UNIVERSITÀ DEGLI STUDI DELLA BASILICATA
SAFE - Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali

Università degli Studi della Basilicata

PhD in
Agricultural, Forest and Food Sciences

Curriculum
Food Sciences and Engineering

TITLE
"Mixed starter yeasts as a biotechnological tool to produce wine with low alcoholic content"

Scientific Area (SSD)
AGR/16 Agricultural Microbiology

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Cycle XXXIII

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Summary

Recently due to climatic changes a progressive increase in the percentage of ethanol in wines was observed. In fact, the ripening phase of the grapes tends to coincide with hot periods resulting in high accumulations of sugars and production of wines characterized by excessive alcoholic content, with negative notes on the quality of the final product. At the same time, national and international markets request well-structured and full-bodied wines, characterized by high aromatic complexity and moderate alcohol content. Alternative approaches have been proposed for the reduction of alcohol content, including viticultural, pre-fermentation, and post-fermentation practices. The microbiological strategies proposed for reducing the alcohol content include the use of genetically modified yeasts or the use of non-conventional wine yeasts belonging to non-*Saccharomyces* group, selected from the microflora usually present on the grapes and/or in the cellar. This technique, appreciated by producers and consumers, is based on the development of controlled multi-starter fermentations that combine the use of these non-*Saccharomyces* yeasts with *S. cerevisiae*. The enological interest in this group of yeasts is correlated to lower yield in ethanol than *Saccharomyces* as the non-*Saccharomyces* yeasts utilize alternative metabolic pathways during the fermentation of the grape must sugars, and to their ability to release aromas, proteins and other active sensory compounds that are not produced by single fermentation with *Saccharomyces cerevisiae*. These characteristics allow improving the aromatic profile of the wine, reducing at the same time its alcohol content. However, some species can produce high concentrations of some secondary compounds which can have undesirable effects on the aromatic quality of the wine, such as ethyl acetate, and high levels of volatile acidity. Therefore, it is necessary to make a careful selection of the species and the inoculation methods to be used. In this thesis we proposed the use of non-*Saccharomyces* wine yeasts in mixed fermentations to select the most promising combination of strains to be used as a tool to reduce the ethanol content in wines. In the first phase, 29 non-*Saccharomyces* strains belonging to the species *Hanseniaspora guilliermondii/osmophila*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia fermentans*, *Saccharomycodes ludwigii*, were tested for parameters of technological interest, such as production level of hydrogen sulphide (H_2S), resistance to ethanol, SO_2 and copper, evaluation of β-glucosidase activity. The strains selected in this first screening phase were tested in mixed fermentations at laboratory scale, using different inoculation protocols (simultaneous and sequential inoculation). The mixed starter culture,

characterized by suitable oenological properties and able of reducing the ethanol content in the produced wine, was tested in different vinification conditions at laboratory scale using yeast as dried form and as immobilized cells in microcapsules. The aim of this step was to evaluate the influence of the starter formulation on the yeast behavior during the fermentation process. In the last research phase, the mixed starter culture selected during laboratory trials was validated at pilot scale in the cellar to identify the optimal fermentation conditions to be proposed to the winemakers for production of wine with a reduced alcohol content and good aromatic complexity.

Chapter I

Screening on oenological properties of non-Saccharomyces yeasts

Abstract

In the last decades the use of non-*Saccharomyces* yeasts in combination with *Saccharomyces cerevisiae* in wine fermentations is one of the most promising strategies to improve the complexity and quality of wines. To achieve this purpose, a careful selection of non-*Saccharomyces "*wild-type" strains is necessary in order to identify those possessing the best oenological properties. At this aim, the research activity of this work started with a first screening among 29 non-*Saccharomyces* strains, belonging to the Fermenting Yeasts Collection of Basilicata University, chosen among five different species (*Hanseniaspora quilliermondii/ osmophila, Saccharomycodes ludwiqii, Torulaspora delbrueckii, Metschnikowia pulcherrima, Pichia fermentans*). The strains have been evaluated for some important technological traits, such as the resistance to some antimicrobial compounds (such as ethanol, sulfur dioxide and copper) as well as for the presence of enzymatic activities of oenological interest (β-glucosidase). Some of the tested strains showed interesting oenological traits, proving to have influences on the production process and the quality of the wine, therefore they represent suitable candidates to be used as mixed starter cultures with *S. cerevisiae*.

1.1 Introduction

In the last decades two contrasting trends have been observed in the wine sector: an increase in the wine alcohol percentage related to the high sugar content in grapes (principally caused by global warming), and on the other hand the increased market demand for low alcohol drinks. This request emerges both from the growing interest of consumers in the healthy aspects of foods and both for fiscal restrictions (EU and non-EU directives) aimed at taxing drinks that exceed a defined ethanol content.

Furthermore, the wines with high ethanol concentration are characterized by particular sensorial profile, as ethanol increases the perception of bitterness, decreases the sensation of astringency and the fruity aroma.

For these reasons, industrial wine sector and research are working to identify techniques and technologies that allow the reduction of the alcohol content, preserving typical wine sensory attributes.

Different strategies have been proposed to reduce the ethanol content of wine, based on two main approaches: technological methods with application of physical methodologies and biotechnological approaches, based on selection and use of specific microorganisms (Goold et al., 2017).

The technological approach includes techniques that act in different stages of the production process. Some viticulture practices, such as the reduction of the leaf surface or other actions addressed to reduce the sugar content of grape must, the dilution of the must (according to national legislation) by nanofiltration or the use of enzymes with oxidative activity (i.e. glucose oxidase) could be used during the pre-fermentation phase.

Post-fermentation techniques involve the use of membrane systems, vacuum systems and extraction techniques using supercritical CO_2; these approaches, although allow to reduce the ethanol content in the wine, have no influence on the improvement of the aromatic and sensory characteristics.

The biotechnological approaches are based on the use of specific microorganisms, with two main strategies: the development of new strains by use of genetic engineering techniques (although the production of genetically modified microorganisms is negatively perceived by public opinion (Gonzalez et al., 2016)), and yeast strains selection characterized by low sugar-alcohol conversion. In this context, the attention was addressed to non-*Saccharomyces* yeasts, species in the past considered as unwanted or deteriorating agents, but recently re-evaluated as a possible biotechnological tool to improve the wine composition.

In this contest, the main researchers' proposal is to develop a mixed starter consisting of one or more selected species of non-*Saccharomyces* yeasts, together with *S. cerevisiae* (to reduce the risks of sluggish alcoholic fermentation) to improve the chemical and sensorial composition of wine quality.

1.2 Non-*Saccharomyces* yeasts

In 1866, Louis Paster explained the role of yeasts in the biochemical process of grape juice conversion in wine.

Yeasts belong to *Saccharomyces cerevisiae* species are individuated as the main actors of wine fermentation. This microorganism plays an important role in the metabolism of grape sugars, with conversion principally into ethanol and carbon dioxide as well as in the production of secondary metabolites related to the wine aroma (Jolly et al., 2014).

Although *S. cerevisiae* is the main actor of the fermentation process, on the surface of grapes other yeast species are dominant (about 10^3 cells/mL), which during ripening and in the first stages of fermentation reaches high cell concentrations (about 10^7 cells/mL). These species present on the grape surface and in cellar environment are transferred into the must during grape crushing.

However, their participation to fermentation process is limited both by cellar hygienic-sanitary practices (to limit the risk of contamination) and for their low fermentation aptitudes. This caused a progressive non-*Saccharomyces* cells death, while *S. cerevisiae* species (indigenous or inoculated) complete the fermentative process.

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

- Yeasts with aerobic metabolism, for example *Pichia* spp., *Debaryomyces* sp., *Rhodotorula* spp., *Candida* spp. and *Cryptococcus albidus*;
- Apiculate yeasts characterized by limited fermentation capacities, such as *K. apiculata* (*H. uvarum), Kloeckera apis, Kloeckera javanica*;
- Yeasts with good fermentation abilities, such as *Kluyveromyces marxianus, Torulaspora spp. (T. delbrueckii) and Zygosaccharomyces* spp.

In the past, the presence of non-*Saccharomyces* yeasts was linked only to the early stages of fermentation. Recent studies have shown the presence of these yeasts even up to about twelve days from the start of fermentation, with high cell concentrations (about 10^6-10^8cells/mL), in particular in spontaneous fermentations, without massive inoculation of *S. cerevisiae*.

The presence of these species was also found during malolactic fermentations of red wines at concentrations of about 10^4 cell/mL.

The non-*Saccharomyces* yeasts that survive during the process should exhibit a good resistance to ethanol; in fact, it has been shown that *C. stellata* is able to grow also in presence of 12% of ethanol (Jolly et al., 2006); a good ethanol tolerance, has also been documented for the genus *Pichia.*

1.3 Non-*Saccharomyces* yeasts in wine fermentation process

The term "non-*Saccharomyces*" includes several genera and species present during the early stages of spontaneous fermentation, characterized by low resistance to high alcohol concentration and

sulfur dioxide and unable to complete the winemaking process in the hostile conditions established during the grape must fermentation.

In the past, these yeasts were considered as spoilage agents and often associated to slow fermentations or anomalous analytical profiles of wines. The negative influence on the wine organoleptic quality is correlated to the production of some metabolites which, if produced in high quantities, lead to the formation of unpleasant odors and flavors. In fact, some yeast species present during wine fermentation produce the same metabolites, but in different amounts, and the different quantitative level determines their positive or negative contribute to the wine composition.

In recent decades, in the wine sector is growing the interest on the use of multi-starter fermentations that include both the *S. cerevisiae* species and some selected non-*Saccharomyces* strains. Non-*Saccharomyces* yeasts are currently being studied particularly for their metabolic activities and their capability to influence the “wine character", offering the possibility of creating a product with greater aromatic complexity that recalls the originality of spontaneous fermentations. The main application of non-*Saccharomyces* yeasts in wine fermentation concerns their positive contribution to aroma.

Non-*Saccharomyces* yeasts can be classified into the so-called “neutral” yeasts, whose presence is linked to a reduced production of aromatic compounds, and yeasts which strongly contribute to the formation of the wine flavor.

In general, most of grapes aromatic compounds are present as "non-volatile" glycosylated form, and only by the hydrolytic action of β-glucosidase enzymes are released as volatile compounds. Unlike *S. cerevisiae*, many species of non-*Saccharomyces* yeasts, such as those belonging to the genera *Debaryomyces, Hansenula, Candida, Pichia, Hanseniaspora*, have genes codifying for this enzymatic activity, and improve the sensory profile of wine by releasing aromatic compounds, such as terpenes and norisoprenoids, that contribute to the formation of the final bouquet (Fia et al., 2005).

In addition to the production of aromatic compounds, non-*Saccharomyces* yeasts play other positive actions for the quality of the wine, including the production of glycerol (compound present in high amounts in wine, after ethanol and carbon dioxide), which contributes to softness, sweetness and complexity of wines. The production of glycerol is a character linked to the yeast species and depends on the chemical-physical conditions of the must. In general, the apiculate yeasts produce lower quantities than other non-*Saccharomyces* species, the genera *Candida* is characterized by a high production of this compound (from 9 to 14 g / L) (Garcia et al., 2018). However, the increase of glycerol concentration is often associated with a high level of acetic acid, therefore it is necessary an accurate yeasts selection to obtain a sensory balance in the production of these two compounds. The production of acetic acid such as glycerol is characteristic of yeast species, but it can also vary among strains belonging to the same species. The presence of some non - *Saccharomyces* yeasts are associated with anomalous values of volatile acidity. The acceptability range for this compound

in wines is 0.3-0.6 g / L, higher concentrations have a negative influence on product flavor. Among the non-*Saccharomyces* species*, T. delbrueckii* has similar fermentative characteristics to that of *S. cerevisiae* and it produces low levels of volatile acidity (Taillandieret a., 2014), with average values of about 150 mg /L. In consequence of these characteristics, this species is considered as one of the best candidates to be used in mixed fermentations to improve aromatic profile.

1.3.1 Some applications of non-*Saccharomyces* in mixed fermentation

Today, mixed fermentations are used as a biotechnological tool in order to enhance the characteristics of wine, consist of inoculations of starter cultures composed by a *S. cerevisiae* strain in combination with non-*Saccharomyces* strains.

In this context the main results obtained by mixed fermentations application are reported below.

Studies have demonstrated that the use of *Schizosaccharomyces pombe* in combination with *S. cerevisiae* could be used to have de-acidifying the grape must or wine by degradation of malic acid, and to improve the color stability by increasing the pH and by the release of secondary metabolites of fermentation that can react with anthocyanins to create more stable forms of colour such as pyranoanthocyanins to preserve intense colour in the wine during aging.

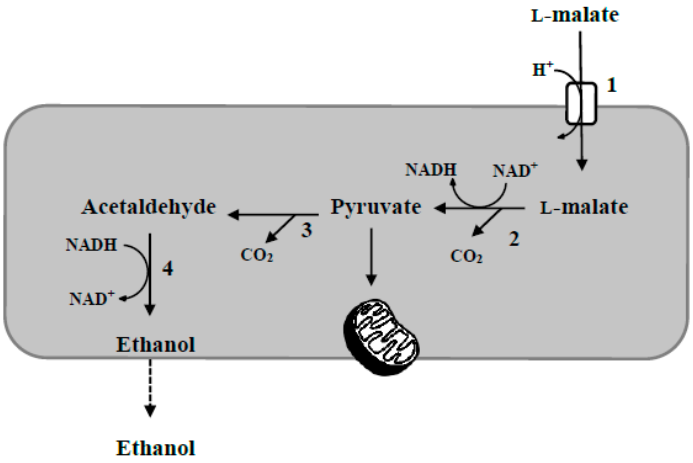


Figure 1.1 Schematic representation of malic acid degradation by *Schizosaccharomyces pombe* (Loira et al., 2018)

An increase in the production of geraniol is obtain by mixed cultures composed of *Debaryomyces vanriji* and *S. cerevisiae* thanks to the high levels of β-glucosidase activity exerted by *Debaryomyces vanriji* (Ciani et al., 2010).

Another non-*Saccharomyces* species with interesting oenological properties is *Metschnikowia pulcherrima*. This yeast is predominant during the early stages of fermentation and is a high producer of β-glucosidase, its presence can cause an increase of volatile acidity, medium chain fatty acids, higher alcohols, esters, terpenoids and glycerol content. This yeast is also able to release polysaccharides from cell walls, reduce the acidity of the wine and sometimes its use can lead to a

reduction in the concentration of ethanol content. It was also investigated a potential natural anti-fungal activity of this specie related to the production of a ferric salt of pulcherriminic acid.
In fact, this yeast shows antimicrobial activity against wine yeasts that could affect the final quality of a wine while they appear to have no effect on *S. cerevisiae*.
The mechanism of this activity is related to the diffusion of pulcherriminic acid as precursor of pulcherrimin which immobilizes the iron in the growth medium (Oro et al., 2014).
The genus *Hanseniaspora (Kloeckera)* is often associated with the production of unpleasant compounds, such as acetic acid and ethyl acetate, which has led for a long time to consider these yeasts as harmful for winemaking (Tristezza et al., 2016). However, recent experiments have shown that mixed fermentations with *H. uvarum* and *S. cerevisiae* increase the isoamyl acetate content, while the use of *Hanseniaspora osmophila* provides improvements in the production of 2-phenylethyl acetate.
The genus *Pichia* in fermentation is instead associated with the presence of isoamyl acetate and high thiols concentrations (Holt et al., 2018).
The principal application of all these indigenous yeasts in mixed fermentations is related by their higher ability to produce extracellular hydrolytic enzymes respect to the specie *S. cerevisiae*.
The greatest number of extracellular enzymes is produced by *C. stellata*, *H. uvarum* and *M. pulcherrima.*
It has also been reported the influence of non-*Saccharomyces* yeasts on concentration of polysaccharides in wine. In fact, the enzymatic degradation of dead yeast cells causes the degradation of some their components such as amino acids, peptides, fatty acids and the release of polysaccharides (mannoproteins) from the cell wall. Polysaccharides improve the sensory properties in wines consistency by increasing its viscosity while the peptides released during yeast autolysis may have antioxidant and other bioactive properties, the most of these products have aromatic positive influence (Domizio et al., 2014). *M. pulcherrima*, *Kloeckera apiculate*, *Wickerhamomyces anomalus*, and *T. delbrueckii* are greater producer of polysaccharides.
This group of yeasts can be considered also a biological mechanism for regulating population dynamics in different microbial ecosystems by the production of some toxins (Liu et al., 2015).
In fact, the killer trait, reported for the first time in *S. cerevisiae*, is well distributed among other yeast genera such as *Candida*, *Hansenula*, *Pichia*, *Debaryomyces*, *Metschnikowia*, *Williopsis*, *Kluyveromyces* and *Zygosaccharomyces.*
It has been demonstrated that the presence of killer trait could be used to inhibit the growth of *B. bruxellensis* in wine and grape juice or against the vine pathogen *Botrytis cinerea*.
In conclusion, the use of selected non-*Saccharomyces* yeasts capable of consuming grape sugars, is a new tool to enhance the production of desirable volatile esters, grape terpenoids, glycerol and other properties of wines (Garcia et al., 2016).

SPECIES	METABOLITES
Candida stellata or Starmerella bacillaris	Acetic acid Dodecanoic acid Ethyl octanoate Glycerol Higher alcohols 2-Methyl propanoic acid Succinic acid Terpenoids
Debaryomyces vanriji	Esters Medium-chain fatty acids Terpenoids
Hanseniaspora uvarum	Acetate and ethyl esters Sulfur compounds Higher alcohols Medium-chain fatty acids
Hanseniaspora vineae	2-Phenylethyl acetate
Hanseniaspora guilliermondii	Acetate and ethyl esters Acetone Heavy sulfur compounds
Hanseniaspora osmophila	Ethyl acetate Ethyl lactate
Pichia anomala	Acetate and ethyl esters Higher alcohols
Issatchenkia orientalis	Color Methanol
Lachancea thermotolerans	Glycerol Lactic acid 2-Phenylethanol
Metschnikowia pulcherrima	Esters
Pichia guilliermondii	Color
Pichia kluyveri	Thiols
Pichia fermentans	Acetaldehyde 2,3-Butanediol Ethyl esters Higher alcohols Polysaccharides
Schizosaccharomyces pombe	Maloalcoholic deacidification Pyruvic acid Color Propanol
Torulaspora delbrueckii	Succinic acid Linalool Polysaccharides
Zygosaccharomyces bailii	Polysaccharides
Zygosaccharomyces florentina	Polysaccharides

Table 1.1 Metabolites produced in wines fermented with non-conventional yeasts (Adapted from Garcia et al. 2016).

