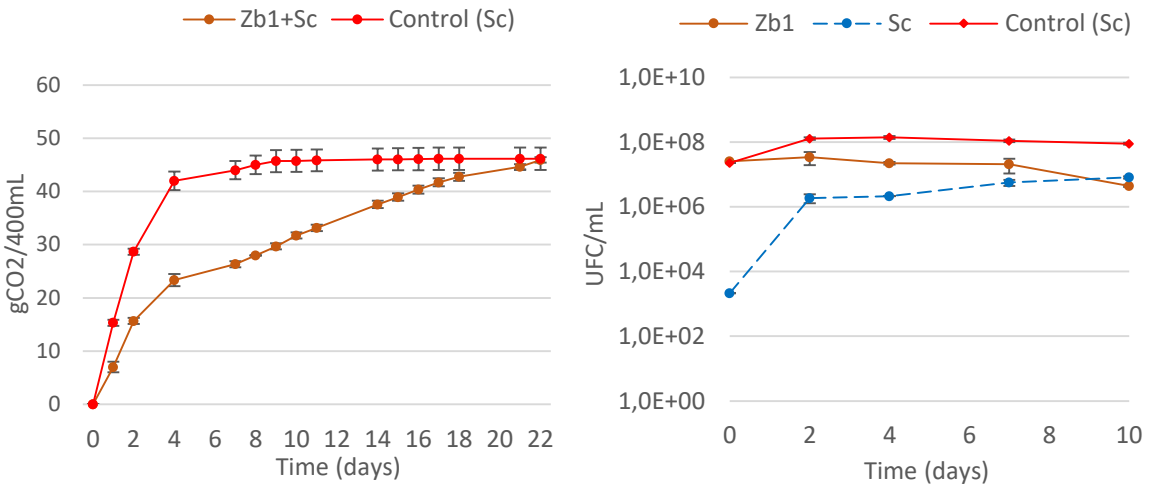

Figure 4.16D| Fermentation kinetics, reported as grams of CO_2 produced during the process, and evolution of yeast populations in mixed fermentations inoculated with *Z. bailii* (Zb1) and *S. cerevisiae* (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of three independent experiments.

The Figure 4.17 shows the sugar consumption during the mixed fermentations in comparison to the control. As expected, the evolution of sugar consumption, reported as reduction of Brix degree during the time, reflects the same trend observed for CO_2 production. In fact, also the reduction of Brix degree was faster in control fermentation (*S. cerevisiae* EC1118) than in mixed starters, in particular for mixed cultures including *St. bacillaris* and *Z. bailii* strains.

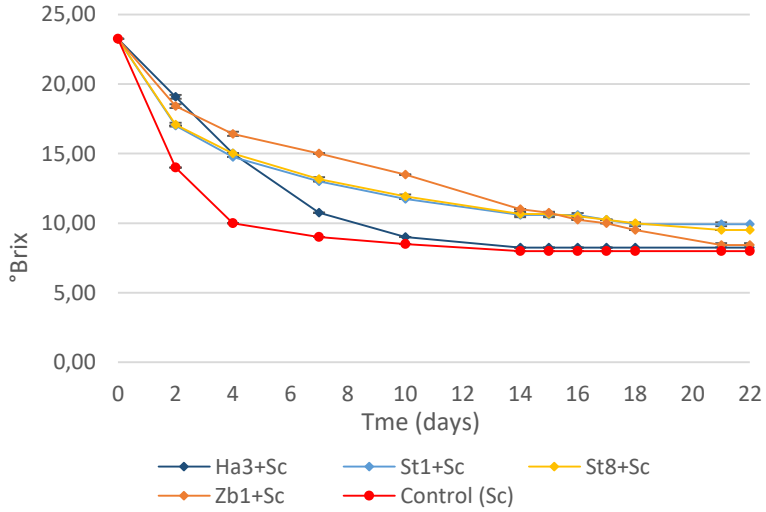

Figure 4.17| Sugar consumption of mixed starters cultures of *H. uvarum* (Ha3), *St. bacillaris* (St1 and St8) and *Z. bailii* (Zb1) strains in mixed fermentations with *S. cerevisiae* EC 1118 (Sc). Pure culture of *S. cerevisiae* EC 1118 (Control Sc) was used as a control. Data are means ± standard deviation of three independent experiments.

The correlation between sugar consumption during the time and ethanol formation is reported in Figure 4.18. As previously reported, in mixed fermentation the sugar consumption and, consequently, ethanol formation was slower than control fermentation,

with exception of mixed starter Ha3+Sc, in which it was observed the same trend of control fermentation, indicating the low participation of non-*Saccharomyces* strain to the fermentative process.

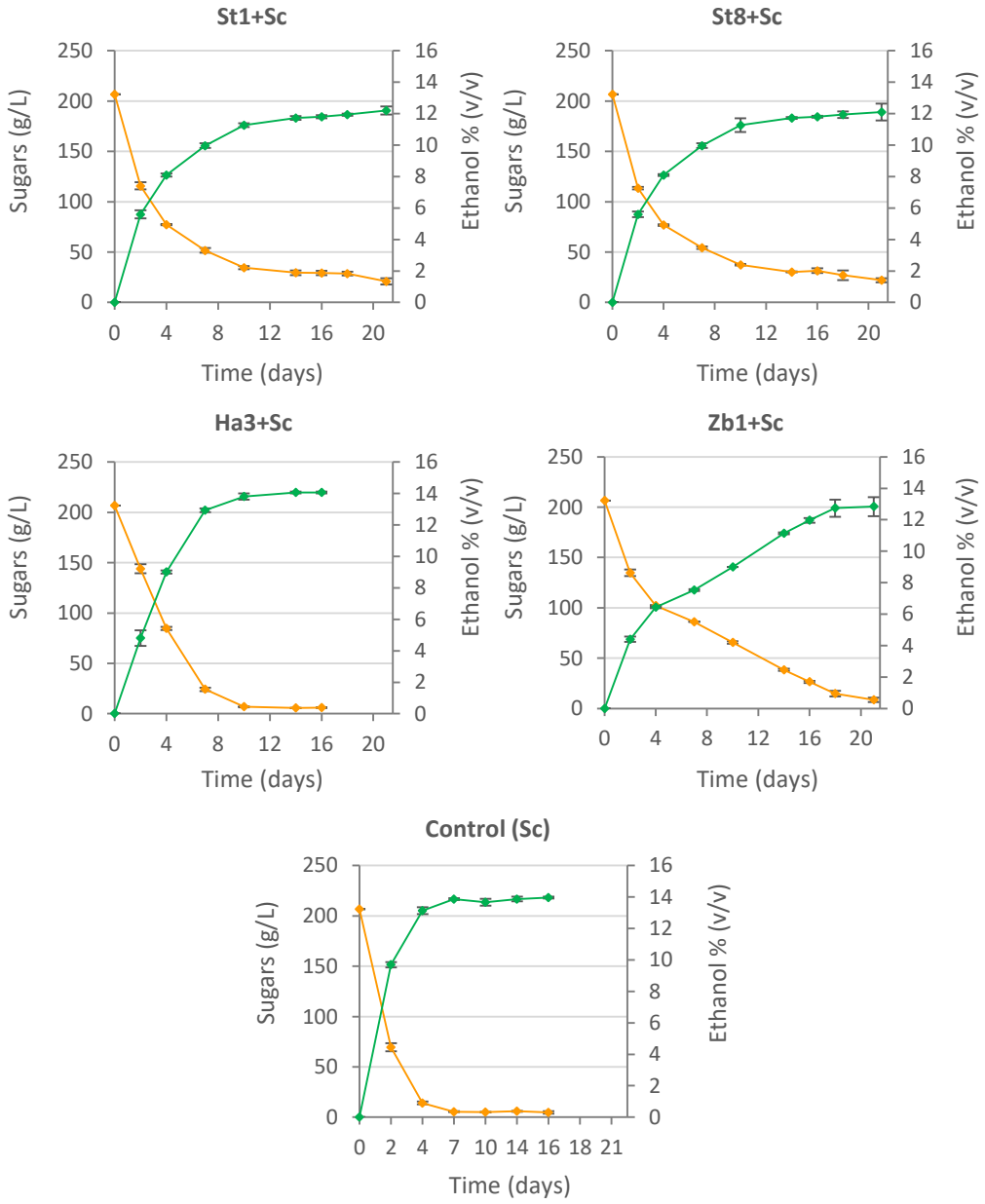

Figure 4.18| Evolution of sugar consumption and ethanol formation during mixed cultures fermentations with *H. uvarum* (Ha3), *St. bacillaris* (St1 and St8) and *Z. bailii* (Zb1) strains co-inoculated with *S. cerevisiae* strain. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are provided as the means ± standard deviation of the results from three independent experiments. (Sugars (g/L), Ethanol % (v/v)).

4.5.2.1| Analysis of experimental wines

The main oenological parameters and secondary compounds of the wines obtained at the end of fermentations are reported in Table 4.4. All the experimental wines contained a negligible content of residual sugars (<3.0 g/L), confirming the ability of starter cultures to

complete the fermentative process, with exception of mixed fermentations with *St. bacillaris* strains. In fact, *St. bacillaris* strains, in mixed fermentations, produced wines containing high levels of residual sugars, represented mainly by glucose (up to 15.43 g/L), while the fructose was almost totally consumed (< 2.0 g/L). This result confirms the fructophilic character of *St. bacillaris* species.

The ethanol concentration in experimental wines obtained by mixed cultures was lower than the control (values of 12.06-12.23 % v/v for mixed starter and 13.34 % v/v for the control), except for wine fermented with Ha3+Sc starter, in which the ethanol content was higher than level detected in pure-culture fermentation. Previous studies revealed that the use of selected non-*Saccharomyces* yeasts can reduce the alcoholic content of wine, by taking advantage of their capacity of redirecting the carbon metabolism away from ethanol production to other metabolites (Benito et al., 2015; Contreras et al., 2014; Quirós et al., 2014).

The glycerol content of final wine was higher in the mixed fermentations than glycerol detected in control wine and the highest value was found in experimental wine fermented with mixed starter including the strain of *S. bacillaris* St1 (average value 3.39 g/L), in agreement with literature data, reporting this species as a high glycerol producer (Comitini et al., 2011). Relevance of glycerol in the sensorial properties will depend on the style of the wine, generally contributing to smoothness, sweetness, and complexity (Comitini et al., 2011; Jolly et al., 2006). An increase in glycerol production is often related to an increase in acetic acid production, which can be detrimental to wine quality (Jolly et al., 2006). This correlation was not found in our results as the highest level of volatile acidity was found in wine produced by Zb1+Sc (0.96 g/L), whereas in wine containing the highest glycerol content, which was fermented with St1+Sc, the lowest level of volatile acidity was detected (0.36 g/L). The values of total acidity were very similar among all the wines (between 9.20 and 9.44 g/L), except sample fermented with Zb1+Sc association, with values of total acidity of 11.24 g/L.

The evaluation of wine samples by gas-chromatography allowed the detection and quantification of the secondary compounds usually present in high concentrations in wines, such as acetaldehyde, ethyl acetate, *n*-propanol, *n*-butanol, isobutanol and amyl alcohols. Experimental wines obtained by mixed fermentations were analysed in comparison with wines produced by single starter. Statistically significant differences were found between wines obtained by mixed and wines produced by single starter, as reported Table 4.4. Differences in the synthesis of aromatic compounds between single and mixed starters were expected as non-*Saccharomyces* species vary from *S. cerevisiae* in the distribution of metabolic flux during fermentation and therefore differ in ethanol production, biomass

synthesis and by-products formation (González et al., 2018). Furthermore, the biosynthesis is strain-dependent (Binati et al., 2020). Differences statistically significant ($p < 0.05$) between wines from single and mixed starters were found for almost all the analysed compounds, except for D-amyl alcohol.

Table 4.4| Oenological parameters and main volatile compounds of experimental wines produced by mixed starters cultures of selected non-*Saccharomyces* strains (Ha3, St1, St8, and Zb1) co-inoculated with *S. cerevisiae* EC1118 (Sc) during lab-scale fermentation in natural grape must. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as control.

Oenological characteristics	Ha3+Sc	St1+Sc	St8+Sc	Zb1+Sc	Control (Sc)
Ethanol	13.67±0.03^{a}	12.23±0.06^{b}	12.06±0.38^{b}	12.16±0.03^{b}	13.37±0.02^{a}
Fructose	1.37±0.12^{a}	1.77±0.32^{ab}	2.03±0.06^{b}	1.47±0.15^{ab}	1.27±0.06^{a}
Glucose	1.87±0.12^{a}	14.23±1.53^{b}	15.43±1.00^{b}	1.23±0.12^{a}	1.33±0.06^{a}
Total acidity	9.17±0.07^{a}	9.44±0.0.8^{b}	9.20±0.13^{a}	11.24±0.06^{c}	9.94±0.06^{d}
Volatile acidity	0.67±0.02^{a}	0.36±0.0.6^{b}	0.43±0.05^{c}	0.96±0.03^{d}	0.61±0.01^{a}
Glycerol	3.01±0.75^{ab}	3.39±0.21^{b}	3.09±0.89^{ab}	3.18±0.25^{ab}	1.77±0.19^{a}
Main volatile compounds					
Acetaldehyde	21.98±4.17^{a}	37.72±9.37^{b}	35.35±1.76^{b}	30.15±0.85^{ab}	30.14±0.95^{ab}
Ethyl acetate	106.91±3.53^{a}	33.21±2.69^{b}	34.09±1.81^{b}	56.50±0.13^{c}	37.92±0.86^{b}
n-Propanol	30.87±1.11^{a}	22.99±0.51^{b}	22.15±0.03^{b}	26.15±0.48^{c}	15.30±0.28^{d}
n-Butanol	20.32±6.01^{a}	133.54±26.89^{b}	141.92±11.46^{b}	27.79±1.33^{a}	20.23±5.95^{a}
Isobutanol	30.08±1.52^{a}	147.29±11.99^{b}	151.27±7.94^{b}	66.16±1.04^{c}	34.96±0.86^{a}
D-amyl alcohol	57.28±9.46	56.25±13.26	46.79±8.72	64.17±1.42	69.00±2.51
Isoamyl alcohol	135.37±14.28^{ab}	146.16±13.06^{b}	109.67±18.49^{a}	197.23±5.74^{c}	230.82±7.02^{c}

Average values of three repetitions ± standard deviations. Different superscript letters in the same column correspond to statistically significant differences (Tukey's test, $p < 0.05$).The oenological characteristic are expressed as g/L, with exception of ethanol, expressed as % (v/v); volatile compound are expressed as mg/L.

The acetaldehyde production of the four mixed cultures was quite similar to the control, whereas high variability was found for ethyl acetate content. For this compound, the highest level was detected in samples fermented with Ha3+Sc, whereas the St1+Sc and St8+Sc starters produced the lowest amounts of ethyl acetate.

As regards the higher alcohols, which represented the most abundant group of secondary compounds detected in this study, the amount varied among wines obtained by mixed fermentations and wine produced by single starter, except for D-amyl alcohol. The two wines obtained by mixed starters including *St. bacillaris* strains were differentiated from control wine for the very high content of *n*-butanol and isobutanol, which was the highest content among all the analyzed wines. The wine obtained by co-inoculation of *H. uvarum* and *S. cerevisiae* (Ha3+Sc) showed a higher level of *n*-propanol and a lower level of amyl alcohols

in comparison to control wine. However, all the wines from mixed starters contained a lower levels of amyl alcohols than wine fermented with pure culture of *S. cerevisiae*.

In the heat-map reported in Figure 4.19 are visualized the differences in aromatic composition between wines from mixed starters and control wine, fermented with EC1118 strain. The wines were grouped in two main clusters, one composed by samples fermented with mixed starters including the two *St. bacillaris* strains (St8 and St1), whereas the other cluster includes the other three wine samples. Control wine was very similar to wine obtained from mixed starter including *Z. bailii* strain (Zb1+Sc).

The two strains of *St. bacillaris* determined a high increase of acetaldehyde, isobutanol and *n*-butanol respect to the control. The wine produced by Ha3+Sc contained the highest concentration of ethyl acetate, but lower level of acetaldehyde, respect to the control and other fermentations.

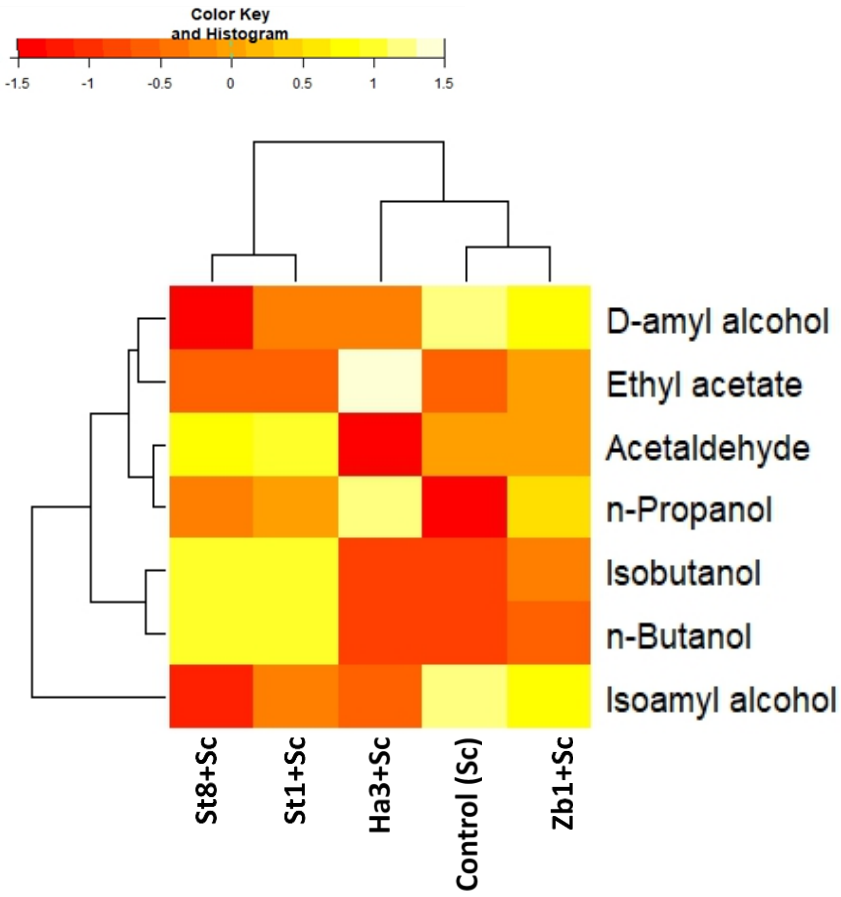

Figure 4.19| Heat-map representing the increased or decreased production of the volatile compounds in wine produced with mixed cultures non-*Saccharomyces* strains and *S. cerevisiae* EC1118 in comparison with the pure culture of *S. cerevisiae* EC1118 (Control SC).

As one of the criteria for mixed starter selection of this work was the starter ability to reduce the ethanol content of wine, this parameter was calculated for the four mixed starters tested in this step (Figure 4.20). Three mixed starters showed an ethanol reduction very similar among them (1.21-1.31), whereas only co-culture Ha3+Sc was not able to reduce the ethanol

content respect to *S. cerevisiae* single starter. These results are in agreement with literature data. Englezos et al. (2017) showed that *St. bacillaris* may be used in mixed fermentation with *S. cerevisiae* to reduce the ethanol content in wine; in this research, the ethanol reduction varied from 0.50 at pilot scale to 0.70-0.90 at laboratory scale using natural must. Di Maio et al. (2012) showed that *St. bacillaris* may be used in mixed fermentation with *S. cerevisiae* to reduce the ethanol content in wine (0.32) and to increase the glycerol content.

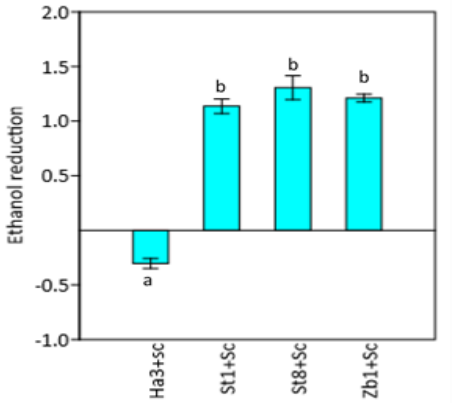

Figure 4.20| Ability of mixed starter cultures to reduce the ethanol content in experimental wines.

4.5.3| Mixed fermentation in double-compartment fermentor

In order to study the interaction mechanisms based on cell-to cell contact between strains composing mixed starters, it was investigated the interaction between *S. cerevisiae* (S) and *H. uvarum* (H) strains in mixed fermentation, by using the double-compartment fermentor pointed out by Renault et al. (2013). The S + H combination was tested in two conditions: NS, in which each compartment was inoculated with both strains, and S condition, in which the two strains were inoculated separately in each compartment.

4.5.3.1| Evolution of fermentative process

As regards the progress of fermentative process, the maximum CO_2 production was similar in both modalities, as shown in Figure 4.21.

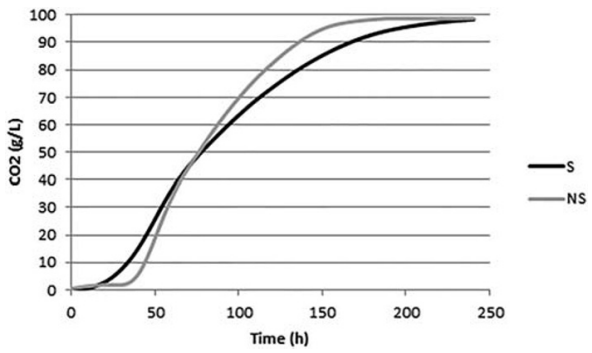

Figure 4.21| Fermentative kinetics in double-compartment fermentor inoculated with *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* in two modalities: each species in separated (S) compartments or two species together (nonseparated [NS]).

In contrast, the duration of lag phase was higher in the fermentation performed by inoculating the strains mixed together (NS modality) than the fermentation inoculated with the two strains physically separated (about 31 and 13 h, respectively, Figure 4.21). However, the fermentation duration was lower in NS than in S modality (about 195 and 227 h, respectively, Figure 4.21). The evolution of yeast population during fermentative process was monitored by viable count on WL medium in both compartments of double fermentor in S and NS modalities. In S modality, the effectiveness of physical separation was confirmed by the absence of contamination from each compartment to the other one (data not shown). The analysis of *H. uvarum* population (Figure 4.22a) revealed that in NS modality, *H. uvarum* cell counts were very similar in both the compartments, by demonstrating the homogeneity in yeast population between the two compartments. The evolution of yeast cells during the process followed the same trend in both the inoculum modalities, with an increase of yeast cells during the first 50 h, after that the *H. uvarum* population starts to decrease. However, in S modality, *H. uvarum* population was higher than yeast cells detected in NS during all the process. Furthermore, after 118 h of fermentation in NS condition, no *H. uvarum* colonies were found on plates, whereas in S condition at the same time, the viable cell count was 1.0×10^2 cells/mL and no growth was observed only after 168 h of fermentation.

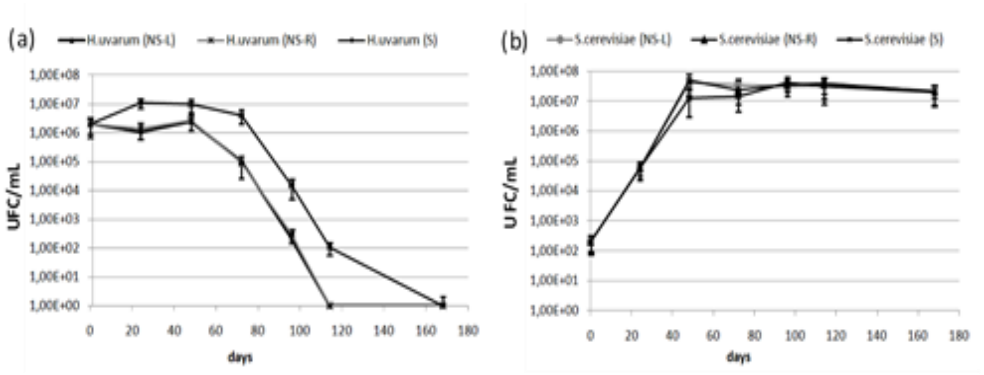

Figure 4.22| Cell evolution, expressed as colony-forming units per millilitre, of (a) *Hanseniaspora uvarum* and (b) *Saccharomyces cerevisiae* in double fermentor by following two modalities of inoculum: each species in separated (S) compartments or two species together, nonseparated (NS)-L (left compartment) and NS-R (right compartment).

The evolution of *S. cerevisiae* population in the two inoculum modalities (Figure 4.22b) confirmed that in NS condition, similar cell count was observed in both the compartment, as already reported for *H. uvarum*. No high differences in *S. cerevisiae* cell count between the two inoculation modalities were found during all the fermentative process, except that population reached a maximum earlier in NS modality. Based on these results, the viability of *H. uvarum* seems affected by the contact with *S. cerevisiae* cells, whereas the physical contact between the two species did not affect *S. cerevisiae* viability.

4.5.3.2| Analysis of wines

The analysis of the main parameters detected in the experimental wines obtained in the two conditions is reported in Table 4.6. As shown in the table, both the starter cultures completed the fermentation; in fact, average residual sugar concentrations varied from 0.7 to 1 g/L, and no statistically significant differences were found between the two modalities and the two compartments. Concentrations of the main fermentation products, that is, ethanol, glycerol and volatile acidity, were similar in both compartments of the double fermentor in NS condition and also similar to those determined in the S modality (no significant differences for final concentrations) (Table 4.6).

Table 4.6| Chemical characteristics of experimental wines obtained in double-compartment fermentor by following two modalities of inoculum: each species in separated (S) compartments or two species together, nonseparated (NS)-L (left compartment) and NS-R(right compartment).

Compounds	Hu S	Sc S	NS-L	NS-R
EtOH(% Vol)	12.33±0.31	12.33±0.33	12.22±0.22	12.25±0.30
Residual sugars (g/L)	1±0.28	0.85±0.21	0.7±0.14	0.8±0.28
Acetic acid (g/L)	0.16±0	0.16±0.01	0.09±0.03	0.11±0.03
Glycerol (g/L)	6.91±0.28	7.01±0.21	7.115±0.25	7.16±0.09
Acetaldehyde	47.22±0.93	47.25±1.05	47.10±0.71	48.55±1.05
Ethyl acetate	45.23±2.12^{a}	48.71±0.84^{a}	27.25±0.01^{b}	29.72±0.71^{b}
n-propanol	22.92±0.77^{a}	24.39±0.40^{a}	15.91±0.10^{b}	16.55±0.41^{b}
isobutanol	30.30±1.29	32.26±0.24	31.12±0.12	32.58±1.08
D-amyl alcohol	60.07±2.42^{a}	63.25±0.67^{a}	80.90±3.08^{b}	82.26±1.19^{b}
Isoamyl alcohol	106.43±7.41^{a}	116.20±2.07^{a}	193.84±3.75^{b}	201.57±5.66^{b}
TPC	237.5±2.14^{a}	256.5±6.36^{a}	287±4.96^{b}	298.5±6.36^{b}
AP	0.51±0.12^{a}	0.55±0.07^{a}	0.66±0.04^{ab}	0.94±0.09^{b}

Note: Average values of two repetitions ± standard deviations. Different superscript letters in the same row correspond to statistically significant differ- ences (Tukey's test, $p < 0.05$). HU and SC, fermentor compartment inoculated with *S. cerevisiae* and *H. uvarum* separately; LS and RS, left and right compart- ments, respectively, of the double fermentor inoculated with *S. cerevisiae* and *H. uvarum* together in each compartment. Acetaldehyde, ethyl acetate, *n*-propanol, isobutanol, D-amyl alcohol and isoamyl alcohol are expressed as mg/L. TPC (total polyphenols content) is expressed as mg gallic acid/L. AP (antioxidant power) is expressed as Trolox Equivalent Antioxidant Capacity (TEAC) mmol/mL.

The experimental wines obtained at the end of the fermentations were analysed also for the content of secondary compounds usually present in high concentrations in wines, such as acetaldehyde, ethyl acetate, *n*-propanol, isobutanol, amyl alcohols and the parameters affecting nutraceutical value of wine, such as TPC and AP (Table 4.7).

Table 4.7| Esters concentrations (µg/L) in wines obtained by inoculating *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* in double fermentor after 40% of the alcoholic fermentation and at the end of process

ESTERS	HU- 40	SC- 40	LS- 40	RS- 40	HU	SC	LS	RS
Major esters								
Ethylpropanoate	42.35±2.25	49.13±1.94	33.75±7.47	33.99±5.41	70.94±9.61	70.83±7.12	64.70±3.11	58.81±8.23
Ethylisobutyrate	4.09±0.46	3.77±0.36	3.78±0.66	4.19±0.88	10.51±0.69	10.59±0.45	9.21±1.85	9.89±2.00
Propyl acetate	21.26±4.57	18.89±1.53	17.96±3.44	20.27±4.39	16.43±4.79	16.28±4.94	13.40±1.23	17.37±4.70
Methylbutyrate	110.29±6.47	119.05±8.51	134.09±10.69	145.89±15.27	67.79±3.71	69.59±11.30	86.38±9.60	88.44±5.12
Ethylbutyrate	57.98±12.32	50.06±2.78	71.58±5.39	76.46±0.22	110.41±2.50^{a}	109.37±4.23^{a}	129.56±7.74^{ab}	137.35±9.21^{b}
Isoamyl acetate	1836.98±82.72^{a}	1857.49±44.29^{a}	2646.54±33.47^{b}	2948.47±59.98^{b}	1907.81±2.44^{a}	1937.57±3.01^{a}	3319.14±66.43^{b}	3515.87±58.36^{b}
Ethylvalerate	25.08±2.25	30.56±4.46	40.71±3.37	45.45±8.05	24.01±7.23^{a}	29.64±0.74^{a}	50.63±2.96^{b}	54.10±6.11^{b}
Ethylhexanoate	170.64±8.56^{a}	218.95±15.02^{a}	307.04±11.38^{b}	345.58±18.79^{b}	172.05±13.92^{a}	171.90±10.28^{a}	269.31±9.56^{b}	276.83±16.39^{b}
Hexyl acetate	68.48±0.48^{a}	77.03±7.14^{a}	111.59±0.59^{b}	120.54±11.33^{b}	21.33±2.39^{a}	21.59±2.92^{a}	35.93±2.33^{b}	37.40±4.79^{b}
Ethyloctanoate	358.97±10.37^{a}	348.59±28.61^{a}	523.49±15.04^{b}	589.10±27.03^{b}	300.74±23.53^{a}	267.85±11.27^{a}	445.72±24.03^{b}	438.27±25.80^{b}
Ethyldecanoate	88.84±7.47^{a}	83.81±4.25^{a}	233.91±14.93^{b}	252.32±16.09^{b}	156.20±10.63^{a}	136.81±7.25^{a}	182.47±9.83^{ab}	193.05±7.60^{b}
Ethyldodecanoate	10.20±0.20^{a}	10.44±0.77^{a}	40.17±5.16^{b}	43.26±6.02^{b}	23.02±5.81	11.03±1.53	19.18±0.86	18.23±2.49
Phenylethyl acetate	296.75±18.06^{a}	231.97±10.04^{a}	722.42±35.19^{b}	800.63±41.58^{b}	340.98±23.49^{a}	347.46±12.87^{a}	808.35±9.67^{b}	833.95±18.76^{b}
∑ **Minor esters**	7.72±3.19^{a}	12.13±1.96^{ab}	18.27±1.39^{b}	20.47±1.26^{b}	18.82±1.51^{a}	17.03±1.35^{a}	26.23±1.22^{b}	26.20±2.14^{b}
∑ **Total esters**	3089.26±139.62^{a}	3111.79±165.19^{a}	4899.77±121.49^{b}	5446.59±178.59^{b}	2941.01±28.31^{a}	3217.53±28.58^{a}	5460.18±145.62^{b}	5705.74±153.09^{b}

Note: Values are expressed as mean ± standard deviation of two independent replicates. HU and SC, fermentor compartment inoculated with *S. cerevisiae* and *H. uvarum* separately; LS and RS, left and right compartments, respectively, of the double fermentor inoculated with *S. cerevisiae* and *H. uvarum* together in each compartment. Different superscript letters in the same row, in function of the time of fermentation process, correspond to statistically significant differences (Tukey's test, $p < 0.05$). Minor esters = ester present in quantities < 10 µg/L.

No statistically significant differences were found between wines from the two sectors in both inoculum modalities for all the compounds, confirming the homogeneity of the medium in both compartments, despite the physical separation of the two yeast populations. By comparing the inoculum modality, significant differences between S and NS modalities were found for ethyl acetate, *n*-propanol and amyl alcohols. When *S. cerevisiae* and *H. uvarum* strains were inoculated in the same compartment, lower amount of ethyl acetate and *n*-propanol and higher amount of amyl alcohols were found than those detected in the wines obtained by the two strains physically separated.

Furthermore, ester concentrations were measured both at the end of the fermentation and at 40% of alcoholic fermentation completion (Table 4.7), in order to better understand the influence of cell contact on esters formation. No significant differences in ester content were found between the wines obtained in the two compartments in both the inoculum conditions confirming the homogeneity of the fermentation medium between left and right sides, whereas high differences were found between S and NS modalities. The inoculum of both yeasts together allowed the highest ester concentration (5,460 and 5,705 μg/L in left and right side, respectively, Table 4.7). This difference was mainly due to the increase of almost all the esters detected in high concentrations (more than 10 μg/L), mainly isoamyl acetate, ethyl exhanoate and phenylethyl acetate, which were found at about double concentration in NS modality than in S condition. Differences were detected also for esters produced at low concentration (less than 10 μg/L), although at lesser extent than other classes of esters (Supplementary Table Sa-b). This behaviour was confirmed also by the analysis of ester concentration at 40% of alcoholic fermentation completion (Table 4.7). Thus, at 40% of alcoholic fermentation, the NS inoculum modality yielded wines containing higher amount of esters than the experimental wines obtained by inoculating the two species separately.

The discrimination of inoculum modalities carried out by PCA based on the 32 ester concentrations represented about 87% of variance for PC1 and PC2 axes (Figure 4.23 and 4.24). Ester concentrations at 40% of alcoholic fermentation were different from ester detected at the end of the fermentative process (right and left side of scatterplot, respectively).

Furthermore, both at 40% and at the end of fermentation, the wines obtained by inoculating the strains mixed together (LS-40, RS-40 and LS and RS, respectively) were separated from the wines produced by the strains inoculated in S compartments (HU-40, SC-40 and HU and SC, respectively), being located in upper and lower part of the scatterplot, respectively. Ester profiles of wines from the two different compartments in the same inoculation modality were quite similar, confirming the homogeneity of samples between the two compartments.

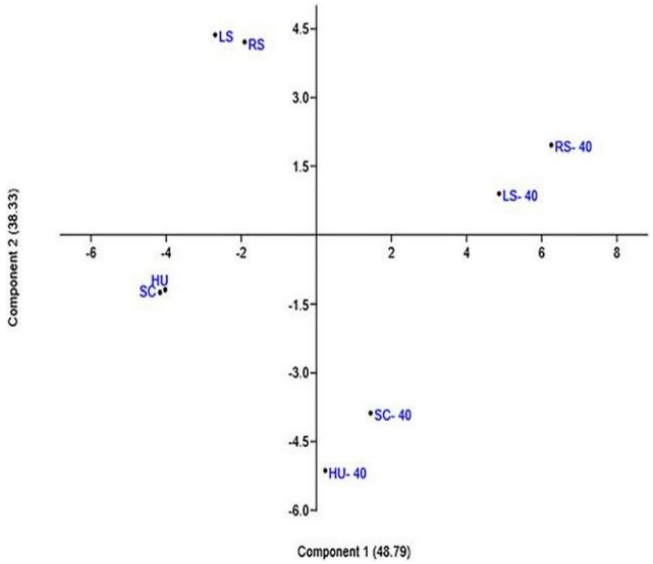

Figure 4.23| Principal component analysis (PCA) based on the ester concentrations detected in wine obtained by inoculating *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* in double fermentor after 40% of the alcoholic fermentation (HU-40, SC-40, LS-40, RS-40) and at the end of process (HU, SC, LS, RS). HU and SC, fermentor compartment inoculated with *S. cerevisiae* and *H. uvarum* separately; LS and RS, left and right compartments, respectively, of the double fermentor inoculated with *S. cerevisiae* and *H. uvarum* together [Colour figure can be viewed at wileyonlinelibrary.com].