1.4 Selection of wine starters

The use of selected starter cultures presents many advantages, principally to guarantee an immediate start of the fermentation process and its completion; furthermore, it allows the control of the development of the microflora naturally present in fermenting must, eliminating the risk of unwanted collateral fermentations. The use of inoculated fermentation with *S. cerevisiae* starter culture to guarantee the reproducibility of the process and the quality of the wines produced addressed the research on the technological characterization of the strains belonging to this species. The growing interest of modern wine consumers to low alcohol wines characterized by peculiar bouquet and aromatic properties has shifted the attention of the wine sector towards the selection of non-conventional yeast strains, able to reduce the ethanol content and at the same time improve the sensory aspects.

Therefore, the selection and identification of the starter cultures, previously addressed only on the *S. cerevisiae* species, nowadays involves the characterization of strains of different species with the aim to select the most promising strains to be used in mixed fermentation. In fact, the development of a mixed starter consisting of the combination of *Saccharomyces*/non-*Saccharomyces* strains currently represents one of the main strategies for modifying the quality of wine (Ranieri et al., 2000).

In the past, the principal attributes considered in the selection of wine starter were fermentation capacity and alcohol tolerance, nowadays the research is interest on new properties, such the presence of enzymatic activities, the production of glycerol, mannoproteins and other compounds influencing organoleptic and sensory quality of the beverages.

1.5 Isolation and starter characterization

Duclaux in 1887 introduced the concept of fermentation in purity, that is a fermentation carried out by a single selected strain.

The selection of yeast strains to be used as a starter culture in winemaking is a long and complex process which aims to study the technological and metabolic characteristics of the different species in order to select those with the desired characteristics. The marketing of starters for the wine industry began in the 1960s.

Today wide range of yeast strains selected for oenological characteristics is currently available on the market.

The production of wine using a starter offers several advantages over spontaneous fermentation. In particular, it ensures a prompt start of the process as well as its completion and guarantees the quality of the finished product.

However, the selection criteria for starters are constantly evolving both according to the modern technologies used in the cellar and to the demands of consumers.

In fact, if in the past the selection of the starter was focused on characteristics related to the technological aspect such as fermentation capacity, alcohol tolerance, today the selection also includes parameters related to the metabolic aspect, the production of compounds that influence the sensory attributes, quality and typicality of the wine.

The first step towards the selection of wine starters is the isolation of the yeasts both from the grapes and from the fermenting must. The isolated species are then identified through molecular biology methods and distinguished at the strain-specific level. The isolated yeasts are subsequently studied based on a series of parameters of oenological interest, such as fermentation capacity, low production of volatile acidity, growth at low or high temperatures, low production of hydrogen sulphide, technological characteristics refer to competitiveness and development methods, the ability to conduct fermentations, inhibiting any other contaminating microbial form present in the must.

The wines obtained are analyzed for both analytical parameters (residual sugars, alcohol, pH, total and volatile acidity, color) and sensory analysis. Finally, the selected strains are used in fermentation at pilot scale in the cellar.

Nowadays the climate changes and the new production technique have incented the selection of the starter based on different new criteria, this has led to broaden the search for starters also among species not belonging to *S. cerevisiae*, natural microorganisms of grapes and musts with interesting qualities and able to enhance the typicality of wines.

This group of microorganisms is named non-*Saccharomyces* yeasts. The selection of these yeasts as starter and their use in mixed fermentations with *S. cerevisiae*, is the new trend of winemaking process.

In the basis of their potential influence on wine quality and the problem related to the global climate changes, the selection of the strains is focused on the increase aromatic properties, the ability to ferment musts with high sugar concentration and the ability to survive in wines with high ethanol content.

Although few commercial preparations of non-*Saccharomyces* yeasts are actually available on the market, they will probably increase in the future.

1.5.1 Technological characteristics to be considered in the selection of wine strains

Table 1.2 Main selection criteria for yeasts starter culture (adapted from Suzzi et Tofalo 2014)

Fermentative properties Rapid start of fermentation High fermentation purity High fermentation vigor High tolerance to ethanol Ability to ferment at a low temperature Low biomass production High osmotolerance	**Properties related to the aroma of wine** Low production of acetic acid Absence of production of hydrogen sulphide Moderate production of higher alcohols High glycerol production Production of aromatic compounds Esterase production High autolytic capacity
Technological properties High genetic stability Low nitrogen requirement Resistance to high concentrations of sulphites Flocculation Resistance to copper High drying survival Proteolytic activity killer capacity Low foaming power	**Metabolic properties with health effects** Absence of production of biogenic amines Low production of sulphites Low production of ethyl carbamate Ability to adsorb Ochratoxin on the cell wall

1.5.2 Focus on principal characters of wine yeasts strains

1.5.2.1 Fermentation power

The fermentation power indicates the maximum percentage of sugar that the yeast can ferment and the relative quantity of alcohol that it can form. In general, it is considered that starting from a grape must with a sugar concentration of about 300 g / L the selected yeasts have a fermentation power equal to or greater than 14% (v / v).

1.5.2.2 Fermentative vigor

Fermentative vigor defines the rate for which the yeast starts the fermentative process. This parameter is expressed as grams of carbon dioxide produced in the first 2-3 days of fermentation.

1.5.2.3 Fermentation purity

Fermentation purity represents the ratio between volatile acidity and ethanol produced at the end of fermentation.

1.5.2.4 Hydrogen sulphide production

The formation of hydrogen sulphide by yeasts during alcoholic fermentation represents a problem as the production of this volatile compound, albeit in low concentration, causes bad smells associated with cabbage, rotten, eggs, etc., because his perception threshold is very low (50-80 mg / L).

This compound derives from the metabolism of some amino acids such as cysteine and methionine and its production depend on the yeast strain, as well as on some factors such as the presence of sulfur in the grape skins, or sulphites and sulphates in the must; therefore, the choice of low producing yeasts of this compound is recommended (Mendes et al., 2002).

1.5.2.5 Production of acetic acid

Acetic acid is the main volatile acid in wine. It is produced at variable level depending on the yeast strain, on the type of wine and beyond a certain concentration negatively affects the sensorial quality.

The optimal concentration for this compound in wine is between 0.2 and 0.6 g/L (Vilela-Moura et al., 2011)

1.5.2.6 Resistance to sulfur dioxide

Sulfur dioxide is used as an antiseptic in the wine industry. Sulphite is added in the first fermentation stages to inhibit unwanted microflora as well as used for sanitizing cellar equipment. The yeasts are endowed with different mechanisms of resistance to this compound, for example among the response mechanisms implemented by *S.* cerevisiae the increase in the production of acetaldehyde which binds to SO_3^{2-} (García-Rios et al., 2019).

The antimicrobial action depends on the pH. At the low pH of the must the very reactive molecular form (SO_2) is prevalent, to which the cells are very sensitive. SO_2 is absorbed by the cell and causes a loss of ATP which leads to an increase in the consumption of glutamine and nitrogen. In general, *S. cerevisiae* strains are able to tolerate concentrations ranging from 50 to 150 mg / L. Since the addition of sulfur is now a consolidated practice in winemaking, the selection of strains with high resistance to this compound is necessary (Lisanti et al., 2019).

1.5.2.7 Ethanol tolerance

Yeast cells undergo various stresses during fermentation, including an increase in ethanol concentration. The production of ethanol is correlated to the ripeness of the grapes; in fact greater is the quantity of sugars present and greater will be the production of ethanol. The increase of ethanol causes alterations in the structure of the cell membrane, inhibits the entry of solutes and the viability of the yeast causing its death. The yeast cell reacts through the synthesis of unsaturated fatty acids and ergosterol as well as trehalose and greater activity of the enzyme alcohol dehydrogenase. (Ding et al., 2009).

1.5.2.8 Killer factor

Killer toxin production was discovered by Bevan and Makower in 1963 (Somers et al., 1969), who classified the yeast strains into three phenotypes: killer, neutral, sensitive.

Killer factor is an unstable macromolecular protein. This toxin binds to the cell membrane and forms channels that release ions and cause cell death. There are 28 types of toxins in *S. cerevisiae* the most common are: K1, K2, K28.

The killer factor represents an antagonism factor among yeasts and can be useful for decreasing the indigenous population during fermentation. Therefore, the presence of the killer factor or resistance is one of the most sought-after characteristics in a starter.

1.5.2.9 Production of volatile compounds

The volatile compounds released by yeasts and bacteria during fermentation are the components of the so-called secondary aroma. The production of secondary aromas depends on the yeast strain as well as on the physical and chemical characteristics of the must. The quantity of these compounds does not exceed 1% of the components of the wine, however they are molecules with a low perception threshold and therefore, even if present in minimal concentrations, they influence the sensory profile (Mina et al., 2017).

The main classes of compounds produced by yeast metabolism are higher alcohols, fatty acids, esters, aldehydes.

Higher alcohols are important as precursors for the formation of esters during aging, are characterized by floral notes and contribute to the smell of wine. Ethyl acetate, 2 phenyl ethyl acetate, isoamyl acetate ethyl hexanoate are the ones that contribute the most.

1.5.2.10 Production of enzymes of oenological interest

Enzymes of oenological interest belong to the following classes: oxidoreductase, pectinase, protease and glycosidase.

The activity of these enzymes positively impacts the flavor and quality of the wine and is often associated with the presence of non-*Saccharomyces* yeasts.
These enzymes come from grapes, yeasts and other microbes associated with vineyards and cellars. Non-*Saccharomyces* yeasts disperse most of these enzymes in the periplasmic space and in the medium, where they can interact with grape precursor compounds to produce active aromatic compounds, and thus carry out an important role in the varietal aroma (Strauss et al., 2001).
Pectinase, protease and glycosidases are some of the enzymes secreted by yeasts of interest in winemaking and contributing to the formation of aroma. Some compounds like terpenes, are bound to sugar molecules in the form of odorless glucoside complexes. The enzyme β-glucosidase can break the bond with sugar molecules and release the volatile compound that will contribute to the final aroma of the wine.
Another important group of enzymes are the pectinolytic enzymes. This class of enzyme is able to break long pectin chains into shorter and more soluble segments and contributes to the clarification of musts, also can influence the color by increasing the extraction of substances during the contact phase of the skins with the must.
Mannoproteins are among the constituents of the cell wall of yeast and are in the form of polysaccharides and proteins. Mannoproteins split during autolysis or by the action of β-glucanase enzyme perform different functions, the main are stabilization of proteins and tartrates of white wines, interact with the aromatic and phenolic compounds of red wines, reduce astringency and bitterness of tannins, increase the body of the wine.

1.6 Aim of the study

In this first phase of research 29 non-*Saccharomyces* yeast strains, isolated from spontaneous fermentations belonging to the Fermenting Yeast Collection of the University of Basilicata, were tested for some physiological characteristics, with the aim to select the strains possessing the best combination of these characteristics and be employed in mixed starter fermentation to produce wines with a low alcohol content, but with complex aromatic characteristics.
The screening was based both on classical technological selection criteria, such as resistance to main antimicrobial compounds used in vineyard gestion (SO_2 and $CuSO_4$), or production of hydrogen sulphide, but also additional criteria, such as the expression of some extracellular enzymes (β-glucosidase), the influence on the organoleptic properties and chemical composition, the yeast growth capacity in the presence of ethanol and high sugar concentrations, the resistance to oxidative stress. These characteristics are useful to predict their behavior and their ability to survive during grape must fermentation.

1.7 Materials and methods

In the first step, twenty-nine non-*Saccharomyces* strains, belonging to the Fermenting Yeast Collection of the University of Basilicata, were chosen, and stored on YPD medium (1% w/v Yeast extract, 2% w/v peptone, 2% w/v glucose, 2% w/v agar).

The strains used, belonging to different species and their isolation origin, are listed in the table 1.3.

Table 1.3 non-*Saccharomyces* strains used in the present work

SPECIE	***STRAIN CODE***	**SOURCE OF ISOLATION**
H.guilliermondii	SNM1-1; SNM1 3-2; SNM3 1-1; SNMH; AP9; TSB; ER 3; TM 5-1; TM 4-1	Grape must
H.osmophila	ND1	Grape must
T.delbrueckii	425; 365; LC 2-1	Grape must
M.pulcherrima	Mpr 2-49; Mpr 1-7; Mpr 2-4; 563; 683; Mpr 1-3; Mpr 2-3; M1; M2; M3; SIA 1; SIA 4	Grape must
S'codes ludwigii	AP G; SIA2	Grape must
P.fermentans	LM 5-3; STG 3-1	Grape must
S.cerevisiae	EC 1118	Commercial starter (Lallemand)

These yeast strains were submitted to a first screening step, based on the evaluation of different technological characteristics.

1.7.1 Resistance to Sulfur dioxide

Sulfur dioxide (SO_2) is often used as a bacteriostatic agent to prevent oxidation and ensure the microbiological stability of the produced wine. Many authors recommend the use of sulfur dioxide up to 150 mg/L. Strains that guarantee the completion of the fermentation process in presence of concentrations of sulfur dioxide until 50 mg/L can be considered suitable for use as starter in wine fermentation.

The yeasts were grown on YPD at a temperature of 25°C for 24h to obtain the cell suspensions, used as inoculum. Cell suspensions containing about 10^6 cells/mL were subsequently prepared for each strain, by assessing cell concentration on the basis of the optical density value at 600 nm.

The resistance to sulfur 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).

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 strain resistance level was evaluated on the basis of highest doses at which strain growth was observed.

1.7.2 Resistance to copper

A common practice is the use of compounds containing copper, such as copper sulfate ($CuSO_4$), as pesticides in the vineyard. However, copper and its derivatives can exert toxic effects on yeast growth during wine fermentation. Some yeast strains have developed different resistance levels in presence of this compound.

The inoculum of yeast strains was performed by following the protocol already reported for SO_2 resistance (paragraph 1.7.1).

The resistance test was performed by evaluating the growth capacity of the non-*Saccharomyces* strains 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 and yeast growth was evaluated after 48 hours at 26°C; the resistance level was calculated as the highest doses allowing the strain growth (Capece et al., 2010; Domizio et al., 2011).

1.7.3 Production of hydrogen sulphide

Hydrogen sulphide (H_2S) is a by-product resulting from yeast metabolism of sulfur, characterized by an unpleasant odor. Low production of this compound is a desirable characteristic for yeast starter. The ability of the strains to produce different amounts of H_2S was tested on Bismuth Sulphite Glucose Glycerin Yeast BIGGY agar, a medium containing bismuth sulphite as indicator. The medium was spot inoculated with fresh yeast cells, and the plates were incubated at 25°C for 48 hours. The production of H_2S was evaluated in function of browning level of yeast colonies by an arbitrary scale from 0 (white color = no production), 1 (hazelnut = low production), 2 (brown = significant production) 3 (coffee = high production).

1.7.4 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. (2000), 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, in comparison to a calibration line, obtained by using 4-p-nitophenol solution.

1.7.5 Growth in presence of high concentrations of ethanol and sugars

In this test, it was evaluated the ability of non-*Saccharomyces* strains to grow in hostile environment, resembling the conditions potentially present during wine fermentation, in particular in presence of high ethanol level and high osmotic pressure (high concentrations of sugars). At this aim, the strains were tested for the ability to grow in synthetic media containing high ethanol and sugar concentrations.

This test was carried out in microplates, following the method reported by Eglezos et al. (2015) with some changes. As growth medium, it was used Yeast Nitrogen Base (YNB) with amino acids, supplemented with a sterile glucose solution (20 g/L), and 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.

1.7.6 Yeasts respiratory activity

A trait useful for selection of yeast strains able to reduce the ethanol content in wines is the ability to metabolize sugars using alternative ways to fermentation, such as the respiratory process.

The ability of yeasts to follow this metabolic pathway is indirectly linked to the presence of some enzymatic activities, such as Catalase activity, Cytochrome C oxidase and tolerance to oxidative stress.

The evaluation of the presence of Cytochrome C oxidase and tolerance to oxidative stress was performed following the protocol reported by of Mestre Furlani et al. (2017) with some modifications.

1.7.7 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 Furlani et al. 2017. The 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).

1.7.8 Cytochrome c oxidase activity

Single strains culture was inoculated on YPD plates, which were incubated at 26 ° C for 7 days. After the incubation period, 10 µL of aqueous solution (1%) of tetramethyl-ρ-phenylenediamine dihydrochloride was added on the microbial cells, developed on the surface. This compound colours the colonies blue.

The presence of Cytochrome C activity is evaluated in function of time required by the colonies to change the colour; the yeast colony changes colour in a time interval between 0- 60 seconds, the reaction is considered positive (presence of Cytochrome C), whereas if it takes a longer time, the reaction is negative (absence of activity).

1.7.9 Catalase activity

To evaluate the presence of Catalase activity, a hydrogen peroxide solution (3 v/v %) was added to 48-h-old yeasts colonies. The presence of this enzymatic activity was related to the bubbles formed on colonies surface and the results were expressed as presence or absence of activity.

1.7.10 Statistical analysis

All the results obtained from the first screening phase were converted into non-dimensional values, assigning the values reported in Table 1.4. The matrix thus obtained was subjected to cluster analysis, using Ward's method with Euclidean distance by the statistical package PAST software ver. 1.90.

Table 1.4 Non-dimensional values assigned for each parameter tested during screening step

Parameters	Value			
	0	**1**	**2**	**3**
Resistance to SO_2 (mg/L)	50	100-125	150-175	200
Resistance to $CuSO_4$ (µM)	< 100	100	200	300
H_2S Production	Absence	Low	Medium	High
β-glucosidase activity. Growth capacity in EtOH /High sugar	V < M -SD	M-SD<V≤M	M<V≤M +SD	V> M+ SD
Oxidative stress (alone of inhibition[mm])	0-4	5-7	8-10	>10
Catalase activity (reaction time [second])	0-29	30-49	50-60	>60
Cytochrome C (reaction time [second])	0-39	40-59	>60-80	80

1.8 Results and discussion

1.8.1 Resistance to SO_2 and $CuSO_4$

As regards the technological characterization for conventional characters, such as SO_2 and copper resistance, 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.1.2). 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 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. 1.2 B).

Generally, *M. pulcherrima* strains were more tolerant than *Hanseniaspora* strains; in fact, *M. pulcherrima* grew in presence of 200 and 300 µM 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 µM of $CuSO_4$. Conversely, *Hanseniaspora* strains tolerated copper content ranging from 100 to 200 µM. Low copper tolerance was exhibited also from two *Torulaspora* and the *S'codes ludwigii* strains.

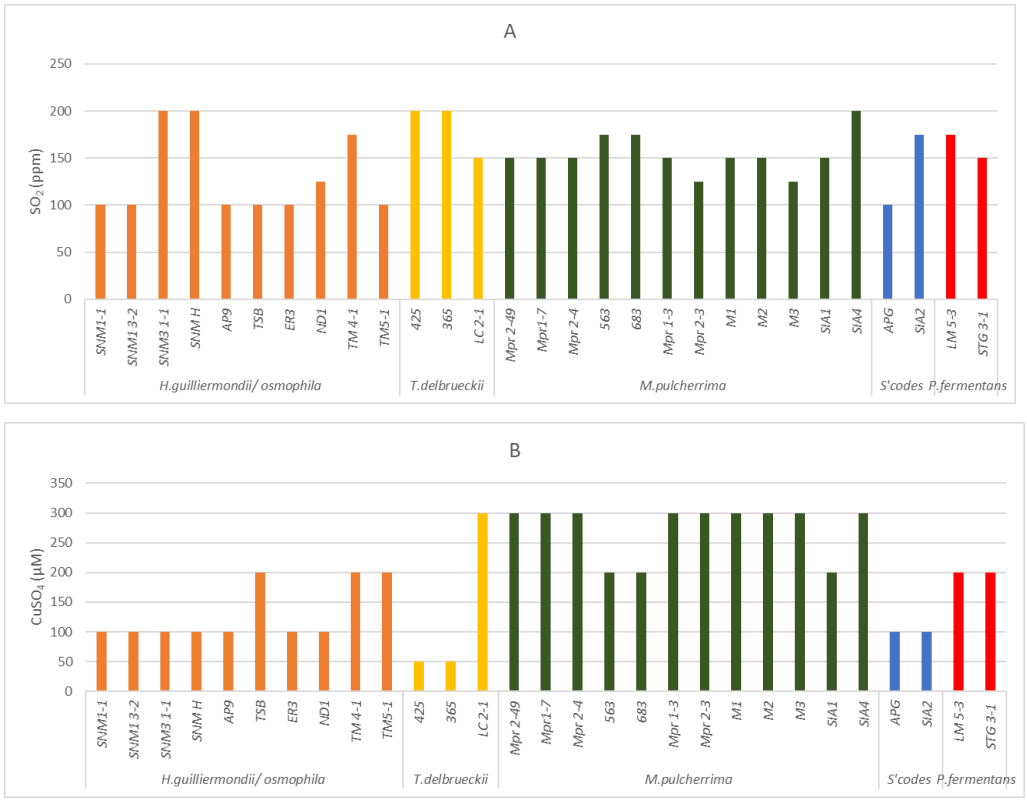


Figure 1.2 Tolerance level exhibited by each non-*Saccharomyces* strain for SO_2 (fig. A) and $CuSO_4$ (fig. B).

1.8.2 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*. The results are reported in the table 1.5.

Table 1.5 Qualitative production of H_2S by selected non-*Saccharomyces* strains

SPECIE	STRAINS CODE	RESULTS
H.guilliermondii	SNM1-1	HAZELNUT
	SNM1 3-2	HAZELNUT
	SNM3 1-1	HAZELNUT
	SBN H	HAZELNUT
	AP9	HAZELNUT
	TSB	HAZELNUT
	ER 3	HAZELNUT
	TM 5-1	HAZELNUT
	TM 4-1	HAZELNUT
H. osmophila	ND1	HAZELNUT
T.delbrueckii	425	BROWN
	365	BROWN
	LC 2-1	HAZELNUT
M.pulcherrima	Mpr 2-49	HAZELNUT
	Mpr 1-7	HAZELNUT
	Mpr 2-4	HAZELNUT
	563	HAZELNUT
	683	HAZELNUT
	Mpr 1-3	HAZELNUT
	Mpr 2-3	HAZELNUT
	M1	HAZELNUT
	M2	HAZELNUT
	M3	HAZELNUT
	SIA 1	HAZELNUT
	SIA 4	HAZELNUT
S'codes ludwigii	AP G	BROWN
	SIA2	BROWN
P. fermentans	LM 5-3	BROWN
	STG 3-1	BROWN

1.8.3 β-glucosidase activity

The presence of β-glucosidase activity for each non-*Saccharomyces* strains was evaluated by plate assay. On the basis of the results shown in the table 1.6 below about qualitative evaluation of enzymatic activity, only the strains that were showed activity on plate were identified and subjected to the quantitative test.

Only for six strains belonging to *M. pulcherrima* (SIA1, SIA 4), *T. delbrueckii* (365, LC 2-1) and *P. fermentans* (STG 3-1, LM 5-3) was found negative results.

Figure 1.3 Browning of colony with β-glucosidase activity.

Table 1.6 Evaluation of β-glucosidase activity among selected non-*Saccharomyces* strains. Results are expressed as presence (+) or absence (-) of the enzymatic activity

SPECIE	STRAINS CODE	β-GLUCOSIDASE ACTIVITY
H. guilliermondii	SNM1-1	+
	SNM1 3-2	+
	SNM3 1-1	+
	SBN H	+
	AP9	+
	TSB	+
	ER 3	+
	TM 5-1	+
	TM 4-1	+
H. osmophila	ND1	+
T.delbrueckii	425	+
	365	-
	LC 2-1	-
M.pulcherrima	Mpr 2-49	+
	Mpr 1-7	+
	Mpr 2-4	+
	563	+
	683	+
	Mpr 1-3	+
	Mpr 2-3	+
	M1	+
	M2	+
	M3	+
	SIA 1	-
	SIA 4	-
S'codes ludwigii	AP G	+
	SIA2	+
P. fermentans	LM 5-3	-
	STG 3-1	-

Therefore, the quantitative test for β-glucosidase activity was carried out on the 23 strains, resulted positive for the qualitative test.

The results, expressed as nmol PNP/mL*h (Figure1.4), 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.

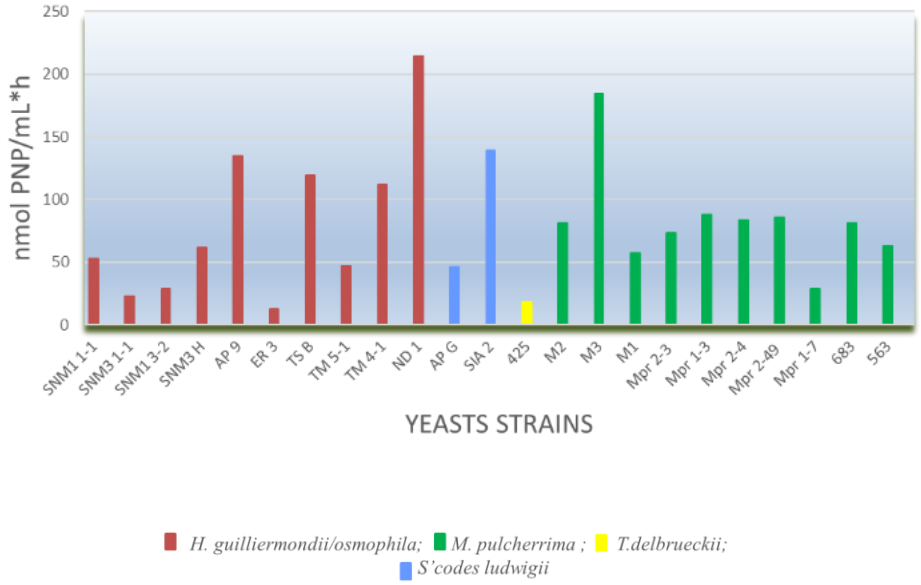


Figure 1.4 β-glucosidase activity exhibited by 23 non-*Saccharomyces* strains

1.8.4 Growth in ethanol and high sugar concentrations

As regards the test 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 graph 1.5 shows the cell growth percentages exhibited by each non-*Saccharomyces* strains in a medium enriched with 40% sugars (orange histograms) and in the presence of ethanol at a concentration of 14% v/v (blue histograms). As shown in the graph, both the strains belonging to the species *H.guilliermondii* , *S'codes lodwigii*, *T.delbrueckii* and *P.fermentans* exhibit high growth capacity in both tested conditions. On the contrary, *M. pulcherrima* species exhibited a high growth capacity in the medium containing high sugar concentration, whereas the growth was reduced in the presence of ethanol concentrations. This species proved to be more tolerant to stress caused by the high osmotic pressure exerted by the solutes (sugars) added in the growth substrate.

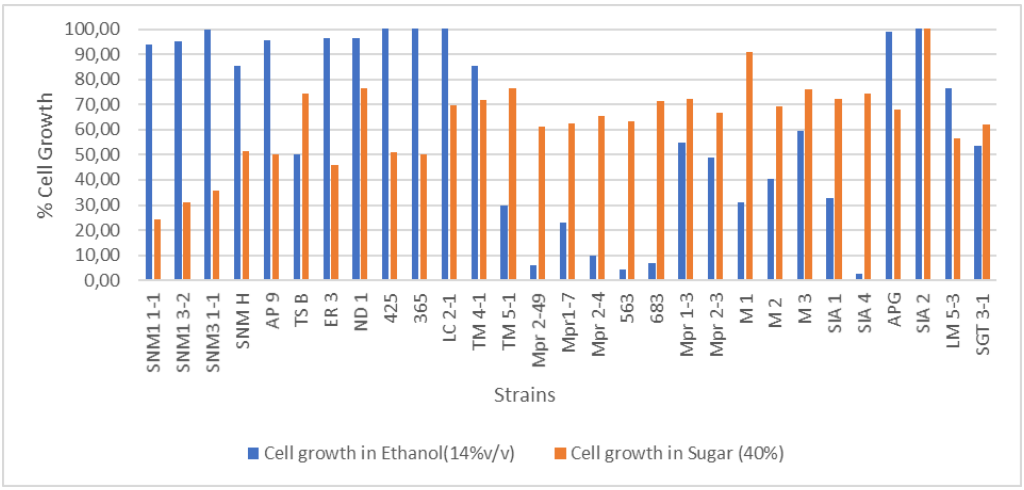


Figure1.5 % Cell Growth in medium enriched by sugar (glucose /fructose 40%) and ethanol (14%v/v)

1.8.5 Oxidative stress tolerance

The strain tolerance to oxidative stress was evaluated by testing their resistance at 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 1.7 reports the results about the oxidative tolerance test obtained by non-*Saccharomyces* species at the higher tested concentration of H_2O_2.

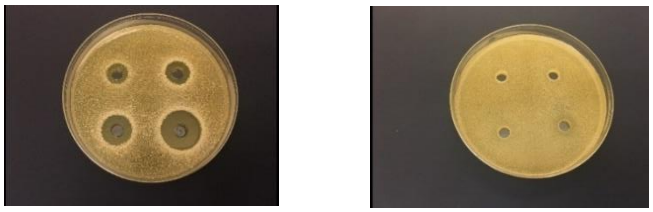


Figure 1.6 Effect of H_2O_2 on sensible and resistant strain

Specie	**Stress tollerance**		
	Low	Medium	High
H. guilliermondii (9)	6	3	-
H. osmophila (1)	-	1	-
T. delbrueckii (3)	3	-	-
S'codes ludwgii (2)	-	2	-
P. fermentans (2)	1	1	-
M. pulcherrima (12)	-	2	10

Table 1.7 Tolerance to oxidative stress of 29 non-*Saccharomyces* strains

The results (Table 1.7) 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.

1.8.6 Analysis of catalase and cytochrome C activities

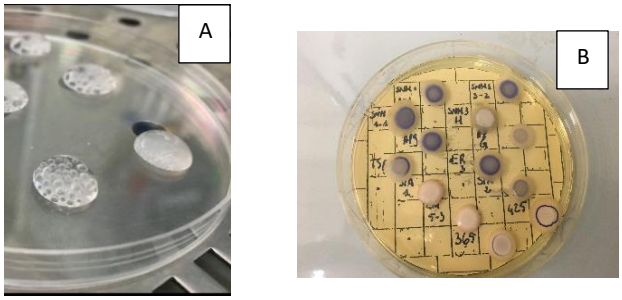


Figure 1.7 Catalase activity (fig. A); Cytochrome C activity (fig. B)

As regards the analysis of the presence of Catalase and Cytochrome C activities no differences were found among the species. All the strains presented both activities. The results are reported in the table 1.8.

Table 1.8 Analysis of Catalase and Cytochrome C activities expressed in function of the time reaction. **Catalase**: **0** (0-29 sec)/**1** (30-49 sec)/**2 (**50-60sec); Cytochrome C: **0** (0-39sec)/**1** (40-59 sec)/**2** (60-80 sec).

SPECIE	STRAINS CODE	CATALASE ACTIVITY	CYTOCHROME C ACTIVITY
H.guilliermondii	SNM1-1	1	0
	SNM1 3-2	1	0
	SNM3 1-1	1	0
	SNM H	1	2
	AP9	1	0
	TSB	1	0
	ER 3	1	0
	TM 5-1	1	2
	TM 4-1	2	1
H. osmophila	ND1	0	0
T. delbrueckii	425	1	0
	365	1	1
	LC 2-1	1	2
M. pulcherrima	Mpr 2-49	1	2
	Mpr 1-7	1	2
	Mpr 2-4	1	2
	563	1	2
	683	2	2
	Mpr 1-3	1	2
	Mpr 2-3	0	2
	M1	2	2
	M2	1	2
	M3	1	2
	SIA 1	0	2
	SIA 4	1	2
S'codes ludwigii	AP G	1	2
	SIA2	1	0
P. fermentans	LM 5-3	1	2
	STG 3-1	2	2

The non-*Saccharomyces* yeast species analyzed showed positive values for all the characteristics correlated to oxidative activity. In fact, although with different reaction times, all the strains possess both activities.

1.8.7 Statistical elaboration of results from technological characterization

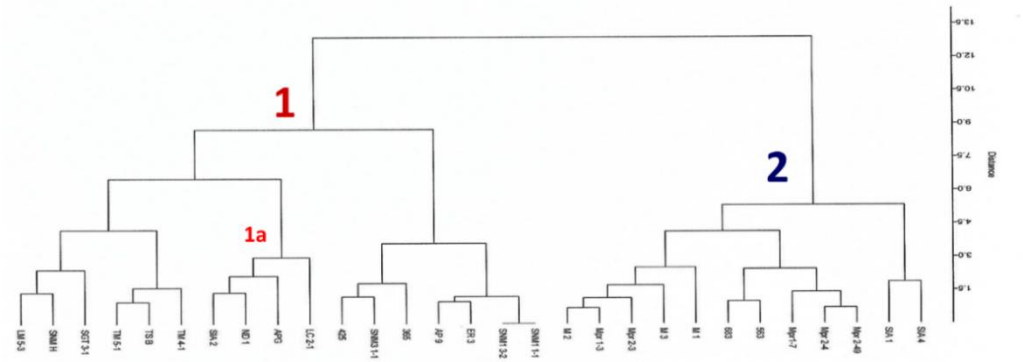
All the results obtained from the first screening phase were converted into non-dimensional values, assigning the values reported in Table 1.9.

Table 1.9 non-dimensional values assigning to the non-*Saccharomyces* strains for all analysed parameters.

STRAIN CODE	Cell Growth in Sugar	Cell Growth in Ethanol	β-glucosidase	Resistance to SO_2	Resistance to $CuSO_4$	H_2S Production	Catalase activity	Oxidative Stress	Cythocrome C
SIA 4	2	0	0	3	3	1	1	0	2
SIA 1	2	1	0	1	2	1	0	0	2
Mpr 2-49	1	0	2	1	3	1	1	0	2
Mpr 2-4	2	0	2	1	3	1	1	0	2
Mpr1-7	1	0	1	1	3	1	1	1	2
563	1	0	2	2	2	1	1	0	2
683	2	0	2	2	2	1	2	0	2
M 1	3	1	1	1	3	1	2	0	2
M 3	2	1	3	1	3	1	1	1	2
Mpr 2-3	2	1	2	1	3	1	0	0	2
Mpr 1-3	2	1	2	2	3	1	1	0	2
M 2	2	1	2	1	3	1	1	0	2
SNM1 1-1	0	2	1	1	1	1	1	2	0
SNM1 3-2	0	2	1	1	1	1	1	2	0
ER 3	0	2	1	1	1	1	1	2	0
AP 9	1	2	2	1	1	1	1	2	0
365	1	1	1	3	0	1	2	2	0
SNM3 1-1	0	2	1	3	1	1	1	2	0
425	1	2	2	3	0	1	1	2	0
LC 2-1	2	2	2	1	3	2	1	2	1
APG	2	2	2	1	1	2	1	1	2
ND 1	2	2	3	1	1	2	0	1	0
SIA 2	3	3	3	1	1	2	1	1	1
TM 4-1	2	2	0	2	2	2	1	1	0
TS B	2	1	1	1	2	1	1	1	0
TM 5-1	2	2	0	1	2	1	1	1	0
SGT 3-1	1	1	0	1	2	2	2	1	2
SNM H	1	2	1	3	1	1	1	2	2
LM 5-3	1	2	0	2	2	2	1	2	2

The statistical elaboration of results obtained by all the tests performed for preliminary screening yielded the dendrogram reported in figure 1.8. The dendrogram subdivided the strains in two main groups (1, 2).