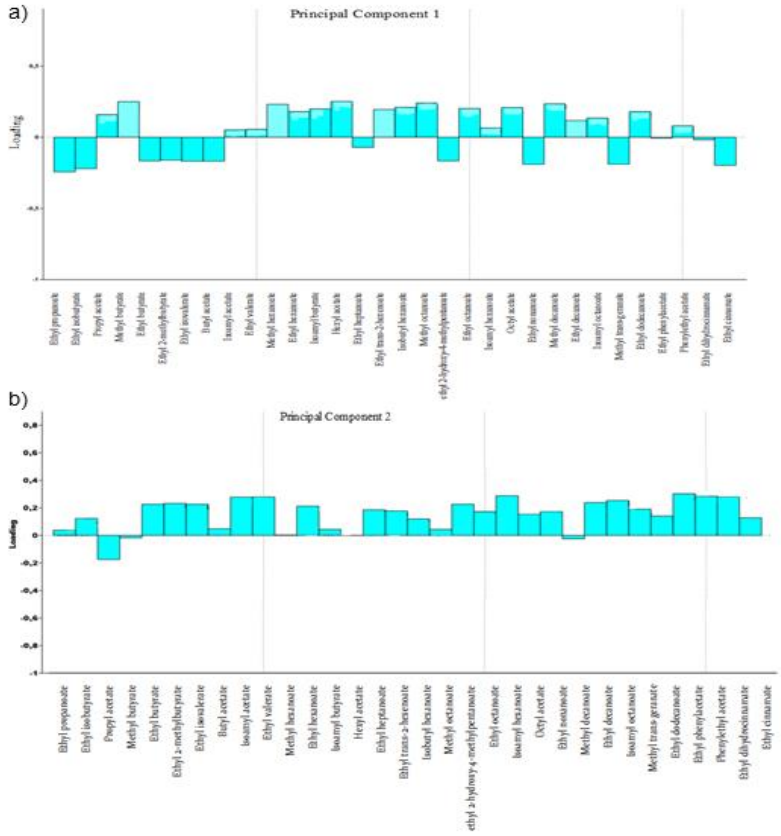

Figure 4.23| Loading plots of the first (a) and the second (b) principal component corresponding to PCA based on the ester concentrations detected in wine obtained by inoculating *S. cerevisiae* and *H. uvarum* in double fermentor after 40% of the alcoholic fermentation and at the end of process.

In conclusion, the results from fermentations showed that the metabolic behaviour of *S. cerevisiae* and *H. uvarum* strains tested in this study seems to be highly influenced by cell to cell contact.

4.6| Conclusions

In this step, eight non-*Saccharomyces* strains (Db2, Ha3, Ha9, St1, St2, St5, St8 and Zb1), selected on the basis of results obtained in the previous step, were tested in simultaneous and sequential inoculation with *S. cerevisiae* EC1118 strain in synthetic must, in order to evaluate their impact on chemical composition and alcohol content of wine.

The data obtained in this step highlighted that the non-*Saccharomyces* yeast strains influence the composition and aroma profile of wine and this influence was correlated with strain ability to survive during the fermentative process together *S. cerevisiae* EC1118.

In fact, the time of permanence of non-*Saccharomyces* yeasts significantly affected ethanol production and chemical composition of wines in both tested modalities, although each yeast strain showed a different behaviour.

In addition, the results obtained in this step showed that the inoculation modality affects the content of aromatic compounds detected in the experimental wines; in fact, the statistical elaboration by PCA of data obtained by gas-chromatographic analysis of experimental wines (Figure 4.13) separated the wines in function of inoculation modality of mixed starter, except wines obtained with mixed starter containing *Z. bailii* (Zb1) strain. This result might be related to different permanence of non-*Saccharomyces* strains during the fermentative process (Figure 4.11 A-D).

Four non-*Saccharomyces* strains (Ha3, St1, St8 and Zb1), selected on the basis of ability to reduce ethanol content of wines were evaluated in simultaneous inoculum in natural grape must. The results obtained in natural grape must showed that the mixed starter affects wine composition and the highest differences were found for experimental wines fermented with *St. bacillaris* strains. As regards the starter ability to reduce the ethanol content of wine, the highest reduction was observed for both *St. bacillaris* strains (mainly for St8) and *Z. bailii* (Zb1). Otherwise, no reduction was found in wine fermented with mixed starter containing *H. uvarum* strain (Figure 4.20), probably in consequence of low permanence of *H. uvarum* strain during the fermentative process (Figure 4.16A).

The results obtained in this step highlight that interactions among yeast strains are likely to occur during the fermentation of grape juice, making very difficult to identify clear trends among different inoculation strategies.

However, the optimal use of mixed yeast cultures is still one of the main challenging tasks for the wine industry, as it requires the selection of the most suitable strains, analysis of the interrelationships between them, trials with different grape musts with different nutritional composition, decision on the timing and consistency of inoculums.

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Chapter 5

Study of two non-*Saccharomyces* selected strains in different winemaking conditions

Abstract

The use of controlled multistarter fermentation using selected cultures of non-*Saccharomyces* and *S. cerevisiae* yeast strains has been encouraged as useful strategies for production of wine with reduced ethanol content. Modification of some fermentative parameters, such as oxygen addition, resulted very useful to address metabolic pathways of yeast strains toward other compounds instead ethanol, resulting in low ethanol yield. The last step of research activity was addressed to a wide investigation on a mixed starter culture, composed by strains showing the best combinations of desirable characteristics investigated in the previous steps. In particular, the mixed starter was composed by three strains (two non-*Saccharomyces* strains together), characterized by good oenological aptitude and highest ability to reduce ethanol content in wine, in combination with the commercial *S. cerevisiae* strain EC1118. This mixed starter was tested in different fermentation conditions, such as co- and sequential inoculation, fermentation with oxygen addition and use of immobilized cells. Furthermore, it was evaluated the influence of stress factors present during fermentation, such as oxidative stress and nutritional deficiency, on these selected strains, by analyzing the effect of these parameters on protein expression.

The final validation of mixed starter culture was performed during pilot scale vinifications in order to individuate the mixed starter culture and fermentation conditions to be proposed to winemakers for production of wine with reduced alcohol content and increased aromatic complexity.

5.1| Introduction

Microbial strategies, based on the selection of starter culture, together with individuation of most suitable fermentation conditions, represent one of the simplest potential approaches for winemakers to produce wine with reduced alcohol content (Canonico et al., 2019a; Contreras et al., 2015; Englezos et al., 2018; Röcker et al ., 2016).

Among non-*Saccharomyces* wine yeasts, some strains/species showed low ethanol yield and sugar consumption by respiration (Crabtree negative) (Contreras et al., 2014; Gobbi et al., 2014; Quirós et al., 2014). Indeed, these strains able to utilise oxygen to oxidise grape sugars could be used to decrease ethanol concentration in wine (Gonzalez et al., 2013; Morales et al., 2015; Shekhawast et al., 2017). Many non-*Saccharomyces* yeasts are able to use oxygen for growth regardless of sugar concentration; in this way, carbon is diverted into other metabolites, and therefore away from ethanol formation (Canonico et al., 2016, 2019b). The use of immobilized cells of non-*Saccharomyces*, such as strains of *St. bombicola*, *M. pulcherrima, H. osmophila* and *H. uvarum* in sequential fermentation with *S. cerevisiae*, could be a suitable strategy to reduce the ethanol content in wine.

In this step, a mixed starter culture composed by two non-*Saccharomyces* strains (*St. bacillaris* St8 and *Z. bailii* Zb1) and *S. cerevisiae* EC1118, was tested during laboratory-scale fermentation in order to evaluate the fermentative fitness, strain influence on the content of the main secondary compounds affecting wine aroma and starter ability to reduce ethanol content. Different inoculation strategies, such as simultaneous and sequential inoculum, oxygen addition and use of immobilized cells of non-*Saccharomyces* strains, were tested. In order to validate the data obtained during laboratory scale fermentations, this mixed starter culture was tested in fermentation trials at pilot scale in the cellar. The evaluation of aromatic characteristics and ethanol content of final wines were used as criteria to test the suitability of these indigenous starters to be used at the cellar level.

5.2| Sugar metabolism of non-*Saccharomyces* yeasts

The possibility of reducing ethanol yields by promoting respiration of sugars by *S. cerevisiae* or other yeast species was suggested by different years as a tool to reduce ethanol content of wine (Contreras et al., 2015; Gonzalez et al., 2013; Morales et al., 2015). A possible way to reach this goal is shown in Figure 5.1.

Some researchers have suggested partial respiration of sugars from grape must as a way to decrease ethanol yield during winemaking (Gonzalez et al., 2013 and references therein). Contrarily to *S. cerevisiae*, which favours fermentative metabolism over aerobic respiration when sugar concentration exceeds 10 g/L (due to the Crabtree effect), many non-

Saccharomyces yeasts are able to use oxygen for growth regardless of sugar concentration and thus divert carbon into other metabolites instead of ethanol formation (García et al., 2016).

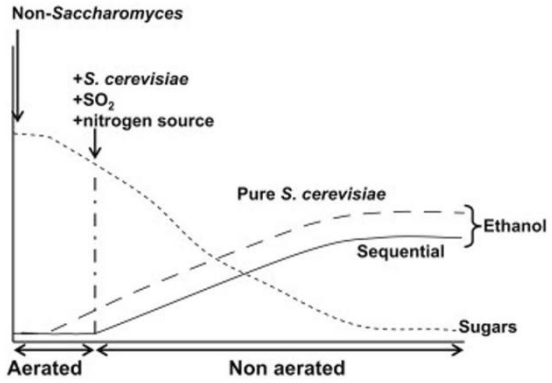

Figure 5.1| Idealized representation of the expected evolution of ethanol production during grape must fermentation in a sequential inoculation with a Crabtree-negative non-*Saccharomyces* yeast strain, followed by *S. cerevisiae* inoculation (Ciani et al., 2016).

On the basis of mechanisms regulating respire-fermentative metabolism, yeasts are classified as Crabtree-positive or Crabtree-negative, or as obligate respiratory. Crabtree-positive yeasts could ferment under aerobic conditions only if sugar concentration is above certain thresholds; an example of Crabtree-positive species is *S. cerevisiae*. This metabolic feature strongly favors fermentative over respiratory metabolism, despite oxygen availability. Only under conditions of very low sugar availability (which is not the case for grape must), respiration is the main metabolic pathway in this species (Figure 5.2).

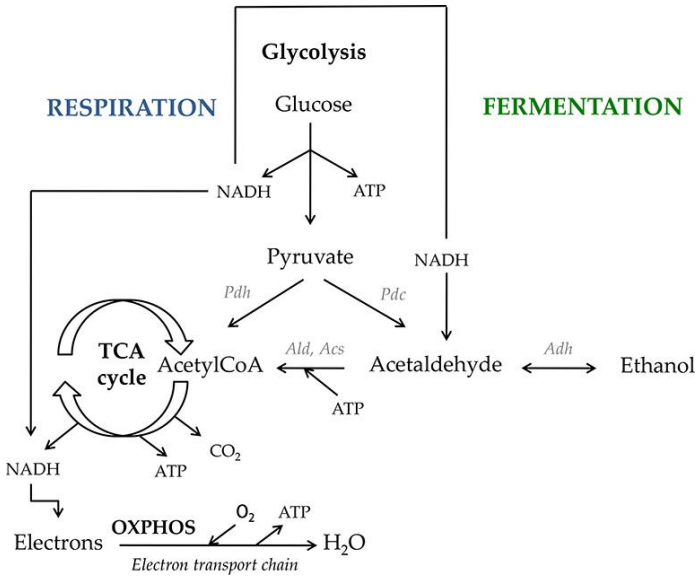

Figure 5.2| Yeast energy metabolism (García et al., 2016).

In contrast, the fermentative metabolism for Crabtree-negative species is very limited whenever a sufficient amount of oxygen is available. *Hanseniaspora uvarum* and *Candida*

species are examples of Crabtree-negative yeasts. Respiratory behaviour of yeast strains seems to be strongly affected by numerous environmental factors, other than sugar abundance or oxygen availability (Rodrigues et al., 2016). The extent to which these environmental factors affect yeast respiro-fermentative metabolism and formation of secondary by-products, such as glycerol or acetic acid, is species or strain-specific.

5.3| Cell Immobilizer as a tool for ethanol reduction

Recent works (Canonico et al., 2016, 2019b) showed that the use of immobilized selected strains of some non-*Saccharomyces* yeasts, such as *Starmerella bombicola*, *Metschnikowia pulcherrima*, *Hanseniaspora osmophila* and *H. uvarum* in sequential fermentation with *S. cerevisiae*, could be a suitable strategy to reduce the ethanol content in wine. Immobilization procedure allows to obtain a high density of cells in confined conditions, with high reaction rate. In these studies, immobilized cells of *S. bombicola* and *M. pulcherrima* under anaerobic conditions led an ethanol reduction of 1.6% and 1.4% respectively, exhibiting an increase of some key aroma compounds.

Yeast immobilization offers numerous opportunities for industrial fermentation processes, such as winemaking or beer production. This technology is aimed to confine intact, active yeast cells to a specific region, thus increasing the cell density, enhancing the production enhancement of some metabolites. Furthermore, the application of this procedure allows a better control and stability of the yeast strain, providing cell protection, and cell recovery and reutilization (Nedović et al., 2015).

However, accurate selection of the immobilization method and the carrier material is essential. In general, for alcoholic beverage production purposes, immobilization supports, has to comply with certain requirements as follows:

- big surface, with functional properties and/or chemical groups favouring cells to adhere;
- high and retained cell viability and operational stability;
- catalytic activity not affected;
- uniform and controllable porosity to allow free exchange of substrates, products, cofactors, and gases;
- good mechanical, chemical, thermal, and biological stability;
- easy, cost-effective, and amenable to scale-up immobilization technique;
- no influence on product quality.

Different methods for yeast immobilization have been developed depending on the mechanism of cell localization, as reported in Table 5.1.

Table 5.1 Methods of yeast immobilization (Moreno-García et al., 2018).

Methods of immobilization	Brief description	Advantages	Disadvantages
Auto-immobilization	Innate ability of cells to aggregate (i.e., adhesion, biofilm formation, filament formation, and flocculation).	Beneficial effects on wine quality and industrially used.	Sensitive to factors like pH, medium, composition, O_2 content.
Immobilization on a support surface	Adsorption of cells to a carrier by cell membrane-immobilizer covalent bonding or by electrostatic forces.	Cheap carrier materials and ease of carrying out the process.	Depth and bonding strength of the cells are not determined. Potential detachment of yeast cells.
Mechanical containment behind a barrier	Cells are entrapped in microporous or ultraporous membrane filters, microcapsules or on an interaction surface of two immiscible liquids.	Useful when minimal transfer of compounds or cell-free products is needed.	Cell loss during mass transfer and possible membrane biofouling
Entrapment in a porous matrix	Cells incorporation to rigid networks	Prevention of cell diffusion and allowance of transfer of substrates and metabolism products.	High cost, low mechanical and chemical stability. The biomass entrapped in a gel matrix is critical for usage of biotechnological processes utilizing viable immobilized yeast cells.
Natural supports	Principle of food-grade purity and used with slightest or no pre-treatment.	High abundance, low cost, and food-grade nature	Degradation process of the supports not evaluated. Industrial scale-up not described.

The “auto-Immobilization” can be defined as the ability of certain yeast species to auto-immobilize in an innate way. For example, some microorganisms, and notably *S. cerevisiae*, can develop various multi-cellular systems of immobilization, as adhesion, biofilm formation, filament formation, and flocculation. The effect of some of these mechanisms on the wine quality is known to be beneficial and it is already industrially applied. For example, the cell flocculation consists of non-sexual aggregation of single-celled organisms in suspension to form aggregates of many cells, known as flocs. This phenomenon is influenced by several factors, such as cell wall composition, pH of medium and dissolved oxygen. In particular, this technique is used in the production of sparkling wines, such as Champagne, in which the utilization of flocculent yeast cells favours the process of removing cell deposit from the bottle, improving clarification of the wine and reducing wine losses (Suárez Valles et al., 2008). The yeast immobilization in biofilms is formed spontaneously in the wine-air interface of wines that are stored in barrels during a process that is known as “biological aging.” This type of biofilm is called “flor” and formed by yeast strains known as “flor yeasts”; it protects wine from oxidation and influences the sensory properties of wines.

Immobilization on a support surface is defined as the binding of yeast cells to a carrier by covalent binding between the cell and the support, or by adsorption. Examples of support

surfaces are cellulosic materials and inorganic materials; this technique has been widely applied due to low cost of used immobilization materials, and the simplicity of the process. Among the cellulosic material, fruit pieces, delignified cellulosic materials, and gluten pellets have been applied in winemaking. Fruit pieces, such as apple and quince, were used as supports, leading an improvement of sensorial traits. Furthermore, grape skins were used to immobilize *S. cerevisiae* yeasts. This support was suitable for winemaking and proposed for the use in combined alcoholic and malolactic fermentations. Yeast cells immobilized on these supports produced wines with enhanced properties in comparison to wines obtained by using free cells, making this application very attractive for industrial use (Bekatorou et al., 2001; Moreno-Grancía et al., 2018).

For the inorganic support surfaces, the researchers recommended the utilization of cellulose (as main carrier), covered with Ca-alginate and an anion-exchange resin, as immobilization supports for winemaking. Other immobilization method is mechanical containment behind a barrier, the most common of which are microporous or ultraporous membrane filters and the microcapsules. The entrapment in a porous matrix is achieved when cells are incorporated in a rigid network, which prevents them from diffusing into the neighboring medium, while still admitting mass transfer of substrates and metabolic products. Some examples are represented by polysaccharide gels, such as alginates, agar, chitosan, and polygalacturonic acid or other polymeric matrixes, like gelatine, collagen and polyvinyl alcohol (Park & Chang, 2000).

Salts like Na-, Ca-, or Ba-alginate are those extensively used for cell immobilization, and among them, Ca-alginate gels are the most suitable for alcoholic fermentation. It was proposed a system entrapping *St. bacillaris* in Ca-alginate gels as system to increase glycerol content in wine; in this study, it was observed also an improvement of fermentation rate (g of CO_2/day) in comparison with free cells, two-fold production of ethanol and a reduction in acetaldehyde and acetoin production (Figure 5.3).

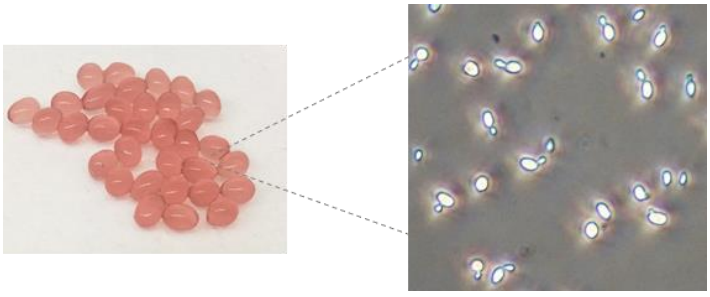

Figure 5.3| Cells immobilized in Na-alginate (left) and section of the inner wall showing yeast cells of *St. bacillaris*, photographed by a microscope (right).

Ca-alginate beads have also been recommended to entrap highly flocculent *S. cerevisiae* strains to perform cell-recycle batch process and optimize must fermentations. Another application of Ca-alginate cell entrapment is the secondary fermentation in sparkling winemaking for easy clarification and removal of cells. Furthermore, *S. cerevisiae* encapsulated in Ca-alginate were utilized with success for the treatment of sluggish and stuck fermentations, giving better results than the traditional method, based on the use of free cells. However, immobilization methods may affect cell growth and physiology, or induce metabolic alterations (Djordjevic et al., 2016). Immobilization on different solid surfaces showed several effects on yeast cells, such as increase in stored polysaccharides, altered growth rates, lower yield of fermentation by-products, activation of yeast energetic metabolism, higher intracellular pH, increased resistance against toxic and inhibitory compounds. In fact, it was reported an enhanced ethanol resistance, due a partial removal of substrate inhibition by cell immobilization. Several authors suggested that the increased ethanol tolerance might be due to a modification in concentration of membrane fatty acids in consequence of limitations in oxygen diffusion or simply due to cell encapsulation by a protective layer of the immobilization material.

In white wine production, it was detected a difference in sensory properties between wines obtained with free and immobilized cells, with a stronger flavor and aroma in wines produced by immobilized yeasts (Moreno-García et al., 2018). Canonico et al. (2016) co-immobilized non-*Saccharomyces* yeasts in Ca-alginate to perform sequential fermentations with a final inoculation of free *S. cerevisiae* cells to reduce ethanol content in wine. The yeasts immobilized were Crabtree negative (sugar consumption by respiration and low ethanol yield) and naturally present on grapes and winemaking equipment. The strategy resulted in high reaction rates, sugar reduction to a 50% in 3 days and the ethanol reduction up to 1.6% (v/v) in comparison to non-immobilized cells. Furthermore, an enhancement of the analytical profile of wine was observed for most of the yeasts immobilized. During the last few years, novel concepts of organism co-immobilization without the need of an external support were diffused. This kind of methodology exploits the ability of the organisms used to adhere to external bodies. This is the case of the co-immobilization of yeasts and filamentous fungus categorized as GRAS. It consists of the attachment of yeast cells to the mycelium of filamentous fungus (e.g., *Rhizopus* sp., *Aspergillus niger*, and *Penicillium* sp.). Co-immobilizing *Penicillium chrysogenum* and yeast cells results in the formation of spherical bodies, known as "yeast biocapsules". This system minimizes changes to the metabolism and yeast viability and enables diffusion of products to and from the biocapsules, due to the porous structure of the hypha framework (García-Martínez et al., 2011).

5.4| Materials and methods

5.4.1| Yeast strains

The strains tested in this step were *St. bacillaris* St8 and *Z. bailii* Zb1. These strains were selected as, among all the mixed starter cultures tested during the previous steps, they determined the highest ethanol reduction during laboratory scale fermentations. In this step, the mixed starter was composed by the two non-*Saccharomyces* strains (St8 and Zb1) and the commercial *S. cerevisiae* strain EC1118 (Lallemand), used also as control. The yeast strains were maintained on yeast extract-peptone-dextrose (YPD) medium (10 g/L yeast extract; 20 g/L peptone; 20 g/L glucose; 20 g/L agar) and stored at 4°C.

5.4.2| Fermentation trials

The selected non-*Saccharomyces* strains were tested in mixed fermentation in different experimental conditions:

- use of different inoculation level;
- addition of oxygen;
- yeast cell immobilization;
- fermentation at pilot scale.

5.4.2.1| Laboratory-scale fermentations with different inoculation levels

The selected strains were tested in mixed fermentations at laboratory scale in natural grape must by using simultaneous (SiF) and sequential (SeF) inoculum and by using different inoculation levels. The two non-*Saccharomyces* strains and *S. cerevisiae* were inoculated at the following concentrations:

- Trial1: *St. bacillaris* 2 x 10^7 cells/mL, *Z. bailii* 2 x 10^5 cells/mL and *S. cerevisiae* 2 x 10^3 cells/mL;
- Trial2: *St. bacillaris* 2 x 10^7 cells/mL, *Z. bailii* 2 x 10^6 cells/mL and *S. cerevisiae* 2 x 10^3 cells/mL;
- Trial3: *St. bacillaris* 2 x 10^7 cells/mL, *Z. bailii* 2 x 10^6 cells/mL and *S. cerevisiae* strain 2 x 10^7 cells/mL.

In Trial1 and Trial2 the three yeast strains were simultaneously inoculated, whereas in Trial3 the grape must was firstly inoculated with the two non-*Saccharomyces* strains and *S. cerevisiae* strain was inoculated when the alcohol content reach 5% v/v (Figure 5.4).

The positive control was represented by pure culture fermentation, obtained inoculating the *S. cerevisiae* EC1118 strain at concentration of 2 x 10^7 cells/mL, whereas non-inoculated

must was used as negative control. The fermentations were carried out in natural red grape must, Aglianico variety, with the grape skin. The grape must was thermally treated at 80°C for 20 minutes in order to inactivate the yeasts population naturally present, and stored at -20°C until the use. Under sterile conditions, 400 mL of the pasteurized must was transferred in 500 mL sterile glass bottle closed with a Müller valve filled with sulphuric acid.

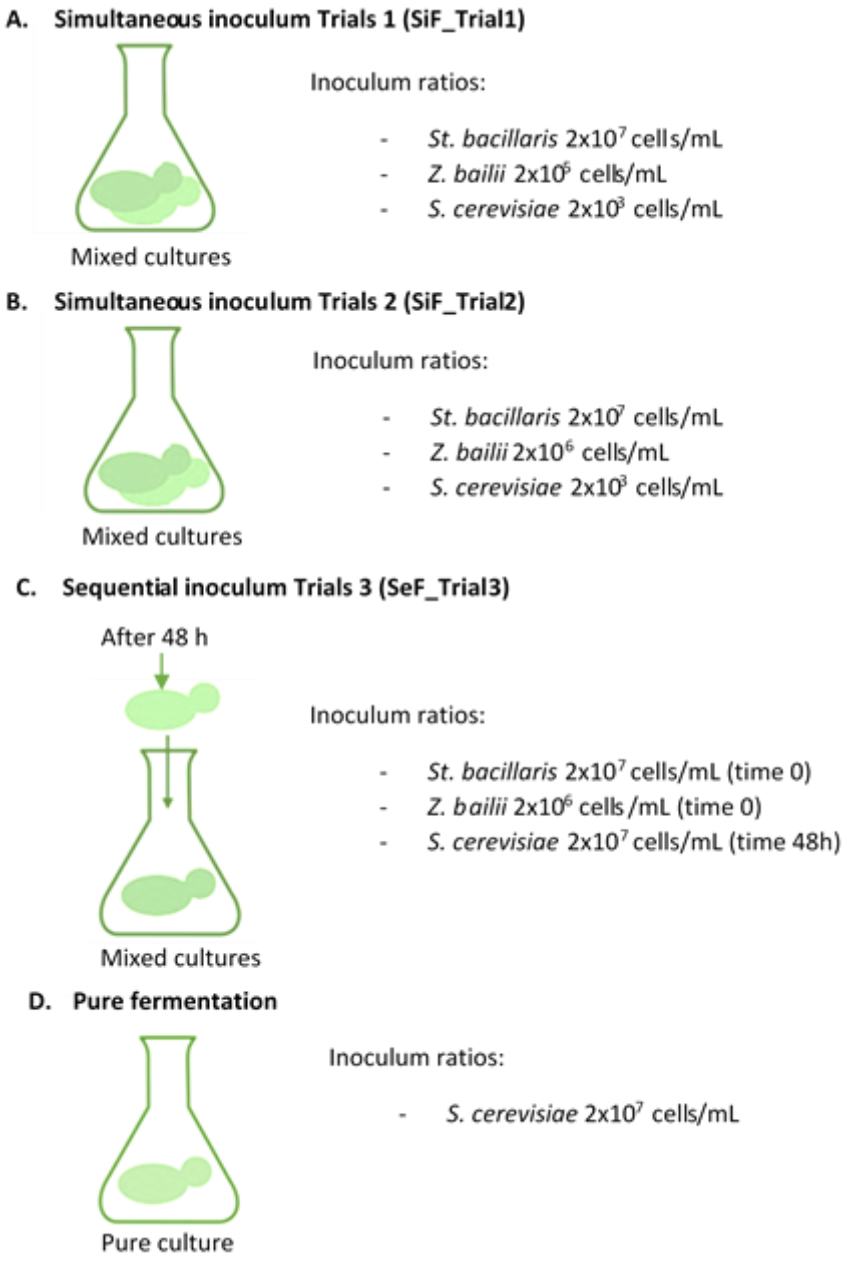

Figure 5.4| Schematic representation of the different fermentations trials.

The grape must composition was the following: 208.7 g/L of sugars, pH value of 3.38, a total acidity of 5.57 g/L, yeast assimilable nitrogen (YAN) was composed of 117.10 mg/L of amino acids and 75.70 mg/L of ammonium. Each fermentation was inoculated with cultures pre-grown in YPD at 28°C for 48 h. All the fermentations were performed at 26°C under

static conditions, and two independent biological replicates were performed. The evolution of the fermentation was evaluated by measuring daily weight loss, caused by carbon dioxide (CO_2) release during the fermentation, and by assessing sugar concentration. The sugar content was determined by a Fourier Transfer Infrared WineScan instrument (OenoFoss, Hillerød, Denmark). Fermentations were considered to be finished when the weight loss of the samples was constant for two-three consecutive days.

For each starter culture, it was calculated the fermentation vigour (FV) and fermentation power (FP); FV was expressed as grams of CO_2 produced in 400 mL of must during the first 48 h of fermentation, while FP was expressed as grams of CO_2 produced at the end of fermentation.

The viable yeast cell population was evaluated by plating fermenting must samples, taken from each flask at different fermentation steps, on Wallerstein Laboratory (WL) Nutrient Agar medium (Sigma-Aldrich), following the protocol reported in the paragraph 4.3.1.3. The three different yeast species included in these mixed starter cultures can be distinguished on the basis of colony colour and morphology. *St. bacillaris* strains metabolize the bromocresol green present in WL medium and therefore form flat colonies, with colour ranging between light to intense green due to the acidogenic nature of this species. On the other hand, *S. cerevisiae* strains do not metabolize this dye in the same way (strain dependent) and as a consequence generally form creamy white colonies, with green umbonate elevation, while *Z. bailii* strains form small white colonies (Figure 5.5). These differences are very useful to follow the evolution of the three strains during mixed starter fermentations.

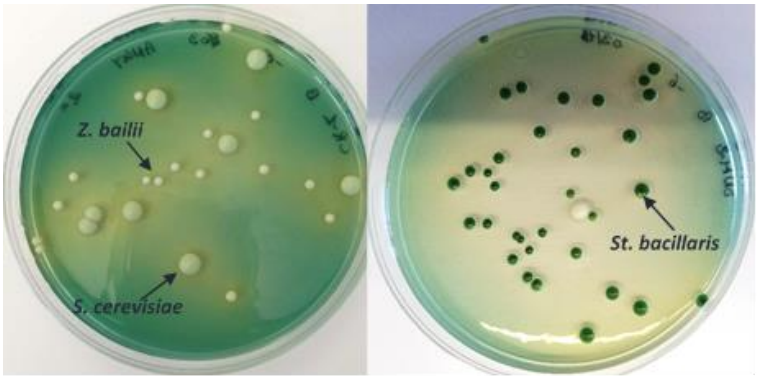

Figure 5.5| Colony morphology of three yeast strains (2 non-*Saccharomyces* and 1 *S. cerevisiae*) on Wallerstein Laboratory (WL) Nutrient Agar medium.

5.4.2.1.1| Analytical determinations

Experimental wines obtained from the inoculated fermentation were analyzed for conventional chemical parameters, such as ethanol, total and volatile acidity, malic and lactic acids, residual sugars (glucose, fructose) and pH, by a Fourier Transfer Infrared WineScan

instrument. The glycerol was determined by enzymatic kit, following the manufacturer's instructions (Megazyme, Ireland).

Furthermore, the content of the main secondary influencing wine aroma, such as acetaldehyde, *n*-propanol, *n*-butanol, isobutanol, acetoin, D-amyl alcohol, Isoamyl alcohol and ethyl acetate, were determined by direct injection gas chromatography of 1 µl sample into a 180 cm × 2 mm glass column packed with 80/120 Carbopack B/5% Carbowax 20 M (Supelco, Bellefonte, PA). The column was run from 70 to 140 °C, the temperature being ramped up at a rate of 7 °C/min. The carrier gas was helium at a flow rate of 20 mL/min. Levels of the secondary compounds were determined by calibration lines, as described by Capece et al. (2013).

5.4.2.1.2| Statistical analysis

Each test was carried out independently in duplicate, and the results are represented as the average with the corresponding standard deviation (±SD). Analysis of variance (ANOVA) was used to evaluate differences in chemical compounds of the experimental wines obtained by different inoculation modalities, by using Tukey's test to compare the mean values. Principal component analysis (PCA) was carried out on the data of wines produced from mixed starters and pure starter at laboratory scale. The PAST3 software ver. 3.20 (Hammer, Harper, & Ryan, 2018) was used for the statistical analyses.

5.4.2.2| Mixed fermentation with oxygen addition.

The selected mixed starter was tested in grape must fermentation with limited aeration by using simultaneous inoculation modality (SiF_Trial1).

Fermentations were carried out in red grape must from Aglianico grapes, previously thermally treated at 80°C for 20 minutes.

The grape must composition was the following: 208.7 g/L of sugars, pH value of 3.38, total acidity 6.29 g/L, yeast assimilable nitrogen (YAN) composed of 110.5 mg/L of amino acids and 105.8 mg/L of ammonium. The fermentations were performed in 2500 mL sterile glass bottles, containing 1800 mL of Aglianico grape must at 26°C without agitation (Figure 5.6). For mixed fermentations, two conditions were tested:

- Condition I: no air addition;
- Condition II: 5 mL/min aeration (0.05 volume of air per volume of culture per minute - VVM) until 50% of the sugar was consumed; in this condition, sterile gas (air) was continuously sparged (66 h) into the bottles, by controlling the aeration rate. As control, pure fermentation without oxygen addition was used.

Figure 5.6| Mixed fermentations in limited aeration conditions.

Each fermentation was inoculated with cultures pre-grown in YPD at 28°C for 24 h. Each strain was grown in small-scale bioreactor under semi-aerobic conditions (Figure 5.7).

Figure 5.7| Small-scale bioreactor.

All the experiments were performed in duplicate. The fermentative course was monitored by measuring weight loss, determined by carbon dioxide evolution during the process, and by assessing reduction of sugar concentration during the time. Fermentations were considered to be finished when the weight loss of the samples was constant for two-three consecutive days, and the level of residual sugars was below 2 g/L. The sugar content was measured by a Fourier Transfer Infrared WineScan instrument (OenoFoss, Hillerød, Denmark).

Fermentation vigour (FV) and fermentation power (FP) of the starters were evaluated. FV was expressed as grams of CO_2 produced in 1800 mL of must during the first 48 h of fermentation, while FP was expressed as grams of CO_2 produced at the end of fermentation. Wines were kept at 4 °C and analysed for chemical and volatile composition. The evolution of yeast cell of each strain included in the mixed starter used for both the fermentation modalities was evaluated by viable yeast count on WL Nutrient agar, as previously reported (paragraph 4.3.1.3). The experimental wines obtained by mixed starter (with and without oxygen addition) and control fermentation were analyzed for main chemical parameters and secondary compounds, as previously reported (paragraph 5.4.2.1.1).

5.4.2.2.1| Statistical analysis

Analysis of variance (ANOVA) was used to evaluate differences in chemical composition of the experimental wines obtained without and with the oxygen addition, by using Tukey's test to compare the mean values. Principal Component Analysis (PCA) was performed on chemical and secondary compounds determined in the all the experimental wines. The PAST3 software ver. 3.20 (Hammer, Harper, & Ryan, 2018) was used for the statistical analyses.

5.4.2.3| Yeast strains immobilization

Fresh cells of St. *bacillaris*, *Z. bailii* and *S. cerevisiae* strains were grown in YPD broth (10 g/L yeast extract; 20 g/L peptone; 20 g/L glucose) at 28°C for 48 h in a rotary shaker (180 rpm). The biomass for the immobilization of the two non-*Saccharomyces* strains were harvested by centrifugation, washed three times with sterile distilled water and added to 2.5% Na-alginate at a ratio of 5% (w/v), following the procedures described in Canonico et al. (2016). Using a sterile syringe, this mixture was then dripped into $CaCl_2$ (0.1 M) to induce gelation (Figure 5.8).

Figure 5.8| Immobilized cells of *Starmerella bacillaris* (St8) and *Zygosaccharomyces bailii* (Zb1).

After 1 h, the formed beads were washed several times with sterile distilled water and then used immediately.