Figure 1.8 Dendrogram obtained after cluster analysis on the data of technological characterization of non-*Saccharomyces* strains.



Group (1) consists of strains belonging to the species *H. guilliermondii; H. osmphila; P. fermentans; S'codes ludwigii* and *T. delbrueckii*. The strains included in this group have the following characteristics:

• High growth capacity in the presence of ethanol;

• Low resistance to $CuSO_4$;

• Low resistance to oxidative stress.

Within group 1, a subgroup (1a) was identified, composed by strains exhibiting a good combination of the tested parameters, such as good growth capacity in the presence of ethanol and high sugar concentrations; presence of β-glucosidase activity; medium resistance to oxidative stress.

The second group (2) is represented by strains belonging to *M. pulcherrima*, characterized by:

• High resistance to osmotic stress;

• Low growth capacity in the presence of ethanol;

• High resistance to oxidative stress.

The strains possessing the best combination of technological characteristics were represented by LC 2-1, 425 (*T. delbrueckii*); LM 5-3 (*P. fermentans*); Mpr2-3, Mpr 2-4, M3, SIA 4 (*M. pulcherrima*); TS B; SNM3 H; AP 9 (*H. guilliermondii*); ND1 (*H. osmophila*); SIA 2 (*S'codes ludwigii*).

These strains were selected for further characterization, focused on evaluation of their oenological aptitude in winemaking trials in combination with *S. cerevisiae*.

1.9 Conclusion

This phase of the research was focused on the preliminary screening among unconventional yeasts to identify strains characterized by traits of oenological interest, to be used as potential candidates in mixed starter cultures to produce low alcohol content wine with peculiar aromatic characteristics.

A total of 29 non-*Saccharomyces* strains belonging to the species: *T. delbrueckii*, *H. guilliermondii / osmophila*, *P. fermentas*, *S'codes ludwigii*, *M. pulcherrima*, were studied for technological characteristics because as it is known that not all strains within a species exhibit the same desirable characteristics. To designate a starter culture that includes one of these native species, in addition to the evaluation of technologically important properties useful for oenological applications of yeasts and conventionally employed in the selection process such as: the high tolerance to antimicrobial compounds commonly used in the winemaking practices (ethanol, sulphite and copper) and the limited production of H_2S, new selection criteria were also considered. In fact, in the present study the objective was to examine the oxidative-fermentative metabolism of non-*Saccharomyces* yeasts, in order to identify the less effective strains in the transformation of grape sugars into ethanol, more tolerant to oxidative stress and possessing characteristics desirable in winemaking (the presence of high levels of enzymes of oenological interest is a required feature as the presence of these activities favor the release of grape terpenoids, influencing the flavor). In this context, with regard to enzymatic activities, a high variability was found for β-glucosidase the highest levels of this activity were exhibited by the ND1 (*H. osmophila*), M3 (*M. pulcherrima*), AP9 (*H. guilliernmondii*) strains thus demonstrating that their presence in fermentations could potentially influence the aroma of the wines produced. Variability of the results was also found for resistance to antimicrobial compounds, particularly for sulphur dioxide, while as regards the resistance to copper the best result was exhibited by almost all strains of *M. pulcherrima*. This species has also shown good results in oxidative stress resistance evaluation tests but presented a higher sensitive to the presence of high concentrations of ethanol than the other species. On the contrary, the strains belonging to the species *H. guilliermondii, S'codes ludwigii, T. delbrueckii* and *P. fermentans* showed both good resistance to the presence of high concentrations of ethanol both to high osmo-tolerability linked to the ability to grow in the presence of sugars. Of the present work from an initial quantity of 29 non-*Saccharomyces* yeasts, we selected 12 strains characterized by interesting oenological traits, such as the presence of β-glucosidase activity, high tolerance to osmotic and oxidative stress as well as good ability to survive in the presence of ethanol. Strains possessing the best combination of these parameters belonging to the species *T. delbrueckii, P. fermentans, M. pulcherrima, H. guilliermondii / osmophila, S'codes ludwigii* were chosen to be used in must with a high alcoholic potential in combination with *S. cerevisiae* to evaluate their positive impact on winemaking.

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Chapter II

Evaluation of the oenological aptitude of selected non-Saccharomyces strains

Abstract

The research activity of this step was addressed to evaluate the fermentative behaviour of non-*Saccharomyces* strains, previously selected on the basis of technological properties, in combination with *S. cerevisiae* commercial strain (EC1118).

Fermentations were carried out on laboratory scale using pasteurized natural must and two different inoculation conditions, sequential and simultaneous inoculums.

In order to evaluate the contribute of each non-*Saccharomyces* strain to the fermentative process, the fermenting samples were monitored over time, by analysing the consumption of sugars during the process, and the evolution of the two inoculated species during fermentation was monitored by plate counting on fermenting must aliquots, sampled at different steps.

The results obtained in this step were useful for selecting the starter combinations possessing the most interesting characteristics for the use as mixed starter cultures able to produce wine with reduced ethanol content and suitable organoleptic characteristics.

2.1 Mixed fermentations to reduce the alcohol content and improve the quality of wines

2.1.1 Effect of Climate change

Recent studies have highlighted the correlation between climate change and the timing of some natural phenomena, such as the migration of birds, life, and the different stages of growth of some animals.

In the last decades, climate change by water deficit and high temperatures has impacted the vine phenology, grape composition and vegetative cycle (Jones et al., 2005). In the vineyard, effects linked to the climatic change are represented by anticipation of the dates of budding, flowering and maturation (in the last 10-30 years, in many regions the tendency is to bring the harvest forward by about 2-3 weeks), the increase in the concentration of sugars in grapes, high percentages of ethanol, modifications of aromatic compounds.

2.1.2 Effect of grape maturation and climate change on wine quality

During the ripening period of the grapes, there is a progressive increase in the concentration of sugars, phenolic compounds, potassium, and a reduction of organic acids content. Therefore, if all the viticultural variables remain constant, climate changes will have a great influence on the quality of the fruits.

Warm climate decrease activity of photosynthesis and high temperatures influence the metabolic activities of the vine, determining an increase of pH of the grapes and a reduction in acidity. In this contest, tartaric acid (the main acid of the grape) remains stable while malic acid content decreases (Adams 2006, Ollat et al., 2002). Sun exposure also influence the accumulation of some compounds related to wine aroma and colour, such as phenolic compounds, or anthocyanins, whose synthesis decreases with high temperature, causing loss of colour. In addition, musts with high concentrations of sugar make fermentations difficult, leading to the formation of unwanted metabolites, such as acetaldehyde and pyruvate. High sugar concentrations in grape must produce in microorganisms, as response to osmotic stress, an up-regulation of glycolytic and pentose phosphate pathways genes, which leads to formation of co-products, such as glycerol and acetic acid, which may affect wine aroma (De Orduna 2010). All these changes could lead to a modification of microbial must ecology, sluggish alcoholic fermentation, and risk of organoleptic alteration.

In consequence of all these aspects, the climate change represents a challenge for winemaking industry, principally for the modification of grape composition and consequently wine quality.

2.1.3 Wine general definition OIV-OENO 18/73

On the basis of OIV (Organization International of Vine and Wine) definition “Wine is the beverage resulting from partial or complete alcoholic fermentation of fresh grapes, whether crushed or not, or of grape must. Its actual alcohol content shall not be less than 8.5% vol.

Nevertheless, taking into account climate, soil, vine variety, special qualitative factors or traditions specific to certain vineyard, the minimum total alcohol content may be able to be reduced to 7% vol. by legislation particular to the region considered."

Actually, in the low alcohol wines category are included (Saliba et al., 2013):

- De-alcoholised wine (0.5%v/v)
- Low alcohol (0.5-1.2% v/v)
- Reduced alcohol (1.2-6.5% v/v)
- Lower alcohol (5.5-10.5% v/v)

2.1.4 Effects of high alcohol content in the wine

Ethanol is one of the main products of alcoholic fermentation; its concentration in wines varies from 8 to 16% (v / v).

The request of beverages with reduced alcohol content is highly increased among modern consumers. The growing demand for this type of product is linked to the health benefits derived from a moderate consumption of alcohol.

The effect of alcohol on health is complex. Although it's well known that the abuse of alcoholic beverages is related to the onset of diseases, such as cirrhosis, cardiovascular problems, diabetes and in extreme cases to death, on the other hand moderate consumption of alcohol, especially red wine, can protect against cardiovascular disease, atherosclerosis, hypertension, some cancers, neurological disorders. The mechanism of action has been attributed to antioxidant, lipid-regulating and anti-inflammatory effects (Guilford et al., 2011).

High alcohol content not only negatively influences the health aspect of the product, but also the sensory attributes. Nowadays, consumer's demand for new drinks with peculiar aromatic properties is increasing and connects to some fundamental sensorial aspects, such as color, taste, sensation in the mouth and preference of fruity aromas and full-body wines (Mestre et al., 2019). The high ethanol content in the wine generally covers the fruity attribute linked to the grape ripeness, which influences aromatic profile of the product.

It modifies the stability of some pigments and enhances the anthocyanin extraction process, masks the fruity character by enhancing the bitter taste, decreases the volatility of most of the compounds linked to the aroma (Escudero et al., 2007) and causes alterations in the cellular structure of microorganisms, in some cases causing cell death and fermentation stuck.

2.1.5 Strategies for ethanol reduction in wine

In order to satisfy the growing market demand for wines with a reduced alcohol content, characterized by greater aromatic complexity and color stability, innovative techniques were investigated.

Wine producers are currently interested in reducing alcohol content in wine by focusing on the use of technologies that allow them to achieve this goal, preserving at the same time the organoleptic balance and quality of the produced wine.

The proposed techniques can be divided into four categories: solutions to be applied in the vineyard; pre-fermentative strategies; fermentation techniques; post-fermentative techniques (Ozturk et al., 2014).

The first two groups include techniques that lead to a reduction in the sugar content of grapes based on the improvement and/or modifications of some viticultural practices. These include the displacement of vines in geographical areas that allow a delay in the ripening times of the berries and the anticipation of the harvest (more widespread technique even if it involves the loss of some qualitative aspects of the wines).

Other industrial strategies are based on the use of enzymes, such as glucose oxidase, a dehydrogenase and other enzymes, that allows to reduce the glucose content and, consequently, the final content of ethanol.

In some countries it is also allowed to dilute the must according to specific legislative regulations, to have a conspicuous reduction of ethanol but this causes a detriment of the aroma, flavor and color of the wines produced.

Other physical technologies, included in post-fermentation strategy, are reverse osmosis technique or membrane filtration systems.

Although the technological approach gives a good result as regard the ethanol reduction, it does not improve the sensory components highly appreciated by consumers.

An alternative to the technological approach is the “biotechnological” strategy. This approach, based on the use of specific microorganisms, includes two main strategies: the development of engineered yeasts (Genetically Modified Organisms - GMO) and the selection of new yeast strains, not conventionally used in the production process.

The common aim of all the proposed strategies is the deviation of the main pathways of carbon metabolism towards the production of fermentation by-products instead of ethanol, avoiding the accumulation of metabolites that can negatively affect the quality of the product (Tilloy et al., 2014).

Among the solutions proposed in the biotechnological approach, the use of non-conventional yeasts has received greater approval from public opinion respect to the use of genetically modified organisms.

In this context, the selection of non-*Saccharomyces* yeasts as a starter is the most investigated strategy to produce wine with a reduced ethanol content, but with improved organoleptic quality.

The table 2.1 resumes the main strategies used to produce wine with reduced ethanol content (Pickering et al., 2000).

PRINCIPLE	METHOD
Reduction of fermentable sugar in grape	➢ Juice dilution ➢ Freeze concentration ➢ Use of enzyme (glucose oxidase)
Removal of alcohol from wine	➢ Distillation under vacuum; evaporation; freeze concentration ➢ Dialysis; reverse osmosis ➢ Extraction by organic solvent
Other methods	➢ Dilution of wine ➢ Stop of fermentation ➢ **Low alcoholic producing yeast**

Table 2.1 The main strategies used to produce wine with reduced ethanol content (Pickering et al., 2000).

2.2 Use of non-*Saccharomyces* yeasts to reduce the alcohol content of wine and improve the synthesis of secondary metabolites

The use of mixed starters of non-*Saccharomyces* yeast strains represents one of the promising strategies used to improve the quality of the wine produced. This strategy exploits the ability of yeasts to produce enzymes and secondary metabolites that enhance the qualitative sensorial properties of wine. In recent years, the use of selected non-*Saccharomyces* yeast strains in mixed fermentations has been proposed not only for their contribution in the formation of the wine bouquet or as a form of biocontrol in the process, but also for the reduction of the ethanol content of wine. This theory is based on the ability of some yeasts to metabolize the sugars present in grapes generating a reduced amount of ethanol and at the same time producing various secondary compounds. Mixed fermentation is particularly suitable for extremely hot regions, where in recent years an increase of about 2 degrees of alcohol in the produced wines has been observed.

In the wine industry, non-*Saccharomyces* yeasts have been used in mixed starters as, conversely to *S. cerevisiae*, non-*Saccharomyces* yeasts are not able to consume all the sugar present in the must, consequently the addition of *S. cerevisiae* is necessary to complete the fermentation. For mixed starter fermentation, two different methods of inoculation, sequential and simultaneous, are used. The fermentation by co-inoculation is carried out by simultaneous inoculation of non-*Saccharomyces* yeast and *S. cerevisiae* strain; usually, high inoculation levels are used for non-*Saccharomyces* strains, mainly for species less competitive than *S. cerevisiae*. On the contrary, the sequential inoculation modality foresees that the non-*Saccharomyces* yeasts are firstly inoculated at high level to ferment alone, after few days *S. cerevisiae* is added to complete the process. In this way, the early inoculation of non-*Saccharomyces* allows the consumption of the sugar present to produce biomass and fermentation by-products, before the addition of the *S. cerevisiae* strain, which complete the fermentation producing ethanol, but in lower amount as a part of sugars were already consumed by non-*Saccharomyces* strains.

Both the strategies are useful for this aim, the choice of the most appropriate modality for the fermentation process depends on the interactions between the yeasts included in the mixed starter (Padilla et al., 2016; Ivit et al., 2020). Garcia et al., 2020 reported that the use of *W. anomalus*, *M. guilliemondii* and *M. pulcherrima* in mixed fermentations with sequential inoculation allows obtaining a reduction between 0.8 and 1.3% v / v of ethanol. Furthermore, the wines obtained showed a higher glycerol content than the control fermentation, probably linked to an overexpression of the glcerol-3-phosphate dehydrogenase gene when the species coexist with *S. cerevisiae*.

The principle on which this strategy is based is the selection of yeasts with low alcoholic yield or those capable of aerobically metabolizing sugar without simultaneous production of ethanol. This strategy aims at reducing the yield in ethanol through the inoculation of different selected species of yeasts that consume the sugars present in the must by respiration pathway rather than fermentation process. By respiration process the yeasts do not produce alcohol and gives rise to higher concentrations of carbon dioxide, the main product released by the metabolism of sugars that has no negative influence on the quality of the wine.

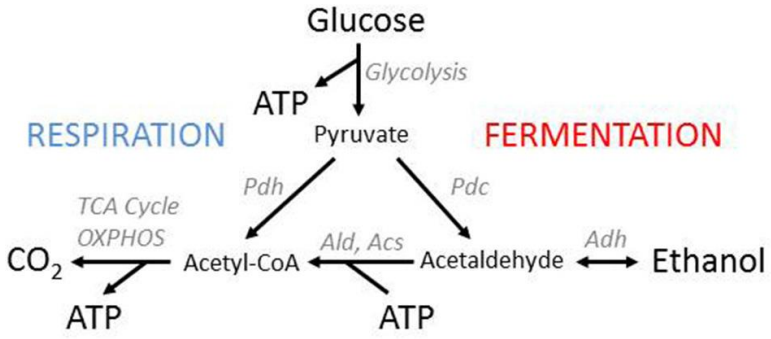


Figure 2.1 Main metabolic sugars pathways

To favor respiration, it is necessary to provide a certain concentration of oxygen in the early stages of the process. For this reason, the selected yeasts must be Crabtree negative, species characterized by a rather limited fermentative metabolism in the presence of oxygen, and therefore able to carry out the respiration process in the specific conditions of the grape must. Non-*Saccharomyces*, unlike *S. cerevisiae*, even when sugar exceeds 10 g / L can use oxygen for growth and divert carbon metabolism towards the formation of secondary compounds other than ethanol.

However, it is necessary to use controlled aeration systems otherwise the risk is that of causing the oxidation of some phenolic compounds and antioxidants, others that promote the formation of some compounds, such as acetic acid which have a negative impact on the sensory aspect. Under these conditions, for example, *T. delbrueckii* and *Zygosaccharomyces balii* show a reduction of the ethanol content of 1.5% (v / v) and 2.0% (v / v) respectively compared to fermentations carried out by pure fermentation with *S. cerevisiae* under aerobic conditions (Contreras et al., 2015). Under

controlled aeration conditions, yeast strains belonging to *M. pulcherrima* and *Zygosaccharomyces* in mixed fermentations reduced the ethanol content by 1.5 and 2.2% v / v respectively (Canonico et al., 2016). *Metschnikowia pulcherrima* in mixed culture and in high aeration conditions leads to a drastic reduction in the alcohol percentage (3.7%), unfortunately associated with unacceptable levels of volatile acidity.

It has been shown that a reduction in air flow to 25% represents a good compromise between the reduction of alcohol levels (2.2% v / v) and an increase in volatile acidity. The application of this practice at an industrial level could represent a significant advantage for warm regions where grapes have a high concentration of sugars by the time of harvest (Morales et al., 2015).

Although aeration is a common practice in wine production, the impact of air on the production of ethanol by non-*Saccharomyces* is still being studied.

2.3 Main non-*Saccharomyces* yeasts used in winemaking

The interest of the market to supply new innovative products for the industry has directed the research of the oenological microbiology sector towards the selection of new species to be used in mixed fermentation as a solution to some technological problems and or to enhance the sensory quality of wine. In 2017, the Organization of Vine and Wine (OIV) publishes a document "Monography of non-*Saccharomyces*" to international Oenological codex (OIV-OENO576B-2017), to underline the role of these species in wine production. In this document, it is specified that these yeasts must be isolated from grapes, musts or wine or result from hybridization of grape / must / wine strains, or have been derived from other wine yeasts (OIV-OENO 576B-2017), and clarifies that for commercialization of these yeasts, some guidelines have to be followed, such as the indication on the packaging of the genus, species and name of the strain, the instructions for use, and the recommended inoculation rate, the storage temperature and, if obtained by specific techniques, the genetic modification.

The main non-*Saccharomyces* species with properties of particular interest in the field of oenological microbiology are listed below.

Schizosaccharomyces pombe

This yeast is used for the deacidification of musts as it can degrade malic acid and gluconic acid. It is present as a commercial product as an alternative method for wine deacidification.

The wines obtained by mixed fermentation with this species showed increased concentration of acetaldehyde, propanol and 2,3-butanediol, but lower concentration of esters and were more appreciated by sensory panel respect to wine obtained with *S. cerevisiae* monoculture (Jolly et al., 2014).

Some studies reported that the final alcohol content of the wines in fermentation by *Schizosaccharomyces* is lower than those fermented by *Saccharomyces*, this confirms the theory

that the sugar consumption could also be employed to produce not only ethanol by also other compounds and to produce yeast biomass.

Schizosaccharomyces spp. can be used as a new tool to assure wine safety because could be used to reduce the urea content (precursor of ethyl carbamate a toxic compound in wine) by the urease activity (Benito et al., 2013).

Metschnikowia pulcherrima

Strains of this species are commercially available, which are characterized by extra-cellular α-arabinofuranosidase activity, good tolerance to ethanol (7% v / v). This yeast is widely used for its ability to improve the production of varietal aromas, such as terpenes and volatile thiols, and ester, mainly ethyl octanoate and the pear-associated esters.

This species was recently proposed for the reduction of ethanol. It has been used in mixed fermentations, both in sequential and simultaneous inoculation conditions, obtaining a reduction of ethanol from to 1.6% (Canonico et al.,2019)**.** This species has also been used in mixed fermentation in combination with *S. uvarum* with excellent results, the produced wines show a reduction of ethanol of about 1.7% and an increase in the concentration of succinic acid and glycerol as well as esters and higher alcohols in comparison single fermentation. A reduction of ethanol was found in mixed fermentation using different aeration regimes, unfortunately often accompanied by an increase in volatile acidity (Morales et al.,2015). Consequently, for the industrial application of this yeast, it is necessary to optimize the ventilation in order to safeguard the quality of the final product.

Lachancea thermotolerans

This species has a positive influence on the sensory characteristics and total acidity of the wine, in fact it reduces the pH, transforms sugars into lactic acid, and it is a good producer of glycerol (> 5g/L).

This species is a good candidate for mixed starter formulation in fermentations with sequential inoculum, in which it allows to obtain a reduction of 0.7% of ethanol (Gobbi et al., 2013). Furthermore, this species can be used to enhance floral and tropical fruit aromas during mixed fermentations of grape musts because it is a good productor of lactic acid, glycerol and 2-phenylethanol.

Candida stellata

This species positively contributes to the production of secondary metabolites and, in particular, it is known as a high glycerol producer with concentrations reported in wine up to 14 g /L (Jolly et al., 2014).

In mixed fermentations, if used in co-inoculation with *S. cerevisiae*, it is not able to consume all the sugar in the must, reaching ethanol levels of about 5.8% v/v (Invit et al., 2020). On the contrary, in fermentations with sequential inoculation, it allows to obtain dry wines with reduced ethanol content.

Studies show a greater production of glycerol in fermentations with sequential inoculation (15.7 g/L) compared to simultaneous fermentations (5.2 g/L) (Invit et al., 2020).

Generally, *C. zemplinina/S.cerevisiae* wines presented significantly increased concentrations of linalool, citronellol, nerolidol, and geraniol, while decreased concentrations of aldehydes and acetate esters, but this is connected to grape variety and must quality.

Torulaspora delbrueckii

T. delbrueckii was one of the first commercial non-*Saccharomyces* starter yeast to be used. This is a yeast with interesting metabolic properties, already present on the market as dry yeast or in frozen form. In mixed fermentations, it allows to increase the glycerol content (4.1-8.9 g/L) and larger quantities of diacetyl, ethyl lactate and phenyl ethyl acetate compared to the control fermentation (Invit et al., 2020).

Many studies suggest that the use of this species in mixed fermentations reduces the ethanol content slightly (<0.2%) or not at all compared to the fermentation conducted by *S. cerevisiae*.

Other metabolites produced include succinic acid and sometimes linalool, which is derived from monoterpene alcohols.

This species modulates wine flavour and aroma, intensity, including 'ripe red fruit 'aroma, increased sweetness, and astringency, particularly when employed in fermentation by co-inoculum with *S. cerevisiae*. (Loira et al., 2014).

Hanseniaspora spp

Hanseniaspora spp. generally show low fermentative power, but these species are important in the production of some wine volatile compounds and influence chemical composition of wines.

Although apiculate yeasts may be associated with the production of high levels of volatile acidity, sulphur compounds, they can have a positive influence on the flavour profile of wine (Jolly et al., 2014). The low frequency of *Hanseniaspora spp*. in fermentation process has also been suggested as a reason for the lack of aroma complexity.

Hanseniaspora vineae (*H. osmophila*) and *H. guilliermondii* are known to produce increased amounts of 2-phenyl-ethyl acetate during vinification, often associated to rose, honey and flowery sensory descriptors (Martin et al., 2018).

Co-fermentation of must with *H. osmophila* and *S. cerevisiae* produced wines that showed an increase of higher fruity' sensory scores than wines produced with *S. cerevisiae* monoculture, while

wines produced with *H. guilliermondii* and *S. cerevisiae* have shown higher concentrations of heavy sulphur compounds associated unpleasant sensory descriptors.
Hanseniaspora uvarum has also been used in mixed fermentations with *S. cerevisiae*. Wines fermented with *H. uvarum/S. cerevisiae* showed increased concentrations of higher alcohols, acetate, and medium-chain fatty acids.

2.3.1 Commercial formulations of non-*Saccharomyces* yeasts

Some formulations of starters that include non-*Saccharomyces* species are already available on the market; the main producers are Laffort; Lallaemand; Enartis; etc.
The main species proposed in the formulation of mixed starters are *T. delbrueckii* (production of thiols, esters, mannoprotein pigments, higher alcohols), *M. pulcherrima* (biocontrol activity, modulation of the sensory profile and the aromatic composition, reduction of the ethanol content); *K. termotolerans* (important role in the production of lactic acid to avoid excessive deacidification of musts), *C. zemplinina* (reduction of the ethanol content and high production of glycerol).
In particular, the majority of starter cultures available on the market include products that claim to improve the aromatic complexity of the wine bouquet as well as improve some structural properties. Secondly, commercial starters have bioprotection and acidity reduction activities, whose use is recommended especially in regions with a very hot climate. The products that have received the greatest interest on the market are listed below: Biodiva® Flavia® and Concerto®, corresponding respectively to the *T. delbrueckii*, *M. pulcherrima* and *K. thermotolerant* species. The use of Biodiva® is indicated for the reduction of volatile acidity and improvement of the aromatic complexity of wines, as well as for the increase in the persistence of the foam in the production of sparkling wines. On the contrary, Flavia® is used with the aim of improving the aromatic complexity of the wine, enhancing floral aromas, notes of pineapple and citrus, producing a large amount of ester thiols and terpenes. Concerto®, is finally proposed to increase acidity of the wine thanks to the production of lactic acid and malic acid with the aim of overcoming the problems linked to climate changes, that have led to an increase in the sugar content and a reduction in the acids present in musts. Promalic® (*S. pombe*), proposed to reduce the process times of alcoholic and malolactic fermentation thanks to a process of de-acidification of the wine. (Roudil et al., 2020)

Although non-*Saccharomyces* yeasts seem to represent a promising solution for the reduction of the alcohol content, different aspects have to be evaluated before the use of these yeasts. In fact, some authors (Viana et al., 2009) have documented an increase in the alcohol percentage in wines produced using mixed cultures.
Therefore, the attention is focused not only on identifying the most correct inoculation methods, but also on the evaluation, according to a transcriptomic and proteomic approaches, of the

metabolic mechanisms underlying the molecular and cellular interactions established during the fermentation process between the different inoculated species and *S. cerevisiae*.

2.4 Focus on principal organic acids and secondary metabolites in wines

2.4.1 Glycerol

Glycerol is one of the major wine components, a polyol, a no volatile sugar alcohol, colourless, odourless, characterized by a sweet taste. In wine, it is formed as a by-product of yeasts metabolism during glycolysis pathway. Glycerol positively contributes to wine sweetness when is presents in quantities above is threshold taste level of 5.2 g/L. It is associated at mouthfeel sensation and confers a peculiar full body of wines (Nieuwoudt et al., 2002).

Its levels in wine are influence by different parameters, such as pH, nitrogen source, temperature, and inoculated yeast species. In general, about 2-3 % of sugar content in must is used for glycerol production.

This compound is synthetized by yeasts by two chemical reactions; the first one involves the reduction of dihydroxy acetone phosphate to glycerol 3 phosphate, followed by dephosphorylation of glycerol 3 phosphate to yield glycerol (Jain et al., 2011, Pahlman et al., 2001).

Nowadays, several attempts were aimed to establish strategy to increase glycerol level in wine in order to improve sensory evaluation of wine consumers.

2.4.2 Organic acids

Total acidity is the result of the sum of all acids present in wine; it influences many aspects, such as taste, aroma and freshness. Many factors are involved in the chemical composition, mainly represented by microbial ecology, vine variety, geographical position, climate, viticultural practices. The primary acids present in grape are malic, citric and tartaric acids, but other acids are present in small amounts (succinic, acetic, pyruvic, lactic acids) deriving from yeasts and bacteria metabolism and often associated to tart, sour, fresh and sometimes metallic wine taste.

Low acidity level supports microbial growth, also of spoilage species, for this reason acidity and pH are fundamental aspects to be considered for structure and sensorial balance of wine quality and represents a good tool to control contamination risks.

2.4.3 Tartaric Acid

This is the principal grape acid, and the main contributor to wine acidity. The level usually varies from 4.5 to 10 g/l, but the concentration depends on vine variety and soil composition. This acid is

characterized by a good stability, in fact, the microorganisms are unable to metabolize it. Usually, it is used for pH adjustment in wine industry.

2.4.4 Malic Acid

Common fruit acid, it is detected in mature grapes in a range from 2 to 6.5 g/L. When the acid malic content reaches too high level, it caused a sour taste and it is necessary the use of lactic acid bacteria to convert this compound in lactic acid, which has a softer taste.

2.4.5 Citric Acid

This is an organic acid generated during TCA cycle, widespread in nature. Some malolactic bacteria can metabolize citric acid, it is generally present in small amount in grape and influence freshness perception in wine, but higher value during fermentation can reduce growth of yeast cells and promote the development of some unwanted species.

The metabolism of citric acid seems have a role in diacetyl production. In general, the content of citric acid is about 0.5-1 g/L.

2.4.6 Succinic acid

This acid is synthesized during fermentation process by yeast metabolism (0.5-1.5g/L in wine). Winemakers pay particular attention on the influence of this compound on the organoleptic properties because it is descripted by bitter-salty flavour, and its threshold is about 35mg/L.

2.4.7 Lactic acid

It is produced by decarboxylation of malic acid during malolactic fermentation, the concentration in the wine ranged between 1 and 3 g/L.

During winemaking, its level is controlled by addition of sulphur dioxide in order to inhibit metabolic activity of *Oenococcus* and *Lactobacillus* genera, able to produce this acid. This acid contributes to mouthfeel sensation by a soft and creamier taste (Chidi et al., 2018).

2.4.8 Acetic acid

It is the main constituent of wine volatile acidity.

Desirable level in wine is about 100-300mg/L, it is considered a defect if present at concentration higher than 0.8 g/L. The aroma threshold varies on the basis of variety but the maximum value for wines is 1.2 g/L (OIV, 2010).

This compound is produced by lactic and acetic bacteria, but also by yeasts during alcoholic fermentation. Enzymatic reactions that can lead to acetic acid formation in yeast include reversible

formation from acetyl coenzyme A through acetyl CoA synthetase, split of citrate by-citrate lyase, production from pyruvate by pyruvate-dehydrogenase, reversible formation from acetyl phosphate by acetyl kinase, and oxidation of acetaldehyde by aldehyde dehydrogenase (Vilela-Moura et al., 2008).

2.4.9 Pyruvic acid

It is one of the most common secondary metabolites of alcoholic fermentation, its range varies from 10 to 500 mg/L in dry wines; it impacts the sensory profile of product in consequence of its tart taste. It is able to bind sulphur dioxide, an antimicrobic agent often used in winemaking practices (Chidi et al., 2008).

2.5 Contribution of non-*Saccharomyces* yeasts on the aromatic quality of wine

Aroma and colour are the main aspects of the quality of wines. The aroma can be defined as a large mixture of volatile compounds, some present in small quantities (mg/L), and represented by a complex blend that includes volatile compounds derived from grapes (primary aroma), those synthesized during fermentation (secondary aroma) and those produced during aging (tertiary aroma)

The synthesis of these compounds during fermentation is linked to the development of different yeast species that produce esters, thiols, terpenes, and other compounds responsible for the floral and fruity notes of the wine bouquet.

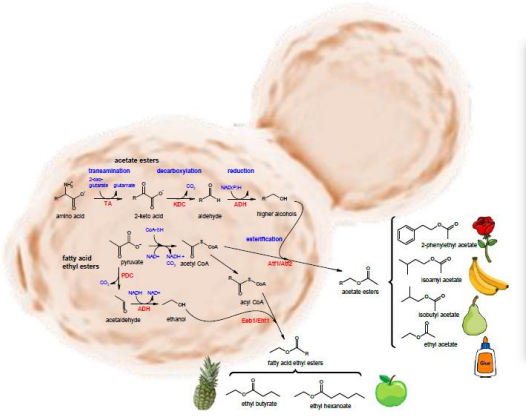


Figure 2.2 Metabolic pathway correlated to production of secondary aroma (Morata et al., 2020)

The use of mixed fermentation is the new biotechnological tool to modulate the chemical aroma profile and enhance colour stability of wines.

In some region, the climate change has determined not only an increment of ethanol content, but also a reduction of total acidity, and consequently, a pH reduction. The use of non-*Saccharomyces* in co-culture with *S. cerevisiae* could be used to enhance the total acidity of wines as a biological acidification in warm region.

Over the last decades, several studies have evaluated the possibility of the use of selected non-*Saccharomyces* yeasts to improve the production of secondary metabolites involved in the formation of wine flavor.

The production of aromatic compounds in wine is linked to the species and strain of yeast used. Non-*Saccharomyces* yeasts, present in high concentrations in the must in the early stages of fermentation, are able to produce a wide range of volatile metabolites compared to *S. cerevisiae*. The production of volatile compounds during fermentation is influenced by the enzymatic profile of the yeast strain, in consequence of the presence of genes coding for enzymes linked to the production of primary and secondary aromatic compounds in wines. Several studies have shown that non-*Saccharomyces* yeasts produce a greater variety of extra cellular enzymes than *S. cerevisiae*, and these enzymatic activities influence the aromatic complexity. The yeasts involved in the formation of the aroma belong to *Hanseniaspora spp*, *Candida spp*., *M. pulcherrima*, present in abundance on the grapes and in the first stages of fermentation, whereas other species, such as *Torulaspora delbrueckii* and *Lanchancea*, can persist also in the later stages of fermentation as they are more resistant to high alcohol concentrations than non-*Saccharomyces* species present in the first steps of the process.

Yeasts belong to the *Hanseniaspora* species are characterized by slow fermentation kinetics; these yeasts tend to consume high quantities of nitrogen in the initial stages of fermentation, limiting the growth of other yeasts including *S. cerevisiae*. The *Hanseniaspora* species is widely used in mixed fermentations as its presence gives a fruity flavour to the wine in consequence of the high production of acetate esters (mainly isoamyl-acetate, isobutyl acetate, which give banana and strawberry aroma, respectively), higher alcohols and sulfur compounds.

Co-fermentations of *H. uvarum* and *S. cerevisiae* produced wine characterized by high concentration of isoamyl acetate and short chain ethyl asters. However, the presence of the *Hanseniaspora* species is also associated with high concentration of acetic acid and ethyl acetate, which negatively impact sensorial quality.

Fermentations with *H. guilliermondii* show an abundant production of acetate esters, 2-phenyl-ethyl acetate and isoamyl acetate, giving rose and honey aroma to the obtained wines.

H. vinae or *osmophila* exhibits good fermentation kinetics thanks to the high ethanol tolerance (> 10% v / v), probably due to the presence of genes coding for the alcohol dehydrogenase enzyme; consequently, these yeasts participate also to the latest stages of fermentation, with a high influence on the aromatic profile. The ability of this yeast to produce large quantities of acetate esters, and in particular high concentrations of 2-phenyl ethyl acetate and short-chain ethyl esters, make it particularly suitable to produce wines with fruity aroma. It is also capable of producing some rare compounds, not found in *S. cerevisiae*, such as N-acetyl tyramine and 1Hindol-3-ethanol (Borren et al., 2021).