5.4.2.3.1| Fermentation trials in natural grape must

In order to evaluate the influence of immobilization on metabolic activity of non-*Saccharomyces* cells, the selected non-*Saccharomyces* strains were tested as mixed starter in co-fermentation trials. In details, the immobilized non-*Saccharomyces* cells were co-inoculated in the grape must with the free *S. cerevisiae* cells (2×10^3 cells/mL). The inoculum for the immobilized cells of the non-*Saccharomyces* was 10% (w/v), which corresponded to an inoculum of approximately 2×10^7 cells/mL for *St. bacillaris* and 5%

(w/v), which corresponded to an inoculum of approximately 2×10^5 cells/mL for *Z. bailii*. In parallel, the same non-*Saccharomyces* strains were tested in free form (by using an inoculum level of 2×10^7 cells/mL for *St. bacillaris* and 2×10^5 cells/mL for *Z. bailii*); furthermore, pure culture fermentation with EC1118 was used as control.

For the fermentation trials, Aglianico grape must, thermally treated at 80°C for 20 minutes, was used; the grape must characteristics were the following: pH 3.38; total acidity 6.29 g/L; initial sugar content 208.7 g/L; yeast assimilable nitrogen (YAN) composed by 110.5 mg/L of amino acids and 105.8 mg/L of ammonium. Under sterile conditions, 800 mL of the pasteurized must was transferred in 1000 mL sterile glass flasks closed in the top (Figure 5.9). All the fermentations were performed at 26°C under static conditions, and two independent biological replicates were performed.

Figure 5.9| Simultaneous fermentation with immobilized non-*Saccharomyces* strains.

5.4.2.3.2 | Fermentation kinetics and yeast enumeration

The fermentation was followed by measuring the weight loss of the glass flasks, due to CO_2 evolution, until the end of the fermentation trials (constant weight for two-three consecutive days), and by assessing sugar concentration, measuring °Brix degrees by using bench-top refractometer.

Fermentative vigour (FV) and power (FP) of the strains were evaluated. FV was expressed as grams of CO_2 produced in 800 mL of must during the first 48 h of fermentation, while FP was expressed as grams of CO_2 produced at the end of fermentation. Samples of media and beads were taken from each flask at different fermentation steps and at the end of the fermentation, and were submitted to chemical and microbiological analysis, respectively.

The viable yeast cell population of each strain included in the mixed starter was evaluated by plating counting on two different agar media, WL Nutrient Agar medium (Sigma-Aldrich) and Lysine Agar medium (Oxoid Unipath Ltd, Hampshire, UK) with addition of bromocresol green, following the protocol reported in the paragraph 4.3.1.3.

Hydrogen Sulphide test strips was used for detection of hydrogen sulphide production by yeasts; a high H_2S production level appears as blackening of the lower part of the strip (level of blackening is directly correlated with production level of H_2S), while a no-production of H_2S is showed as no blackening.

5.4.2.3.3| Analytical procedures

Experimental wines obtained from the inoculated fermentation were analyzed for conventional chemical and aromatic parameters, as previously reported (paragraph 5.4.2.1.1).

5.4.2.3.4| Data analysis

Each test was carried out independently in duplicate, and the results are represented as the average with the corresponding standard deviation (±SD). Levels of secondary compounds and chemical parameters detected in wines from laboratory scale fermentations were submitted to statistical analysis by one-way analysis of variance (ANOVA); the statistical significance was set at $p \leq 0.05$. Tukey's test was used to compare the mean values of secondary compounds between experimental wines obtained by free and immobilized cells. Principal component analysis (PCA) was carried out on products of alcoholic fermentation with free and immobilized cells. The PAST3 software ver. 3.20 (Hammer, Harper, & Ryan, 2018) was used for the statistical analyses.

5.4.2.4| Effect of stress factors

In this step, the three selected yeast strains (*St. bacillaris* St8, *Z. bailii* Zb1 and *S. cerevisiae* EC1118) were tested for evaluate the influence of stress factor potentially present in winemaking. The trials were performed at the Institute for Integrative Systems Biology (I2SysBio, Paterna, Valencia, Spain).

5.4.2.4.1| Oxidative stress tolerance

The yeast strains were grown overnight in YPD broth and pre-cultures of each strain (2 OD_{600} units of cells) was poured into Petri plates. Sensitivity to hydrogen peroxide was assayed by pouring 5 µL of H_2O_2 (0.5 M) on sterile paper filter (0.5 cm diameter), put on the center of the YPD agar plate and Petri dishes were incubated at 30 °C for 24h. The tolerance to oxidative stress (hydrogen peroxide) was defined in function of size of the inhibition zone surrounding the paper filter for each yeast, according to Mestre Furlani et al. (2017), with some modifications.

5.4.2.4.2| Growth in medium with different nutritional composition

The strain growth on media with different nutritional composition was evaluated by collecting, cells from stationary phase, which were subjected to a series of tenfold dilutions; 5 µL of each dilution were spotted on the plates containing the different chemical compounds, added to YPD or SD media. The minimal medium SD contained a 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% glucose. In details, the following media were tested:

- YPD (control);
- YP_Sucrose (2% sucrose);
- YP_ Glycerol (2% glycerol);
- YP_Sucrose+2DG (2% glucose and 200 µg/mL 2-Deoxyglucose);
- SD_ura (0.5% ammonium sulfate);
- YP_Pro+ura (0.5% proline and 0.5% ammonium sulfate);

where YP means YPD medium, added with compounds indicated in the brackets, and the same for SD. The plates were incubated at 30 °C for 2 days. Experiments were performed in duplicate.

***5*.4.2.5| Fermentations in synthetic must**

Fermentation experiments were carried in 250 mL bottles, filled with 150 mL of synthetic must MS300, previously described (paragraph 4.3.1.4). In each fermentation, the *St. bacillaris* St8 and *Z. bailii* Zb1 strains were co-inoculated with *S. cerevisiae* EC1118 strain, by using an inoculation ratio of 1 x 10^7 cells/mL for non-*Saccharomyces* and 1 x 10^6 cells/mL for *S. cerevisiae*. As control, pure fermentations with the *S. cerevisiae* strain (1 x 10^6 cells/mL) was used. All the fermentations were performed at 26°C under static conditions, and three independent biological replicates were performed. The flasks were inoculated with 48-h pre-cultures grown in YPD broth at 28°C with shaking. After incubation, the pre-cultures of each strain were harvested by centrifugation (3000 rpm, 5 min), and re-suspended in synthetic grape juice.

5.4.2.5.1| Fermentation kinetics and cell growth measurements

The fermentation kinetics were monitored daily by measuring the weight loss of the flasks due to the carbon dioxide (CO_2) release, and by assessing sugar concentration. The process was considered completed when a constant weight of the samples was recorded for two-three consecutive days. The sugar consumption was determined by reaction with DNS (dinitro-3,5-salycilic acid), a colorimetric method, where the DNS is an oxidizing agent

(Santos et al., 2017; Vallejo et al., 2020) (Figure 5.10). Furthermore, was evaluated the growth kinetics of starter cultures by spectrophotometric measurements at OD600nm,during the fermentations in synthetic must.

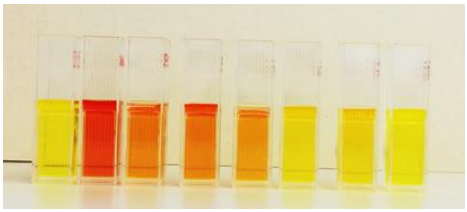

Figure 5.10| Evaluation of sugar consumption by DNS (dinitro-3,5-salycilic acid).

In mixed cultures, yeast growth was determined by plate counting on Wallerstein Laboratory (WL) Nutrient Agar medium (Sigma-Aldrich). Samples were taken at specific times during the fermentation, diluted in sterile MilliQ water, plated on WL medium and incubated at 28°C for 3-5 days. WL medium was used for differential cell counts between non-*Saccharomyces* and *S. cerevisiae* yeasts, following the protocol reported in the paragraph 4.3.1.3.

5.4.2.5.2| Analytical Determinations

Experimental wines obtained from the inoculated fermentation were analyzed for the main chemical parameters (reducing sugars, total and volatile acidity, pH, malic and lactic acid) WineScan instrument, as already described. The glycerol content was determined by enzymatic kit, following the manufacturer's instructions (Megazyme, Ireland), and it was determined in samples taken after 48, 72h and at the end fermentation for each trial.

The main volatile compounds (acetaldehyde, ethyl acetate, *n*-propanol, Isobutanol, *n*-butanol, acetoin, D-amyl and isoamyl alcohols) of wines were determined by direct injection gas chromatography, as already described.

5.4.2.6| Analysis of the proteins

5.4.2.6.1| Proteins extraction

During the fermentations, proteins extraction was performed with the aim of studying the expression of the Tsa1, Tsa1-SO_3 protein and visualizing the phosphorylation of the Snf1 protein, by using a method based on fast cell lysis with tricholoacetic acid (TCA) (Orlova et al., 2008). TCA (5.5%) was added to 5 OD600 units of cells, which were incubated on ice for 15 min. before centrifuging cells. After that, the cells were harvested by centrifugation at 12000 x g for 2 min at 4°C, the supernatants were eliminated and the pellet was washed

twice with acetone, after that it was air-dried and frozen at -80 °C. Subsequently, pellets were resuspended in 150 µl 1× TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) and added with 150 µL of 0.2 M NaOH, followed by incubation for 5 min at room temperature. Extracts were clarified by centrifugation (12000 × g for 1 min) and, after quantification by the Bradford method (Biorad Inc. Hercules, CA, USA), were diluted in loading buffer. The volume of the loading buffer used was 30 µl/1.0 OD600 cells collected. The samples were boiled for 5 min, air-cooled and submitted to centrifugation in a microfuge for 5 min at 12000 × g. The supernatants were loaded on SDS–PAGE at 10 µl/lane (Orlova et al., 2008; Vallejo et al., 2020).

5.4.2.6.2| SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

The extracts were subjected to SDS-PAGE (10% and 6.25% polyacrylamide gels), by using an Invitrogen mini-gel device (Figure 5.11).

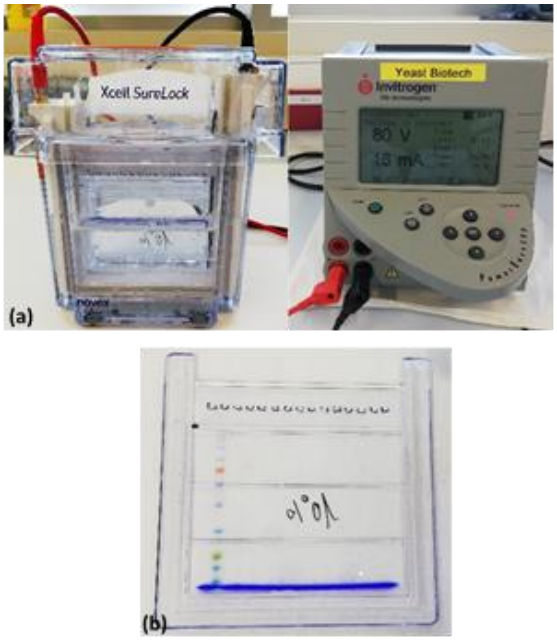

Figure 5.11| Invitrogen mini-gel device (a) and gel electrophoresis (SDS-PAGE) (b).

The first dimension gels (9 mL) contained buffer Tris-HCl 1.5M pH 8.8, 40% acrylamide (10%, with an acrylamide to bisacrylamide ratio of 37.5:1), 10% SDS, 1% Temed (N, N, N', N'-tetramethyl-ethylenediamine), and 10% ammonium persulfate, whereas second dimension gels or pregel (3 mL) contained buffer Tris-HCl 1.5M pH 6.8, 40% acrylamide (6.25%, with an acrylamide to bisacrylamide ratio of 37.5:1), 10% SDS, 1% Temed and 10% ammonium persulfate.

The electrophoresis was performed in buffer Tris-glicina (Tris-HCl 25 mM pH 8.8, glicina 192 mM), by using the program Trys-Gly Gel (1h and 30 min, 125V, 35 mA and 5.0 W).

5.4.2.6.3| Western blot analysis

The SDS-PAGE gel was blotted onto polyvinylidene difluoride (PVDF) membranes for the immunodetection analysis with a Novex semy dry blotter (Invitrogen, Carlsbad, CA, USA) (Figure 5.12) (Gamero-Sandemetro et al., 2013; Trotter et al., 2008; Vallejo et al., 2020), the program was as follows: 1h, 5 V, and 125 mA.

Figure 5.12| Polyvinylidene difluoride membranes (PVDF).

Subsequently, the membrane was incubated in the primary and second antibody solutions. The used antibodies were described in Table 5.2.

Table 5.2 Antibodies used in this work.

Primary antibody	Source	Dilution	Secondary antibody	Source	Dilution
Anti-AMPKα	Cell Signaling Technologies	1:1000	Anti-rabbit	BioRad	1:3000
Anti-Prx	Abcam	1.1000	Anti-mouse	Santa Cruz Biotechnology/BioRad	1:3000
Anti-SO3-Prx	Abcam	1:2000	Anti-mouse	Santa Cruz Biotechnology/BioRad	1:3000
Anti-SOD1	Chemicon AB5448	1:3000	Anti-rabbit	BioRad	1:3000

The detection of the protein was performed by chemiluminescence, using detection kit ECL TM Prime/Select Western Blotting Reagent (GE Healthcare), following the manufacturer´s instructions; mix detection solutions A (luminol) and B (peroxide) were used in a ratio of 1:1. The software LAS-500 (GE Healthcare) was used for capture the image (Figure 5.13).

Figure 5.13| Western blotting detection system: ImageQuant LAS 500.

5.4.2.7| Pilot-scale vinification in cellar

In the final step of research activity, the two non-*Saccharomyces* strains (St8 and Zb1) were tested during fermentations at pilot scale in the cellar "Azienda Agricola Vitivinicola, Cerrolongo", located in Nova Siri (Matera), in 2019 vintage. In the winery, two trials were performed in parallel: a pure culture fermentation with the commercial starter *S. cerevisiae* EC 1118, and a mixed culture fermentation with the mixed starter selected in lab-scale fermentation. The two trials were repeated in two fermentation processes, performed in two different grape musts, Primitivo and Cabernet, both red varieties.

The fermentations were performed at 25°C in 500 L vessels, containing 400 L of grape must; the physical-chemical characteristics of the two different grape musts were the followings:

- Primitivo, 199.9 g/L of sugars, total acidity 5.47 g/L, pH 3.62, yeast assimilable nitrogen (YAN) composed by 142.3 mg/L of amino acids and 121.9 mg/L of ammonium;
- Cabernet, 198.05 g/L of sugars, total acidity 4.70 g/L, pH 3.85, YAN composed by 107.45 mg/L of amino acids and 81.10 mg/L of ammonium.

The grapes were crushed and the obtained musts were supplemented with 10 mg/L total SO_2, added as potassium metabisulphite. The strains were grown in YPD broth at 28 °C for 2 days with shaking.

During pilot-scale vinification, the same inoculation procedure used in laboratory fermentations was applied. The two non-*Saccharomyces*, *St. bacillaris* St8 and *Z. bailii* Zb1, were inoculated with an initial cell population of 2×10^6 cells/mL and 2×10^5 cells/mL respectively, with S. *cerevisiae* EC1118 (initial cell population of 2×10^4 cells/mL), as previously described for laboratory-scale trials. The control fermentation was inoculated with a pure culture of the commercial starter *S. cerevisiae* EC 1118, with an initial cell population of 2×10^6 cells/mL.

5.4.2.7.1| Fermentation kinetics and yeast enumeration

The fermentation course was monitored by determining sugar consumption, both by using WineScan instrument and by checking Babo degree. Must was pumped up twice a day and the fermentations were considered finished when residual sugars were less than 2 g/L.

In order to evaluate the presence of each starter, during the vinification process at different fermentation stages (beginning, middle and at the end of the process), Primitivo and Cabernet of must/wine samples were collected and submitted to plate count on WL medium, as already reported.

5.4.2.7.2| Wine analyses

The must samples collected during the process and wine samples obtained at the end of the vinifications were analyzed for the main chemical parameters (reducing sugars, total and volatile acidity, pH, malic and lactic acid) WineScan instrument, as already described. The glycerol content of final wines was determined by enzymatic kit, following the manufacturer's instructions (Megazyme, Ireland), whereas the main volatile compounds (acetaldehyde, ethyl acetate, *n*-propanol, isobutanol, *n*-butanol, acetoin, D-amyl and isoamyl alcohols) of wines were determined by direct injection gas chromatography, as already described. Furthermore, the wines obtained during pilot-scale vinifications were analyzed for the content of minor volatiles compounds, such as some esters and terpenes, by SPME-GC-MS (Headspace solid phase micro extraction - gas chromatography/mass spectrometry), at a laboratory (Isvea s.r.l., Poggibonsi, Italy) officially accredited for analysis of musts and wines.

5.4.2.7.3 Statistical analysis

The main volatile compounds and oenological parameters detected on wines obtained in the cellar were submitted to PCA, by using PAST3 software ver. 3.20 (Hammer, Harper, & Ryan, 2018), whereas the data of minor volatile compounds were analyzed by heat-map, using the RStudio software.

5.5| Results and Discussions

In this phase, one mixed starter culture, composed by *St. bacillaris* St8, *Z. bailii* Zb1 and *S. cerevisiae* EC1118 and selected on the basis of results obtained in the previous steps, were tested in different conditions.

5.5.1| Laboratory-scale fermentations with different inoculation levels

5.5.1.1| Fermentative kinetics and population dynamics

In this step, the mixed starter was tested in Aglianico grape must fermentation by using different inoculation strategies, which were simultaneous (SiF) and sequential (SeF) inoculum; furthermore, for SiF modality two different inoculation levels for Zb1 strain were tested. As control, single fermentation with EC1118 was used. In details, four fermentation experiments were performed:

- SeF_Trial3, in which EC1118 was inoculated sequentially respect to St8 and Zb1;
- SiF_Trial1 and SiF_Trial2, in which St8 and Zb1 were inoculated together EC1118, by using two different inoculation levels for Zb1 (10^5 and 10^6 cells/mL, respectively)

- Control, represented by pure culture fermentation of EC1118.

The fermentative kinetics, represented by sugar consumption and CO_2 release, and sugar consumption during lab-scale fermentations of grape must are presented in Figure 5.14. A regular trend of fermentative process was observed for all the fermentations, although the duration of the process differed between the pure and all the mixed fermentations. All the fermentations trials were completed in 11-15days, with significant differences among them. Fermentations inoculated with pure culture of *S. cerevisiae* EC 1118 or with sequential inoculum of *S. cerevisiae* finished faster (within 11 days) than mixed fermentation with co-inoculation. All the mixed fermentations exhibited a similar fermentative vigour, producing from 15.66 to 17.17 g of CO_2 per 400 mL within 48 h, whereas the maximum level of CO_2 per 400 mL produced after 48 h was 30.02 g in pure fermentation with *S. cerevisiae*.

The highest CO_2 production at the end of the process was 38.44 g/400 mL, found for St8/Zb1 co-culture (SeF_Trial3), whereas the lowest production (35.83/400 mL) was observed for St8/Zb1 co-culture in SiF_Trial2. All the all mixed fermentations exhibited a slow rate of fermentation, taking 2 days to consume 50% of initial sugar.

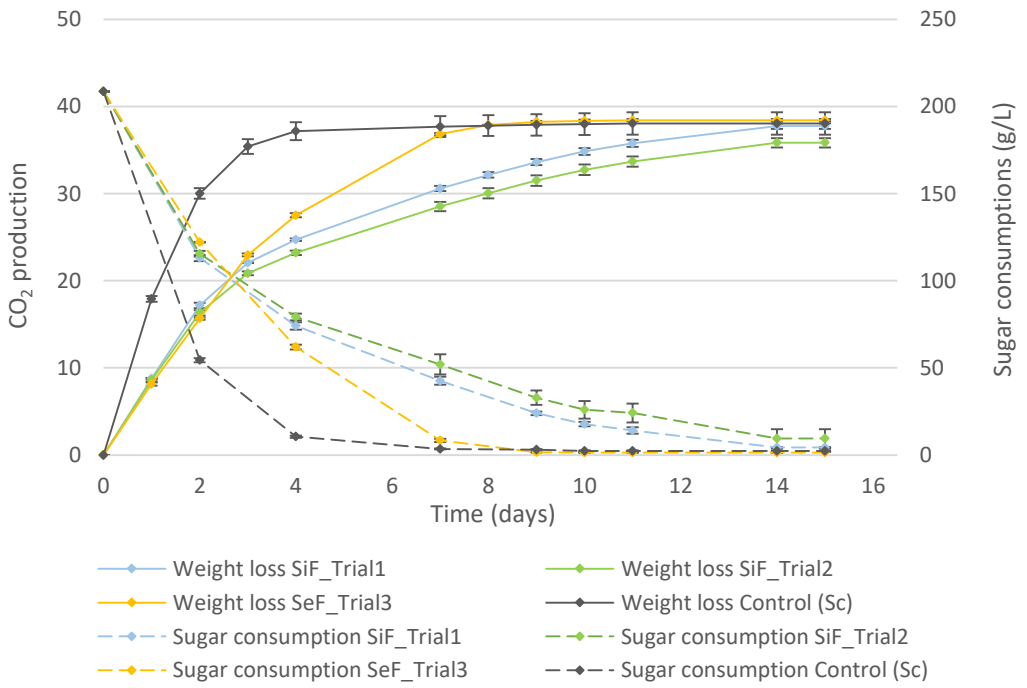

Figure 5.14| CO_2 production (g/400 mL) and sugar consumption of mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously (SiF_Trial1 and SiF_Trial2) and sequentially (SiF_Trial3) inoculated with *S. cerevisiae* EC1118. Pure culture of *S. cerevisiae* EC 1118 (Control Sc) was used as a control. Data are means ± standard deviation of two independent experiments.

The yeast population dynamics in the four fermentations are reported in Table 5.3. Yeast cell viability of *S. cerevisiae* strains in pure culture fermentations achieved a maximum population of 2.13×10^8 UFC/mL after 4 days, maintaining high levels of viable cells along all the fermentative process, as expected. In co-inoculation SiF_Trial1, *St. bacillaris* and *Z.*

bailii were present at detectable levels (3.10 x 10^6 UFC/mL and 1.00 x 10^5 UFC/mL, respectively) up to day 7, after that the viable counts decreased and at the end of the process a low number of viable cells was detected for Zb1 strain (3.00 x 10^4 UFC/mL). In the mixed fermentations, *St. bacillaris* and *Z. bailii* population was higher than that of *S. cerevisiae* until 2nd of fermentation, as a consequence of high percentage of inoculum used for non-*Saccharomyces* strains respect to *S. cerevisiae*, after that dominance of *S. cerevisiae* strain was observed during all the fermentative process (Table 5.3).

Table 5.3| Evolution of yeast populations in mixed fermentations inoculated with *St. bacillaris* (St8), *Z. bailii* (Zb1) and *S. cerevisiae* (Sc) in SiF_Trial1, SiF_Trial2 and SeF_Trial3. Values are mean of two independent duplicates. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control.

Fermentation trials	**Time (days)**					
	0	**2**	**4**	**7**	**11**	**14**
SiF_Trial1						
St8	7.60±7.07	7.81±7.45	6.67±6.17	6.49±5.93		<4.00±0.00
Zb1	5.89±4.89	5.82±5.17	5.30±5.15	5.00±0.00		4.48±4.15
Sc	4.46±4.05	6.39±5.97	6.72±5.85	6.94±5.89		6.61±5.80
SiF_Trial2						
St8	7.64±6.19	7.63±6.80	6.94±6.77	6.06±4.85		<4.00±0.00
Zb1	6.64±5.80	6.80±5.80	6.62±6.45	5.18±4.85		<4.00±0.00
Sc	4.37±3.89	5.88±5.08	6.59±6.10	6.85±6.31		6.67±5.96
SeF_Trial3						
St8	7.65±6.52	8.66±8.26	6.51±5.45	5.70±5.63	<4.00±0.00	
Zb1	6.38±5.15	7.50±6.80	6.18±5.15	5.30±0.00	<4.00±0.00	
Sc		7.32±6.45	7.46±6.34	7.45±6.96	7.22±6.74	
***S. cerevisiae* (Control Sc)**						
Sc	7.74±6.75	8.26±7.35	8.33±6.55	8.30±6.45	7.72±7.21	

Similar results were found for co-fermentations SiF-Trial2, in which the two non-*Saccharomyces* strains reached a maximum of yeast cells after 2nd day (4.25 x 10^7 UFC/mL for St8 and 6.25 x 10^6 UFC/mL for Zb1), after that the viable count decreased and at the 14th day of the fermentation no *St bacillaris* and *Z. bailii* cells were found.

Yeast cell viability of *S. cerevisiae* strain in mixed culture fermentations achieved a maximum population after 7 days (8.65 x 10^6 UFC/mL in SiF_Trial1 and 7.05 x 10^6 UFC/mL in SiF_Trial2), maintaining similar levels until the end of process. These results indicate that the cell number of *S. cerevisiae* was lower in co-inoculation than in control fermentation, probably in consequence of low inoculum level or as a consequence of the presence, with potential competition mechanisms, of St8 and Zb1 strains. Similar results for the evolution of non-*Saccharomyces* population were found for sequential inoculation SeF_Trial3, with highest cell numbers of *St. bacillaris* and *Z. bailii* at 2nd fermentation day and presence of

viable cells until day 7 (5.00 x 10^5 UFC/mL and 2.00 x 10^5 UFC/mL, respectively). Also for this fermentation, no viable cells of non-*Saccharomyces* strains were found at the end of the process and the number of viable cells of these non-*Saccharomyces* strains decreased when *S. cerevisiae* was inoculated in grape must fermentation. Yeast cell viability of *S. cerevisiae* EC1118 strain in this mixed culture fermentations remained at high levels throughout the fermentation process, comparable to yeast cell viability of *S. cerevisiae* strain in pure culture fermentations.

The results obtained in this step showed that *St. bacillaris* and *Z. bailii* strains tested are able to survive during the fermentation, mainly in the first phases of the process. The presence of non-*Saccharomyces* yeasts in the early stages of fermentation could affect the metabolic activity of *S. cerevisiae*, probably as a consequence of competition for nutrients or cell-cell contact mechanisms. On the basis of these results, the viability of non-*Saccharomyces* strains seems to be affected by the inoculum modalities, inoculum ratio and the contact time with *S. cerevisiae* cells.

5.5.1.2| Chemical profile of wine produced in laboratory scale fermentations

The experimental wines obtained at the end of the fermentations were analysed for chemical and aromatic composition. The ethanol accumulation during pure and mixed culture fermentations is presented in Figure 5.15.

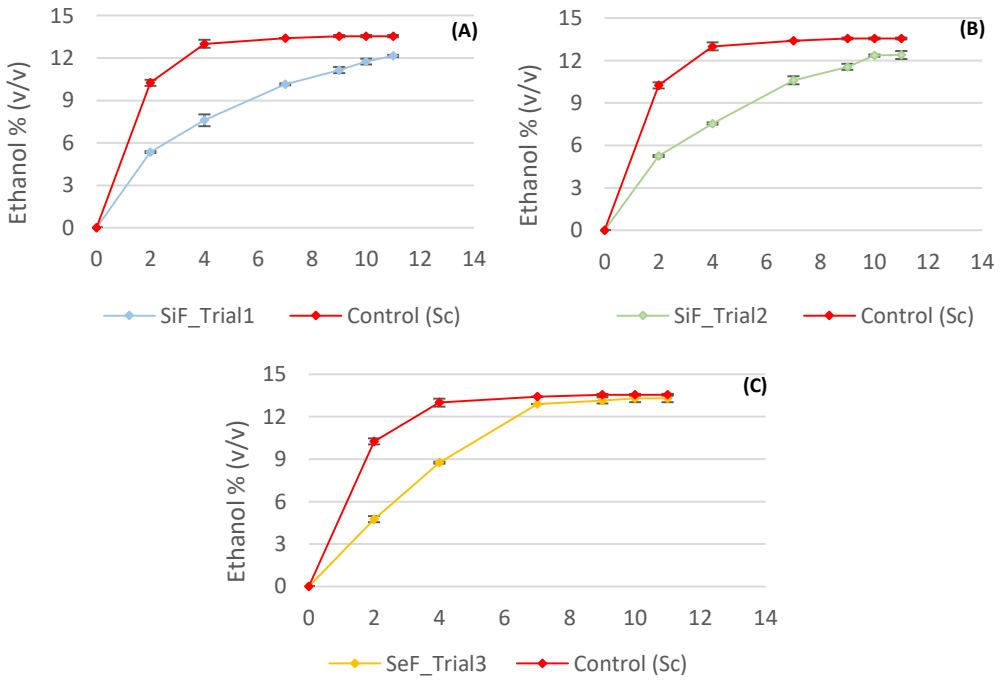

Figure 5.11| Ethanol accumulation (% v/v) during SiF_Trial1 (A), SiF_Trial2 (B), and SeF_Trial3 (C) mixed fermentations. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are provided as the means ± standard deviation of the results from three independent experiments.

As shown in the Figure, in all the mixed fermentations, the ethanol content of final wines was lower than the level detected in control wine (12.15% in SiF_Trial1, 12.40% in SiF_Trial2 13.30 % in SeF_Trial3, 13.55% in control wine). The highest ethanol reduction was obtained by using simultaneous inoculation modality, for both inoculation levels of Zb1 strain.

In fact, wines obtained with simultaneous inoculum showed approximately 0.71 and 0.49% (for SiF_Trial1 and SiF_Trial2, respectively) lower ethanol concentration than wine produced with *S. cerevisiae* EC1118 alone, while wine from SeF_Trial3 contained approximately 0.28% less ethanol than control wine (Figure 5.16).

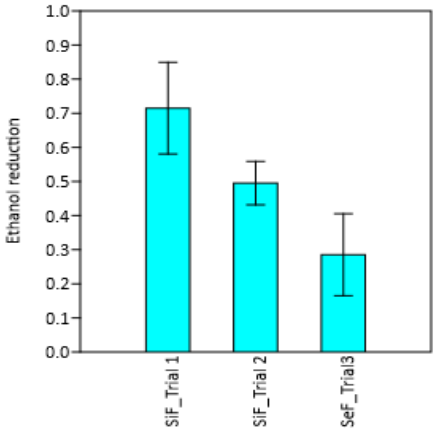

Figure 5.16| Reduction ethanol in experimental wines obtained from mixed fermentations.

These results are consistent with data reported in literature. Several studies reported the reduction of ethanol levels with sequential inoculations of non-*Saccharomyces* and *Saccharomyces* yeasts and the influence of different winemaking conditions on this characteristic (Contreras et al., 2014; Englezos et al., 2016; Tristezza et al., 2016).

The analyses of the main chemical parameters of experimental wines are summarized in Table 5.4. All the trials produced wines with residual sugar below 3 g/L, indicating that the fermentations were successfully completed.

As previously reported, wines obtained with mixed fermentations SiF_Trial1, SiF_Trail2 and SeF_Trial3 showed ethanol concentration significantly lower than wine from control fermentation. No significant differences were observed for fructose and glucose content, whereas the pH values were highest in wines produced with co-cultures compared to control (Sc) wine. As regards the volatile acidity, malic and lactic acids, differences were observed among the trials, the wines from SiF_Trial1 and SiF_Trail2 exhibited the lowest concentration of total acidity, malic and lactic acids and the highest concentration of volatile acidity. All the trials inoculated with mixed starters (SiF_Trial1, SiF_Trial2 and SeF_Trial3) exhibited the highest concentration of glycerol, with values between 2.55 g/L and 3.16 g/L.

Table 5.4| Concentration of major chemical compounds of experimental wines obtained from mixed starters cultures of selected non-*Saccharomyces* strains (St8 and Zb1) simultaneous (SiF_Trial1 and SiF_Trial2), and sequentially (SeF_Trial3) inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control.

Oenological characteristics	**SiF_Trial1**	**SiF_Trial2**	**SeF_Trial3**	**Control (Sc)**
Ethanol % (v/v)	12.22±0.12^{a}	12.45±0.06^{ab}	12.65±0.13^{bc}	12.94±0.01^{c}
Fructose (g/L)	0.55±0.07	0.55±0.21	0.90±0.00	0.85±0.07
Glucose (g/L)	1.30±0.57	2.65±1.06	0.70±0.14	0.75±0.07
Total acidity (g/L)	9.10±0.04^{a}	9.07±0.01^{a}	9.58±0.05^{b}	9.25±0.08^{a}
Volatile acidity (g/L)	0.77±0.01^{a}	0.66±0.12^{ab}	0.58±0.02^{ab}	0.43±0.02^{b}
pH	3.67±0.01^{a}	3.66±0.00^{a}	3.64±0.01^{a}	3.52±0.01^{b}
Malic acid (g/L)	0.93±0.02^{a}	0.98±0.06^{a}	1.26±0.04^{b}	1.47±0.04^{c}
Lactic acid (g/L)	1.23±0.04^{a}	1.25±0.07^{a}	1.30±0.00^{a}	1.00±0.00^{b}
Glycerol (g/L)	3.16±0.01	2.55±0.06	3.06±0.02	1.92±0.02
Main volatile compounds				
Acetaldehyde (mg/L)	21.91±4.84	31.32±2.65	31.00±1.55	31.22±2.12
Ethyl acetate (mg/L)	38.13±0.18	36.53±0.00	35.11±0.40	39.16±1.97
n-Propanol (mg/L)	22.36±1.29^{a}	22.82±0.13^{a}	23.60±0.24^{a}	14.85±0.62^{b}
Isobutanol (mg/L)	104.12±3.06^{a}	107.44±5.35^{a}	99.22±3.52^{a}	40.78±2.17^{b}
n-Butanol (mg/L)	14.20±1.71^{a}	48.81±4.94^{b}	23.10±1.49^{a}	12.96±3.77^{a}
D-amyl alcohol (mg/L)	37.76±1.79^{a}	38,26±0.95^{a}	60,36±0.98^{b}	75.87±0.49^{c}
Isoamyl alcohol (mg/L)	111.32±7.06^{a}	108.49±2.76^{a}	161.67±0.73^{b}	249.20±10.08^{c}

Note: Data are means ± standard deviation of two independent experiments. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$).

As regards the secondary compounds usually present in high concentrations in wines, high variability was found mainly for *n*-propanol, isobutanol, *n*-butanol, D-amyl and isoamyl alcohols. The highest production of *n*-propanol and isobutanol was detected in wine obtained by mixed inoculums, whereas wines obtained by mixed fermentations showed significantly lower concentrations of D-amyl and isoamyl alcohols than control wine.

Principal Component Analysis (PCA) was applied to visualize the differences in the profiles of wines produced through different inoculation protocols (Figure 5.17). The two principal components, PC1 and PC2, accounted for 86% of the total variance (66 and 20%, respectively). This analysis was able to separate control wine from wines obtained by mixed culture fermentations. In fact, wine from monoculture fermentation was located in the left side, bottom quadrant of the PCA plot, whereas the wines obtained by co-culture fermentations were distributed in the right side of the bottom quadrant. Furthermore, the analysis revealed that the inoculation modality affects wine composition; in fact, wine produced by SiF_Trial1 is located very near to the wine obtained by mixed culture

fermentation by SiF_Trial2, whereas it was very far by from wine obtained with sequential fermentation (SeF_Trial3).

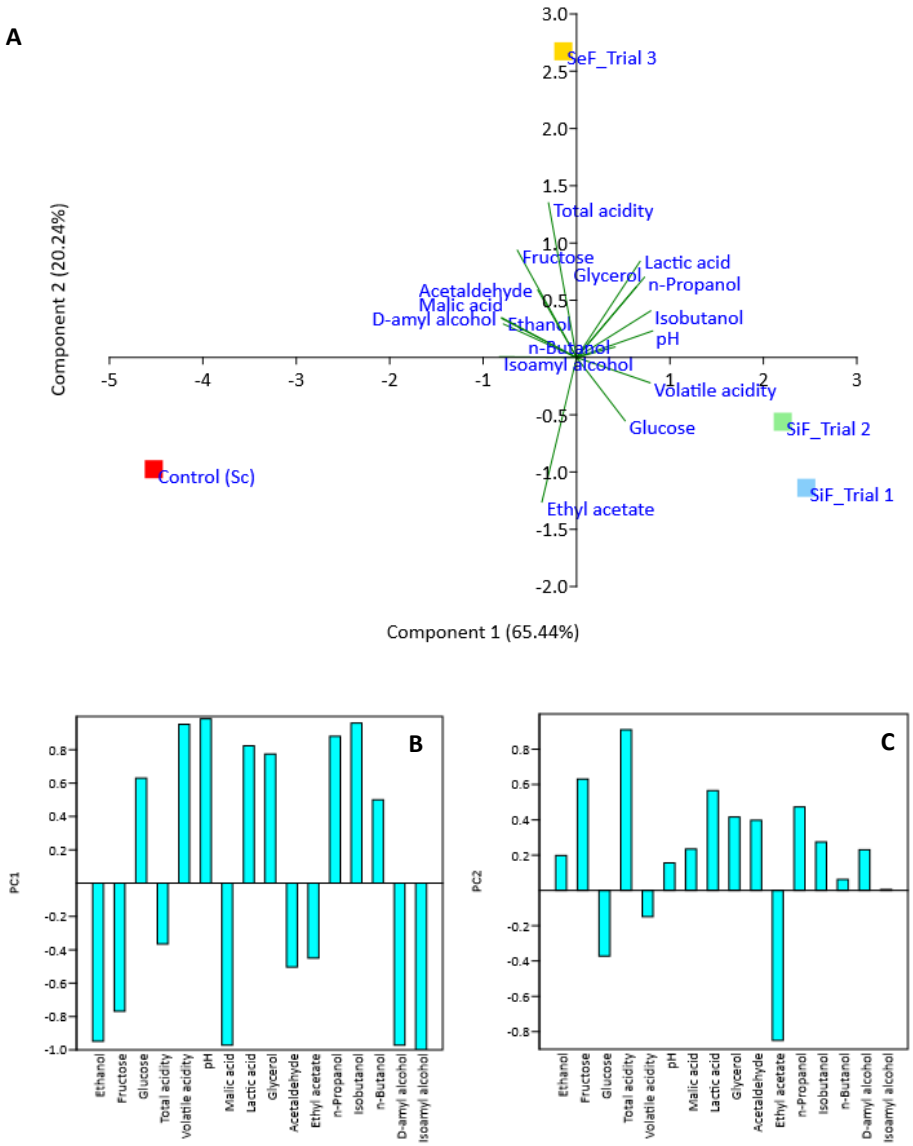

Figure 5.17| Scatter Plot (A) and loading plots of the first (B) and second (C) principal component corresponding to Principal component analysis (PCA) of the oenological parameters and main volatile compounds detected in experimental wines produced at laboratory scale through the following inoculation schemes: Control (Sc): commercial *S. cerevisiae* EC1118; SiF_Trial1 and SiF_Trial2: *St. bacillaris*, *Z. bailii* and *S. cerevisiae* added simultaneously; SeF_Trial3: *St. bacillaris*, *Z. bailii* and *S. cerevisiae* added sequentially.

These results confirmed the metabolic interaction among strains included in mixed starter cultures. During mixed fermentations yeast strains can metabolically interact each other, producing wines characterized by a composition different from the wine obtained by single starter. Therefore, yeasts modify their metabolism during growth in mixed fermentation, where interaction among strains composing mixed starter cultures can determine sharing of some secondary metabolites.

5.5.2| Impact of aeration on fermentation performance and yeast population dynamics

In this step, it was evaluated the influence of oxygen addition on selected mixed starter. In particular, the two non-*Saccharomyces* yeast strains, *St. bacillaris* and *Z. bailii* were evaluated in simultaneous inoculation with *S. cerevisiae* under aeration conditions (0.5 VVM aeration for 66 h, followed by anaerobic conditions). The aim of this trial was to promote sugar respiration by non-*Saccharomyces* yeasts in the first steps of the fermentation, reducing the amounts of sugars potentially available for fermentation and, consequently, reducing the potential ethanol content of resulting wines. The course of fermentations, represented by CO_2 release, for pure and mixed starters is shown in Figure 5.18.

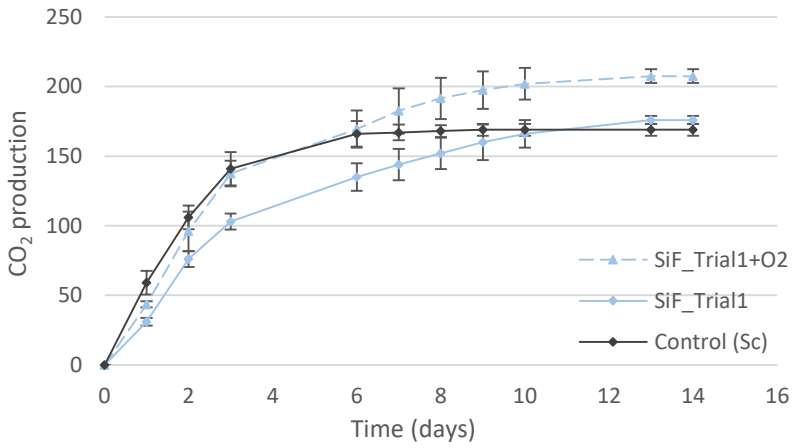

Figure 5.18| Fermentation kinetics of mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC 1118 (Sc), under different conditions: anaerobic conditions (—◆—), aeration 0.5 VVM (- -▲- -). Pure culture of *S. cerevisiae* EC 1118 (Control Sc) was used as a control. Data are means ± standard deviation of two independent experiments.

Both mixed fermentations were completed in 14 days, while control fermentation finished within 10 days, indicating that the mixed starter slowed down the process. Analogous results on the time courses of mixed fermentations were recently found by other several authors. For example, Englezos et al. (2018) described that a sequential fermentation with *S. bacillaris* and *S. cerevisiae* in white grape must took 14 days to finish, while 9 days were needed for the single inoculation with *S. cerevisiae*. The trend of CO_2 production was very similar in all the fermentations, although CO_2 production was very similar and higher for control and aerated fermentations until the sixth fermentation day in comparison to mixed fermentation in anaerobic conditions. After this point, the CO_2 production was stopped for control fermentation, whereas a constant increase until the end of the process was observed for both the mixed fermentations. However, the CO_2 produced at the end of the process was higher for fermentation with oxygen addition than for process performed in anaerobic conditions. The fermentative kinetics, reported as sugar consumption (expressed as °Brix

degree) during the time, is reported in Figure 5.19. Also for this parameter, the mixed starters finished the process later than control fermentation (*S. cerevisiae* EC1118). Furthermore, mixed starter with oxygen addition reached the same sugar residual of control fermentation, whereas in fermentation performed in anaerobic conditions the residual sugars were higher than the other two fermentations.

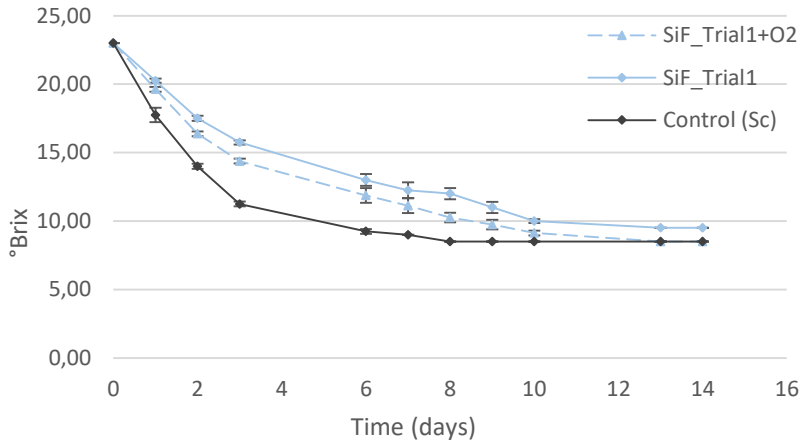

Figure 5.19| Sugar consumption of mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC1118 (Sc), under different conditions: anaerobic conditions (—◆—), aeration 0.5 VVM (- -▲- -). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of two independent experiments.

The yeasts growth dynamics in different oxygenation conditions are reported in Table 5.5. The oxygen addition did not affect the growth of *S. cerevisiae* strain included in mixed starter cultures; in fact, the evolution of yeast cells with and without oxygen addition was very similar.

Table **5.5**| Yeast growth kinetics in mixed fermentations with *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC1118 (Sc) under different conditions: without and with oxygen addition. Fermentations were carried out in duplicate and the mean Log UFC/mL values ± standard deviations are shown. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control.

Fermentation trials	**Time (days)**				
	0	**1**	**2**	**3**	**10**
SiF_Trial1+O₂					
St8	7.36±6.00	7.46±5.75	7,57±7.00	7.41±6.15	<3.00±0.00
Zb1	5.04±4.13	5.23±4.45	5,30±4.45	5.98±5.55	<3.00±0.00
Sc	3.88±3.33	6.01±4.33	7.06±6.00	7.27±6.21	7.07±5.33
SiF_Trial1					
St8	7.34±5.89	7.45±5.66	7.55±6.95	6.57±6.00	<3.00±0.00
Zb1	5.07±4.38	5.23±4.40	5.30±4.25	4.30±4.39	<3.00±0.00
Sc	3.90±3.35	5.99±5.45	7.06±5.89	7.09±6.85	7.25±6.93
***S. cerevisiae* (Control Sc)**					
Sc	7.05±3.35	8.06±3.35	8.18±3.35	8.00±3.35	7.89±3.35

The trend of yeast cells counts of *S. cerevisiae* in these two conditions was very similar to those observed for pure culture of *S. cerevisiae*, although a low number of cells was present along all the fermentative process for mixed fermentations.

Conversely, the aeration affected the growth of both *St. bacillaris* and *Z. bailii*, with high differences between the two conditions. As shown in Table 5.4, oxygen addition enhanced the growth of both non-*Saccharomyces* strains, especially at the third fermentation day, with viable counts of about 2.0×10^7 UFC/mL for *St. bacillaris* and 9.0×10^5 UFC/mL for *Z. bailii*. After the ninth fermentation day, no viable cells of both the non-*Saccharomyces* strains was found in both the conditions.

5.5.2.1| Composition of experimental wines obtained from fermentation in different aeration conditions

The results obtained in this step showed that the aeration enhanced biomass formation for the two non-*Saccharomyces* strains evaluated. It was reported that oxygen affects yeast physiology and metabolism (Shekhawat et al., 2018; Tronchoni et al., 2018). Conversely to *S. cerevisiae*, for which respiration is repressed by high concentrations of hexoses also in presence of oxygen, different non-*Saccharomyces* species are able to aerobically respire sugar, thereby metabolising sugars without the concomitant production of ethanol. This behaviour provides a potential strategy to be used for reducing the ethanol content of wine. Although ethanol concentration in wine can be reduced by providing oxygen to non-*Saccharomyces* yeasts, this practice sometimes has undesirable side-effects, such as increased production of acetic acid by some non-*Saccharomyces* species (Contreras et al., 2015; Röcker et al., 2016; Shekhawat et al., 2017), a compound that in high concentrations is considered detrimental for wine quality (Ribéreau-Gayon et al., 2006). This effect was not observed for the mixed starter culture evaluated in this study; in fact, the content of volatile acidity of wine obtained with mixed starter was not increased after oxygen addition (Table 5.6), indicating that the composition of the media/grape juice might affect its formation.

As regards the other parameters detected in the experimental wines, the sugar residual, both as glucose and fructose, was higher in wine obtained by control fermentation than wines obtained by mixed starters, in both the conditions tested.

As regards the ethanol content, the highest concentration was found in control wine (13.61 % (v/v)), whereas the wines obtained with mixed starter with and without oxygen addition contained 12.21 and 13.06 % (v/v), respectively.

This result, other to confirm the ability of selected mixed starter to reduce the ethanol content of wine, showed the influence of aeration on this parameter. In fact, the maximum ethanol

reduction (1.40) was found in experimental wine obtained by mixed starter with oxygen addition.

Table 5.6| Oenological characteristics and main volatile compounds of experimental wines obtained from mixed starters cultures of selected non-*Saccharomyces* strains (St8 and Zb1) simultaneously inoculated with *S. cerevisiae* EC1118 (Sc) under different conditions: without and with oxygen addition. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control.

Oenological characteristics	**SiF_Trial1+O_2**	**SiF_Trial1**	**Control (Sc)**
Ethanol % (v/v)	12.21±0.01^{a}	13.06±0.06^{b}	13.61±0.18^{c}
Fructose (g/L)	0.80±0.00^{a}	0.70±0.07^{a}	1.40±0.00^{b}
Glucose (g/L)	0.95±0.21^{ab}	0.70±0.07^{a}	1.50±0.07^{b}
Total acidity (g/L)	9.73±0.50	10.32±0.35	9.20±0.74
Volatile acidity (g/L)	0.45±0.03^{a}	0.84±0.13^{b}	0.55±0.01^{a}
pH	3.61±0.03^{a}	3.51±0.01^{ab}	3.45±0.03^{b}
Malic acid (g/L)	1.19±0.10	1.12±0.07	1.41±0.12
Lactic acid (g/L)	1.30±0.42	1.50±0.28	0.70±0.28
Glycerol (g/L)	4.20±0.07^{a}	4.00±0.06^{a}	3.07±0.06^{b}
Main volatile compounds			
Acetaldehyde (mg/L)	53.76±2.93^{a}	52.05±2.16^{a}	40.51±1.33^{b}
Ethyl acetate (mg/L)	29.72±1.11^{a}	36.12±1.58^{b}	32.51±0.44^{ab}
n-Propanol (mg/L)	26.24±2.11^{a}	22.73±1.71^{ab}	16.02±0.61^{b}
Isobutanol (mg/L)	110.11±1.75^{a}	101.13±1.86^{b}	29.23±0.25^{c}
n-Butanol (mg/L)	30.77±3.08^{a}	45.84±2.83^{b}	31.67±0.56^{a}
Acetoin (mg/L)	14.20±0.21	13.34±1.20	10.58±0.90
D-amyl alcohol (mg/L)	61.57±7.17^{a}	65.11±5.66^{a}	90.47±4.33^{b}
Isoamyl alcohol (mg/L)	138.24±10.32^{a}	150.19±12.73^{a}	233.74±5.21^{b}

Data are means ± standard deviation of two independent experiments. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$) between mixed and control fermentation.

As regards the glycerol content, the use of mixed starter increased significantly the content of this compound (3.07 g/L in single starter wine, and 4.20 and 4.00 g/L in wine from mixed starter with and without oxygen addition, respectively), also the aeration increased the glycerol content, although the differences were not statistically significant.

As regards the influence of oxygen addition on the formation of main volatile compounds detected by gas-chromatographic analysis of experimental wines, the main differences were found among wines from pure and mixed culture fermentations, independently from oxygen addition (Table 5.6). In fact, wine obtained by pure fermentation with *S. cerevisiae* EC1118 contained significantly lower concentrations of acetaldehyde, *n*-propanol and isobutanol

compared to wines from mixed starter obtained in both aeration conditions, whereas higher concentrations of amyl alcohols were found in wine obtained by EC 1118 single strain in comparison to wines fermented with mixed starter (both with and without oxygen addition). These results are confirmed by the analysis of radar plot reported in Figure 5.20, in which the volatile profiles of wines obtained from different fermentations were shown.

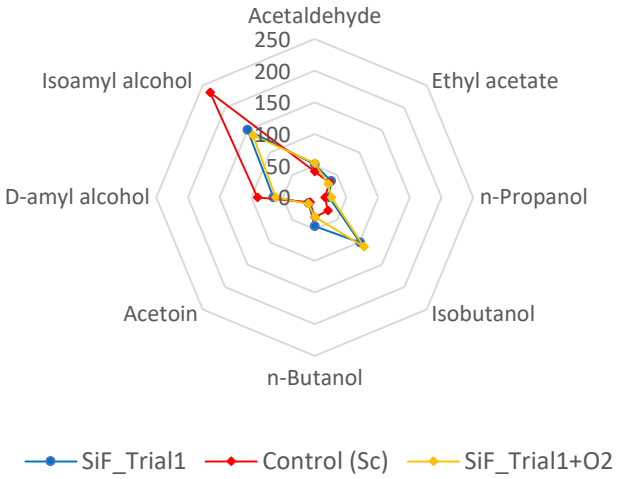

Figure 5.20| Radar plots of volatile compounds detected in wines fermented with mixed starter with and without oxygen addition (SiF_Trial1+O_2 and SiF_Trial1, respectively) in comparison to single starter wine, Control (Sc).

The wine sample fermented by mixed culture under aerobic conditions showed a similar profile of wine obtained by mixed culture in anaerobic conditions, indicating that the application of oxygen not influence the metabolic pathways involved in the formation of aromatic compounds detected in this step. Conversely, both the wines obtained from mixed culture showed aromatic profile different from profile of control wine, fermented with *S. cerevisiae* EC1118.

Data Reported in Table 5.6 were submitted to Principal Component Analysis (PCA), to assess the overall effect of aeration on wine characteristics. The obtained scatter plot is reported in Figure 5.21, in which the wines obtained by the three different fermentation trials (mixed starter with and without oxygen addition, single starter fermentation) were located in three different quadrants. The first principal component (PC1, 75.21% of the variance) was mainly correlated to lactic acid, glycerol, acetaldehyde, *n*-propanol, isobutanol and acetoin, and negatively correlated to residual sugar concentration, malic acid, D-amyl and isoamyl alcohols. The second principal component (PC2, 24.78% of the variance) was positively correlated to volatile acidity, ethyl acetate and *n*-butanol, and negatively correlated to pH.

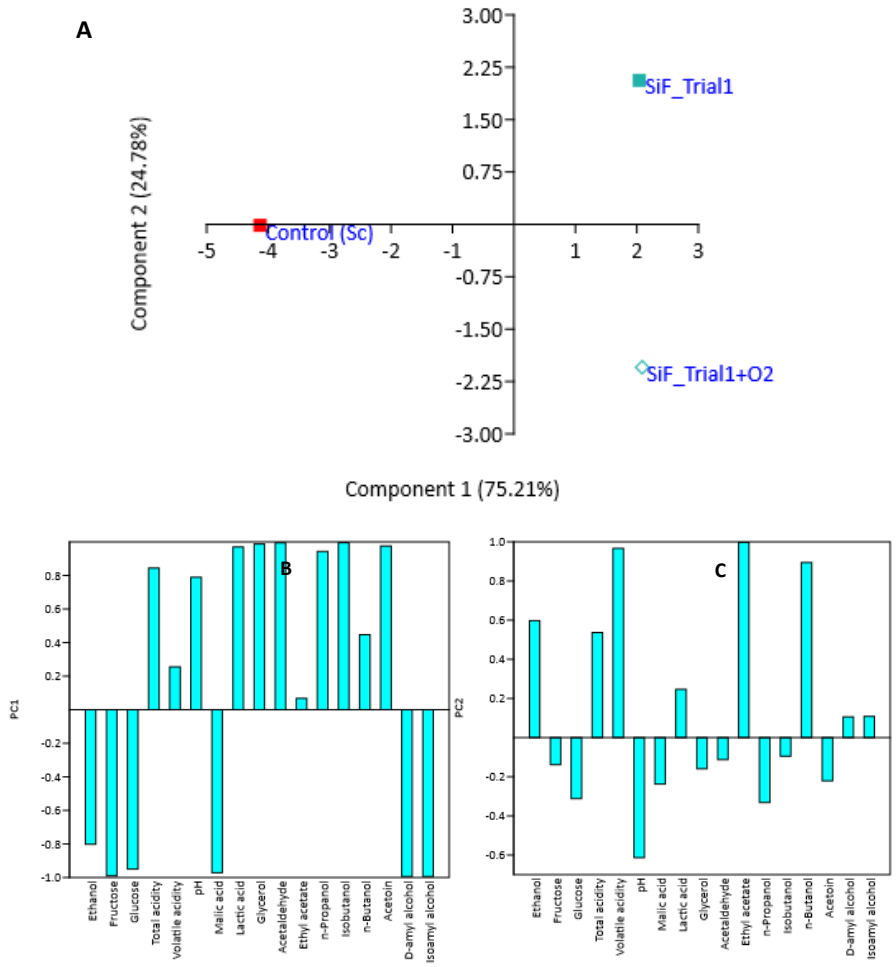

Figure 5.21| Scatter Plot (A) and loading plots of the first (B) and second (C) principal component corresponding to Principal component analysis (PCA) of the oenological parameters and main volatile compounds detected in experimental wines obtained by simultaneous inoculum of selected non-*Saccharomyces* strains, with and without oxygen addition. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as control.

5.5.3| Main fermentative parameters with immobilized non-*Saccharomyces* strains and evolution of cell viability during the fermentation

The last step for laboratory-scale screening of selected mixed starter culture was addressed to evaluate the influence of cell immobilization on behaviour of the two non-*Saccharomyces* strains, *St. bacillaris* and *Z. bailii*, in simultaneous fermentation with *S. cerevisiae* on ethanol reduction and volatile profile of wines. In this experiment, the immobilized non-*Saccharomyces* cells were co-inoculated with free *S. cerevisiae* cells. The course of fermentations, represented by CO_2 release, is shown in Figure 5.22.

Fermentation trial with immobilized non-*Saccharomyces* cells showed a trend similar to control fermentation; both the fermentations were completed in about 14 days, while the fermentation trial with free cells of non-*Saccharomyces* strains was completed later (within 20 days). The control fermentation started the process firstly, with the highest CO_2 production until the fifth fermentation day, after that single starter and mixed starter with

immobilized cells showed a similar trend. The mixed starter with free cells started the process before the mixed culture composed by immobilized cells, but this tendency was inverted from the second fermentation day.

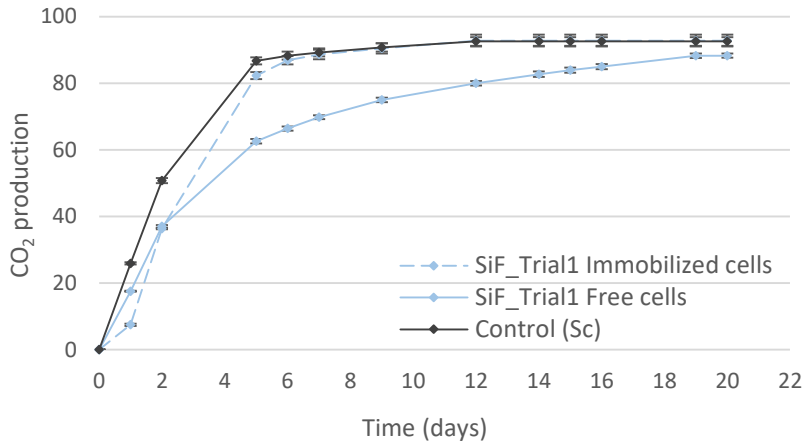

Figure 5.22| Fermentation kinetics of mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated as immobilized ( ) and free ( ) cells with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of two independent experiments.

The fermentation trial with immobilized cells showed overlapping fermentation kinetics to that exhibited by *S. cerevisiae* pure culture after fifth day and until the end of the fermentation process. At the end of the process, the maximum CO_2 production was found in the mixed fermentation with immobilized cells and in the fermentation inoculated with *S. cerevisiae* pure culture (about 92.80 g CO_2/800 mL and 92.56 g CO_2/800 mL, respectively), whereas the lowest amount (88.89 g CO_2/800 mL) was detected in the fermentation inoculated with non-*Saccharomyces* free cells.

The evolution of sugar consumption, reported as reduction of °Brix degree during the time, was similar to the trend observed for CO_2 production, as reported in Figure 5.23.

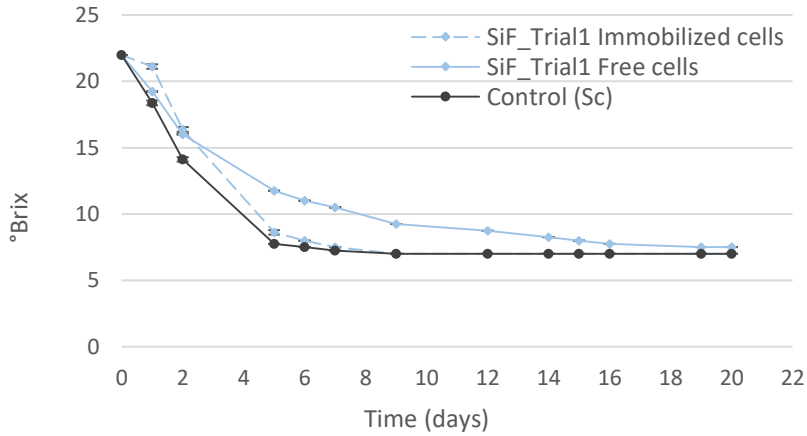

Figure 5.23| Sugar consumption of mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated as immobilized ( ) and free ( ) cells with *S. cerevisiae* EC 1118 (Sc). Pure culture of *S. cerevisiae* EC 1118 (Control Sc) was used as a control. Data are means ± standard deviation of two independent experiments.

Also for this parameter, the fermentation with mixed starter in free cells finished the process later than trials inoculated with immobilized non-*Saccharomyces* cells and control fermentation (*S. cerevisiae* EC1118). Furthermore, EC 1118 strain and mixed starter with immobilized cells showed the similar trend of sugar reduction after the fifth fermentation day, as already reported for CO_2 production.

The yeast cells viability of each strain included in the mixed starter, both as free and immobilized cells, was evaluated at different fermentation days in comparison to cell evolution of control fermentation. The data reported in Table 5.7 show that in grape must the number of viable cells released from the beads was around 1 x 10^6 cell/mL for both non-*Saccharomyces* strains, with significant loss of cell viability after 5th day. The evaluation of yeast cells present in the beads recovered at the end of fermentative process showed that *St. bacillaris* cells were not viable, whereas a high decrease of viability was found for *Z. bailii* cells (about 3.5×10^3 UFC/mL).

Table 5.7| Yeast cell viability of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated as immobilized and free cells with *S. cerevisiae* EC1118 (Sc) at 2nd and 5th day of fermentations. Fermentations were carried out in duplicate and the values are reported as mean Log UFC/mL ± standard deviations.

Fermentation trials	**Viable cells Log UFC/mL**	
	2nd day	**5th day**
SiF_Trial1 Immobilized cells		
St8	6.72±0.34	<5.00±0.00
Zb1	6.15±0.21	<5.00±0.00
Sc	7.91±0.12	7.89±0.07
SiF_Trial1 Free cells		
St8	8.21±0.14	7.74±0.07
Zb1	6.85±0.10	6.56±0.09
Sc	6.29±0.02	6.27±0.02
Control (Sc)		
Sc	8.15±0.01	8.43±0.10

5.5.3.1| Analysis of experimental wines obtained with immobilized cells of selected mixed starter culture

In order to evaluate the influence of cell immobilization on metabolic activity of selected non-*Saccharomyces* strains and, consequently, on wine characteristics, the experimental wines were analyzed for main oenological parameters and volatile compounds (Table 5.8).

As regards the ethanol content, as already found in the previous experiments, the wine form mixed starter, both as free and immobilized cells, contained a level of ethanol significantly lower (11.43 and 12.13 % (v/v), respectively) than ethanol content of control wine (13.07 % v/v).

Table 5.8| Oenological characteristics and main volatile compounds of experimental wines obtained from mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated as immobilized and free cells with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control.

Oenological characteristics	SiF_Trial1 Immobilized cells	SiF_Trial1 Free cells	Control (Sc)
Ethanol % (v/v)	12.13±0.26^{a}	11.43±0.13^{b}	13.07±0.22^{c}
Fructose (g/L)	0.67±0.06^{a}	1.00±0.10^{b}	0.63±0.06^{a}
Glucose (g/L)	0.47±0.06^{a}	7.37±0.67^{b}	0.60±0.17^{a}
Total acidity (g/L)	7.66±0.07^{a}	8.78±0.01^{b}	8.81±0.07^{b}
Volatile acidity (g/L)	0.23±0.01^{a}	0.70±0.01^{b}	0.27±0.09^{a}
pH	3.61±0.01^{a}	3.63±0.01^{a}	3.55±0.01^{b}
Malic acid (g/L)	1.22±0.04^{a}	1.37±0.03^{b}	1.72±0.04^{c}
Lactic acid (g/L)	0.70±0.00^{a}	1.13±0.12^{b}	0.57±0.06^{a}
Main volatile compounds			
Acetaldehyde (mg/L)	42.92±2.37	41.34±3.32	33.28±1.40
Ethyl acetate (mg/L)	19.42±0.29^{a}	18.03±0.62^{a}	23.29±0.39^{b}
n-Propanol (mg/L)	17.06±0.34^{a}	26.07±0.56^{b}	11.43±0.10^{c}
Isobutanol (mg/L)	35.36±1.05^{a}	86.87±2.28^{b}	31.52±1.09^{a}
n-Butanol (mg/L)	11.77±0.60^{a}	70.73±1.58^{b}	12.04±0.62^{a}
Acetoin (mg/L)	6.52±0.87^{a}	24.57±0.25^{b}	4.46±0.84^{a}
D-amyl alcohol (mg/L)	62.26±1.91^{a}	47.96±4.26^{b}	79.41±3.02^{c}
Isoamyl alcohol (g/L)	150.72±0.63^{a}	97.66±3.22^{b}	216.56±5.57^{c}

Data are means ± standard deviation of two independent experiments. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$) between mixed and control fermentation.

However, the lowest ethanol content (11.43 % v/v) was found in wine from mixed starter inoculated as free cells, indicating that immobilization procedure did not enhance the ability of the two non-*Saccharomyces* strains (St8 and Zb1) to reduce the ethanol content of wine (Figure 5.24).

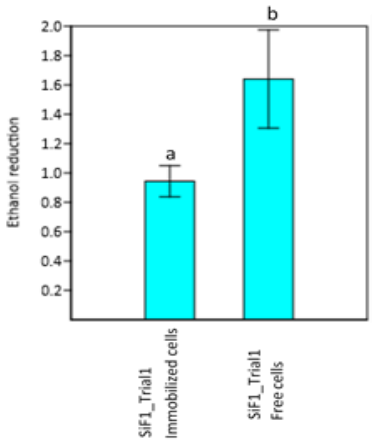

Figure 5.24| Ability of mixed starter cultures to reduce the ethanol content in experimental wines.

As regards sugar residual in the final wines, the fructose residual was very low and very similar in all the three samples, with a level slightly higher in wine fermented with free cells of non-*Saccharomyces* strains. Otherwise, significant differences were found for the glucose residual, with a level significantly higher in wine from mixed starter with free cells compared both to wine from immobilized cells and control wine. These results are summarized in Figure 5.25.

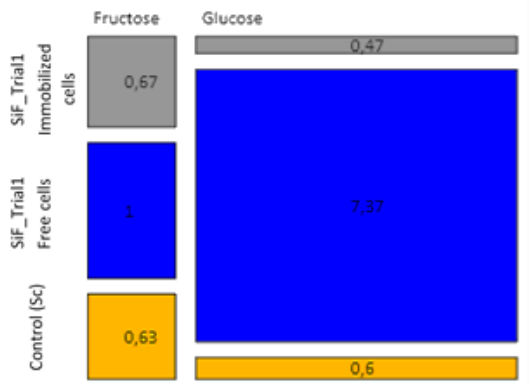

Figure 5.25| Residual sugar (fructose and glucose) in simultaneous fermentation trials, using free and immobilized cells of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. The data are expressed as mg/L.

The volatile acidity was very similar in wine from immobilized cells and EC1118 strains, whereas higher level was detected in wine obtained by inoculating *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains as free cells.

As regards the main volatile compounds detected in the experimental wines (Table 5.8), the content of acetaldehyde and ethyl acetate was very similar among all the three wine samples, whereas significant differences were found for the other compounds. In details, levels very high were found for *n*-propanol, isobutanol, *n*-butanol and acetoin in wine fermented with free cells of the two non-*Saccharomyces* strains, whereas for the other two samples (wine fermented with immobilized cells and control wine) the levels of these compounds were very similar among them. As regards amyl alcohols, the highest values were detected in samples fermented with *S. cerevisiae* strain, but the level found in wines produced by inoculating free and immobilized cells of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains were different among them.

The qualitative level of H_2S production was directly correlated with browning level of filter paper strips during the fermentative process (Figure 5.26). After 48 h of fermentation, in mixed fermentations (both with free and immobilized cells of non-*Saccharomyces* strains) the amount of H_2S produced was lower than level detected in pure fermentation with *S. cerevisiae* EC1118 (Figure 5.26a). At the end of the process, the production of H_2S remains low for process performed by mixed starter as free cells, whereas it was increased for mixed

starter including immobilized cells of non-*Saccharomyces* strains. No differences were observed between 48 h and end of fermentation for control fermentation (Figure 5.26b).

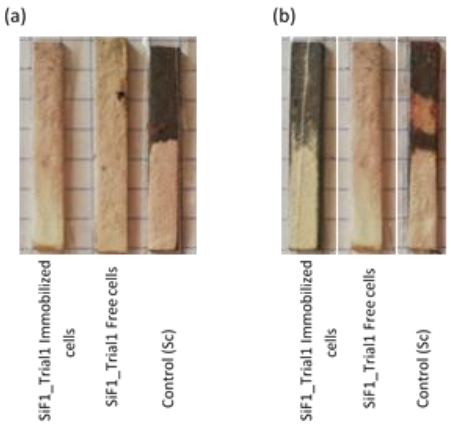

Figure 5.26| Hydrogen sulphide production evaluated on Hydrogen Sulphide test strips in fermentations with *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated as immobilized and free cells with *S. cerevisiae* EC 1118 (Sc) in comparison with pure culture of *S. cerevisiae* EC1118 (Control Sc) at 48h (a) and the end of fermentation (b).

5.5.4| Oxidative stress tolerance

In this step, the response to some stress factors potentially present in winemaking was evaluated in the three selected strains, *S. cerevisiae* EC1118 and the non-*Saccharomyces* strains *St. bacillaris* St8 and *Z. bailii* Zb1.

The strains tolerance to oxidative stress was evaluated by testing strain growth in medium added with H_2O_2. As showed in the figure 5.27, all the three strains was tolerant to H_2O_2 and only slight differences were found among the strains.

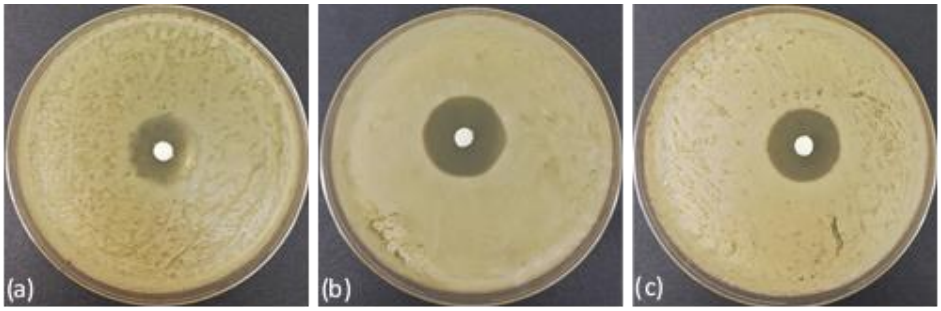

Figure 5.27| Tolerance to oxidative stress of *St. bacillaris* St8 (a), *Z. bailii* Zb1 (b) and *S. cerevisiae* EC1118 (c) strains.

5.5.4.1| Growth in medium with different nutritional composition

The aim of this step was to screen nutrient requirements of the three selected strains by using as probes some compounds that target nutrient signaling pathways. The behaviour was evaluated by comparing strain response in YPD and media added with different compounds (Figure 5.28).