As reported by Viana (2009), mixed culture of *H. osmophila* and *S. cerevisiae* can be used to increase 2-phenyl ethyl acetate level, a compound characterized by a peculiar fructate flavour and floral aroma.

Another non-*Saccharomyces* yeasts able to persist until the end of the fermentation process is *Candida zemplinina*, a yeast characterized by high alcohol tolerance (> 10%). Studies have shown that the coexistence between *C. zemplinina* and *S. cerevisiae* in mixed fermentations has positive effects on the fermentation kinetics of both species. *C. zemplinina* is a fructophilic yeast, whereas *S. cerevisiae* prefers glucose, their simultaneous presence in fermentation favours the consumption of all the sugars present in must.

Studies report the use of *C. zemplinina / S. cerevisiae* in mixed fermentations for the production of wines with reduced alcohol content and high level of glycerol (Giaramida et al., 2013).

Unlike *C. zemplinina*, *Metschnikowia pulcherrima* species is characterized by a low fermentation power, resulting able to survive up to only 4% v / v of alcohol.

However, this species possesses important oenological characteristics, among of which the production of "pulcherrimin", a pigment with antimicrobial properties against other non-*Saccharomyces* yeasts and some fungi, such as *Botrytis* cinerea and *Penicillium*, whereas *M. pulcherrima* is often indicated as a good potential candidate in the formulation of mixed starters.

It also shows good proteolytic activity, β-glucosidase, high production of esters (among which the most abundant is ethyl octanoate), thiols, terpenes (limonene, alpha-terpineol). Its presence in mixed fermentation has also been associated with high levels of ethyl acetate, which is responsible of negative odour notes (Borren et al., 2021).

Lachancea thermotollerans has good oenological properties, it is used to reduce the pH of wines in consequence of its ability to produce lactic acid. This yeast was found with low frequency on grapes, vines and soil, but it was reported that it plays an important role in creating regionally specific aroma in some regions of Portugal (Borren et al., 2021).

Among the non-*Saccharomyces* yeasts, *Torulaspora delbrueckii* show a fermentative behaviour very similar to *S. cerevisiae*. Studies have shown that the sequential fermentation with *T. delbrueckii* and *S. cerevisiae* showed a reduction in glycerol content and 2-3 butanediol and at the same time a higher content of diacetyl, ethyl lactate, 2 phenylethyl acetate compared to the single fermentation with *S. cerevisiae* (Loira et al., 2014).

The results obtained in numerous studies suggest that non-*Saccharomyces* yeasts produce different concentrations of extracellular enzymes and a wider range of volatile compounds than *S. cerevisiae* species; for these characteristics, these yeasts are able to influence the fermentation kinetics and the aromatic profile of the produced wine.

Evaluation of the oenological aptitude of selected non-*Saccharomyces* strains

2.6 Materials and methods (I)

In this step, the oenological aptitude and enzymatic profile of the previously selected strains were analysed. The analyzed yeasts were LC 2-1, 425 (*T. delbrueckii*); LM 5-3 (*P. fermentans*); Mpr2-3, Mpr 2-4, M3, SIA 4 (*M. pulcherrima*); TS B; SNM3 H; AP 9 (*H. guilliermondii*); ND1 (*H. osmophila*); SIA 2 (*S'codes ludwigii*).

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.

2.6.1 Fermentation trials

In order to test the fermentation ability of the previously selected non-*Saccharomyces* yeast strains, micro-fermentations at laboratory scale were performed in flasks containing 100 mL of natural grape must. The fermentation tests were carried out by inoculating the single strain, using an inoculum level of 10^7cell/mL. The fermentative processes were followed by evaluating the sugar consumption during the time, whereas the experimental wines were analyzed for main analytical parameters, such as residual sugars, ethanol, volatile acidity by a Fourier Transfer Infrared WineScan instrument (OenoFoss).

2.6.2 Evaluation of enzymatic activities

These strains were submitted to screening for the presence of 19 enzymatic activities by using API®ZYM system (BioMérieux, France), a semi-quantitative micromethod for the research of 19 enzymatic reactions. This system is composed by strip with 20 microwells (capsules) filled with synthetic substrate and its buffer.

The enzymatic strip tests were inoculated with a microbial suspension containing $5*10^8$ cells/mL corresponding to 5-6 McFarland turbidity. Suspensions were inoculated in microwell at level for 65 µl for each couple and the strips were incubated at 37C° for 4-5 hours. Later in each couple ZYM A and ZYM B reagents were added, and the metabolic reactions produced during the incubation period (5_min) were detected with coloured reactions after reagent addiction.

Figure 2.3 API®ZYM system

2.7 Results and discussion (I)

2.7.1 Fermentations trials

In this step, the main parameters related to the fermentative process have been assessed for each strain, such as the fermentative vigor, expressed as amount of CO_2 produced in the first days of fermentation, and the ability to consume a high quantity of sugars at the equal amount of produced ethanol. The sugar consumption/ethanol production ratio is useful to evaluate the different ability of non-*Saccharomyces* yeasts to use the sugar present in grape must.

As expected, all non-*Saccharomyces* yeast strains in pure culture showed a lower fermentation activity than the *S. cerevisiae* strain. As shown in the table 2.2, some strains belonging to the species *H. osmophila*, *T. delbrueckii*, *S'codes ludwigii* exhibited a good fermentative vigor, indicating a potential ability of these strains to start quickly the fermentative process. However, they are also characterized by a low fermentative power, consequently they finish the process later respect to *S. cerevisiae*.

In order to identify the strains potentially capable of reducing the wine alcohol content, the unit ratio of sugar / ethanol conversion was calculated, as shown in the table 2.2. It has been shown that for *S. cerevisiae* strains the conversion ratio is between 16.83 and 19 g/L (Furlani et al., 2017), whereas the non-*Saccharomyces* species generally utilize more than 16.83 g/L to produce 1% of ethanol, in consequence, these strains probably contribute to ethanol reduction. As regards non-*Saccharomyces* strains tested in this study, some of them (such as *P. fermentans* LM 5-3; *M. pulcherrima* Mpr 2-4; *H. guilliermondii* TSB) exhibited a conversion ratio higher than that found for *S. cerevisiae*.

Another parameter evaluated in this step was the level of volatile acidity as it's well known that some non-*Saccharomyces* yeasts are high producers of this character. All the experimental wines obtained at this step contained values of volatile acidity that fall within the desirable range of volatile acidity without negative repercussions on the organoleptic characteristics of the product.

The strains exhibiting the better fermentative performances, which were good fermentative vigor and optimal sugar alcohol conversion ratio, were evidenced in the table 2.2.

SPECIE	STRAIN CODE	FERMENTATIVE VIGOUR (CO_2/gg)	SUGAR/ALCOHOL CONVERSION (g/L)	VOLATILE ACIDITY (g/L)
T. delbrueckii	LC 2-1	4.21	16.79	0.31
	425	3.74	16.9	0.46
P. fermentans	LM 5-3	2.2	17.7	0.59
M. pulcherrima	M3	1.54	17.03	0.49
	Mpr 2-4	1.73	17.27	0.51
	Mpr 2-3	1.67	16.24	0.56
	SIA 4	1.58	16.8	0.49
S'codes ludwigii	SIA 2	4.67	16.9	0.47
H. guilliemondii	SNM3 H	4.74	17.8	0.42
	TS B	3.34	18.37	0.49
	AP 9	2.25	18.64	0.51
H. osmophila	ND1	5.02	16.67	0.56
S. cerevisiae	EC 1118	5.95	17.74	0.33

Table 2.2 Fermentative traits of non-*Saccharomyces* selected strains
The strains showing the bests results for the tested parameters are highlighted in grey.

2.7.2 Evaluation of enzymatic activities

Various enzymatic activities can be employed to improve the winemaking process; the addition of pectinases increase the extraction of juice from grapes, by favouring wine clarification and filtration, whereas the presence of glycosidases hydrolyze non-volatile glycosidic precursors, affecting aromatic characteristics of the wine.

In the wine industry to obtain these reactions, commercial preparations and enzymes are used which are added directly to the must or wine. Although the addition of exogenous enzymes is a common practice in wine making, the potential of indigenous wine yeasts to produce extracellular enzymes to modify the properties of wine seems to be an excellent tool that contributes to the formation of the aroma and taste of wine.

S. cerevisiae is not recognized as a significant producer of extracellular enzymes, but non-*Saccharomyces* species contribute to the enzymatic activity with reactions that occur in the must during the early stages of winemaking (Mateo and Maicas, 2016). Extracellular protease activity has been reported in some strains of *Kloeckera apiculata* and glucosidase activity in *Candida, Pichia* and *Hanseniaspora* strains.

Terpene compounds represent aromatic compounds which are present in large quantities as odourless precursors in grapes and can be released thanks to the action of some enzymes. Glycosidases, such as β-glucosidase, β-xylosidase, -apionidase, α-rhamnosidase and α-arabinofuranosidase have been described as involved in the release of flavor compounds. At this

purpose, high attention is paid to glucosidase activity, enzyme capable of releasing aromatic substances in wine, present in various strains of *Hanseniaspora vineae, Candida, Kluyveromyces, Metschnikowia, Pichia, Saccharomycodes,* the genera *Schizosaccharomyces* and *Zygosaccharomyces* (Zarzoso et al., 1998).

Other yeast enzymes, such as esterases are also involved in the formation of aromatic compounds. Esterases catalyse the hydrolysis of ester bonds in triacylglycerides to glycerol and fatty acids, ethyl acetate is the most abundant ester in wine, which can have positive effects on wine aroma if present in little amount.

The principal enzymatic activities evaluated by API® ZYM system were β-glucosidasese, the groups of esterases- lipases, the group of aminopeptidases (leucine arylamidase, valine arylamidase and cystine arylamidase), which are involved in the production of some amino acids, such as leucine, isoleucine, valine, and phenylalanine, known for being precursors of active aroma compounds produced by the yeasts and involved in wine final aroma (Escribano et al., 2017).

The results obtained by this test were reported in the matrix plot (figure 2.4), in which numeric values were assigned to the expression of the single activity, by using a scale from 0 (negative reaction) to 2 (high enzymatic activity). This figure shows that as the non-*Saccharomyces* yeasts exhibited more enzymatic activities respect the *S. cerevisiae* strain (EC1118). In fact, non-*Saccharomyces* strains show a good presence of enzymatic activities, such as leucine arylamidase, valine arylamidase and cystine; in particular, the genus *Hanseniaspora, Metschnikowia*, and *Saccharomycodes* exhibited a good presence of α-glucosidase and β-glucosidase, activities involved in the release of volatile compounds and aromatic notes in wines bouquet.

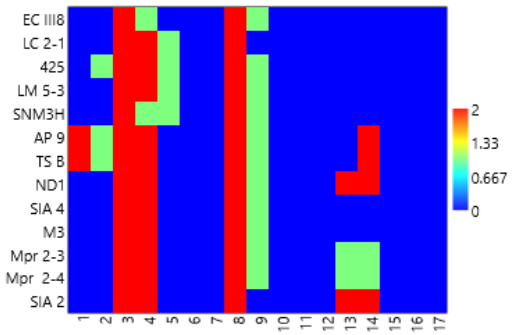


Figure 2.4 Matrix plot based on data obtained by the evaluation of enzymatic activities using API® ZYM system. Enzymatic activity codes: (**1**) ALKALINE PHOSPHATASE, (**2**) ESTERASE LIPASE, (**3**) LEUCINE ARYLAMIDASE, (**4**) VALINE ARYLAMIDASE, (**5**) CISTINE ARYLAMIDASE, (**6**) TRYPSIN, (**7**) α-CHYMOTRYPSIN, (**8**) ACID PHOSPHATASE, (**9**) NAFTOL-AS-BI-PHOSPHOHYDROLASE, (**10**) α-GALACTOSIDASE, (**11**) β-GALACTOSIDASE, (**12**) β-GLUCURONIDASE, (**13**) α-GLUCOSIDASE, (**14**) β-GLUCOSIDASE, (**15**) N-ACETIL-β-GLUCOSAMINIDASE, (**16**) α-MANNOSIDASE, (**17**) α-FUCOSIDASE.

0=negative reaction; 1= medium enzymatic activity; 2= high enzymatic activity

On the basis of results obtained in this step, some non-*Saccharomyces* strains were selected for further characterization. The selected strains were *T. delbrueckii* strains (425; LC 2-1); *P. fermentans*

(LM 5-3); *M. pulcherrima* (Mpr 2-4); *H. guilliermondii* (TSB); *H. osmophila* (ND1); *S'codes ludwigii* (SIA2).

Evaluation of the oenological aptitude of 7 non-*Saccharomyces* strains in microfermentations assays

2.8 Materials and Methods (II)

2.8.1 Mixed fermentations

In this phase, 7 strains of non-*Saccharomyces* previously selected [*(T. delbrueckii* strains (425; LC 2-1); *P. fermentans* (LM 5-3); *M. pulcherrima* (Mpr 2-4); *H. guilliermondii* (TSB); *H. osmophila* (ND1); *S'codes ludwigii* (SIA2)] were used as mixed starters in combination with *S. cerevisiae* commercial strain (EC1118). The mixed starters were tested in fermentation tests at laboratory scale according to the inoculation methods described below:

THESIS 1 Simultaneous inoculum	10^7 Cell/mL non-*Saccharomyces* + 10^3 cell/mL *S.cerevisiae*
THESIS 2 Sequential inoculum	10^7 Cell/mL non-*Saccharomyces* + 10^5 cell/mL *S.cerevisiae*

Table 2.3 Inoculation methods of mixed fermentation at laboratory scale (Invit et al.,2020)

Only for the strains belong to *T. delbrueckii* (425; LC2-1) species, the inoculum level was 10^7 cell/mL non-*Saccharomyces* + 10^7 cell/mL *S. cerevisiae* as this species has a fermentative behaviour similar to *Saccharomyces* species.

For each thesis, a pure culture of *S. cerevisiae* EC1118 (inoculum level of 10^7 cell/mL) was used as control.

The fermentations were performed in duplicate, using pasteurized natural must (Temperature=90°C for 20 minutes).

The must contained in each flask was contemporary inoculated with the two species in thesis 1 (simultaneous inoculum), while in the thesis 2 (sequential inoculum) the *S. cerevisiae* strain was added when it was produced 5% v/v of ethanol and the sugar content (Glucose/Fructose g/L) was reduced to 50% (Contreras et al., 2014).

Fermentation process was conducted at constant temperature of 26 °C and monitored by °Brix measurement (sugar consumption) until when the °Brix values were constant for three consecutive days.
The experimental wines were refrigerated and stored at 4°C in sterile falcon.

2.8.2 Analysis of yeasts viable population in fermentation process

The evolution of yeasts population during fermentative process was monitored by viable count on WL and LYS media (Domizio et al., 2011).
Aliquots of must samples were analysed at different intervals to monitor the evolution of the two species included in the mixed starters during alcoholic fermentation. The samples were diluted in saline solution and plated on WL Nutrient Agar (Oxoid, Hampshire, UK) to differentiate the yeast species on the base of the colony morphologies and colors, while LYSINE agar medium (Oxoid, Hampshire, UK) was used for non-*Saccharomyces* cells count, because this substrate contains lysine as only carbon source and *Saccharomyces* cannot utilize this carbon source.
For the isolation, 0.5mL of each sample appropriately diluted were taken and spread onto the plates containing the substrates; cells count was performed after incubation at 26 °C for 5 days.

2.8.3 Analytical determination

2.8.3.1 Analysis of secondary compounds in wines by gas chromatography

Gas-Chromatography (GC) analysis was used to analyse volatile components of experimental wines.
The chromatographic instrument is composed by an injector, a column, and a detector.
As carrier is used a gas to transport the volatile compounds trough the column stationary phase of the column and finally the data that arrives to the detector are collect by a software in a chromatogram for each sample. The choice of the technique depends on physical and chemical characteristics of the samples. In general, the identification of single compounds is based on retention times of the interest peaks (Qualitative analysis).
The quantification of identified peaks must be made by the evaluation of the peaks area that is proportional to the compound content (Quantitative analysis) (Vilanova et al., 2012).

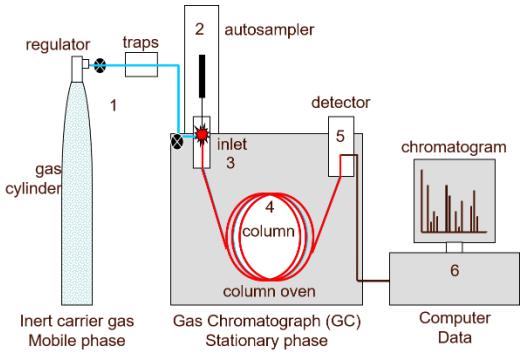


Figure2.5 Schematic representation of gas- chromatographic instrument

2.8.3.2 Gas chromatographic analysis

The experimental wine samples were analysed by gas-Chromatography (GC) with flame ionization detector analysis to quantify in a single run the principal secondary compounds present at relatively high concentrations, such as higher alcohols; acetaldehyde; ethyl acetate (Capece et al., 2013).

The analysis was performed by gas-chromatograph AGILENT 7890 A (FID), equipped with an autosampler and connected to the software "Agilent ChemStation". One microliter of the sample was injected into a glass column BAW 5% and CARBOWAX 20% (internal diameter=2mm; external diameter=6mm, Supelco). The temperature was increased from 80°C to 200°C, the gas (He) flow was 20mL/minute, and the duration of the run was about 25 minutes.

2.8.4 Determination of principal oenological parameters

Some oenological parameters were also monitored by Fourier transform Infrared Oenofoss™ instrument (OenoFoss™, Hillerød, Denmark). The sample absorbs light according to the appearance of wine constituents, such as sugars and acids etc. The absorption is converted through the Fourier transform Infrared mathematical model to a prediction of the concentration of various constituents. The Oenonofoss ™ analyser offers the possibility of performing quick analysis of multiple key parameters of winemaking in a few minutes. The parameters detected are reported in the table 2.4.