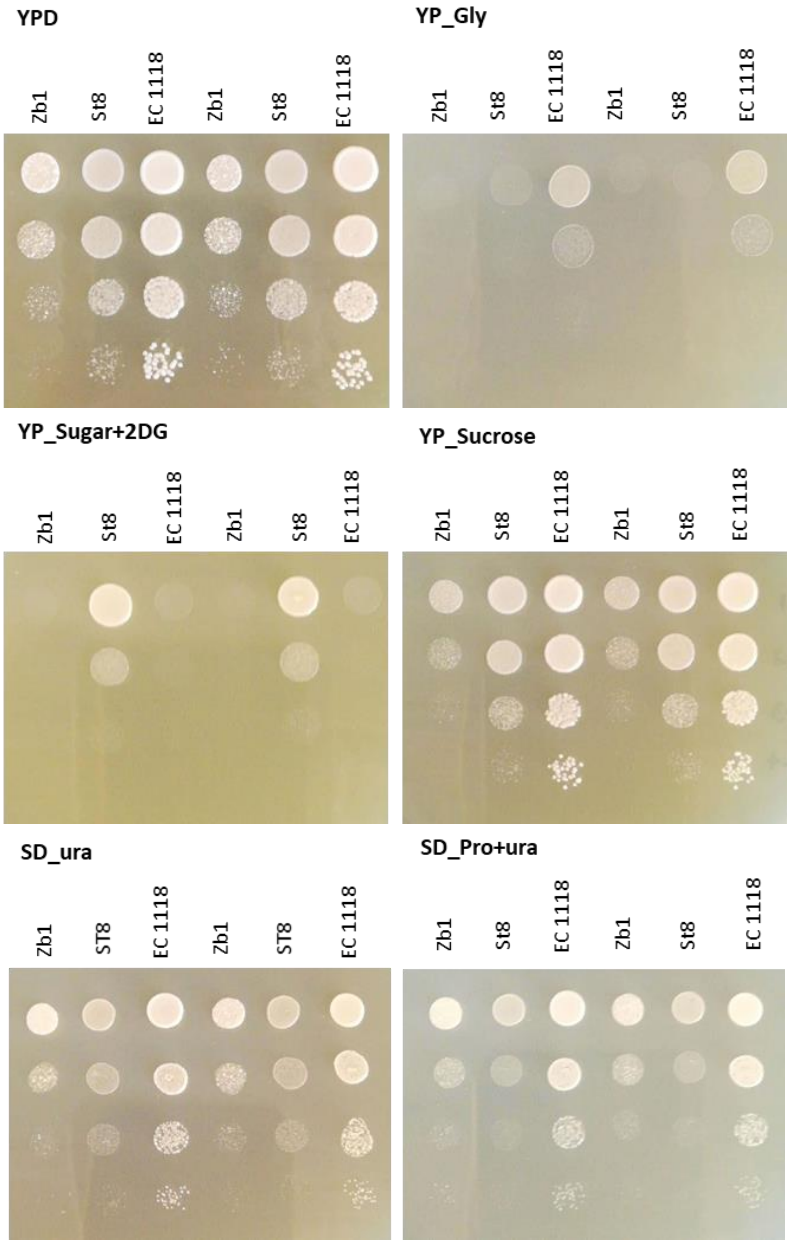

Figure 5.28| Spot growth analysis of the selected strains of *St. bacillaris* St8, *Z. bailii* Zb1 and *S. cerevisiae* EC1118. Serial dilutions were spotted on the plates containing: 2% Glycerol in YPD; 2% Sucrose in YPD; 200 µg/mL of 2-deoxyglucose (2DG) in YPD, 0.5% ammonium sulfate (SD_ura) in SD; 0.5% proline (SD_Pro+ura) in SD.

The results revealed that the response to chemicals was variable in function of the strain. The response to carbon sources can be studied by using the glucose analogous 2-deoxyglucose (2DG), which causes glucose repression blocking growth in sucrose. 2DG completely blocked the growth of the *Z. bailii* Zb1 and *S. cerevisiae* EC1118 strains, but only partially blocked the growth of the *St. bacillaris* St8 strain. The increased tolerance to 2DG suggests that the strain present a less strong glucose repression. Moreover, results show that cell growth is affected from other carbon sources, i.e. glycerol. As shown in the Figure 5.28, only EC1118 strain exhibited high growth level in presence of glycerol, whereas the other two strains did not growth in medium containing this compound. As regards the growth

of the strains in medium containing nitrogen source, such as ammonium sulfate (SD_ura), a preferred nitrogen source, and prolina and ammonium sulfate (SD_Pro+ura) (Figure 5.28), the results showed that the growth of the two non-*Saccharomyces* strains St8 and Zb1 was lower than *S. cerevisiae*.

5.5.4.2| Fermentation behavior of pure and simultaneous cultures and evaluation of yeast population dynamic

The two non-*Saccharomyces* strains and *S. cerevisiae* strain were tested in synthetic must fermentation, by inoculating simultaneously each non-*Saccharomyces* strain with EC1118. The fermentations were performed in synthetic must in consequence of the higher reproducibility and constant composition of this medium compared to natural grape must, also in order to make easier comparisons to the available global data obtained in the same medium. The results related to fermentation kinetics, measured as CO_2 release, were presented in Figure 5.29. The trend of CO_2 production was very similar in the two mixed fermentations, with constant increase in CO_2 production from the first fermentation day; this trend was similar to those of pure culture of *S. cerevisiae* EC1118 (control fermentation, Sc). All the fermentations were completed in 11 days, without significant differences among them. At the end of the process, the maximum CO_2 production was found in the fermentation inoculated with Zb1+Sc starter (about 10.12 g/150 mL).

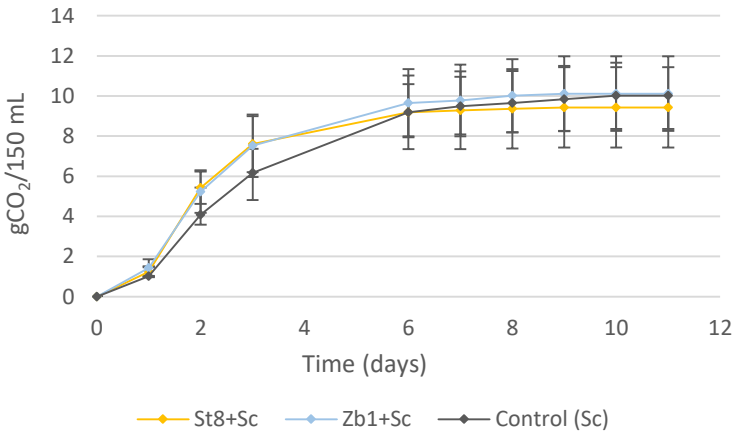

Figure 5.29| Fermentation kinetics of mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of three independent experiments.

At the same time points, the growth evolution of starter cultures was evaluated by spectrophotometric measurements at OD_{600nm} (Figure 5.30). The highest levels of optical density was found for mixed culture composed by *St. bacillaris* St8 and *S. cerevisiae* EC1118, whereas the lowest levels was found for mixed culture composed by *S. cerevisiae* EC1118 and *Z. bailii* Zb1, whereas control fermentation showed intermediate values.

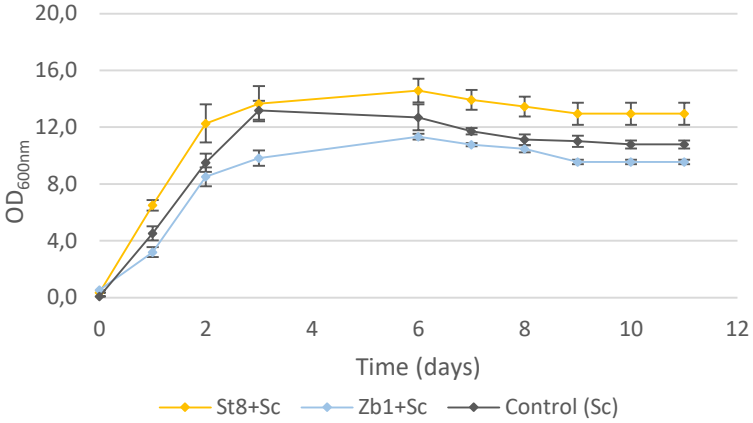

Figure 5.30| Growth kinetics of different starter cultures (OD_{600nm}) in synthetic must during the fermentations in synthetic must. Results represent the mean value ± standard deviation of three independent experiments.

The evolution of sugar consumption showed different kinetics in simultaneous and pure fermentations. The evolution of sugar consumption, reported as reduction of DNS during the time, reflects the same trend observed for CO_2 production, as expected. During the first 24h of fermentation, the sugar consumption was faster in *S. cerevisiae* pure culture than mixed fermentations, after that sugar reduction was slightly faster in mixed starter in comparison to control fermentation (Figure 5.31). The concentration of sugars residual was below 2.5 g/L in samples from mixed starters, whereas the sugar level in sample from *S. cerevisiae* pure culture was 3.23 g/L.

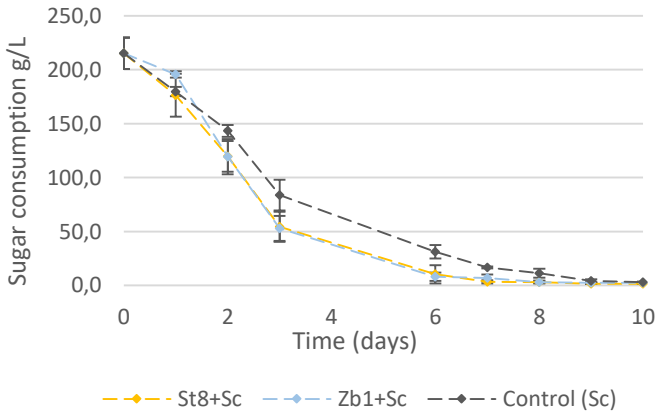

Figure 5.31| Sugar consumption of mixed starters cultures of *St. bacillaris* St8 and *Z. bailii* Zb1 strains simultaneously inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control. Data are means ± standard deviation of three independent experiments.

Yeast growth dynamics were monitored for single and mixed starter fermentations by plate counts on WL medium, able to distinguish non-*Saccharomyces* and *S. cerevisiae* strains, in order to evaluate the presence level of non-*Saccharomyces* strains during the fermentation. The microbial population dynamics of the fermentations are shown in Figure 5.32 and 5.33. In mixed fermentations inoculated with *St. bacillaris* strain, cells count decreases at 7.0 x

10^5 UFC/mL after 3 days, after that the viable count decreased and at the 6th day of the fermentation no *St. bacillaris* cells were found strain at this point, with counts less 10^5 UFC/mL, such as shown in (Figure 5.32). As regards *S. cerevisiae* population, no differences were found between mixed and control fermentations and the maximum population reached was 3.40×10^7 UFC/mL after 3 days, and this cell number was maintained until the end of fermentation.

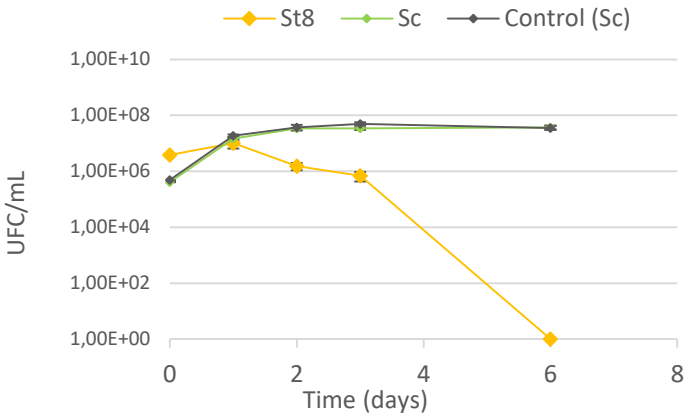

Figure 5.32| Evolution of yeast populations in mixed fermentations simultaneously inoculated with *St. bacillaris* (St8) and *S. cerevisiae* (Sc). Values are mean of three independent duplicates. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control.

A different behaviour was found for *Z. bailii* in mixed fermentation, in which the population of Zb1 increased during the first fermentation days, by reaching the maximal population after 2 days (1.81×10^7 UFC/mL), after that a reduction in number of viable cells was observed (Figure 5.33), although a number quite high of viable cells was found at the end of the fermentation (1.67×10^6 UFC/mL). As regards the evolution of *S. cerevisiae* EC1118 population, similar trend was observed both in mixed and single fermentations, with the presence of high cell number until the end of the fermentations.

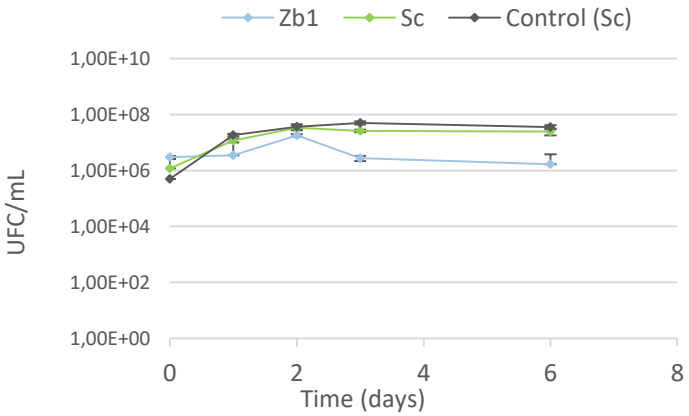

Figure 5.33| Evolution of yeast populations in mixed fermentations simultaneously inoculated with *Z. bailii* (Zb1) and *S. cerevisiae* (Sc). Values are mean of three independent duplicates. Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control.

5.5.4.3| Chemical analysis

The experimental wines obtained at the end of the fermentations were analyzed for the content of the main analytical components, reported in Table 5.9.

All of the wines produced had a residual sugar content <3.5 g/L, confirming the ability of yeast starters used were able to complete the fermentations.

Table 5.9| Concentration of major chemical compounds of experimental wines obtained from mixed starters cultures of selected non-*Saccharomyces* strains (St8 and Zb1) simultaneous inoculated with *S. cerevisiae* EC1118 (Sc). Pure culture of *S. cerevisiae* EC1118 (Control Sc) was used as a control.

Oenological characteristics	St8+Sc	Zb1+Sc	Control (Sc)
Ethanol % (v/v)	13.44±0.0^{a}	13.07±0.1^{a}	13.92±0.3^{b}
Fructose (g/L)	1.43±0.3^{a}	1.90±1.0^{a}	4.20±1.0^{b}
Glucose (g/L)	1.0±0.1	1.33±0.3	1.10±0.3
Total acidity (g/L)	6.30±0.1	6.60±0.3	6.26±0.2
Volatile acidity (g/L)	0.59±0.1	0.82±0.4	>1.0
pH	3.50±0.0	3.54±0.0	3.51±0.0
Malic acid (g/L)	1.60±0.1	1.60±0.1	1.73±0.1
Lactic acid (g/L)	0.00±0.0	0.00±0.0	0.00±0.0
Main volatile compounds			
Acetaldehyde (mg/L)	45.34±3.26^{a}	42.38±3.58^{a}	73.37±6.47^{b}
Ethyl acetate (mg/L)	23.32±1.98	23.74±3.48	20.65±1.85
n-Propanol (mg/L)	29.94±0.75^{a}	34.18±2.38^{ab}	39.76±5.72^{b}
Isobutanol (mg/L)	21.58±1.82^{a}	20.81±1.67^{a}	15.92±1.79^{b}
n-Butanol (mg/L)	12.00±1.41^{a}	16.70±4.45^{a}	24.93±3.18^{b}
Acetoin (mg/L)	3.80±0.62^{a}	4.57±0.50^{a}	8.90±0.56^{b}
D-amyl alcohol (mg/L)	165.91±3.49^{a}	22.43±0.65^{b}	28.19±4.84^{b}
Isoamyl alcohol (mg/L)	51.40±9.00^{a}	37.20±4.71^{ab}	35.60±1.68^{b}

Note: Data are means ± standard deviation of three independent experiments. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$).

Ethanol concentration was slightly reduced in all mixed inoculations when compared to the control, and presented the lowest values in the wines obtained with mixed starter containing *Z. bailii* Zb1 (average value 0.85), whereas the mixed starter containing *St. bacillaris* St8 reduced the content level of ethanol respect to single starter of 0.48 (Figure 5.34).

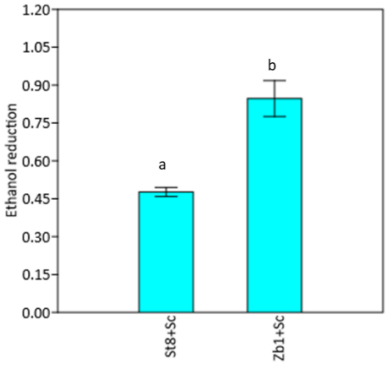

Figure 5.34| Reduction of ethanol content in experimental wines obtained from mixed fermentations.

As regards the fructose content, significant differences were observed for fructose content between wines from single and mixed starters, with the highest level in control wine (Sc), whereas no significant differences were observed for glucose content. As regards the volatile acidity, malic and lactic acids, no differences were observed among the trials.

As regards the secondary compounds usually present in high concentrations in wines, significant differences among the samples were found for acetaldehyde, acetoin and higher alcohol. The highest production of D-amyl and isoamyl alcohols was detected in wine obtained by mixed inoculum St8+Sc, whereas wines obtained by mixed fermentation Zb1+Sc showed significantly lower concentrations and similar to control wine.

As regards the glycerol content, in all the samples collected at the different fermentation days, *St. bacillaris* and *Zb. bailii* mixed culture produced a higher amount of glycerol than single starter. Furthermore, during the process in each fermentation an increase of glycerol content was observed (Figure 5.35).

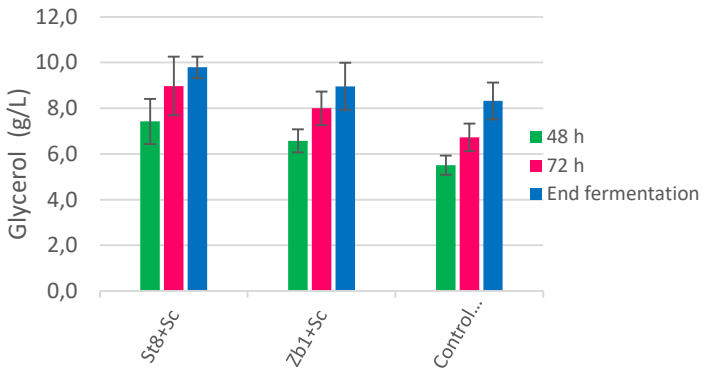

Figure 5.35| Glycerol production in experimental wines obtained from mixed and pure fermentations.

5.5.4.4| Western blot analysis of regulatory proteins.

During the fermentation in synthetic must, the analysis proteins Tsa1, Tsa-SO_3 and Snf1 was analyzed at the different time points, in particular the samples were taken at 24 h, 48 h and 72h (Figure 5.36).

Furthermore, the single strains were grown until to exponential phase in YPD medium containing H_2O_2 (1mM) for 1 h at 28 °C in order to analyze the protein peroxiredoxin Tsa1 and kinase Snf1 in cells exposed to oxidative stress.

Peroxiredoxins are ubiquitous thiol-specific proteins that have multiple functions in stress protection, including protection against oxidative stress. Tsa1 is the major yeast peroxiredoxin and it was shown that it functions as a specific antioxidant to protect the cell against the oxidative stress (Weids et al., 2014). The Snf1 protein kinase of the yeast was first identified by its roles in responses to glucose limitation, but recent evidence indicates

that Snf1 is also activated by other stresses, including nitrogen limitation, sodium ion stress, oxidative stress and alkaline pH (Orlova et al., 2008).

Western blot against peroxiredoxin Tsa1 under oxidative stress shows that there is a protein similar in *Z. bailii* than in *S. cerevisiae*, whereas this ortholog is not present in *St. bacillaris*, indicating that this peroxiredoxin system in this yeast may be absent or it is not present under these conditions (Figure 5.36). In synthetic must fermentation performed with single culture of *S. cerevisiae*, alone, the peak in the levels of this protein appears at 48 h, whereas later the levels are lower, but as seen in the actin loading control, also the total amount of proteins is lower at 72 hours compared to 24 and 48 hours. Co-cultivation with *St. bacillaris* does not trigger an increase in the protein, indicating that probably this yeast did not increase oxidative stress situation. In the *S. cerevisiae*/*Z. bailii* co-fermentation, the levels of this protein are higher, but this result may reflect the fact that both yeast species contribute with peroxiredocin. Again, the peak is produced after two days, as shown in Figure 5.36.

As regards hyperoxidized Tsa1-SO_3, this protein is produced in presence of H_2O_2, but not during synthetic must fermentation.

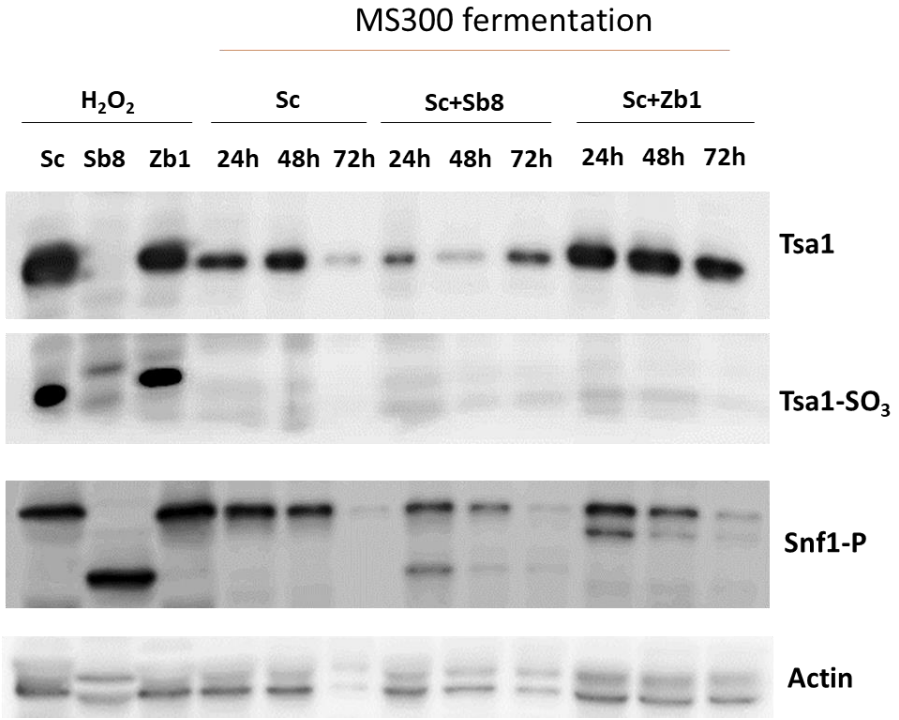

Figure 5.36| Western blot analysis of Tsa1, Tsa-SO_3 and Snf1 during fermentation in synthetic must M300, using a protein extraction method. Actin was used a loading control.

Snf1 kinase activity may provide information on the overall carbon source metabolism. When glucose is exhausted, Snf1 kinase is activated to promote the use of other carbon sources. Therefore, Snf1 lies at the core of events known as glucose repression where the presence of glucose shuts down respiration, as well as the use of unfermentable carbon

sources like glycerol, but also the use of alternative sugars sources and gluconeogenesis. Glucose derepression kinase Snf1 is phosphorylated, therefore is active in all three yeasts during oxidative stress. In *S. cerevisiae* EC1118 single starter an early Snf1 activation was observed, Snf1 phosphorylation in MS300 was showed at the first time point (Figure 5.36), despite the high levels of sugars, as expected. The total amount of Snf1 was fairly constant up to day 2, after that the levels drop.

In the mixed fermentation with *St. bacillaris*, the pattern is similar, indicating that the co-cultivation did not affect this parameter. Therefore, also in *St. bacillaris* Snf1 is active in the initial stages of fermentation, a results never described before. In the mixed fermentation with *Z. bailii* and *S. cerevisiae*, Snf1 proteins share the same size for the two strains, consequently it is not possible to distinguish them. Moreover, the co-cultivation triggers the apparition of a new band, suggesting a posttranscriptional effect due to the presence of the two different species. Also in this case, Snf1 was fully activated in the first steps of fermentation.

5.5.5| Pilot-Scale Vinification

In order to validate the results obtained at laboratory scale, the selected mixed starter, composed by St8 and Zb1 strains simultaneously inoculated with *S. cerevisiae* EC1118, were tested during pilot-scale vinifications in the cellar on two different grape varieties (Primitivo and Cabernet). Pure fermentation with *S. cerevisiae* EC1118 was used as control. Inoculation levels very similar to those used during laboratory scale fermentations were tested. In order to evaluate the presence of inoculated strains during the fermentative process, yeast isolation on WL medium (Figure 5.37) was performed at different fermentation times (beginning, middle, end) in each vinification trial.

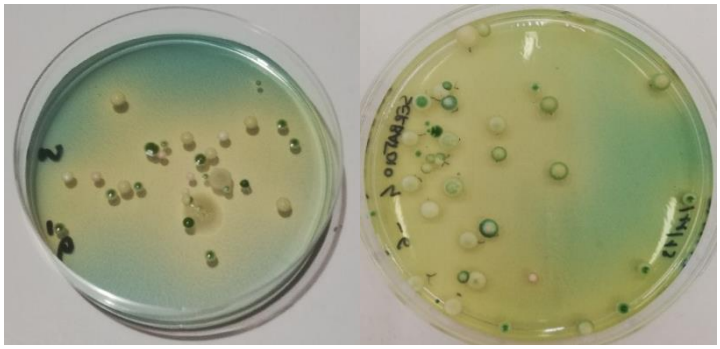

Figure 5.37| Colony morphology on Wallerstein Laboratory (WL) Nutrient Agar medium.

The yeast population dynamics during pilot scale fermentations of Primitivo and Cabernet grape musts are presented in Figure 5.38. In our experiments, other strains, different from inoculated starters, participated in the fermentative process. As expected, the highest number

of yeast cells was found in the middle step of fermentation (day 3) and differences among yeast counts determined in the four inoculated fermentations were found.

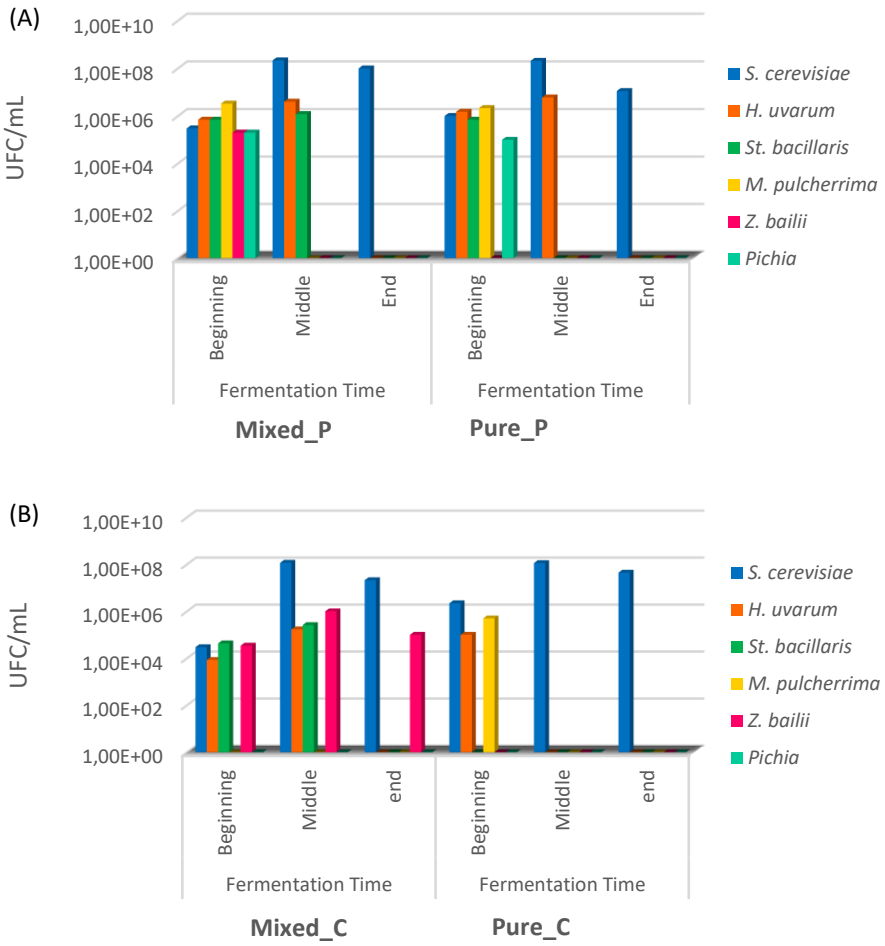

Figure 5.38| Yeast population (UFC/mL) at different sampling times during pilot scale fermentations of Primitivo (A) and Cabernet (B) grape musts in mixed and pure fermentations.

All the colonies isolated from the fermentations performed in Primitivo and Cabernet grape musts belonged to *H. uvarum*, *M. pulcherrima*, *St. bacillaris*, *S. cerevisiae* and *Z. bailii* species, whereas in Primitivo grape must, other than these four species, yeasts belonging to *Pichia* genus was also found, although only in the first stage of fermentation, in both fermentation trials. These results indicate the presence of higher number of yeast species in Primitivo fermentation than Cabernet.

On the other hand, the inoculated strains showed different dynamics in the mixed fermentation performed in the two grape musts. Although inoculated at the same initial level in both the grape musts, the evolution of non-*Saccharomyces* strains during the fermentative processes was different. In Primitivo grape must, in the middle step of the process only *St. bacillaris* yeasts were found, probably composed both by wild and inoculated strains, as this species was found in this fermentation step also in fermentation inoculated with pure culture of *S. cerevisiae*. No *Z. bailii* cells were found in the middle step of Primitivo grape must

fermentation and at the end of the process only *S. cerevisiae* cells were found, both in mixed and single starter fermentation.

In mixed fermentation performed in Cabernet grape must, *St. bacillaris* was present until the middle phase of the process and, probably, the isolated yeasts belonged to inoculated St8 strain, as this species was not found in single starter fermentation. As regards *Z. bailii*, this yeast was found until the end of fermentation, together *S. cerevisiae*. By comparing the two fermentations, higher number of yeast cells and species was found in Primitivo grape must than in Cabernet grape must, mainly during the first steps of fermentative process. Other the inoculated yeast species, *H. uvarum* was present in all the four fermentations until the middle phase of the process, with exception of pure fermentation in Cabernet must, in which the presence of this species was limited to the beginning of fermentative process.

The duration of fermentative process was similar in the two grape musts. In fact, fermentations performed in Primitivo and Cabernet were completed in 7-8 days, both control and mixed fermentations (Figure 5.39 a-d).

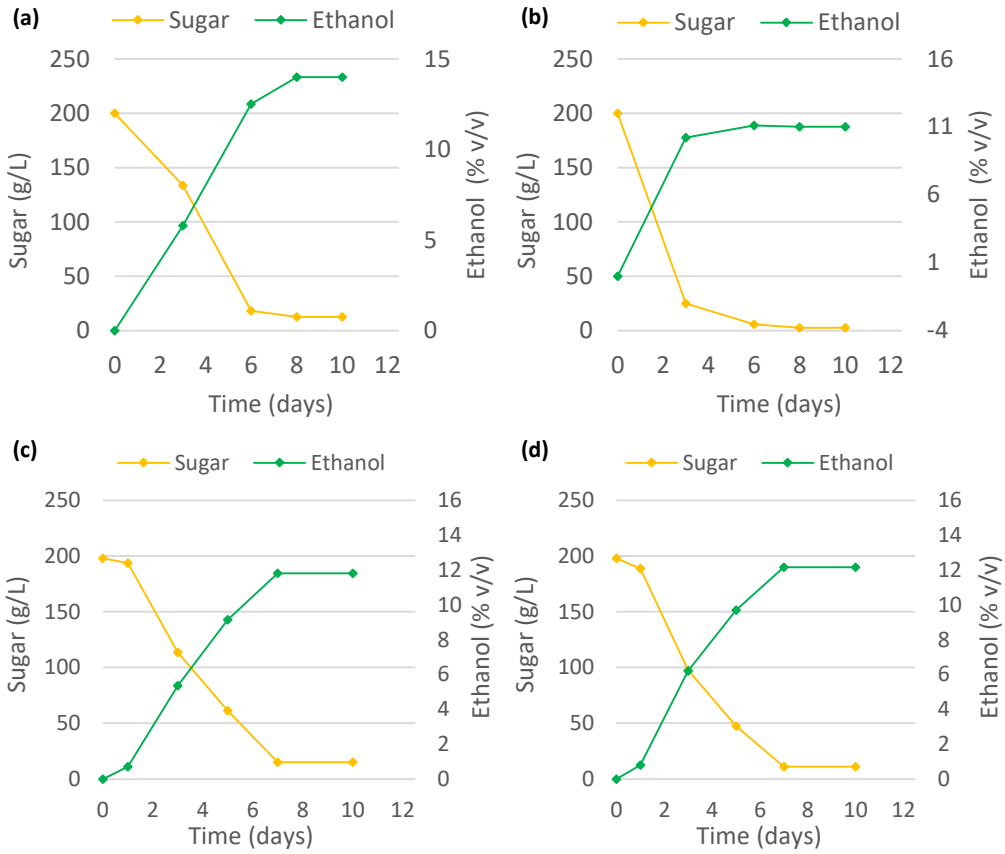

Figure 5.39| Sugar utilisation (g/L) and ethanol production (% v/v) during fermentations in Primitivo (a, b) and Cabernet (c, d) varieties, inoculated with selected mixed starter (a, c) and EC1118 single strain (b, d).

After three fermentation days, in Primitivo grapes mixed fermentation exhibited a lower sugar consumption (33.07%) in comparison to *S. cerevisiae* pure fermentation (87.44%).

Similar result was obtained in Cabernet fermentation, although the differences in sugar consumption between mixed and single starter was less evident.

5.5.5.1| Chemical profile of wines produced in pilot scale fermentations

Chemical parameters detected in the wines obtained by the four pilot-scale vinifications, performed in the two different grape musts, are shown in the Table 5.10.

Table 5.10| Oenological characteristics and main volatile compounds of wines obtained during pilot-scale vinifications in different grape musts (Primitivo and Cabernet) by using mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC1118 ("Mixed" code). Single fermentations with *S. cerevisiae* EC1118 ("Pure" code) was used as control.

Oenological characteristics	**Primitivo**		**Cabernet**	
	Mixed_P	**Pure_P**	**Mixed_C**	**Pure_C**
Ethanol % (v/v)	14.08	10.89	12.64	13.83
Fructose (g/L)	1.85	0.75	1.00	0.50
Glucose (g/L)	1.75	1.05	0.00	0.30
Total acidity (g/L)	7.90	7.38	6.17	6.17
Volatile acidity (g/L)	0.16	0.16	0.35	0.18
pH	3.68	3.58	3.89	3.86
Malic acid (g/L)	1.70	1.51	1.69	1.74
Lactic acid (g/L)	0.00	0.05	0.00	0.00
Glycerol (g/L)	3.99	3.75	3.48	3.88
Main volatile compounds				
Acetaldehyde (mg/L)	34.08	34.63	118.98	71.96
Ethyl acetate (mg/L)	42.26	30.83	24.58	27.25
n-Propanol (mg/L)	18.96	14.33	11.60	10.61
Isobutanol (mg/L)	45.67	44.01	38.16	33.19
n-Butanol (mg/L)	19.72	17.05	65.21	21.96
Acetoin (mg/L)	13.78	23.01	19.52	14.24
D-amyl alcohol (mg/L)	58.72	67.20	103.95	87.46
Isoamyl alcohol (mg/L)	175.92	194.88	234.43	236.41

The ethanol content ranged between 10.89 and 14.08 % (v/v), with the highest levels in Primitivo wines. Differently from laboratory trials, ethanol reduction by using mixed starter cultures was observed only in wines produced with Cabernet, in which the sample fermented with mixed starter contained approximately 1.19 less ethanol than wine from single fermentation.

All four wines contained a similar level of volatile acidity, with exception of Cabernet wine obtained with mixed starter culture, containing a volatile acidity level slightly higher than the content detected in other wines; however, all the values of volatile acidity falls within the desired range. It has been reported that the optimal concentration of acetic acid in wine is 0.2-0.7 g/L, and the acceptability level of this parameter is comprised between 0.7-1.1 g/L,

depending on the style of wine (Álvarez-Pérez et al., 2014), whereas the OIV states that the maximum acceptable limit for volatile acidity in most wines is 1.2 g/L of acetic acid (Capece et al., 2019). The glycerol content was quite similar among all the wines.

As regards the main volatile compounds detected by gas-chromatographic analysis of wines (Table 5.10), the main differences were correlated to grape must and not starter culture.

As regards acetaldehyde, it's well known that this compound represents more than 90% of the total aldehyde content in wine. Its aroma threshold value is 100 mg/L; low levels of this compound give a desirable fruity aroma to the wines, whereas an excessive content produces an apple-like off-flavor in the wine, and levels more than 200 mg/L cause wine flatness (Capece et al., 2019). The highest amount of acetaldehyde was found in the wine produced in Cabernet, in particular in sample inoculated with mixed starter. Primitivo wines inoculated with mixed and single starters contained very similar levels of acetaldehyde.

As regards ethyl acetate, the content detected in the wines was in the usual range (10-75 mg/L), similar values were found in all the wines, although the highest amount of ethyl acetate was found in Primitivo wines, in particular in sample inoculated with mixed starter.

Higher alcohols represent the largest group of volatile metabolites, synthesised by yeast during alcoholic fermentation (Dzialo et al., 2017). For *n*-propanol and isobutanol, the varaibility was correlated to grape must. In fact, similar level of these alcohols was found in wines produced from both pure and mixed fermentations, for each grape variety used. As regards *n*-butanol, similar levels was found in wines produced with Primitivo variety, whereas for Cabernet variety values very differtent were found for wine fermented with mixed and single starter. Amyl alcohols were found in higher levels in wine inoculated with EC1118 than wine inoculated with mixed starter in Primitivo variety, whereas opposite results were found in wines from Cabernet.

These results showed that from fermentation of the different grape must, similar strains can produce significantly different amounts of aromatic compounds, as a consequence of both the differential ability of wine yeast strains to release varietal volatile compounds from grape precursors and to synthesize de novo volatile compounds.

Principal Component Analysis (PCA) was applied to better visualize the differences in the chemical profiles of wines produced in the two grape musts. The total variability of the first two principal components was 89.04 % (62.82 % and 26.22 % for PC1 and PC2, respectively) and the plot of the four wines on the plane defined by these first two components is shown in Figure 5.40. The PCA of the wines revealed that the wines obtained inoculating the same mixed starters and commercial strain *S. cerevisiae* EC1118 , were located in three different quadrants, indicating differences in the chemical composition of

wines obtained by using the same yeast strains, but in different grape varieties. Only wines obtained by Cabernet grape must with both starters were located in the same quadrant.

Although the same mixed starter was used in the two grape musts, the grape variety can affect the composition of wine, i.e., the precursors content. In fact, factors that are also related to the vineyard growing area, such as seasonal weather differences, soil composition, and vineyard management were reported to affect the development and retention of grape aroma compounds, and consequently the aroma of the wines produced (Capece et al., 2019). This result emphasizes that the effective impact of yeast strains on the aroma properties is dependent on strains metabolisms and other factors, such as raw material composition.

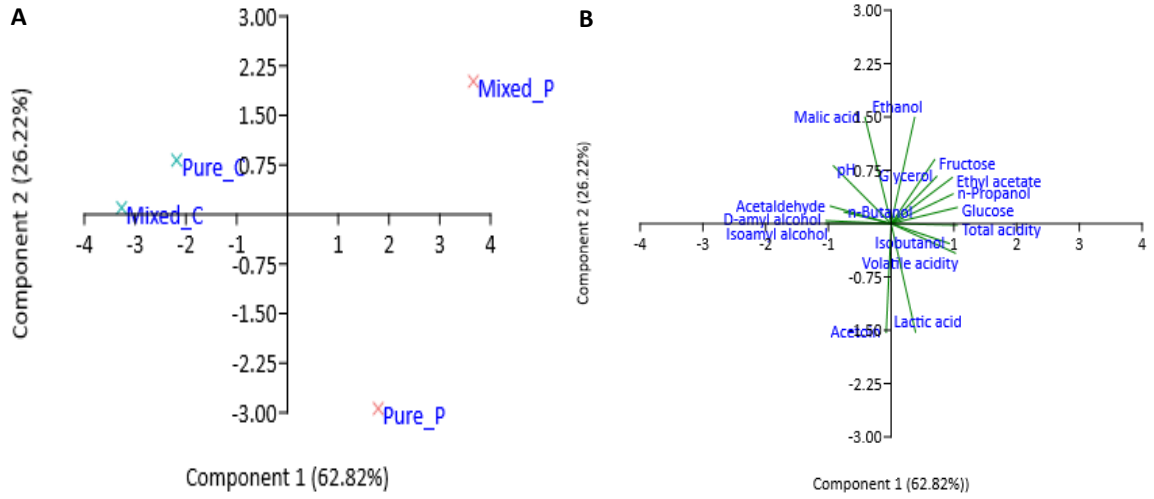

Figure 5.40| Principal Component Analysis (PCA) based on the chemical characteristics of wines produced at the pilot-scale vinifications. PC1 and PC2 account for 62.82 % and 26.22 % of the total variation, respectively. (A) PCA score plot and (B) PCA loading plot of the technological characteristics.

The aromatic compounds detected determined by SPME-GC-MS in the four wines were reported in Table 5.11A-B and Figure 5.41.

Identification and quantification of the volatile metabolites was carried out in order to determine the effect of the inoculation protocol on wine aroma. As shown in Table 5.11, a total of 45 volatile compounds was identified and subsequently divided into 2 families, including 30 esters and 15 terpenes. Differences for content of esters and terpens were found both as a function of starter and grape must.

Esters. Fermentation-derived esters are an important group that can significantly affect wine aroma, and are responsible for the fruity character of the wines (Dzialo et al., 2017, Englezos et al., 2018). The fermentation esters associated with wine fruitiness are divided in two groups: acetate esters (mainly ethyl acetate, 2-phenyl ethyl acetate, 3-methyl-1-butanol acetate or isoamyl acetate, hexyl acetate) and ethyl fatty acid esters. In general, lower levels

of esters were detected in wine obtained from mixed starter than in wines produced with EC 1118, both in Primitivo and Cabernet varieties (Figure 5.41A-B).

Table 5.11A| Concentration of esters determined by SPME-GC-MS in wine obtained by the pilot-scale vinifications in Primitivo and Cabernet grape musts by using mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC 1118 ("Mixed" code). Single fermentations with *S. cerevisiae* EC1118 ("Pure" code). Values are expressed in µg/L, except those indicated with superscript letter "a" (mg/L) and "b" (ng/L).

Esters	**Primitivo**		**Cabernet**	
	Mixed_P	**Pure_P**	**Mixed_C**	**Pure_C**
Hexil Acetate	0.005	0.025	0.130	0.121
Isoamyl Acetate[a]	0.039	0.389	0.092	0.051
Isobutyl Acetate	12.3	12.1	12.7	15.8
Phenyl Ethyl Acetate[a]	0.186	0.154	0.134	0.077
Ethyl Hydrocinnammate[b]	0	0	1	1
Ethyl Decanoate	0.018	0.134	n.r.	0.006
Ethyl Eptanoate	0.047	0.041	0.201	0.433
Ethyl Hexanoate	201.6	111.8	63.9	65.6
Ethyl Laurate	0	0.290	0	0
Ethyl Nonanoate	0.184	0.106	0.002	0.250
Ethyl Octanoate	18.48	21.22	9.87	9.98
Ethyl Propionate	18.3	337.4	63.3	49.0
Ethyl Tetradecanoate	0.506	1.883	0.084	0.129
Ethyl Undecanoate	0.387	1.577	0.113	0.167
Ethyl 2-Hydroxy-4-Methylpentanoate	51.9	30.3	8.6	14.0
Ethyl 2-Methylbutyrate	1.59	3.00	5.96	11.57
Ethyl Isobutyrate	0.9	22.9	30.5	27.8
Ethyl Isovalerate	3.16	6.68	8.98	9.63
Diethyl Succinate[a]	29.5	73.2	0.7	0.8
Ethyl 3-Hydroxybutyrate[a]	0.314	0.257	0.094	0.141
Ethyl Lactate (L)[a]	17.31	11.14	4.75	9.25
Ethyl Phenylacetate	14.33	19.84	1.83	2.53
Ethyl 2-Furoate	9.2	25.2	4.0	3.4
Ethyl Cinnamate	0.19	12.11	0.06	0.13
Ethyl Valerate	0.85	1.30	3.54	3.50
Ethyl Vanillate	199.2	457.7	2.5	4.5
Methyl Dihydrojasmonate	0.177	0.111	0.044	0.077
Methyl Salicylate	0.561	0.310	0.102	0.148
Methyl Vanillate	1.35	2.03	1.66	1.21
Isoamyl Caprylate	0.650	0.372	0.071	0.077

Among the identified esters, the isoamyl acetate, phenyl ethyl acetate, ethyl hexanoate, ethyl propionate, diethyl succinate, ethyl 3-hydroxybutyrate, ethyl lactate (L), ethyl vanillate were the most representative esters in all the wines produced, both in the pure and mixed fermentations, in Primitivo wine, whereas lower levels of these compounds were found in

Cabernet wines (Table 5.10A). Diethyl succinate was the most representative ester found in wine obtained with pure fermentation of Primitivo (73.20 mg/L) .

***Terpenes*.** Other compounds detected in this step, were terpenes, which are responsible for the characteristic floral and fruity aroma of wines, although they are not present at high levels in wine. Generally, they are present in grape berries in free or bound form and synthesised from glucose via the isoprenoid pathway (Lin et al., 2020); however, it has been reported that yeasts are also involved in the production of terpenes. As shown in Table 5.11B, several terpenes were identified in these wines.The terpenes with high odour activity are linalool, geraniol and nerol. Geraniol has aromas described as rose-like and linalool aroma was described as floral, whereas oxidised linalool is described as camphoraceous.

Table 5.11B| Concentration of terpenes determined by SPME-GC-MS in wine obtained by the pilot-scale vinifications in Primitivo and Cabernet grape musts by using mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC 1118 ("Mixed" code). Single fermentations with *S. cerevisiae* EC1118 ("Pure" code). Values are expressed in µg/L, except those indicated with superscript letter "b" (ng/L).

Terpenes	**Primitivo**		**Cabernet**	
	Mixed_P	**Pure_P**	**Mixed_C**	**Pure_C**
Piperitone[b]	32	30	7	10
Cymene <Para-	4.24	15.27	0.42	0.36
(±)-Cis-Nerolidol	2.50	0.54	0.11	0.23
(±)-Trans-Nerolidol	0.287	0.081	0.169	0.163
Citronellol	200.0	212.3	32.1	38.0
Eucalyptol (1,8-Cineole)	0.34	2.11	1.35	1.44
Geraniol	3.19	4.07	0.36	0.48
Linalyl Acetate	74.3	78.7	3.1	2.5
Linalol	7.4	10.3	13.9	17.7
Linalool Oxide <Cis-	3.3	7.4	1.1	1.8
Linalool Oxide <Trans-	6.7	14.2	1.0	1.6
Nerol	29.4	42.7	1.6	3.4
Rose Oxide Cis[b]	62	0	6	8
Rose Oxide Trans	0.010	0.676	0.005	0.006
Terpineol <Alpha-	13.3	18.8	0.5	0.6

For almost all these compounds, wine produced by Primitivo, expecially in pure fermentation, contained higher levels of terpens than Cabernet wines (Figure 5.41C).

The most represented terpene in both the wines was citronellol, followed by linalyl acetate and nerol in Primitivo wine (Table 11B), whereas in wines obtained by Cabernet fermentations also linalol was found at high concentration (13.90-17.70 ng/L). However, the level of terpenes detected in these wines was higher in single than in mixed fermentations,

contrarily to our expectations, revealing a scarce activity of non-*Saccharomyces* strains included in the mixed starter.

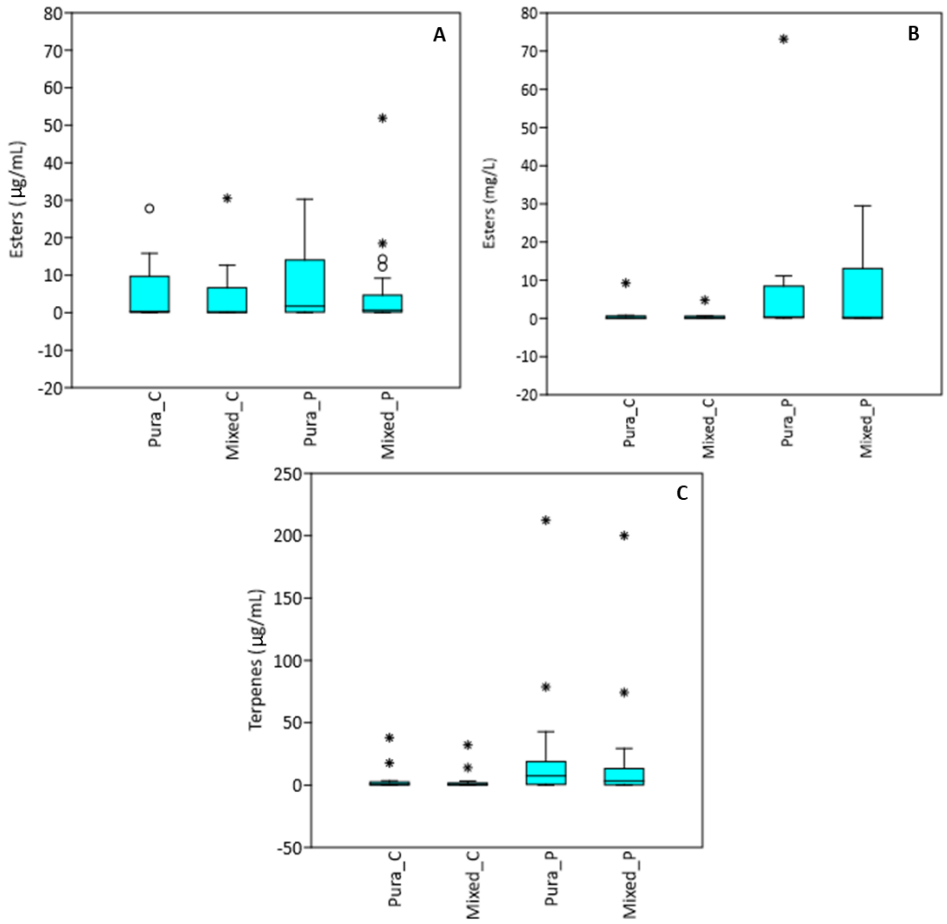

Figure 5. 41A-C| Box plot representing the variability of volatile compounds determined by SPME-GC-MS in Primitivo and Cabernet wines obtained by using mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC1118 (Mixed_P and Mixed_C, respectively) and single fermentations with *S. cerevisiae* EC1118 (Pure_P and Pure_C, respectively). As regards ester, the values are expressed in µg/L (A) and in mg/L (B), for terpenes, values are expressed in µg/L (C).

Data related to esters and terpenes were elaborated by Heat-map, reported in Figure 5.42. This analysis confirms the highest content of these aromatic compounds in wines obtained by fermentations of Primitivo grape must.

Furthermore, this analysis did not separate the wines obtained by mixed and pure fermentations in Cabernet grape must, which are included in the same group. Otherwise, wine obtained by fermentation of Primitivo grape must inoculated with selected mixed starter was separated from wine obtained by pure fermentation of this grape must, which is characterized by the highest content of main of the volatile compounds detected by SPME analysis. This result is in agreement with results obtained by the PCA analysis of main chemical and volatile compounds (Figure 5.40), in which Primitivo wines were located in two different quadrants, whereas Cabernet wines were grouped in the same quadrant.

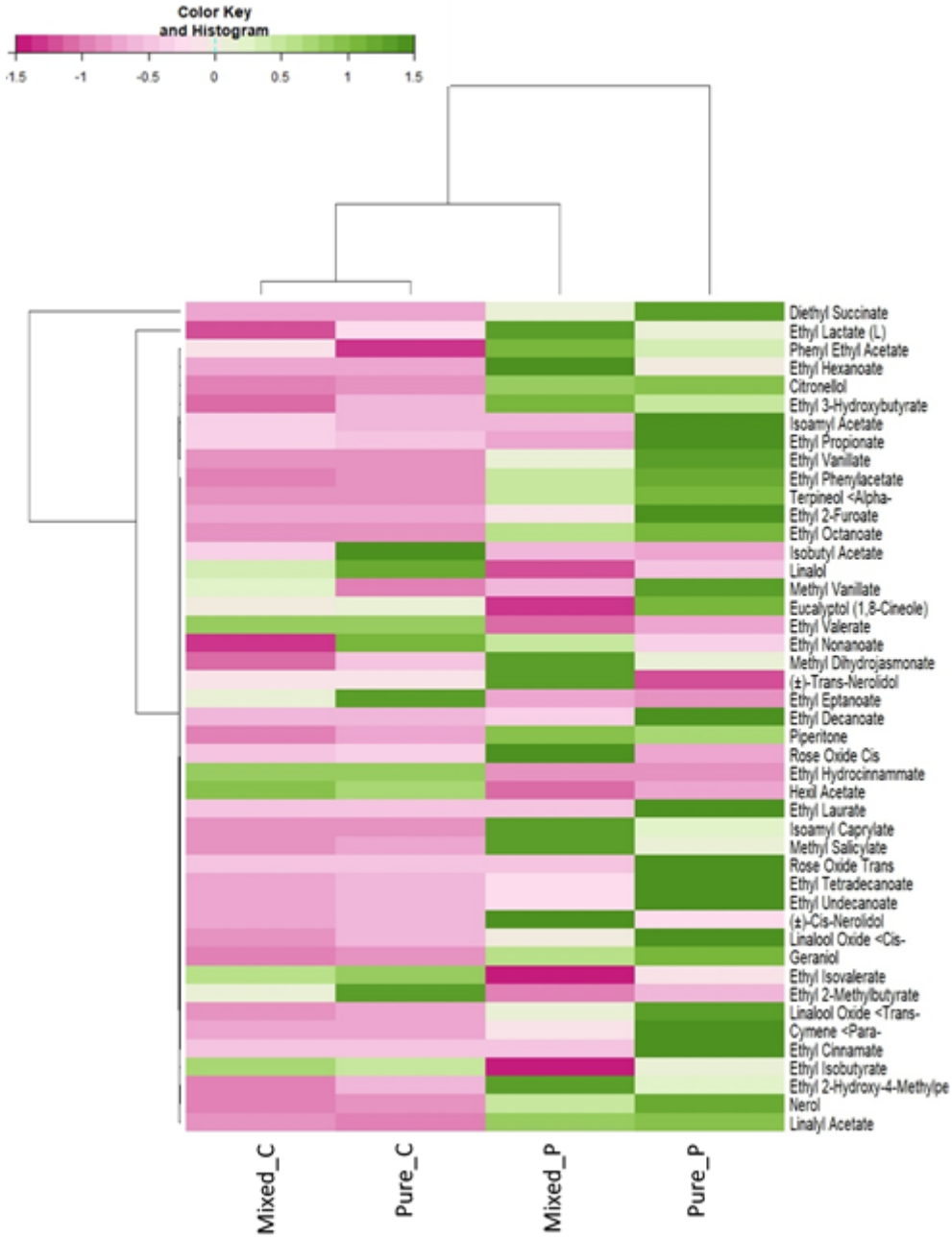

Figure 5.42| Heat-map indicating the increased or decreased concentration of esters and terpenes determined by SPME-GC-MS in wines obtained by using mixed starters cultures of *St. bacillaris* (St8) and *Z. bailii* (Zb1) strains simultaneously inoculated with *S. cerevisiae* EC1118 (Mixed_P and Mixed_C, respectively) and single fermentations with *S. cerevisiae* EC1118 (Pure_P and Pure_C, respectively).

5.6| Conclusions

In this step, one mixed starter culture (composed by *St. bacillaris* St8 and *Z. bailii* Zb1 with *S. cerevisiae*) selected on the basis of positive oenological traits and ability to produce wine with reduced ethanol content (respect to *S. cerevisiae* commercial starter) was tested in different fermentation conditions with aim to optimize some fermentative parameters.

In the first phases, different inoculation procedures were compared, such as simultaneous and sequential inoculation of non-*Saccharomyces* strains with EC1118 strain. The obtained results showed that the three microbial species were compatible and able to persist during

the fermentative process, producing wines characterized by reduced ethanol concentration. Among the different inoculation protocol, the best results were obtained with simultaneous inoculum, in which the wine obtained was characterized by an ethanol level 0.72 lower than value found in wine form single starter and higher glycerol level (3.16 g/L).

As it was reported that these non-*Saccharomyces* wine yeasts was used to promote sugar consumption via respiration rather than fermentation, through partial aeration of the grape juice, in this step it was tested the effect on ethanol reduction of the addition of limited amounts of oxygen during the first stages of fermentation. It was found that limited aerated conditions, determined the production of wine containing 1.40 less ethanol than control wine, whereas in the wine obtained by mixed starter under anaerobic condition the content was reduced of 0.55.

The addition of oxygen during the early stage of fermentation favoured yeast growth, viability and fermentation activity of *St. bacillaris* and *Z. bailii*, other than an increased sugar utilization kinetics. Moreover, wines obtained under aerobic conditions showed a promising balance between ethanol reduction and volatile profile. However, the aeration of grape juice requires further investigations.

The final step was the validation of selected mixed starter and fermentation conditions during pilot scale vinification at cellar level in two different grape varieties. The performance of *St. bacillaris* St8 and *Z. bailii* Zb1 strains and the profiles of the wines produced in pilot scale fermentations were affected both by grape variety and starter inoculum. The influence of mixed starter on wine quality was higher in Primitivo than in Cabernet wine, as both PCA analysis of main chemical and aromatic compounds and Heat-map on minor volatile compounds differentiated wines obtained with mixed starter from single starter wine only in Primitivo fermentation.

These findings confirm that the aromatic quality of wine is the result of a strict interaction between grape must composition and starter performing the fermentation. However, an accurate selection program at laboratory scale is a fundamental step for the individuation of most promising starter culture to be used at cellar level.

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General conclusions

Warm climate and lengthy maturation periods can lead to grapes with high sugar concentrations, and this, in turn, leads to wines with high concentrations of ethanol. High alcohol content can compromise wine quality, including increasing the perception of hotness, body, viscosity, and, to a lesser extent, sweetness and acidity. In addition, it can lead to a decrease in aroma and flavor intensity.

In this context, biotechnological approaches based on the use of selected non-*Saccharomyces* yeast species have shown great potential to produce wine with reduced ethanol content. Over the last few years, several studies have re-evaluated the use of non-*Saccharomyces* yeasts in controlled mixed fermentations with *S. cerevisiae*, indicating that rational selection of non-*Saccharomyces* yeasts can lead to the obtainment of selected cultures that can be used in association with *S. cerevisiae* for production of wines characterized by decreases in the ethanol concentration, compared to the ethanol levels achieved with single *S. cerevisiae* inoculum.

This research activity was aimed to select mixed starter cultures able to produce wine with reduced ethanol content and desirable organoleptic characteristics. The goal of an efficient selection program is to find the best strains within determined species that are able to maximize the advantages and minimize the disadvantages. A preliminary screening among wide numbers of indigenous non-*Saccharomyces* yeasts was an useful tool for the selection of a new generation of co-starters to be integrated in innovative and rational mixed fermentation strategies, with the final goal of production of low-alcohol wines safeguarding organoleptic quality.

Our selection protocol started with the evaluation of 33 non-*Saccharomyces* wild strains, belonging to *Debaryomyces polymorphus*, *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Zygosaccharomyces bailii* species, for parameters of technological interest, such as production level of H_2S, resistance to ethanol, SO_2 and copper, evaluation of enzymatic activities with enological impact (ester-hydrolase, β-glucosidase and β-xylosidase activities) In particular, *D. polymorphus* strains exhibited interesting and desirable properties to improve wine sensory profile, such as high β-glucosidase and β-xylosidase activities, and

resistance to high concentrations of antimicrobial compounds present in winemaking, being able to survive in fermentation must conditions. Also *H. uvarum* strains showed interesting technological traits, but with high variability among the analyzed strains. All *St. bacillaris* and *Z. bailii* strains exhibited high tolerance to antimicrobial compounds, and the *Z. bailii* strains showed also β-glucosidase and β-xylosidase activities.

The technological screening used in the present study confirmed that non-*Saccharomyces* yeasts represent a sink of unexplored biodiversity that might be of great value for the winemaking industry. The results obtained among the 33 non-*Saccharomyces* yeasts indicated that some of these strains can be used in association with *S. cerevisiae* starter cultures to improve the complexity of wine, such as non-*Saccharomyces* strains possessing high level of enzymatic activities could be profitable used in specific grape musts, where the precursors of aromatic compounds are widely present. In fact, the combinations in mixed starter cultures of strains characterized by different oenological characteristics is an useful strategy for obtaining final products with specific traits.

On the basis of results obtained in the first screening, eight strains, one *D. polymorphus* (Db2), two *H. uvarum* strains (Ha3 and Ha9), four *St. bacillaris* strains (St1, St2, St5 and St8) and one *Z. bailii* strain (Zb1,) were selected.

The selected non-*Saccharomyces* strains were tested as mixed starters with the commercial *S. cerevisiae* EC1118 strain during laboratory scale fermentations. These two different cultures were inoculated simultaneously or sequentially and the fermentation dynamics were studied in both fermentations. From the results of this series of tests, different inoculation modalities (sequential and co-inoculation) and ratio between non-*Saccharomyces* and *S. cerevisiae* strains were used during laboratory scale fermentations. In these steps, results related to kinetics of growth and fermentative activity, supported by analytical data on fermenting musts and final wines, were useful to individuate the conditions allowing the best performance of selected strains.

The data obtained in this step highlighted that the non-*Saccharomyces* yeast strains influence the chemical characteristics and aroma profile of wine, such as the ethanol content, and this influence was correlated with ability of non-*Saccharomyces* strains to survive during the fermentative process together *S. cerevisiae* EC1118.

Moreover, it was found that co-inoculation was more efficient than sequential fermentation for ethanol reduction. In fact, all the experimental wines obtained by simultaneous fermentations contained lower concentrations of ethanol (between 0.19-0.80) than wines obtained by sequential fermentations (between 0.18-0.45). The mixed starter including the strains St8 (*St. bacillaris*) and Zb1 (*Z. bailii)* produced the wines characterized by the lowest

ethanol content (i.e. 0.57 and 0.80 ethanol reduction values, respectively). These results were further confirmed in a laboratory-scale fermentations performed in natural grape must, in which it was observed also an increased content of glycerol in wines obtained by mixed starter cultures.

On the basis of the findings obtained in these preliminary steps, co-inoculation represents an alternative approach in commercial winemaking and its success strongly depends on the selection of suitable yeast strain combinations.

The last step of research activity was addressed to a wide investigation on a mixed starter culture, composed by the two non-*Saccharomyces* strains showing the best combinations of desirable characteristics investigated in the previous steps (St8 and Zb1, used together) and EC1118. On this selected starter different fermentation conditions were tested, such as co- and sequential inoculation, fermentation with oxygen addition and use of immobilized cells. In fact, modification of some fermentative parameters, such as oxygen addition, resulted very useful to address metabolic pathways of yeast strains toward other compounds instead ethanol, resulting in low ethanol yield. Also the use of immobilized cells of selected strains of some non-*Saccharomyces* yeasts, could be a suitable strategy to reduce the ethanol content in wine.

The results obtained in this step showed that the three microbial species were compatible and able to persist during the fermentative process, producing wine characterized by ethanol concentration lower than wine obtained by pure culture of EC1118. Among the different inoculation protocol, the best results were obtained with simultaneous inoculum, in which the wine obtained was characterized by an ethanol level 0.72 lower than value found in wine form single starter and higher glycerol level (3.16 g/L). Furthermore, our results confirmed that the aeration is an useful strategy to reduce the ethanol content in wine as this approach promotes sugar consumption via respiration rather than fermentation. In fact, the addition of limited amounts of oxygen during the first steps of fermentations, determined the production of wine containing 1.4 less ethanol than control wine, whereas lower reduction level was observed in the wine obtained by mixed starter under anaerobic conditions. The addition of oxygen during the early stage of fermentation favoured yeast growth, viability and fermentation activity of *St. bacillaris* and *Z. bailii*, other than an increased sugar utilization kinetics. Moreover, the wine characteristics, i.e. volatile acidity, were not negatively affected by aeration.

The final validation of mixed starter culture, composed by St8, Zb1 and EC1118, was performed during pilot scale vinifications at cellar level in two different grape varieties, Primitivo and Cabernet. The performance of *St. bacillaris* St8 and *Z. bailii* Zb1 strains and

the profiles of the wines produced in pilot scale fermentations were affected both by grape variety and starter inoculum. The influence of mixed starter on wine quality was higher in Primitivo than in Cabernet wine, demonstrating that the aromatic quality of wine is the result of a strict interaction between grape must composition and starter performing the fermentation.

All the results obtained in this research confirmed that the optimal use of mixed yeast cultures is still one of the main challenging tasks for the wine industry, but it needs the support of an accurate selection program at laboratory scale, which is a fundamental step for the individuation of most promising starter culture to be used at cellar level. In fact, the selection of most suitable starter requires the study of biological factors, such as the analysis of the interrelationships between strains composing the mixed starter, and technological aspects, such as trials with different grape musts characterized by different nutritional composition, evaluation of timing and consistency of inoculum, oxygen addition.

Publications

Focus

Fermentazioni miste controllate per migliorare il profilo analitico e sensoriale dei vini

Starter costituiti da colture non-Saccharomyces e S. cerevisiae sfruttano contemporaneamente le caratteristiche uniche di entrambe le tipologie di lievito

Data:*11 set 2020*

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La fermentazione del vino è un processo biologico complesso, e si caratterizza per la presenza di diversi gruppi di microrganismi tra cui lieviti, funghi filamentosi e batteri, i quali interagiscono con i composti presenti nel mosto d'uva trasformandolo in vino.
Per molti anni, la fermentazione è stata condotta spontaneamente dalla microflora naturalmente presente sulla superfice delle uve e residente nell'ambiente di cantina. Nella fermentazione spontanea si succedono diverse specie di lieviti, che vengono tradizionalmente divisi in lieviti non-*Saccharomyces* e *Saccharomyces cerevisiae*, che rappresenta il "lievito vinario per eccellenza".

Nel corso degli anni, la fermentazione spontanea è stata sostituita, nella maggior parte dei casi, dalla fermentazione controllata, condotta utilizzando starter commerciali di *S. cerevisiae*. Tale applicazione garantisce un maggiore controllo della vinificazione, producendo vini con caratteristiche costanti e riproducibili. L'uso di colture starter di *S. cerevisiae* ha rappresentato una tra le più importanti applicazioni biotecnologiche nel processo fermentativo. Nonostante i significativi vantaggi della fermentazione controllata,

l'utilizzo di colture pure di *S. cerevisiae* ha portato all'ottenimento di vini molto uniformi, che mancano della complessità organolettica, che invece si osserva nei vini ottenuti dalla fermentazione spontanea, poiché ognuno dei lieviti che sviluppa in questo processo apporta il proprio contributo alle caratteristiche aromatiche del vino. In conseguenza di ciò, negli ultimi anni si assiste ad una rivalutazione dei lieviti non-*Saccharomyces*, in passato considerati di importanza secondaria o lieviti indesiderabili, per la loro bassa efficienza di fermentazione e maggiore produzione di sostanze indesiderate rispetto a *S. cerevisiae*.

I lieviti non-*Saccharomyces* sono stati rivalutati poiché diversi studi hanno dimostrato che questi lieviti secernono enzimi e producono composti aromatici diversi da quelli prodotti da *S. cerevisiae*, mettendo in evidenza il loro ruolo rilevante sul profilo analitico e sensoriale dei vini. In questo contesto, fermentazioni miste controllate, costituite da lieviti non-*Saccharomyces* e *S. cerevisiae*, che sfruttano le caratteristiche uniche di entrambe le tipologie di lievito, possono essere uno strumento biotecnologico per incrementare la complessità aromatica dei vini.

Applicazione di uno starter misto su scala di laboratorio

Al fine di individuare un lievito non-*Saccharomyces* da proporre come starter misto per la produzione di vini con caratteristiche aromatiche peculiari, è stata condotta un'accurata fase di screening preliminare nell'ambito della numerosa collezione di lieviti, isolati da matrici naturali, presente nel Laboratorio di Lieviti Fermentativi dell'Università degli Studi della Basilicata. Al termine di questa fase di screening, sono stati selezionati due ceppi di lievito non-*Saccharomyces*, indicati con le sigle **Ha** (specie *Hanseniaspora uvarum*, Figura 1a) e **Sb** (specie *Starmerella bacillaris*, Figura 1b). Ognuno dei due ceppi è stato testato in prove di fermentazione in associazione con un ceppo di *S. cerevisiae* (sigla **Sc**, Figura 1c) solitamente usato come starter in cantina. A confronto, è stata condotta una prova di fermentazione con il solo ceppo Sc.

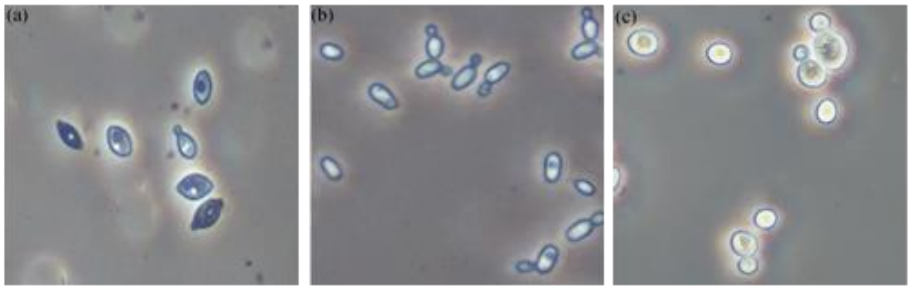

Figura 1. Cellule di *H. uvarum* (a), *St. bacillaris* (b) e di *S. cerevisiae* (c) osservate al microscopio ottico.

L'analisi dei vini sperimentali ottenuti, basata sulla determinazione del contenuto di alcuni dei composti che influenzano l'aroma del vino, come alcoli superiori (alcol **isoamilico**, *n*-butanolo e isobutanolo) e esteri (acetato di etile) ha messo in evidenza l'influenza della coltura starter sul profilo aromatico del vino (Figura 2).

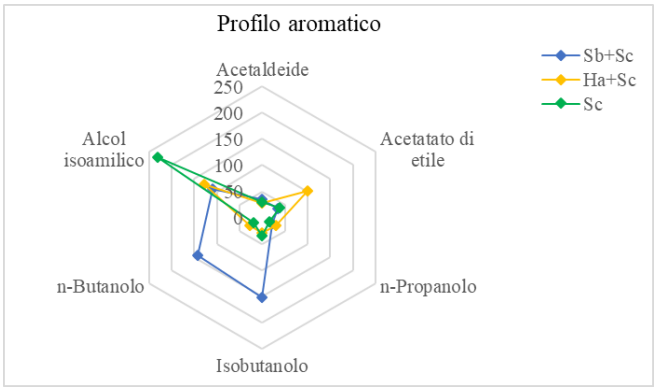

Figura 2. Profilo aromatico dei vini sperimentali

Infatti, i vini ottenuti dall'inoculo di entrambi i lieviti non-*Saccharomyces* in coltura mista con *S. cerevisiae* (Sb+Sc e Ha+Sc, Figura 2) erano caratterizzati da un profilo aromatico diverso dal vino ottenuto con il solo ceppo di *S. cerevisiae* (Sc).

Conclusioni

Sebbene in questa prova sia stato determinato un numero molto limitato di composti aromatici, considerando che il vino contiene centinaia di composti aromatici, già questi risultati preliminari mettono in evidenza il ruolo dei lieviti non-*Saccharomyces* sul profilo organolettico del vino e la grande potenzialità delle colture starter miste selezionate come strumento per esaltarne la componente aromatica.

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SPECIAL ISSUE ARTICLE

Yeast WILEY

Saccharomyces cerevisiae and *Hanseniaspora uvarum* mixed starter cultures: Influence of microbial/physical interactions on wine characteristics

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Abstract

The growing trend in the wine industry is the revaluation of the role of non-*Saccharomyces* yeasts, promoting the use of these yeasts in association with *Saccharomyces cerevisiae*. Non-*Saccharomyces* yeasts contribute to improve wine complexity and organoleptic composition. However, the use of mixed starters needs to better understand the effect of the interaction between these species during alcoholic fermentation. The aim of this study is to evaluate the influence of mixed starter cultures, composed by combination of different *S. cerevisiae* and *Hanseniaspora uvarum* strains, on wine characteristics and to investigate the role of cell-to-cell contact on the metabolites produced during alcoholic fermentation. In the first step, three *H. uvarum* and two *S. cerevisiae* strains, previously selected, were tested during mixed fermentations in natural red grape must in order to evaluate yeast population dynamics during inoculated fermentation and influence of mixed starter cultures on wine quality. One selected mixed starter was tested in a double-compartment fermentor in order to compare mixed inoculations of *S. cerevisiae*/*H. uvarum* with and without physical separation. Our results revealed that physical contact between *S. cerevisiae* and *H. uvarum* affected the viability of *H. uvarum* strain, influencing also the metabolic behaviour of the strains. Although different researches are available on the role of cell-to-cell contact-mediated interactions on cell viability of the strains included in the mixed starter, to our knowledge, very few studies have evaluated the influence of cell-to-cell contact on the chemical characteristics of wine.