Figure 2.6 OenoFoss™ instrument

Table 2.4 Principal oenological parameters detected by Oenofoss™ instrument

Must	Must under fermentation	Wine
°Brix, pH, total acidity, volatile acidity, tartaric acid, malic acid, gluconic acid, density	Ethanol, acidity total, malic acid, volatile acidity, glucose/fructose, pH	Ethanol, total acidity, malic acid, lactic acid, volatile acidity, glucose/fructose, pH, density

2.8.5 Glycerol determination

Glycerol can be detected using different methodologies. In this work, glycerol level in wine samples was analysed by KIT GROL 08/18 (Megazime®), a very rapid method.

The principle of this method is based on the reaction of glycerol production during glycolysis pathway.

The reactions involved are reported below:

(I) Glycerol + ATP $\xrightarrow{(GK)}$ L-Glycerol-3-phoshate + ADP

(II) ADP + PEP $\xrightarrow{(PK)}$ ATP + Pyruvate

(III) Pyruvate + NADH + H+ $\xrightarrow{(L\text{-}LDH)}$ L-Lactate + NAD(+)

ATP= Adenosine-5'-triphospate

GK= Glycerokinase

ADP= Adenosine-5'-diphospate

PEP= Phosphoenolpyruvate

PK= Pyruvate kinase

NADH = Nicotinamide- adenine-dinucleotide

L-LDH = L-Lactate dehydrogenase

The amount of NAD (+) is stoichiometric with the glycerol concentration in the sample. The NADH consumption is measured by determination of absorbance decrease at 340nm. The measurement was performed in microplate (96 well, clear flat-bottomed, plastic). For each well, the different amount of the mix components reported in the table 2.5 was pipetted in the microplate.

Pipette into wells	Blank	Sample	Standard
• Distilled water • Sample solution • Buffer • Solution 2(NADH/ATP/PEP) • Suspension 3(PK/L-LDH)	• 0.200 mL • - • - • 0.020 mL • 0.010 mL • 0.002 mL	• 0.190 mL • O.010 mL • - • 0.020 mL • 0.010 mL • 0.002 mL	• 0.190 mL • - • O.010 mL • 0.020 mL • 0.010 mL • 0.002 mL
Read the solution absorbance (A1) after about 4 minutes and start the reaction by addiction of:			
• Suspension 4 (GK)	• 0.002 mL	• 0.002 mL	• 0.002 mL
Read the solution absorbance (A2) after about 5 minutes, or when the reaction will end			

Table 2.5 Procedure for glycerol determination by KIT GROL 08/18 (Megazime®)

Calculation:

g/L= $\frac{\Delta A\ sample}{\Delta A\ Standard}$ * g/L Standard * F

F= dilution factor

2.9 Results and discussion (II)

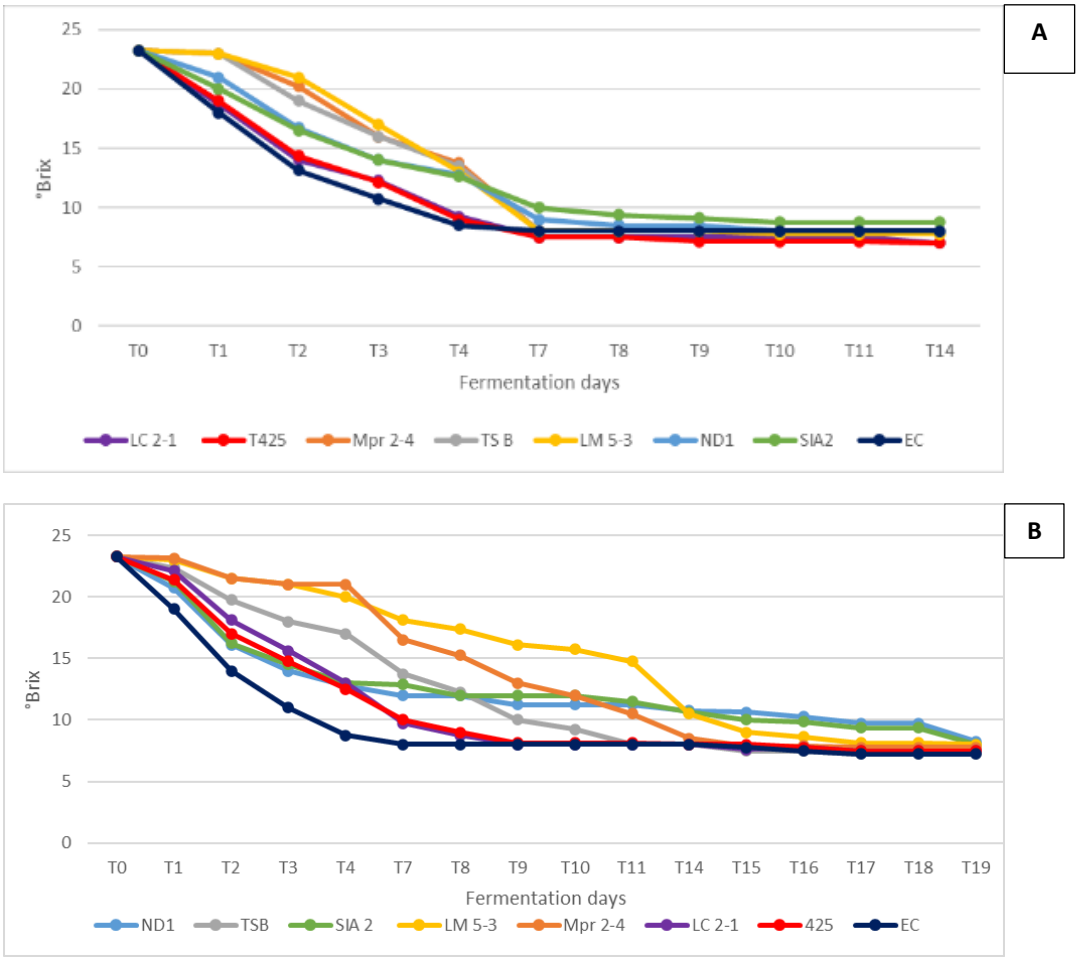
2.9.1 Analysis of fermentation kinetics

In order to analyze the behaviour of the selected non-*Saccharomyces* strains as mixed starters, mixed fermentations at laboratory scale were performed to verify the fermentative kinetic of the non-*Saccharomyces* strains preliminary selected. At this aim the evolution of °Brix degree during fermentations was evaluated to control the sugar consumption by yeasts; furthermore, yeast isolation during the process was useful to monitor the evolution of the different species composing the mixed starters.

The figure 2.7 represented the evolution of sugar consumption in the two tested conditions. As showed in the figures, the duration of fermentation was shorter in thesis 1 (simultaneous fermentation), in which the two different species were contemporary added to the must, than sequential inoculation (thesis 2). In thesis 1, all the starters consumed the total sugars faster than the corresponding sequential fermentation (thesis 2), in which in the first step of fermentation process are present only non-*Saccharomyces* yeast cells and sugar consumption resulted reduced.

Furthermore, in the first days of fermentation, in sequential inoculation the different mixed starters showed high variability in kinetics of sugar consumption, whereas fewer differences were found for simultaneous inoculations

Figure 2.7 Kinetics of sugar consumption of mixed starters cultures of *T. delbrueckii* (LC2-1, 425); *M. pulcherrima* (Mpr2-4); *H. guilliermondii* (TSB); *P. fermentans* (LM5-3); *S'codes ludwigii* (SIA2) strains simultaneously [A] and sequentially [B] inoculated with *S. cerevisiae* EC1118 (EC). Pure culture of *S. cerevisiae* EC1118 (EC) was used as a control.



In conclusion, the mixed starters show a good fermentative behaviour when the non-*Saccharomyces* strain and *S. cerevisiae* are simultaneous added in the grape must (Thesis 1).

2.9.2 Analysis of living yeast population evolution during the fermentation

With aim to understand the interactions between yeast strains included in the mixed starter the microbial population dynamics of the mixed fermentations were analysed at different times during the fermentations. The evolution of microbial cell growth of the analysed species during the fermentative process was evaluated by viable count on WL and LYS media of samples collected from mixed fermentations conducted in the two different inoculum conditions.

Similar behaviour was observed in all the samples, in fact the non-*Saccharomyces* species in all the cases exhibited higher growth values in the sequential inoculation than in simultaneous. Furthermore, in the sequential inoculum the presence of the non-*Saccharomyces* strains was observed for longer time during fermentation than simultaneous fermentation.

❖ ***P. fermentans* (LM5-3)**

As regard the mixed fermentations with *P. fermentans* the results obtained were showed in the figure 2.8.

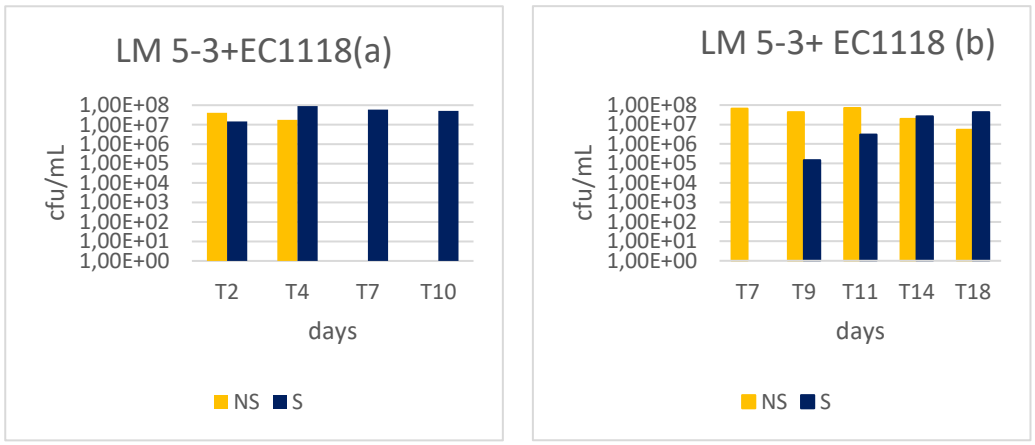


Figure 2.8 Evolution of yeast populations in mixed fermentations inoculated with *P. fermentans*-LM5-3 (NS) and *S. cerevisiae* EC1118 (S) in simultaneous (a) and sequential (b) modalities. Data are the means of two independent experiments.

The evolution of yeast cells of *P. fermentans* strain (LM 5-3) during the mixed fermentations with EC 1118 presented different trend in the two tested inoculum conditions.

For simultaneous inoculum, a slight increase of cell count of *P. fermentans* was observed in the first four days of fermentations, after that cell count decrease until the end of the process when only *S.cerevisiae* cells were found.

On the contrary, as regards sequential modality, the first isolation was carried out after the inoculation of *S. cerevisiae* cells. Viable cells of *P. fermentans* (ranging between $4*10^7$ and $7*10^7$ cfu/mL) was found until T11 (eleventh day of fermentation) after the *S. cerevisiae* inoculation.

The viable cells of *P. fermentas* decreased after T11 days of fermentation and non-*Saccharomyces* colonies were found on plates until T18 days of fermentation.

❖ ***H. guilliermondii* (TSB)**

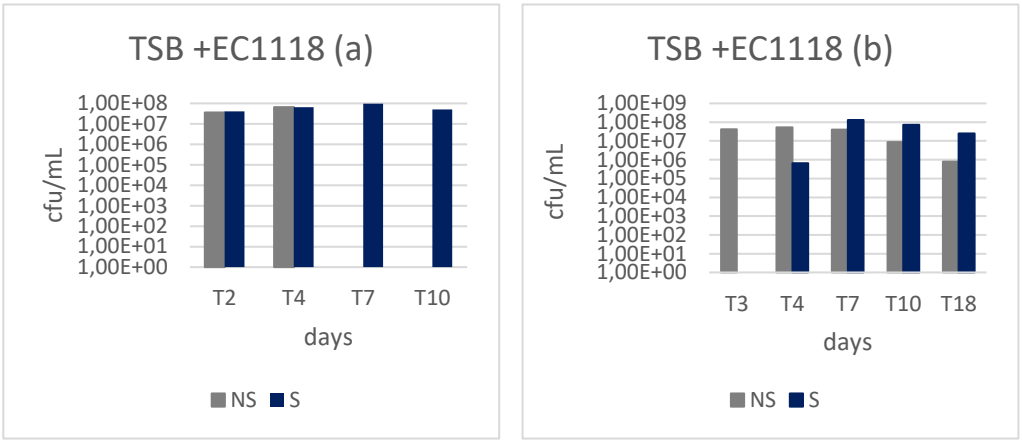


Figure 2.9 Evolution of yeast populations in mixed fermentations inoculated with *H. guilliermondii*- TSB (NS) and *S. cerevisiae* EC1118 (S) in simultaneous (a) and sequential (b) modalities. Data are the means of two independent experiments.

The evolution of yeast cells of *H. guilliermondii* strain (TSB) during the mixed fermentations with EC 1118 presented the same trend of *P. fermentans* (LM5-3) in the two tested inoculum conditions. In fact, for simultaneous inoculum fig. 2.9 (a) a slight increase of cell count is observed in the first four days of fermentations (6.5 10^5 cfu/mL), while after 7 days of fermentation non-*Saccharomyces* cell count on plate decrease until the end of the process, when only *S. cerevisiae* cells were found. Same results were found also in sequential mixed fermentation, the fig. 2.9 (b) shows that non-*Saccharomyces* viable cells were high until eighteenth day of the fermentation.

❖ ***M. pulcherrima* (Mpr 2-4)**

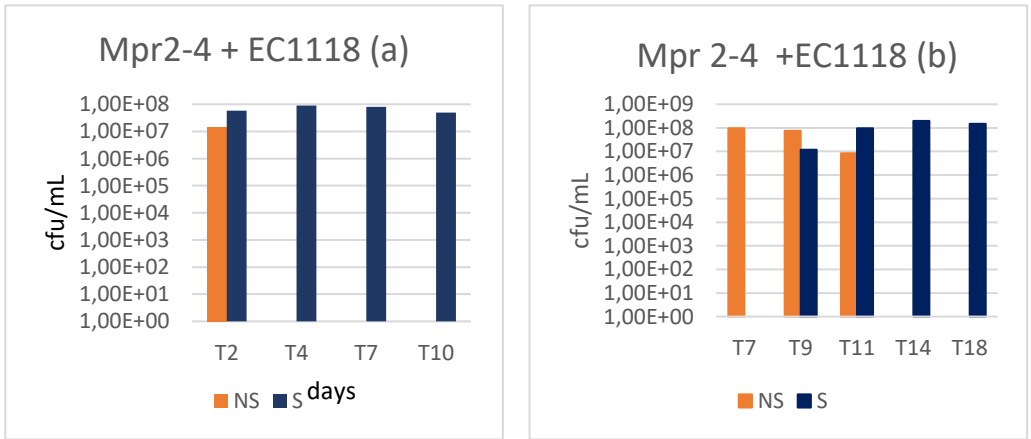


Figure 2.10 Evolution of yeast populations in mixed fermentations inoculated with *M. pulcherrima* – Mpr 2-4 (NS) and *S. cerevisiae* EC1118 (S) in simultaneous (a) and sequential (b) modalities. Data are the means of two independent experiments.

As regards mixed fermentation with *M. pulcherrima* (Mpr 2-4) a different trend in the evolution of *M. pulcherrima* cells was found as function of inoculation modality.

In simultaneous inoculum, the number of Mpr 2-4 cells remains constant in the first two days of the process, after that a decrease in number of viable cells of non-*Saccharomyces* was observed and the fermentation process was conducted only by *S. cerevisiae* until the end.

For sequential inoculum, high number of viable cells of *M. pulcherrima* was observed on plate also several days after inoculation of the S*. cerevisiae* strain (about 10^6 cells/mL after eleven days from the start of the fermentation).

As regards the evolution of *S. cerevisiae* EC1118 population in mixed fermentations, the results reflect the typical growth kinetic; in fact, the presence of high cell number was found until the end of the fermentations.

The results obtained showed that *M. pulcherrima* (Mpr 2-4) strain tested in this study is able to survive during the fermentation for more time, if this non-*Saccharomyces* strain is inoculated sequentially with *S. cerevisiae*, whereas in simultaneous inoculation this strain is unable to survive until the end of the process.

- ***S'codes ludwigii* (SIA2)**

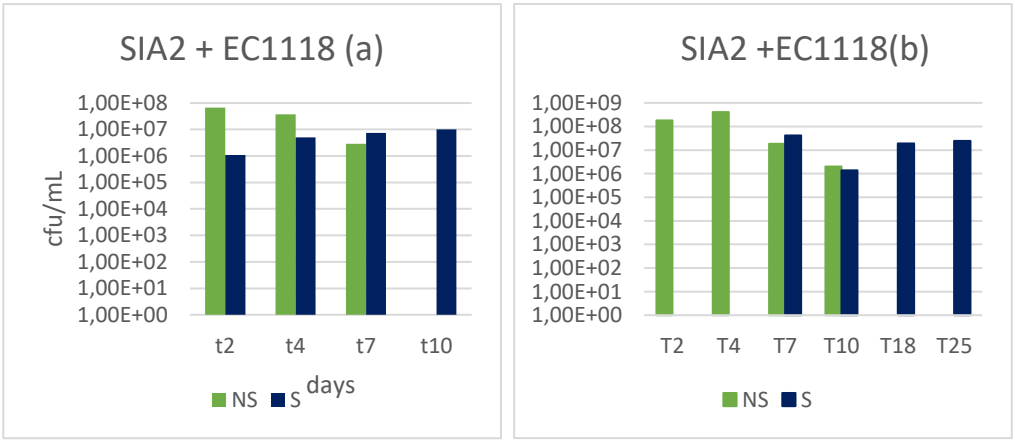


Figure 2.11 Evolution of yeast populations in mixed fermentations inoculated with *S'codes ludwigii*-SIA2 (NS) and *S. cerevisiae* EC1118 (S) in simultaneous (a) and sequential (b) modalities. Data are the means of two independent experiments.