KE YWOR DS

cell-to-cell contact, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, wine composition

1 | INTRODUCTION

Traditional wine fermentation is a complex biological process, which takes place through different interactions between the compounds present in the grape must and the various microorganisms, which transform the must into wine. Spontaneous alcoholic fermentation is carried out by the sequential action of different populations of numerous yeast species. The first stages of alcoholic fermentation are promoted by numerous and different non-*Saccharomyces* species, which are replaced, in the final stage of the process, by *Saccharomyces* species (predominantly *Saccharomyces* cerevisiae), which are more strongly fermentative and more alcohol tolerant and complete the fermentation (Jolly, Varela, & Pretorius, 2014;

Padilla, Gil, & Manzanares, 2016; Varela, 2016; Wang, Mas, & Esteve-Zarzoso, 2015). Besides ethanol, during fermentation, the different yeast species produced several metabolites, exerting a metabolic impact on wine flavour (Capece & Romano, 2019). In particular, the involvement in wine production of non-*Saccharomyces* is very important, because they contribute to improve wine complexity and organoleptic composition (Comitini, Capece, Ciani, & Romano, 2017; Capozzi et al., 2019). Consequently, the continuous research for specific oenological properties, in association with new trends in winemaking industry, has increased the interest to non-*Saccharomyces*, by addressing the focus on the selection and characterization of indigenous yeasts. In last decades, the positive role of non-*Saccharomyces* yeasts has been reported, highlighting interesting characteristics on the final quality of the wine, which are absent in *Saccharomyces* species (Dutraive et al., 2019; Gschaedler, 2017; Padilla et al., 2016), such as extracellular enzymes, which exert a positive impact on the quality of the final product (Basso, Alcarde, & Portugal, 2016; López, Mateo, & Maicas, 2016; Mendoza, Vega-Lopez, Fernández de Ullivarri, & Raya, 2019).

In this context, the widespread approach to the use of selected monoculture of *S. cerevisiae* appears not sufficient to confer the stylistic distinction determined by the contribution of non-*Saccharomyces* indigenous yeasts (Gschaedler, 2017; Padilla et al., 2016). These non- conventional yeasts, used as starter culture in conjunction with *S. cerevisiae*, through their distinctive production of secondary metabolites, have the potential to improve and diversify wine organoleptic characteristics (Jolly et al., 2014). Indeed, the winemaking industry is showing a growing interest in new yeast species, capable of obtaining wines with innovative organoleptic characteristics, considering that the use of a mixed starter culture, non-*Saccharomyces/Saccharomyces*, allows the modulation of wine sensory and organoleptic parameters, such as ethanol, glycerol, acidity and fermentation by-products.

Among non-*Saccharomyces*, the apiculate yeast *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*) is one of the most frequent species present on grapes and found at high numbers in grape must (Bezerra-Bussoli, Baffi, Gomes, & Da-Silva, 2013; Combina et al., 2005; Fleet, 2003; Jolly et al., 2014). These nonconventional yeasts exhibit low fermentative power, but they play a significant role in the first stages of alcoholic fermentation producing enzymes and volatile compounds, contributing to modify wine organoleptic quality (López et al., 2016; Martin, Valera, Medina, Boido, & Carrau, 2018; Tristezza et al., 2016). This species was tested in mixed starter cultures as an alternative to the inoculation of only *Saccharomyces* yeast species, exploiting the exclusive characteristics of both the two species. The use of *H. uvarum* in mixed fermentation with *S. cerevisiae* has been reported to produce significantly different wines from those obtained by pure cultures of *S. cerevisiae* (Capece & Romano, 2019; Hu, Jin, Mei, Li, & Tao, 2018). In details, an increased concentration of higher alcohols, acetate and the varietal aroma was recorded. However, as *Hanseniaspora* yeasts can be associated with the production of relevant and unfavourable concentrations of some compounds, such as acetic acid, yeast selection is crucial, as a high variability among the different *Hanseniaspora* strains has been reported (Capece, Fiore, Maraz, & Romano, 2005; Romano, Fiore, Paraggio, Caruso, & Capece, 2003; Tristezza et al., 2016). However, to take all the advantages from the use of mixed starter cultures, it is crucial to gain a deeper understanding of the interactions between non-*Saccharomyces* and *Saccharomyces* species, during alcoholic fermentation. For a long time, the early death of non-*Saccharomyces* yeasts during fermentation was believed to be primarily due to their low ability to withstand the selective conditions of wine environment, such as high levels of ethanol, low pH, SO_2 addition and oxygen deficiency. More recent studies demonstrated that microbial interactions, such as cell–cell contact, affect the persistence of non-*Saccharomyces* along the wine fermentation (Albergaria, Francisco, Gori, Arneborg, & Gírio, 2010; Branco et al., 2014; Gschaedler, 2017; Kemsawasd et al., 2015; Nissen & Arneborg, 2003; Renault, Albertin, & Bely, 2013; Wang et al., 2015).

The purpose of this study is to evaluate the influence of mixed starters, composed by *S. cerevisiae* and *H. uvarum*, on the characteristics of wine (secondary compounds, total polyphenols and antioxidant power [AP]) and to study the interaction between the two species during alcoholic fermentation. To evaluate the influence of cell–cell contact on the fermentation course and metabolites produced, one selected mixed starter culture was tested in a double-compartment fermentor in order to compare mixed inoculations of *S. cerevisiae/H. uvarum* with and without physical separation.

2 | MATERIALS AND METHODS

2.1 | Yeast strains

The wild yeasts used in this study were the following:

- Two *S. cerevisiae* strains, 5TB8-60 (work code S3) and SA6-31 (work code S5).
- Three *H. uvarum* strains, H2, H7 and H19.

All the wild strains belong to the UNIBAS Yeast Collection of the School of Agricultural, Forestry, Food and Environmental Sciences (University of Basilicata, Potenza) and were isolated during spontaneous grape must fermentations, performed at lab scale from grapes of different varieties and directly collected in the vineyard. They were identified by 5.8S-ITS-RFLP analysis (Esteve-Zarzoso, Belloch, Uruburu, & Querol, 1999) and sequence analysis of D1/D2 domain of 26S rDNA (Kurtzman & Robnett, 1998).

The two *S. cerevisiae* strains were previously selected, on the basis of interesting enological traits (Capece, Romaniello, Siesto, & Romano, 2012; Siesto, Capece, Sipiczki, Csoma, & Romano, 2013). The three *H. uvarum* strains were selected for their low production level of acetic acid and high β-glucosidase activity (Capece et al., 2005; Guaragnella et al., 2020). All the yeasts were maintained on yeast extract-peptone-

dextrose (YPD) medium (1% yeast extract; 2% peptone; 2% glucose; 2% agar) at 4° C.

2.2 | Laboratory-scale fermentations

The three *H. uvarum* and two *S. cerevisiae* strains were tested in mixed fermentations at laboratory scale by using simultaneous inoculation. In each fermentation, one *S. cerevisiae* strain was co-inoculated with one *H. uvarum* strain, by using different inoculation ratio (10^3 cell/ml for *S. cerevisiae* and 10^7 cell/ml for *H. uvarum*). As control, pure fermentations with the two *S. cerevisiae* strains (10^7 cell/ml) were used. The fermentations were performed in 100 ml of natural red grape must, Merlot variety, (pH 3.24, sugar concentration 258 g/L, available nitrogen 147 mgN/L), supplemented with 50 mg/L of free SO_2, following the protocol previously described (Capece et al., 2013). The absence of viable cells of indigenous yeasts was checked by plate counting on Wallerstein Laboratory (WL) Nutrient Agar medium (Pallmann et al., 2001) after 30 min from treatment of grape must with 50 mg/L of free SO_2. All the experiments were performed in triplicate at 26° C. The fermentation course was monitored by evaluating CO_2 evolution and yeast viable populations. Fermenting must samples were taken from each flask at days 0, 1, 2, 5, 6, 8, 12 and 15 of fermentation. Each sample was diluted in saline solution and plated on WL medium and the plates were incubated at 26° C for 5 days. On this medium, yeast species can been distinguished by different colony morphologies and colours. Statistically representative dilution plates were counted, and around 30 colonies from each fermentation sample (10 colonies from each triplicate) were randomly selected and purified on YPD plates for further yeast identification. Fermentation process was considered completed when a constant weight of the samples was recorded.

The experimental wines were analysed for total polyphenol con- tent (TPC) and AP. TPC was determined by using the Folin–Ciocalteau reagent, and data were expressed as mg gallic acid/L. A calibration curve for gallic acid was prepared for concentrations ranging from 10 to 100 mg/L. The AP was determined by ABTS [2,29-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid)] assay, and the obtained results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) (Re et al., 1999).

Furthermore, concentration of secondary compounds (acetaldehyde, *n*-propanol, isobutanol, amyl alcohols, ethyl acetate and acetic acid) in wines was determined by direct injection gas chromatography of 1 μl of sample, as previously described (Capece et al., 2013).

2.3 | Mixed fermentation in double-compartment fermentor

With the aim to examine the interactions between the two yeast species, S3 and H2 strains were tested in mixed fermentation by using a 2.4-L double-compartment fermentor (Renault et al., 2013) in two experimental conditions. In one condition (nonseparated [NS]), each fermentor compartment was inoculated with both strains (2×10^6 cell/ml of H2 and 2×10^2 cells/ml of S3). In the second condition (separated [S]), the two strains were inoculated separately, by adding H2 at 2×10^6 cell/ml in the right compartment and S3strain (2×10^2 cells/ml) in the left side. In both cases, natural red grape must (Merlot) was used (pH 3.34, sugar concentration 214 g/L, available nitrogen 122 mgN/L); before yeast inoculation, the must was sterilized by filtration (0.45-μm nitrate cellulose membrane). All the fermentations were carried out at 26° C. The fermentation kinetic was monitored by CO_2 release (Renault et al., 2013). The viable yeast cell population was evaluated by plate counting on WL at different fermentation steps. This experiment was performed in duplicate.

Samples of wines were taken from each compartment of the double fermentor at different times. The fermentation course and cell counts of the two species in S and NS modalities were evaluated as described previously in the point 2.2 laboratory-scale fermentations.

2.3.1 | Analysis of experimental wines obtained in double-compartment fermentor

The obtained wines were analysed for ethanol concentration (volume %) by infrared refractance (Infra Analyser 450, Technicon, Plaisir, France). Sugar (expressed as gramme per litre) and volatile acidity (expressed in grammes per litre of acetic acid) were determined chemically by colorimetry (460 nm) in continuous flux (Sanimat, Montauban, France), whereas TPC and AP were measured following the protocol previously described.

Acetaldehyde, *n*-propanol, isobutanol, amyl alcohols and ethyl acetate were determined by direct injection gas chromatography (Capece et al., 2013).

The wines were analysed also for esters content, both on final wines and at 40% of alcoholic fermentation completion, by solid-phase microextraction followed by gas chromatography mass spectrometry (SPME-GC-MS), following the protocol reported by Renault, Coulon, de Revel, Barbe, and Bely (2015). Samples of wines were taken from each compartment of the double fermentor.

2.4 | Statistical analysis

Analysis of variance (ANOVA) was used to evaluate differences in chemical and volatile compounds of the experimental wines obtained by different inoculation modalities, which was done after the verification of variance homogeneity (Levene test, $p < 0.05$). Tukey's test was used to compare the mean values between mixed and pure fermentations at laboratory scale and wines from double- compartment fermentor. Principal component analysis (PCA) was carried out on the data of wines produced from single and mixed starters at laboratory scale and esters detected in wines from double-compartment fermentor (both at 40% of alcoholic fermentation completion and at the end of the process). The PAST software ver. 1.90 (Hammer, Harper, & Ryan, 2001) was used for the statistical analyses.

3 | RESULTS

3.1 | Laboratory-scale mixed fermentations

Six co-fermentations were performed by inoculating three *H. uvarum* strains with two *S. cerevisiae* strains.

3.1.1 | Yeast growth and fermentation kinetics

During overall the fermentative process, fermentation kinetic was monitored by measuring the CO_2 evolution, whereas cell growth of both inoculated species was evaluated at different steps during the process (Figure 1). The general trend was similar in all the fermentations, although the cell count of each strain was variable in function of the other strain included in the mixed starter. In all the tests, the cell count of *S. cerevisiae* strains reached the maximum population after 5–6 days; then, it was kept constant, and only at the end of the pro- cess, it decreased slightly. As regards *H. uvarum* strains, a reduction of cell count was observed during the first 2 days for all the fermentations, after that an increase of cell count was observed until 5–6 days of fermentation. At the end of the process, no *H. uvarum* cells were found, except for S5 + H2 starter, in which *H. uvarum* count was 1×10^2 cells/ml. In some mixed fermentations, during the first 4–5 days, cell count of *H. uvarum* was higher (S3 + H19 and S5 + H7) or very similar (S3 + H7 and S5 + H19) to *S. cerevisiae* count. After this period, a fast decrease of *H. uvarum* cell population was recorded. Conversely, in both the mixed fermentations with the H2 strain, mainly in the mixed fermentation with S5, the cell count of the *Hanseniaspora* strain was lower than *S. cerevisiae* cells from the second to third fermentation day.

The trend of CO_2 production was very similar in all the fermentations, with an increase after the second fermentation day, except for the mixed fermentation with S3 and H7 starters, which starts already after the first day. About *S. cerevisiae*, the S5 strain exhibited higher fermentative power than S3; in fact, at the end of the process, the maximum CO_2 production was about 18 g/100 ml in the fermentation inoculated with S5, whereas about 14 g/100 ml were produced in the fermentations inoculated with S3.

3.1.2 | Analysis of experimental wines

Experimental wines obtained by mixed fermentations were analysed in comparison with wines produced by single starters S3 and S5. The content of secondary compounds usually present in high concentrations in wines, such as acetaldehyde, *n*-propanol, isobutanol, amyl alcohols, ethyl acetate and acetic acid, is reported in Table 1. Further- more, the wines were analysed by TPC and AP.

One-way ANOVA was applied to evaluate, for each parameter, the differences between mixed starter and the corresponding single starter fermentation. As regards the mixed fermentations including S3 strain, differences statistically significant ($p < 0.05$) between wines from single and mixed starters were found mainly for the levels of acetaldehyde, isobutanol and amyl alcohols, whereas no differences were found for *n*-propanol level.

About the fermentation including S5 strain, single starter wine was significantly different from mixed starter wines for almost all the detected compounds, except ethyl acetate, acetic acid and D-amyl alcohol. It is worthwhile to note that the use of mixed starter including *H. uvarum* strains did not determine an increase of acetic acid content of wines. In fact, only the wine from mixed starter composed of S3 + H19 contained a higher level of acetic acid (statistically significant difference) than the wine from S3 strain, whereas in all the other wines, no statistically significant differences were found for the acetic acid content between single and mixed starter wines. As regards TPC and AP, the level of these parameters were higher

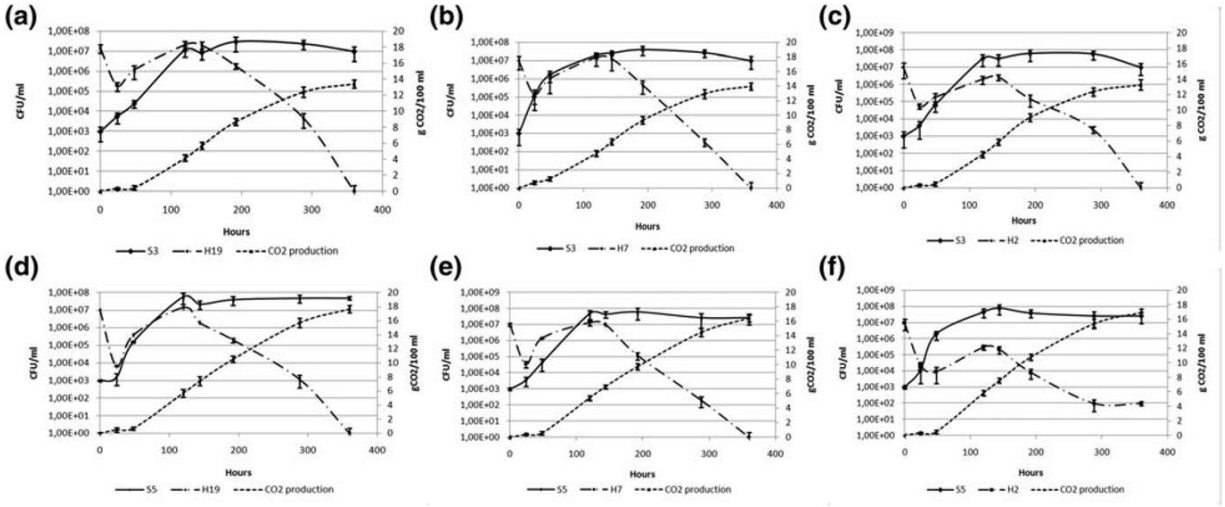

FIGURE 1 Evolution of yeast population and CO_2 production during *Hanseniaspora uvarum*/*Saccharomyces cerevisiae* mixed fermentation. In each trial, one *H. uvarum* strain (H19, H7, H2) was simultaneously inoculated with one *S. cerevisiae* strain, (a–c) S3 and (d–f) S5

TABLE 1 Major volatile compounds, TPC and AP detected in wines produced by mixed fermentations of *Hanseniaspora uvarum* (H2, H7 and H19) and *Saccharomyces cerevisiae* (S3 and S5) in comparison with single fermentation wines.

	S3 + H2	S3 + H7	S3 + H19	S3 (pure)	S5 + H2	S5 + H7	S5 + H19	S5 (pure)
Acetaldehyde	74.32 ±6.87^{a}	63.82±10.12^{a}	62.74±3.47^{a}	40.79 ±2.03^{b}	41.08±2.24^{a}	35.54 ± 5.33^{ab}	38.14 ± 2.29^{a}	33.54 ±1.40^{b}
Ethyl acetate	18.58 ±0.54^{a}	21.39 ±0.97^{a}	26.36 ±3.31^{b}	19.40±2.10^{a}	13.35 ±0.31	13.91 ± 0.25	13.29 ± 1.99	11.79 ±0.23
n-Propanol	28.86 ±2.18	27.49 ±3.24	26.93 ± 2.23	28.47 ±2.03	35.38 ±3.47^{a}	31.33 ± 1.06^{a}	36.88 ±4.13^{a}	24.29 ±0.42^{b}
Isobutanol	47.96±3.85^{ac}	42.20 ±1.93^{b}	52.79 ±2.10^{c}	44.58 ±1.58^{a}	36.99 ±0.40^{a}	35.64 ± 1.48^{a}	34.92 ±2.30^{a}	40.19 ±1.04^{b}
Acetic acid	326.91 ±24.50^{a}	346.99 ± 43.70^{a}	438.45 ±27.56^{b}	421.14±21.46^{a}	413.30 ±9.55	412.32 ± 11.70	398.87 ±8.94	434.50 ± 20.62
D-amilic alcohol	102.37±2.98^{a}	94.96 ± 4.24^{ac}	87.93 ± 8.20^{BC}	101.70±4.34^{a}	112.94 ±1.26	104.71 ± 2.83	105.46 ±2.50	102.26 ±2.81
Isoamyl alcohol	240.74 ±10.37^{a}	209.31 ± 7.79^{b}	185.38 ± 5.65^{c}	222.62 ±10.69^{a}	226.65±7.57^{a}	187.69 ± 8.94^{b}	194.97 ± 12.63^{c}	193.22±10.57^{ab}
TPC	433±8.49^{b}	342.5±17.68^{ab}	384.5±47.38^{ab}	317.5±19.09^{a}	442.5±4.95^{b}	413.5±44.55^{ab}	329 ± 31.11^{ab}	308 ± 26.87^{a}
AP	0.95 ±0.01^{c}	0.70 ± 0.03^{b}	0.61 ±0.05^{ab}	0.55 ±0.01^{a}	0.67±0.03	0.63 ± 0.11	0.65 ± 0.19	0.51 ±0.04

Note: The data are reported as mean values for three independent experiments and standard deviations. Different letters indicate significant differences (Tukey's test $p < 0.05$) between mixed starter and the corresponding single starter fermentation. TPC (total polyphenols content) is expressed as mg gallic acid/L. AP (antioxidant power) is expressed as Trolox Equivalent Antioxidant Capacity (TEAC) mmol/ml.

in all the experimental wines obtained by the mixed fermentations, both with S3 and S5 strains, than the level detected in single starter fermentation, although the differences for both the parameters were statistically significant in all the wines obtained by inoculating S3 strain. Content of main secondary compounds, TPC and AP in the experimental wines obtained from single and mixed fermentations were subjected to PCA, as shown in Figure 2. The first two components explain 75% of the variance (43.5% and 31.5% for PC1 and PC2, respectively). As shown in Figure 2, the first two components could be used to distinguish samples obtained from the different fermentations. Along the first component, the samples were separated on the basis of *S. cerevisiae* starter; in details, the wines from fermentation inoculated with S5 strain (both as single and mixed starter) were located on left side, whereas the wines obtained by inoculating S3 strain, alone and by inoculating S3 strain, alone and mixed with the three *H. uvarum* strains, were located on right side. Compounds, such as acetaldehyde, ethyl acetate and isobutanol, were correlated with the PC1, which separated the wines inoculated with S5 strains from wines obtained by S3. Furthermore, the PC2 distinguished the wine obtained from the single S5 strain from those obtained from S5 in co-fermentation with the three *H. uvarum* strains. As regards wines obtained from S3 (single and mixed fermentations), only the wine from the mixed starter S3 + H2 was separated from the other samples obtained by inoculating S3 strain. The factors affecting mainly the variability along PC2 were acetic acid, amyl alcohols and AP. In summary, PCA allowed to demonstrate significant differences in the wine parameters analysed in this study for the different starter cultures tested. Both the *S. cerevisiae* strains exhibited a high influence on wine composition, but also the *H. uvarum* strains, co-inoculated with *S. cerevisiae*, contributed significantly to wine organoleptic quality, mainly when in co-culture with the S5 strain.

3.2| Mixed fermentation in double-compartment fermentor

On the basis of the obtained results, the mixed starter composed of S3 + H2 was selected to study the interaction between *S. cerevisiae* and *H. uvarum* during mixed fermentation. This mixed starter was chosen as it produced the wine characterized by the highest antioxidant activity and the lowest acetic acid content (Table 1). Furthermore, S3 + H2 is separated (Figure 2) from all the wines produced with S3 (both as single and mixed starters), indicating a potential higher influence of H2 strain on wine characteristics than the other two *H. uvarum strains*.

In order to study the interaction among the two yeast species, the S3 + H2 combination was tested in double-compartment fermentor previously described (Renault et al., 2013) to evaluate the influence of cell-to-cell contact during mixed fermentation. During this step, two conditions were tested: NS, in which each compartment was inoculated with both strains, and S condition, in which the two strains were inoculated separately in each compartment.

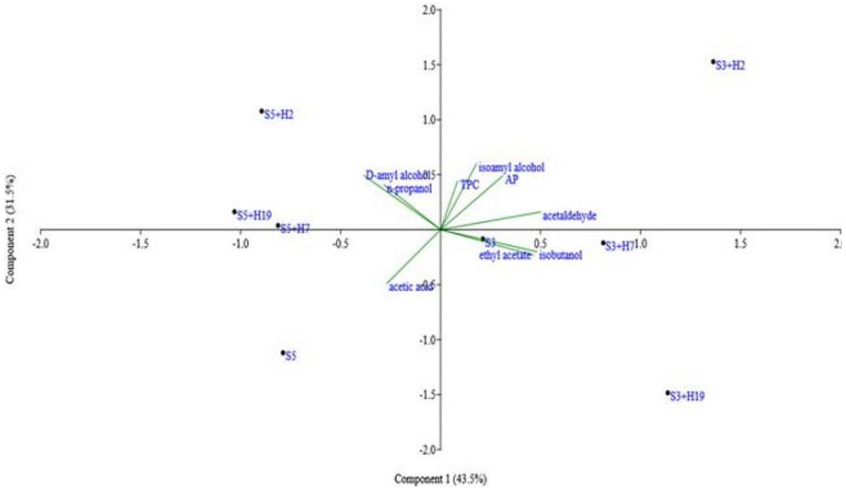

FIGURE 2 Principal component analysis of wines obtained by inoculating two *S. cerevisiae* strains alone (S3 and S5) and in co-fermentation with three *H. uvarum* strains (H2, H7 and H19) [Colour figure can be viewed at wileyonlinelibrary.com]

3.2.1 | Evolution of fermentative process

As regards the progress of fermentative process, the maximum CO_2 production was similar in both modalities, as shown in Figure 3. In contrast, the duration of lag phase was higher in the fermentation per- formed by inoculating the strains mixed together (NS modality) than the fermentation inoculated with the two strains physically separated (about 31 and 13 h, respectively, Figure 3). However, the fermentation duration was lower in NS than in S modality (about 195 and 227 h, respectively, Figure 3). The evolution of yeast population during fermentative process was monitored by viable count on WL medium in both compartments of double fermentor in S and NS modalities. In S modality, the effectiveness of physical separation was confirmed by the absence of contamination from each compartment to the other one (data not shown). The analysis of *H. uvarum* population (Figure 4a) revealed that in NS modality, *H. uvarum* cell counts were very similar in both the compartments, by demonstrating the homogeneity in yeast population between the two compartments. The evolution of yeast cells during the process followed the same trend in both the inoculum modalities, with an increase of yeast cells during the first 50 h, after that the *H. uvarum* population starts to decrease. However, in S modality, *H. uvarum* population was higher than yeast cells detected in NS during all the process. Furthermore, after 118 h of fermentation in NS condition, no *H. uvarum* colonies were found on plates, whereas in S condition at the same time, the viable cell count was 1.0×10^2 cells/ml and no growth was observed only after 168 h of fermentation.

The evolution of *S. cerevisiae* population in the two inoculum modalities (Figure 4b) confirmed that in NS condition, similar cell count was observed in both the compartment, as already reported for *H. uvarum*. No high differences in *S. cerevisiae* cell count between the two inoculation modalities were found during all the fermentative process, except that population reached a maximum earlier in NS modality.

Based on these results, the viability of *H. uvarum* seems affected by the contact with *S. cerevisiae* cells, whereas the physical contact between the two species did not affect *S. cerevisiae* viability.

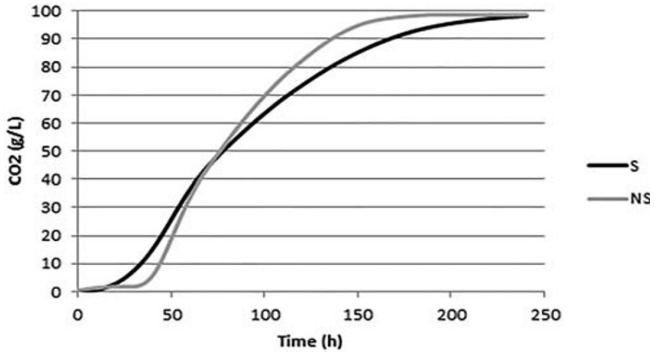

FIGURE 3 Fermentative kinetics in double-compartment fermentor inoculated with *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* in two modalities: each species in separated (S) compartments or two species together (nonseparated [NS])

3.2.2 | Analysis of wines

The analysis of the main parameters detected in the experimental wines obtained in the two conditions is reported in Table 2. As shown in the table, both the starter cultures completed the fermentation; in fact, average residual sugar concentrations varied from 0.7 to 1 g/L, and no statistically significant differences were found between the two modalities and the two compartments. Concentrations of the main fermentation products, that is, ethanol, glycerol and volatile acidity, were similar in both compartments of the double fermentor in NS condition and also similar to those determined in the S modality (no significant differences for final concentrations) (Table 2).

The experimental wines obtained at the end of the fermentations were analysed also for the content of secondary compounds usually present in high concentrations in wines, such as acetaldehyde, ethyl acetate, *n*-propanol, isobutanol, amyl alcohols and the parameters affecting nutraceutical value of wine, such as TPC and AP (Table 2). No statistically significant differences were found between wines

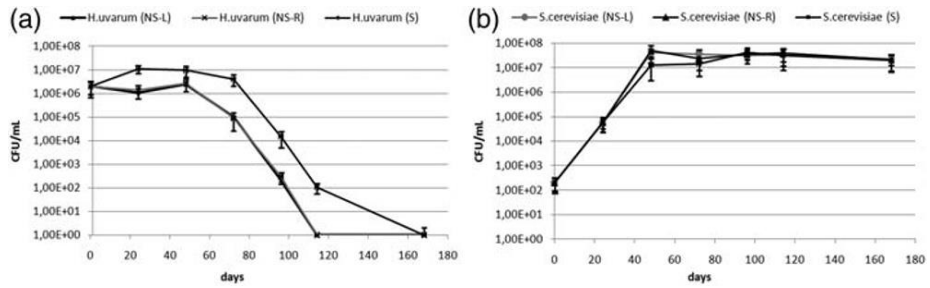

FIGURE 4 Cell evolution, expressed as colony-forming units per millilitre, of (a) *Hanseniaspora uvarum* and (b) *Saccharomyces cerevisiae* in double fermentor by following two modalities of inoculum: each species in separated (S) compartments or two species together, nonseparated (NS)-L (left compartment) and NS-R (right compartment)

TABLE 2 Chemical characteristics of experimental wines obtained in double-compartment fermentor by following two modalities of inoculum: each species in separated (S) compartments or two species together, nonseparated (NS)-L (left compartment) and NS-R (right compartment)

Compounds	HU S	SC S	NS-L	NS-R
EtOH (% Vol)	12.33 ± 0.31	12.33 ± 0.33	12.22 ± 0.22	12.25 ± 0.30
Residual sugars (g/L)	1.01 ± 0.28	0.85 ± 0.21	0.70 ± 0.14	0.80 ± 0.28
Acetic acid (g/L)	0.16 ± 0	0.16 ± 0.01	0.09 ± 0.03	0.11 ± 0.03
Glycerol (g/L)	6.91 ± 0.28	7.01 ± 0.21	7.12 ± 0.25	7.16 ± 0.09
Acetaldehyde	47.22 ± 0.93	47.25 ± 1.05	47.10 ± 0.71	48.55 ± 1.05
Ethyl acetate	45.23 ± 2.12^{a}	48.71 ± 0.84^{a}	27.25 ± 0.01^{b}	29.72 ± 0.71^{b}
n-Propanol	22.92 ± 0.77^{a}	24.39 ± 0.40^{a}	15.91 ± 0.10^{b}	16.55 ± 0.41^{b}
Isobutanol	30.30 ± 1.29	32.26 ± 0.24	31.12 ± 0.12	32.58 ± 1.08
D-amyl alcohol	60.07 ± 2.42^{a}	63.25 ± 0.67^{a}	80.90 ± 3.08^{b}	82.26 ± 1.19^{b}
Isoamyl alcohol	106.43 ± 7.41^{a}	116.20 ± 2.07^{a}	193.84 ± 3.75^{b}	201.57 ± 5.66^{b}
TPC	237.50 ± 2.14^{a}	256.51 ± 6.36^{a}	287.00 ± 4.96^{b}	298.50 ± 6.36^{b}
AP	0.51 ± 0.12^{a}	0.55 ± 0.07^{a}	0.66 ± 0.04^{ab}	0.94 ± 0.09^{b}

Note: Average values of two repetitions ± standard deviations. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$). HU and SC, fermentor compartment inoculated with *S. cerevisiae* and *H. uvarum* separately; LS and RS, left and right compartments, respectively, of the double fermentor inoculated with *S. cerevisiae* and *H. uvarum* together in each compartment. Acetaldehyde, ethyl acetate, *n*-propanol, isobutanol, D-amyl alcohol and isoamyl alcohol are expressed as mg/L. TPC (total polyphenols content) is expressed as mg gallic acid/L. AP (antioxidant power) is expressed as Trolox Equivalent Antioxidant Capacity (TEAC) mmol/ml

from the two sectors in both inoculum modalities for all the compounds, confirming the homogeneity of the medium in both compartments, despite the physical separation of the two yeast populations. By comparing the inoculum modality, significant differences between S and NS modalities were found for ethyl acetate, *n*-propanol and amyl alcohols. When *S. cerevisiae* and *H. uvarum* strains were inoculated in the same compartment, lower amount of ethyl acetate and *n*- propanol and higher amount of amyl alcohols were found than those detected in the wines obtained by the two strains physically separated.

Furthermore, ester concentrations were measured both at the end of the fermentation and at 40% of alcoholic fermentation completion (Table 3), in order to better understand the influence of cell con- tact on esters formation. No significant differences in ester content were found between the wines obtained in the two compartments in both the inoculum conditions confirming the homogeneity of the fermentation medium between left and right sides, whereas high differences were found between S and NS modalities. The inoculum of both yeasts together allowed the highest ester concentration (5,460 and 5,705 μg/L in left and right side, respectively, Table 3). This difference was mainly due to the increase of almost all the esters detected in high concentrations (more than 10 μg/L), mainly isoamyl acetate, ethyl exhanoate and phenylethyl acetate, which were found at about double concentration in NS modality than in S condition. Differences were detected also for esters produced at low concentration (less than 10 μg/L), although at lesser extent than other classes of esters (Table Sa-b). This behaviour was confirmed also by the analysis of ester concentration at 40% of alcoholic fermentation completion (Table 3). Thus, at 40% of alcoholic fermentation, the NS inoculum modality yielded wines containing higher amount of esters than the experimental wines obtained by inoculating the two species separately.

The discrimination of inoculum modalities carried out by PCA based on the 32 ester concentrations represented about 87% of

TABLE 3 Esters concentrations (μg/L) in wines obtained by inoculating *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* in double fermentor after 40% of the alcoholic fermentation and at the end of process

Esters	HU-40	SC-40	LS-40	RS-40	HU	SC	LS	RS
Major esters								
Ethylpropanoate	42.35 ± 2.25	49.13 ± 1.94	33.75 ± 7.47	33.99 ± 5.41	70.94 ± 9.61	70.83 ± 7.12	64.70 ± 3.11	58.81 ± 8.23
Ethylisobutyrate	4.09 ± 0.46	3.77 ± 0.36	3.78 ± 0.66	4.19 ± 0.88	10.51 ± 0.69	10.59 ± 0.45	9.21 ± 1.85	9.89 ± 2.00
Propyl acetate	21.26 ± 4.57	18.89 ± 1.53	17.96 ± 3.44	20.27 ± 4.39	16.43 ± 4.79	16.28 ± 4.94	13.40 ± 1.23	17.37 ± 4.70
Methylbutyrate	110.29 ± 6.47	119.05 ± 8.51	134.09 ± 10.69	145.89 ± 15.27	67.79 ± 3.71	69.59 ± 11.30	86.38 ± 9.60	88.44 ± 5.12
Ethylbutyrate	57.98 ± 12.32	50.06 ± 2.78	71.58 ± 5.39	76.46 ± 0.22	110.41 ± 2.50^{a}	109.37 ± 4.23^{a}	129.56 ± 7.74^{ab}	137.35 ± 9.21^{b}
Isoamyl acetate	1836.98 ± 82.72^{a}	1857.49 ± 44.29^{a}	2646.54 ± 33.47^{b}	2948.47 ± 59.98^{b}	1907.81 ± 2.44^{a}	1937.57 ± 3.01^{a}	3319.14 ± 66.43^{b}	3515.87 ± 58.36^{b}
Ethylvalerate	25.08 ± 2.25	30.56 ± 4.46	40.71 ± 3.37	45.45 ± 8.05	24.01 ± 7.23^{a}	29.64 ± 0.74^{a}	50.63 ± 2.96^{b}	54.10 ± 6.11^{b}
Ethylhexanoate	170.64 ± 8.56^{a}	218.95 ± 15.02^{a}	307.04 ± 11.38^{b}	345.58 ± 18.79^{b}	172.05 ± 13.92^{a}	171.90 ± 10.28^{a}	269.31 ± 9.56^{b}	276.83 ± 16.39^{b}
Hexyl acetate	68.48 ± 0.48^{a}	77.03 ± 7.14^{a}	111.59 ± 0.59^{b}	120.54 ± 11.33^{b}	21.33 ± 2.39^{a}	21.59 ± 2.92^{a}	35.93 ± 2.33^{b}	37.40 ± 4.79^{b}
Ethyloctanoate	358.97 ± 10.37^{a}	348.59 ± 28.61^{a}	523.49 ± 15.04^{b}	589.10 ± 27.03^{b}	300.74 ± 23.53^{a}	267.85 ± 11.27^{a}	445.72 ± 24.03^{b}	438.27 ± 25.80^{b}
Ethyldecanoate	88.84 ± 7.47^{a}	83.81 ± 4.25^{a}	233.91 ± 14.93^{b}	252.32 ± 16.09^{b}	156.20 ± 10.63^{a}	136.81 ± 7.25^{a}	182.47 ± 9.83^{ab}	193.05 ± 7.60^{b}
Ethyldodecanoate	10.20 ± 0.20^{a}	10.44 ± 0.77^{a}	40.17 ± 5.16^{b}	43.26 ± 6.02^{b}	23.02 ± 5.81	11.03 ± 1.53	19.18 ± 0.86	18.23 ± 2.49
Phenylethyl acetate	296.75 ± 18.06^{a}	231.97 ± 10.04^{a}	722.42 ± 35.19^{b}	800.63 ± 41.58^{b}	340.98 ± 23.49^{a}	347.46 ± 12.87^{a}	808.35 ± 9.67^{b}	833.95 ± 18.76^{b}
Σ Minor esters	7.72 ± 3.19^{a}	12.13 ± 1.96^{ab}	18.27 ± 1.39^{b}	20.47 ± 1.26^{b}	18.82 ± 1.51^{a}	17.03 ± 1.35^{a}	26.23 ± 1.22^{b}	26.20 ± 2.14^{b}
Σ Total esters	3089.26 ± 139.62^{a}	3111.79 ± 165.19^{a}	4899.77 ± 121.49^{b}	5446.59 ± 178.59^{b}	2941.01 ± 28.31^{a}	3217.53 ± 28.58^{a}	5460.18 ± 145.62^{b}	5705.74 ± 153.09^{b}

Note: Values are expressed as mean ± standard deviation of two independent replicates. HU and SC, fermentor compartment inoculated with *S. cerevisiae* and *H. uvarum* separately; LS and RS, left and right compartments, respectively, of the double fermentor inoculated with *S. cerevisiae* and *H. uvarum* together in each compartment. Different superscript letters in the same row, in function of the time of fermentation process, correspond to statistically significant differences (Tukey's test, $p < 0.05$). Minor esters = ester present in quantities < 10 μg/L..

variance for PC1 and PC2 axes (Figures 5 and S1). Ester concentrations at 40% of alcoholic fermentation were different from ester detected at the end of the fermentative process (right and left side of scatterplot, respectively). Furthermore, both at 40% and at the end of fermentation, the wines obtained by inoculating the strains mixed together (LS-40, RS-40 and LS and RS, respectively) were separated from the wines produced by the strains inoculated in S compartments (HU-40, SC-40 and HU and SC, respectively), being located in upper and lower part of the scatterplot, respectively. Ester profiles of wines from the two different compartments in the same inoculation modality were quite similar, confirming the homogeneity of samples between the two compartments.

In conclusion, the results from fermentations showed that the metabolic behaviour of *S. cerevisiae* and *H. uvarum* strains tested in this study seems to be highly influenced by cell to cell contact.

4 | DISCUSSION

In this paper, we studied fermentative behaviour of mixed starter cultures composed by wild *S. cerevisiae* and *H. uvarum* strains. Although different non-*Saccharomyces* yeasts are present in the first days of fermentation, we have chosen *H. uvarum* as it is one of the most abundant yeast species found on grapes, irrespective of location and grape variety, and in grape must throughout the fermentation process (Albertin et al., 2016; Martin et al., 2018). This species has been also proposed as wine starter in mixed fermentation with *S. cerevisiae* (Masneuf-Pomarede, Bely, Marullo, & Albertin, 2016; Tristezza et al., 2016) and in some situations *H. uvarum* could be found until the end of fermentation (Hu, Jin, Xu, & Tao, 2018). Therefore, studies on yeast population dynamics during inoculated fermentation with mixed starter cultures will help in understanding the interactions between yeast strains included in the mixed starter and the final impact of *H. uvarum* on wine quality. In this study, the growth pattern of yeast populations during wine fermentations per- formed at lab scale with different strain combination of *H. uvarum* and *S. cerevisiae* was established. Our results showed that *H. uvarum* strains were not detected in the middle-final phases of fermentation, although in our case the persistence of non-*Saccharomyces* strains was higher than the levels usually reported. In fact, in our study, a high decrease of the cell count of *H. uvarum* was observed after 5–6 days of fermentation, with a similar behaviour for all the mixed starters tested, whereas usually non-*Saccharomyces* yeasts are not detected after the first 3–4 days of fermentation (Fleet, 2003; Moreira, Mendes, Guedes de Pinho, Hogg, & Vasconcelos, 2008). The experiments performed with the three indigenous *H. uvarum* strains, co-inoculated with two different *S. cerevisiae* strains, underlie the fact that apiculate yeasts can survive throughout the alcoholic fermentation for longer periods than previously thought, in agreement with other reported results (Moreira et al., 2011). During the first 2 days of fermentation, in all the mixed starters, *H. uvarum* population exhibited a particular trend that is a high reduction of cell count. This behaviour might be correlated with the use of sulphited grape must for fermentation, as sulphite is an abiotic factor affecting significantly the growth of this species (Albertin et al., 2014; Cocolin & Mills, 2003; Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). Contrary to *Saccharomyces*, among *Hanseniaspora* strains, the ability to tolerate this antiseptic is undoubtedly a rare characteristic (Grangeteau et al., 2016). The *H. uvarum* strains tested in this study were already selected for sulphur dioxide tolerance (Guaragnella et al., 2020), and consequently, we tested these three *H. uvarum* strains in sulphited grape must in co-culture with *S. cerevisiae*. The decrease in *H. uvarum* cells in the first 2 days of fermentation might be correlated to the presence of *S. cerevisiae* strain, that, although at low cell numbers, can compete with *H. uvarum* cells, determining a reduction in cell count, after that the cell count of *H. uvarum* starts to increase (Figure 1). This initial reduction of *H. uvarum* cell number may be attributed to the time needed by *H. uvarum* strains to adapt to the contemporary presence of SO_2 and

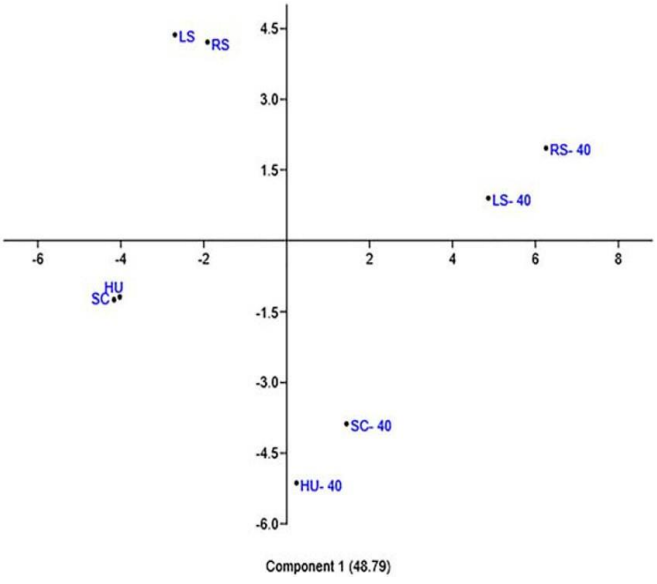

FIGURE 5 Principal component analysis (PCA) based on the ester concentrations detected in wine obtained by inoculating *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* in double fermentor after 40% of the alcoholic fermentation (HU-40, SC-40, LS-40, RS-40) and at the end of process (HU, SC, LS, RS). HU and SC, fermentor compartment inoculated with *S. cerevisiae* and *H. uvarum* separately; LS and RS, left and right compartments, respectively, of the double fermentor inoculated with *S. cerevisiae* and *H. uvarum* together [Colour figure can be viewed at wileyonlinelibrary.com]

S. cerevisiae strain.. In addition, in mixed fermentation, *H. uvarum*, although present at lesser extent than *S. cerevisiae*, has determined differences statistically significant among the wines obtained by mixed and single starters (Table 1). These results confirmed that when yeast strains developed together in mixed fermentations, they do not grow together passively, but rather, they interact each other, producing different compounds or different amounts of compounds affecting the chemical and aromatic composition of wines (Anfang, Brajkovich, & Goddard, 2009; Ciani et al., 2016; Ciani, Comitini, Mannazzu, & Domizio, 2010; Howell, Cozzolino, Bartowsky, Fleet, & Henschke, 2006).

Different authors reported that apiculate yeasts play a significant role in producing aroma compounds, contributing to the development of 'flavour phenotypes' different from those obtained by *S. cerevisiae* single starter cultures (Martin et al., 2018; Swiegers, Bartowsky, Henschke, & Pretorius, 2005). Although we have demonstrated that apiculate strains affect wine aroma, we did not observe high level of acetic acid in the experimental wines, a characteristic usually associated to the metabolism of apiculate yeasts (Romano et al., 2003). In fact, the maximum level of acetic acid detected in the experimental wines was 438 mg/L (Table 1). This level was below the legal limit of 1.2 g/L of acetic acid (Office Internationale de la Vigne et du Vin, 2009), established as higher values of this compound, which can confer to wine a detrimental acidic flavour (Bely, Rinaldi, & Dubourdieu, 2003). The high biodiversity among *H. uvarum* strains was reported also by other authors (Andorrà et al., 2010; Suzzi et al., 2012; Tofalo et al., 2016), thus representing an useful tool to design the most suitable starter strain. Although the influence of non- *Saccharomyces* yeasts on aromatic compounds of wine is well-documented, few data on chemical modifications induced by these yeasts on phenolic compounds are available until now. Some authors (Medina, Boido, Dellacassa, & Carrau, 2018) reported for the first time the ability of species of the genera *Hanseniaspora* and *Metschnikowia* to produce phenolic compounds. In our study, all the experimental wines obtained by the mixed starters with both the *S. cerevisiae* strains were characterized by higher TPC than wines from single starter fermentation, underlying the role of *H. uvarum* strains in improving this characteristic. Considering that phenolic compounds can influence the sensory characteristics of wine quality, such as colour indexes, these mixed starters might represent a useful tool to enhance the red wine colour perception.

The comparison among overall characteristics of the experimental wines obtained by the two *S. cerevisiae* strains and the six mixed starter cultures (Figure 2) revealed, as expected, a major role of *S. cerevisiae* on wine characteristics. However, also the *H. uvarum* strains affected wine parameters at different level in function of apiculate strain.

In consequence of these results, we decided to investigate the interaction between the two species testing S3 and H2 strains in mixed fermentation, by using the double-compartment fermentor pointed out by Renault et al. (2013). Our aim was to evaluate the influence of physical contact between *S. cerevisiae* and *H. uvarum* strains on cell viability and strain metabolic activity.

In our experiments, the same trend in evolution of yeast cells during the process was found both for S cells and cells in physical contact, but we found a high H2 cells number when the two strains were physically separated, indicating an influence of physical contact of *S. cerevisiae* cells on the viability of *H. uvarum* cells. Several studies (Nissen, Nielsen, & Arneborg, 2003; Pérez-Nevado, Albergaria, Hogg, & Girio, 2006; Renault et al., 2013; Taillandier, Lai, Julien-Ortiz, & Brandam, 2014; Wang et al., 2015; Wang, Mas, & Esteve-Zarzoso, 2016) have raised evidence that the early death of non-*Saccharomyces* yeasts, for a long time assigned to their low capacity to withstand the selective growth factors of the wine environment, is correlated to the interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts.

The interactions directly involved in the antagonism exerted by *S. cerevisiae* against non-*Saccharomyces* yeasts involve the production of killer-like toxins, such as antimicrobial peptides (AMPs) (Albergaria et al., 2010; Branco et al., 2014; Comitini, Ferretti, Clementi, Mannuzzu, & Ciani, 2005; Nehme, Mathieu, & Taillandier, 2010; Osborne & Edwards, 2007) and the death mediated by a cell-to-cell contact.

The early death of *Hanseniaspora* species in mixed cultures with *S. cerevisiae* was explained as induced by unknown toxins produced by *S. cerevisiae* (Pérez-Nevado et al., 2006) or unknown metabolites excreted to the medium and not by cell-to-cell contact with *S. cerevisiae* (Wang et al., 2015). Renault et al. (2013), investigating the effect of physical separation on the growth and fermentation kinetics of *Torulaspora delbrueckii*/*S. cerevisiae* mixed cultures, proposed that cell–cell contact may involve the direct physical contact through receptor/ligand-like interactions or the production by *S. cerevisiae* of soluble molecules, lethal for non-*Saccharomyces* yeasts. This might be a defensive strategy used by *S. cerevisiae* to combat other microorganisms and become prevalent during alcoholic fermentation. The researches investigating the cell-to-cell contact mediated mechanisms were mainly addressed to study the direct influence of these phenomena on cell viability or the production of metabolites affecting cell cultivability. To our knowledge, very few studies have been conducted to evaluate the influence of cell-to-cell contact on chemical characteristics of wine. In our study, the inoculation modality, that is, cell physical contact, affected significantly the content of some aromatic compounds, such as ethyl acetate, *n*-propanol, amyl alcohols (Table 2) and some esters (Table 3).

In particular, we found that the wine produced by inoculating H2 and S3 strains in the same compartment contained very higher levels of some esters, mainly isoamyl acetate, ethyl exhanoate and phenylethyl acetate, than the amounts detected in the wine obtained by inoculating each strain in an S compartment. The same behaviour was observed by analysing the ester concentration at 40% of alcoholic fermentation completion.

Also nutrient level has been reported to play a role in yeast inter- action, because of different preferences for nitrogen sources by *Saccharomyces* and non-*Saccharomyces* during wine fermentation (Andorrà, Berradre, Mas, Esteve-Zarzoso, & Guillamón, 2012). Further- more, changes detected in the

transcriptome of *S. cerevisiae* appear to result from a cellular response to changes in nutrient availability in the fermenting must as a consequence of *H. guilliermondii* metabolic activity (Barbosa, Mendes-Faia, Lage, Mira, & Mendes-Ferreira, 2015). It is well known the effect of nitrogen availability on production of the metabolites affecting wine aroma profile, such as esters. These authors demonstrated that the presence of *H. guilliermondii* dramatically influenced the expression patterns of genes associated to various flavour-active compounds. The same effect could be supposed in our study, in which *H. uvarum* strain in physical contact with *S. cerevisiae* might influence the expression of genes related to the production of aromatic compounds, whereas this influence cannot be registered when the two strains are physically separated. In agreement with Barbosa et al. (2015), we can speculate that the influence of non- *Saccharomyces* yeasts on wine aroma is related not only to the production of aromatic compounds by non-*Saccharomyces* yeasts different from those produced by *S. cerevisiae* but also in consequence of the influence of non-*Saccharomyces* yeasts on *S. cerevisiae* through modulation of the grape must nutritional properties.

5 | CONCLUSION

In sum, this work confirms the role of interactions between *S. cerevisiae* and non-*Saccharomyces* yeasts in affecting metabolic activity of yeast strains included in mixed starter, leading to new findings regarding the role of interactions on wine quality. The rational design of mixed starter cultures should take into account not only the screening addressed to exploit positive features of non- *Saccharomyces* strains, but it also is of utmost importance to carefully characterize the microbial interactions established among the specific strains composing the mixed starter cultures. By considering the increasing interest towards the use of mixed starter cultures to produce wines with peculiar traits, these new knowledges can help in the successfully formulation of mixed starter cultures able to improve the wine quality, which represents an useful tool for winemakers interested in differentiating their wines in a hugely competitive market.

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SUPPORTING INFORMATION

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Exploitation of technological variability among wild non-*Saccharomyces* yeasts to select mixed starters for the production of low alcohol wines

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Abstract. Increase of the sugar content in grape must, and consequently, alcohol levels in wine are some of the principal problems affecting the winemaking industry. High alcohol content can compromise wine quality, creating sensory imbalances, as well as decreasing the perception of some flavors. The technological approaches proposed at this aim, although allowing achievement of the purpose, can determine negative influence on quality of wine. A promising strategy is based on the use of specific microorganisms, such as selected yeast strains, mainly non-*Saccharomyces*, able to convert grape must sugars towards secondary metabolites rather than ethanol. This study aims at screening of wild non-*Saccharomyces* strains in order to identify those suitable for the use in mixed starter for the production of wine with reduced alcohol content and, at the same time, with improved aromatic characteristics.

1. Introduction

In recent decades there has been an increase in the alcohol content of wine mainly linked to climate changes, which have led to the production of grapes with high sugar content and consequently the production of wines with high level of ethanol.

High alcohol content not only compromises wine quality (increasing hotness and bitterness perception) [1], but also represents an economic and social problem. This is related both to taxes imposed in some countries for the higher alcohol drinks and to demands of modern consumers, preferring wines with a low percentage of ethanol and fruity favour. To meet consumer expectations, winemaking industry is focused on the production of wine with a moderate ethanol level and a peculiar organoleptic profile [2].

The technological approaches, proposed at this aim, include the use of strategies in vineyard (optimization of the harvest date to obtain a reduction of sugar in grape), application of pre-fermentation or winemaking practices (removal of sugar from grape must) and post- fermentation practices (distillation, nanofiltration, reverse osmosis). Although those techniques allow achieving this purpose, they can have a negative influence on quality of wine [3].

To avoid this inconvenience, research aims to apply biotechnological approaches, principally based on the selection of new starter cultures possessing specific technological properties, such as lower sugar-alcohol conversion and high enzymatic activity to improve organoleptic quality.

In this context, non- *Saccharomyces* yeasts deserve special attention. Yeasts "non-*Saccharomyces*" include different genera and species present in the early stages of spontaneous fermentation. Generally, they are not very tolerant to high ethanol level and unable to complete the fermentation process. They were considered in the past as undesirable or spoilage agents. Recently, these yeasts have been re-evaluated as a potential biotechnological tool to improve wine composition. Their ability to produce hydrolytic enzymes, glycerol, mannoproteins and other metabolites of oenological relevance allows to enhance the quality of wine [4,5].

A promising approach to reduce wine alcohol content could be to exploit the oxidative fermentative metabolism of some non-*Saccharomyces* yeasts to consume partly the sugar present in the must by respiration rather than fermentation process. As a consequence, the co- inoculation practice of grape must with a selected mixed starter (non-*Saccharomyces*/*S. cerevisiae*) with partial and controlled aeration of grape juice [6, 7] can be considered as one of the best strategies to obtain wine with a reduced content of alcohol and enhanced aromatic quality.

This work is addressed to the characterization of indigenous non-*Saccharomyces* strains by physiological and biochemical analysis, such as resistance to antimicrobial compounds, growth capacity in presence of ethanol and high sugar concentration, β-glucosidase activity, in order to identify the strains suitable for the use as mixed starter for the production of low alcohol wine.

2. Materials and methods

2.1. Yeast strains

Twenty-nine strains of non-*Saccharomyces* species, belonging to the Collection of Fermenting Yeasts of Basilicata University, were used (Table 1). They were isolated from spontaneous fermentation of grapes of

Table 1. Non-*Saccharomyces* strains used in the present study.

Yeast species	Strain code
Hanseniaspora guilliermondii	SNM1 1-1, SNM1 3-2, SNM3 1-1, SNM H, AP 9, TS B, ER 3, TM 4-1, TM 5-1 ND 1
H. osmophila	425, 365, LC 2-1
Torulaspora delbrueckii	
Metschnikowia pulcherrima	Mpr 2-49, Mpr1-7, Mpr 2-4, 563, 683, Mpr 1-3, Mpr 2-3, M 1, M 2, M 3, SIA 1, SIA 4
Saccharomycodes ludwigii	APG, SIA 2
Pichia fermentans	LM 5-3, SGT 3-1

different origin. The strains were maintained on YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, 2% w/v agar).

2.2. Technological characterization

2.2.1. Resistance to SO_2 and $CuSO_4$, and H_2S production

The resistance to sulphur dioxide (SO_2) was tested by evaluating the growth of non-*Saccharomyces* yeasts on pasteurized agarized grape must added with increasing doses of SO_2 (0, 25, 50, 100, 125, 150, 175, 200 mg/L), whereas the copper resistance was evaluated on agarized Yeast Nitrogen Base (YNB) without amino acids, added with increasing concentrations of $CuSO_4$ (0, 50, 100, 200, 300, 400, 500 µM). The yeast strains were spot-inoculated on the medium (concentration of about 10^6 cell/ml) and yeast growth was evaluated after 48 hours at 26 °C.

The ability of the strains to produce different amounts of hydrogen sulphide (H_2S) was tested on bismuth-containing indicator medium BIGGY agar. The medium was spot-inoculated, and plates incubated at 25 °C per 48 hours. The production of H_2S was evaluated in function of yeast spot browning by an arbitrary scale from 0 (white color = no production), 1 (hazelnut = low production), 2 (brown = significant production) 3 (coffee = high production).

2.2.2. Growth in Ethanol and high sugars concentration

The growth test in ethanol was carried out in microplates following the method reported by Eglezos et al. [9] with some changes. As medium, it was used YNB with amino acids, supplemented with a sterile glucose solution (20 g/l), added with different ethanol amounts to obtain final concentrations of 0, 8, 12, 14% (v/v).

The same procedure was used to evaluate the growth in presence of high sugar concentrations, adding to the substrate (YNB) increasing amounts of glucose and fructose, in the same ratio, to reach the final concentrations of 2, 20, 40%.

Yeast cells (about 10^6 cell/ml) were inoculated in the medium and the microplates were incubated at 26 °C (two days for ethanol test and three days for sugar test). The optical density was measured at 630 nm and the cell growth was calculated as a ratio (%) between the strain growth in the medium with and without addition of ethanol or sugars.

2.2.3. β-glucosidase activity

This enzymatic activity was evaluated both by qualitative and quantitative methods.

The qualitative β-glucosidase activity was determined on a synthetic medium containing 0.67% YNB with amino acids, 0.5% arbutin, added with 4 ml of ferric ammonium citrate and 2% agar. The strains were spot-inoculated, and the plates incubated at 25 °C for 5 days. The presence of β-glucosidase activity was related to the browning of strain colony.

The quantitative β-glucosidase activity was measured following the method previously described Manzanares et al. [11], with minor changes. Yeasts strains were grown in 25 ml of YNB broth (without ammonium sulphate and amino acids) added with glucose (2%) and ferric ammonium citrate (1%). After incubation at 26 °C for 24 hours in an orbital shaker, cell suspension (10^6 cell/ml) was centrifuged (3000 rpm for 10 minutes) and 0.2 ml of supernatant was mixed with 0.2 ml of p-nitrophenyl-β-D- glucoside (pNPG). The samples were incubated at 30 °C for 1 hour and the reaction was stopped by adding 1.2 ml of sodium carbonate. The amount of p-nitrophenol released in the reaction was measured spectrophotometrically at 400 nm and the enzymatic activity was expressed as nmol PNP/mL*h comparing to a calibration line of 4-p-nitophenol solution.

2.2.4. Oxidative stress tolerance

The tolerance to oxidative stress was tested by evaluating strain growth in agarized YPD medium, added with different concentration of H_2O_2 (25, 50, 100, 250 mM), following the protocol reported by Mestre et al. [12]. The different level of strain sensitivity to oxidative stress was correlated to diameter dimension (mm) of the inhibition zone in correspondence of the highest concentration of H_2O_2 tested (250 mM).

2.3. Statistical analysis

All data of the technological parameters were converted into non-dimensional values, assigning the values reported in Table 2.

These values were submitted to cluster analysis, using Ward's method with Euclidean distance by using the statistical package PAST software ver. 1.90 [13].

3. Results

3.1. Technological characterization

3.1.1. Resistance to SO_2 e $CuSO_4$

All the twenty-nine non-*Saccharomyces* strains exhibited the ability to grow in presence of 100 mg/L of SO_2. Significant variability was detected among the strains, even within the same species (Fig. 1). In general, *T. delbrueckii* strains exhibited the highest resistance; in fact, two strains tolerated 200 and one strain 300 mg/L of SO_2 (the highest tolerance level found among the strains). Major variability was recorded within the species *H. guilliermondii*, with strain resistance ranging from 100

Table 2. Adimensional values assigned to technological parameters.

Parameteters	Values			
	0	**1**	**2**	**3**
SO_2 resistance[a]	50	100–125	150–175	200
$CuSO_4$ resistance[b]	<100	100	200	300
H_2S production	no	low	medium	high
β-glucosidase; EtOH /high sugar	V < M-SD	M-SD < V ≤ M	M < V ≤ M + SD	V > M + SD
Oxidative stress[c]	> 10	8–10	5–7	0–4

a = reported as mg/L; b= reported as µM; c = reported as diameter dimension (mm) of inhibition zone.

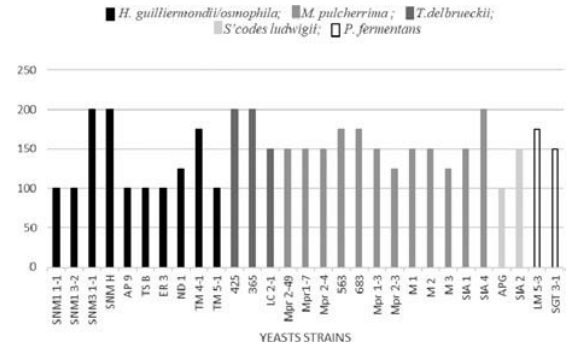

Figure 1. Tolerance level to SO_2.

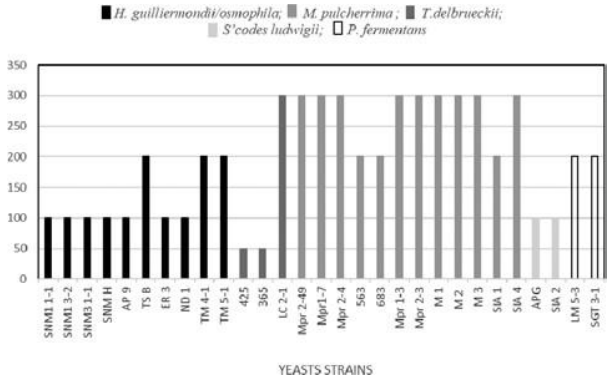

Figure 2. Tolerance level to $CuSO_4$.

to 200 ppm of SO_2, and *M. pulcherrima* strains, which tolerated SO_2 concentrations ranging between 125 and 200 mg/L.

As regards the copper resistance, the 29 non-*Saccharomyces* strains tolerated concentration of $CuSO_4$ between 100 and 300 µM and all the strains did not grow in presence of concentrations higher than 300 ppm of $CuSO_4$ (Fig. 2).

Generally, *M. pulcherrima* strains were more tolerant than *Hanseniaspora* strains; in fact, *M. pulcherrima* grew in presence of 200 and 300 mM of $CuSO_4$, with 9 strains (Mpr 2–49, Mpr 1–7, Mpr 2–4, Mpr 1–3, Mpr 2–3, M1, M2, M3, SIA 4) exhibiting the highest resistance to the compound. All strains did not grow in presence of concentrations higher than 300 ppm of $CuSO_4$. Conversely, *Hanseniaspora* strains tolerated copper content ranging from 100 to 200 mM. Low copper tolerance was exhibited also from two *Torulaspora* and the *S'codes ludwigii* strains

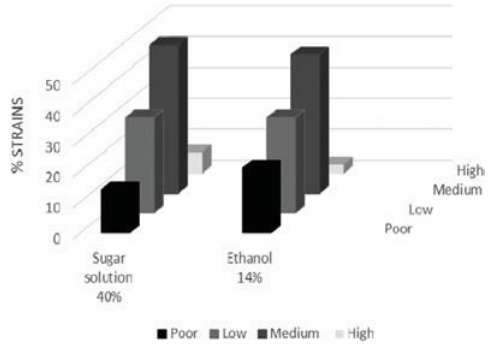

Figure 3. Strain growth in high sugar (40%) and ethanol (14%) concentrations.

3.2. Qualitative production of H_2S

The test for the evaluation of qualitative production of H_2S demonstrated that 79% of yeasts exhibited low production of the compound (hazelnut colonies), mainly strains of *M. pulcherrima* and *H. guilliermondii*, and 21% of strains showed a medium production (brown colour of colonies), exhibited by strains of *P. fermentans*, *T. delbrueckii*, *H. osmophila*, *S'codes ludwigii*.

3.2.1. Growth in ethanol and high sugar concentrations

As regards the test addressed to evaluate the strain tolerance toward high sugar and ethanol concentration, the strains exhibited significant differences among them only in presence of the highest doses of the tested compounds, (14% v/v and 40% for ethanol and sugar, respectively). The strains grew at similar levels for the other doses, both in ethanol and sugar growth test.

About half of the strains exhibited a good cell growth in 14% ethanol (medium growth by 13 strains and high by 1 strain) (Fig. 3).

Similar results were found for growth in high sugar concentrations, 14 strains exhibited medium growth and 2 strains high growth (Fig. 3).

3.2.2. β-glucosidase activity

As regards the qualitative assay of the enzymatic activity, six strains gave negative results (SIA1, SIA 4, 365, LC 2–1, STG 3–1, LM 5–3). Therefore, the quantitative test for β-glucosidase activity was carried out on the 23 strains, resulted positive for the qualitative test.

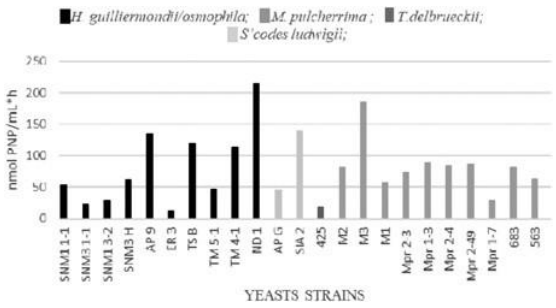

Figure 4. β-glucosidase activity exhibited by 23 strains.

Table 3. Tolerance to oxidative stress of 29 non-*Saccharomyces* strains.

Species	Stress tolerance		
	Low	Medium	High
H. guilliermondii (9)	6	3	–
H. osmophila (1)	–	1	–
T. delbrueckii (3)	3	–	–
S'codes ludwigii (2)	–	2	–
P. fermentans (2)	1	1	–
M. pulcherrima (12)	–	2	10

The results, expressed as nmol PNP/mL*h (Fig. 4), demonstrated that the strains showed a different level of β-glucosidase activity. Some strains exhibited a high enzymatic activity, three strains of *H. guilliermondii* (AP-9, TS-B, TM 4-1), the *S'codes ludwigii* SIA 2, *M. pulcherrima* M3 and *H. osmophila* ND1. These six strains could potentially be used in mixed fermentation to improve the flavor of wines.

3.2.3. Oxidative stress tolerance

The strain tolerance to oxidative stress was evaluated by testing different concentrations of H_2O_2. However, only at the highest dose tested (250 mM) a variability in strain response was found, whereas the concentrations lower than 250 mM did not affected strain growth.

The table reports the results about the oxidative tolerance test obtained by non-*Saccharomyces* species at the higher tested concentration of H_2O_2.

The results (Table 3) show that the strains belonging to *M. pulcherrima* exhibited the highest tolerance to the compound, while the strains of the other tested species presented a low-medium tolerance to 250 mM of H_2O_2.

3.2.4. Statistical elaboration of results from technological characterization

The data obtained by all the tests were converted in adimensional values and the obtained matrix was submitted to cluster analysis in order to differentiate the non-*Saccharomyces* strains. Figure 5 reports Ward's method hierarchical clustering. The dendrogram clearly subdivided the strains in two main groups (a, b).

The group "a" includes strains belonging to the *H. guilliermondii* and *T. delbrueckii* species, whereas the other strains were distributed in the group "b". The principal factors discriminating the two main groups are the ability to grow in presence of high sugar concentration,

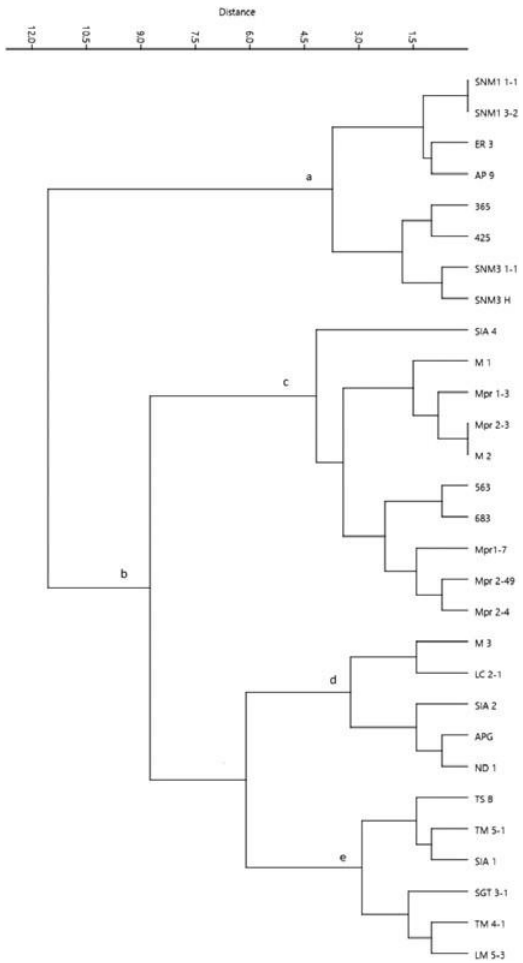

Figure 5. Dendrogram obtained after cluster analysis on data of technological characterization of 23 non-*Saccharomyces* strains.

copper resistance and tolerance to oxidative stress. In fact, the group "a" includes strains exhibiting these traits at the lowest level, whereas strains grouped in "b" were characterized by medium-high ability to grow in presence of high sugar concentration and medium-high tolerance to copper sulphate and oxidative stress.

The ability to tolerate H_2O_2 was more exhibited by the group "b", mainly by the strains of *M. pulcherrima*.

The group "b" can be divided in three subgroups, "c", "d" and "e". The subgroup "c" is the only group composed by strains belonging to the same species, that is *M. pulcherrima*. The characteristics differentiating this cluster are the highest level of tolerance to H_2O_2 and the lowest tolerance to high ethanol concentration.

The other subgroups are composed by strains belonging to different species, such as *P. fermentans, H. guilliermondii, H. osmophila, S'codes ludwigii,* two *M. pulcherrima* strains and one *T. delbrueckii* strain. The characteristic mainly differentiating "d" from "e" subgroup is the β-glycosidase activity, which was low/very low in "d" and medium/high in "e". Furthermore, the subgroup "d" includes the strains showing the best combination of technological parameters tested. In fact, the strains grouped in "d" were characterized by medium/high ability to tolerate high concentration of sugar and ethanol, medium/high β-glycosidase activity and medium level of tolerance to oxidative stress.

4. Conclusions

This preliminary screening of indigenous non-*Saccharomyces* yeasts might be an useful tool to individuate some strains characterized by traits of oenological interest and potential candidates in pure or mixed starter cultures for the production of low alcohol wine. Our results confirm that non-*Saccharomyces* yeasts, previously considered as spoilage microorganisms, can be considered as an interesting source of biodiversity, with positive applications to grape-must fermentation.

In particular, *H. osmophila* and *S'codes ludwigii* exhibited interesting and desirable properties to improve wine sensory profile, such as the highest β-glucosidase activity and the good resistance to osmotic stress, being able to survive in fermentation must condition.

Also *M. pulcherrima* strains showed interesting technological traits, but, due to its sensibility to high concentrations of ethanol, it could be used only in mixed culture with *S. cerevisiae* in order to complete the fermentation process. In particular, all *M. pulcherrima* strains exhbited a high tolerance to hydrogen peroxide, that is one of reactive oxygen species produced by the sugar respiratory catabolism, which is potentially toxic to yeast cell. Non-*Saccharomyces* strains able to tolerate H_2O_2 are suitable to be used in the first stage of fermentation process, under aerobic controlled condition, in order to oxide sugars present in the grape must and consequently to reduce ethanol production.

In conclusion, mixed starter cultures with non-*Saccharomyces* strains, carefully selected in function of wine characteristics and market trends, can be considered an innovative biotechnological tool not only to improve wine quality complexity, but also to satisfy the current challenge of wine industry addressed to "lower alcohol wines.

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