As regards mixed fermentation with *S'codes ludwigii*, the evolution of yeast cells of SIA2 during the process by simultaneous inoculation with EC 1118 presented similar trend of *P. fermentans* (LM5-3).

The number of SIA2 cells increased in the early steps of the fermentation until four days of the process ($2.9*10^6$ cfu/mL), while after seven days a decrease in number of viable cells of non-*Saccharomyces* was observed and simultaneously a higher number of *S. cerevisiae* cells were found until the end of the fermentation.

The results obtained for sequential inoculum showed a good competition between the two yeast species.

As showed in the figure 2.11 (b), high viable cell number of SIA2 were found also after the addiction of *S. cerevisiae* strain, but from the tenth fermentation day a high reduction of number of viable cells of *S'codes loudwigii* was observed and the process was completed by EC1118.

- ***H. osmophila* (ND1)**

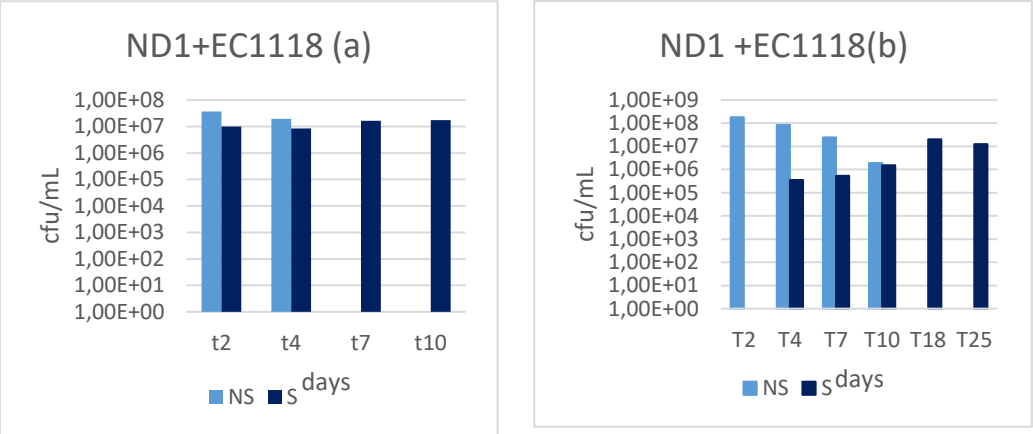


Figure 2.12 Evolution of yeast populations in mixed fermentations inoculated with *H. osmophila* -ND1 (NS) and *S. cerevisiae* EC1118 (S) in simultaneous (a) and sequential (b) modalities. Data are the means of two independent experiments.

As regard the mixed fermentations with *H. osmophila* -ND1 the results obtained were showed in the figure 2.12; in fermentation with simultaneous inoculum this strain, as *P. fermentas* and *S'codes,* exhibited a constant number of viable cells in the first days of the process, after that a reduction in number of viable cells was observed.

The evolution of the two species population in sequential inoculum condition followed the same trend found for the strain SIA2; in fact, similar cell count was observed for non-*Saccharomyces* strains until tenth fermentation days, after that the same behaviour was observed at the end of the process, where only the number of S*. cerevisiae* viable cells increased at the end of the fermentation.

❖ ***T. delbrueckii (425; LC 2-1)***

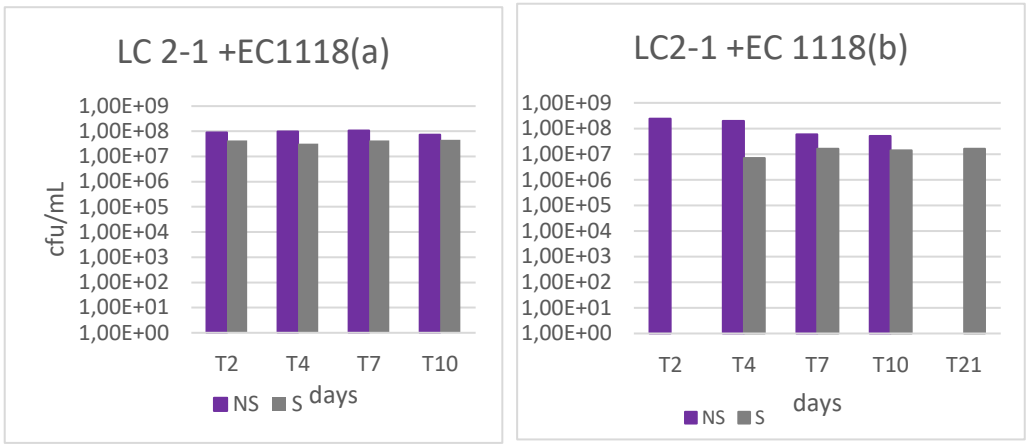


Figure 2.13 Evolution of yeast populations in mixed fermentations inoculated with *T. delbrueckii*-LC 2-1 (NS) and *S. cerevisiae* EC1118 (S) in simultaneous (a) and sequential (b) modalities. Data are the means of two independent experiments.

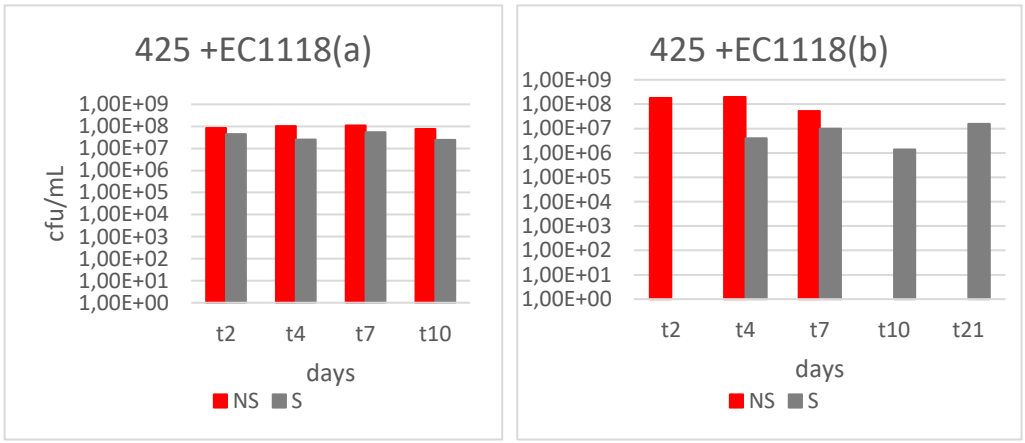


Figure 2.14 Evolution of yeast populations in mixed fermentations inoculated with *T. delbrueckii*-425 (NS) and *S. cerevisiae* EC1118 (S) in simultaneous (a) and sequential (b) modalities. Data are the means of two independent experiments.

The behaviour observed for the two strains (425, and LC 2-1) belonging to *T. delbrueckii* in mixed fermentation was similar. For simultaneous inoculum, the specie is very competitive against the inoculated *S. cerevisiae* strain. Both the two *T. delbrueckii* strains presented high cell number until the end of the fermentations, comparable to cell number of *S. cerevisiae*.

Also, for the evolution of two yeasts population in sequential inoculum condition the same behaviour was observed, the non-*Saccharomyces* cells were found almost until to the end of the process.

The analysis of microbial cells evolution of *S. cerevisiae* and non- *Saccharomyces* yeasts as mixed starter highlighted that during the vinification, they are subject to some stress factors, the most common is the increase in ethanol concentration. For this reason, to survive the yeasts act different defence mechanisms, such as the modification of cellular lipid composition, killer toxin production,

acids fatty production. Therefore, sometimes the non-*Saccharomyces* yeasts are more sensitive to certain growth-inhibitory compounds than *S. cerevisiae* (Garcia et al., 2016).
The results obtained in this step showed that ND1 (*H. osmophila*) and 425 (*T. delbrueckii*) strains are able to survive during the fermentation and have exhibited a good cell growth both in sequential and co-inoculum condition. In fact, in either inoculum modalities these strains showed a high competition with *S. cerevisiae* inoculated strain. Probably they act some defence mechanisms against *S. cerevisiae* that cause a significant loss of its cell viability during the fermentation.

2.9.3 Chemical analysis of experimental wines

In order to determine the influence of non-*Saccharomyces* strains on the aromatic profile of the experimental wines, the main volatile compounds were analysed by gas-chromatographic technique.
The main secondary compounds detected in wine samples in the two tested inoculum conditions (simultaneous and sequential inoculum) are reported in the tables 2.6 and 2.7.

Table 2.6 Main volatile compounds detected in wines obtained by the mixed starters (**thesis 1 simultaneous inoculum**) in comparison to single starter wine with EC1118. For each compound data are expressed as mean value ±SD of two independent experiments. The values were expressed as mg/L. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$) between mixed and control fermentation.

COMPOUNDS	EC1118	LM 5-3	Mpr 2-4	TS-B	SIA 2	ND1	425	LC 2-1
Acetaldehyde	49.39 ± 3.56	53.1±4.90	54.12 ± 0.53	69.42 ±3.66	58.78±10.05	59.98±3.25	120.07 ±36.69	116.23±9.41
Ethyl acetate	7.80±0.38^{a}	11.84±0.51^{b}	10.34± 0.76^{b}	19.97±0.34^{d}	13.48±0.10^{cb}	10.51±0.5^{b}	10.49 ± 1.23 ^{b}	10.91±0.17^{b}
n-propanol	12.89 ± 0.57^{a}	17.82±0.04^{c}	14.28±0.15^{ad}	17.00±0.27^{ce}	20.28± 0.13^{b}	16.26±0.21^{be}	13.19±0.58^{ad}	14.34±0.39^{bd}
Isobutanol	32.40± 0.96^{a}	26.30±0.24^{ad}	42.24c±5.12^{ac}	46.12±4.46^{bc}	37.29±2.09^{ac}	26.68±0.24^{ad}	31.32 ±1.86^{d}	32.03 ± 0.16^{a}
n-butanol	14.86 ±5.99^{a}	20.07±0.43^{ab}	16.48±8.28^{ab}	13.63 ±6.10^{a}	40.13±11.74^{b}	21.23±1.08^{ab}	19.96± 2.40^{ab}	17.72±0.44^{ab}
Acetoin	9.09± 0.92^{a}	19.56±1.51^{bc}	19.89 ±1.34^{bc}	14.39±1.00^{ac}	17.55±3.28^{bc}	8.9± 0.24^{a}	6.95± 0.70^{ad}	10.41 ± 2.61^{a}
Acetic acid	291.83±26.89^{a}	405.64±5.56^{a}	134.58±43.85^{a}	213.20±13.8^{a}	987.70±392.65^{bc}	740.35±6.83^{ac}	259.01±36.94^{a}	321.04±0.60^{a}
D-amyl alcohol	71.74± 3.81^{a}	46.13 ± 1.20^{b}	54,44± 4,96^{ab}	52,93±2,36^{ab}	62,83± 11,40^{ab}	50,89 ±5,06^{ab}	53,20±10,75^{ab}	57,65±1,50^{ab}
Isoamylic alcohol	211.60±10.21^{a}	119.16±3.52^{bc}	170.78±19.02^{ac}	144.87±0.02^{bc}	163.13± 30.58^{ac}	136.60±0.87^{bc}	166.08±9.56^{ac}	177.23±3.89^{ac}

Table 2.7 Main volatile compounds detected in wines obtained by the mixed starters (**thesis 2 sequential inoculum**) in comparison to single starter wine with EC1118. For each compound data are expressed as mean value ±SD of two independent experiments. The values were expressed as mg/L. Different superscript letters in the same row correspond to statistically significant differences (Tukey's test, $p < 0.05$) between mixed and control fermentation.

COMPOUNDS	EC1118	LM 5-3	Mpr 2-4	TS-B	SIA 2	ND1	425	LC 2-1
Acetaldehyde	60.44±0.42^{a}	34.43±0.54^{b}	39.70±6.84^{b}	45.50±0.57^{b}	44.57 ±7.99 ^{b}	38.60±2.04^{b}	33.91±0.22^{bc}	47.44±2.58^{ab}
Ethyl acetate	8.79± 0.41 ^{a}	19.30±4.95^{b}	16.62±2.87^{bc}	19.18±2.7 ^{b}	8.65 ± 0.74 ^{a}	9.77±0.52^{ac}	8.77 ± 0.53 ^{a}	8.67 ± 0.05 ^{a}
n-propanol	13.56±0.39^{a}	18.95±0.00^{ab}	22.63±6.81^{ab}	26.52±0.87^{b}	25.20 ±0.03 ^{b}	20.42±0.41^{ab}	20.44±0.34^{ab}	21.28±0.31^{ab}
Isobutanol	34.11±1.33^{a}	55.64 ±3.45 ^{b}	94.29±12.59^{c}	39.20±1.20^{ab}	29.71±0.91^{ac}	25.45±3.12^{ac}	51.49±2.45^{ab}	41.78±1.40^{ab}
n-butanol	11.52±0.32^{a}	12.83 ± 1.57^{b}	18.04± 0.02 ^{c}	17.22±1.01^{ab}	20.85±2.11^{ac}	30.59±7.50^{ac}	17.32±0.46^{ab}	12.53±3.72^{ab}
Acetoin	7.53 ±2.14	11.17 ±5.90	15.17± 0.68	13.64 ± 0.51	15.15 ± 0.06	19.07 ± 4.01	8.66 ± 7.24	8.84 ± 1.16
Acetic acid	313.76±4.65^{a}	911.13±18.40^{b}	356.86±31.13^{a}	121.20±8.21^{c}	697.28±33.76^{d}	1123.98±546.4^{e}	239.17±28.94^{ac}	234.38±46.94^{ac}
D-amyl alcohol	55.91 ± 2.44^{a}	52.73 ± 1.10 ^{a}	50.68± 0.16 ^{a}	215.61±1.71^{b}	37.22 ± 0.07 ^{c}	37.51 ± 1.46 ^{c}	35.44 ± 4.74 ^{c}	35.87 ± 2.08 ^{c}
Isoamyl alcohol	197.14±7.87^{a}	157.27 ± 0.51^{b}	161.78 ± 4.22^{b}	87.46 ± 3.15^{c}	135.92± 1.37 ^{b}	138. 16 ±18.56^{b}	126.07 ± 8.32^{bc}	116.47 ± 2.19^{bc}

In order to determine the effect of yeast inoculum modalities on the final composition of the wine, experimental wines were analysed for oenological parameters and main volatile compounds, and the data are shown in Tables (Tab. 2.6- simultaneous inoculum) and (Tab. 2.7- sequential inoculum). As reported in the tables 2.6 and 2.7**,** statistically significant differences were found between wines obtained by mixed and wines produced by single starter for the majority of the analyzed compounds.

As regards co-inoculum (Table 2.6), all the samples from mixed fermentations contained higher level of ethyl acetate than control sample (pure culture of EC1118), the highest value was found for the mixed fermentations inoculated with EC+TSB.

Ethyl acetate, if present at low concentrations, may add fruity aroma to the wine bouquet, whereas a content higher than 150 mg/L affects negatively the flavour (Ciani et al., 2005).

The content of isobutanol and n-butanol was similar for all the mixed fermentations and control sample (EC1118), except for the samples inoculated with EC+TSB and EC+Mpr2-4, which presented the highest content. Similar values were found also for amyl alcohols in all the samples, only the wine obtained with LM5-3 showed a low content.

In some mixed fermentations, the activity of non-*Saccharomyces* strains increases volatile acidity, measured as acetic acid content (mg/L). As showed in the table, the wines obtained with ND1 and SIA 2 contained the highest concentrations of acetic acid, while the values detected in the other wines from mixed fermentations are very similar to level found in control wine, particularly for the samples inoculated with LC 2-1, 425, Mpr 2-4, TSB.

However, in all the experimental wines, the volatile acidity value is below the acceptability level (1.2 g/L).

Similar results were exhibited in the fermentations by sequential inoculation (Table 2.7). As showed in the table 2.7**,** differences statistically significant ($p < 0.05$) between wines from single and mixed starters were found for almost all the analysed compounds, except for acetoin.

As expected, also for this inoculum modality, the content of ethyl acetate is higher in wines from mixed fermentation and the highest value was detected in sample inoculated with the strains LM5-3 and TSB.

The wines inoculated with LM5-3, Mpr2-4, ND1, 425 presented higher acetaldehyde levels than the control, whereas in wines from other mixed cultures the content was similar to the control (EC1118).

As regards the higher alcohols, the amount varied among wines obtained by mixed fermentations and wine produced by single starter. The wines obtained by sequential inoculation with 425, LM5-3, Mpr2-4 showed higher level of isobutanol, while n-butanol content was highest in wine fermented with ND1.

However, all the wines from mixed starters contained a similar level of amyl and isoamyl alcohols than that found in the wine obtained with pure culture, except for the fermentation with TSB that showed the lowest value for isoamyl alcohol and the highest for amyl alcohol.

As regards volatile acidity, the activity of non-*Saccharomyces* strains also in this case increases acetic acid content. The wines obtained by mixed starters including LM 5-3, SIA2, and ND1 were differentiated from control wine for the very high value of acetic acid, while the fermentation including 425, LC 2-1 and TSB presented similar content to the control wine.

2.9.4 Chemical analysis of experimental wines by Oenofoss™ instrument

The experimental wines were analysed also for the content of some basic oenological parameters by Oneonofoss™ instrument.

Table 2.8 Main oenological parameters detected in wines obtained by the mixed starters (thesis 1-simultaneous inoculation) in comparison to single starter wine with EC1118. For each compound data are expressed as mean value ±SD 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.

SAMPLE	Alcohol % (v/v%)	Glucose/ Fructose (g/L)	Fructose (g/L)	Glucose (g/L)	Total Acidity (g/L)	pH	Ac. Malic (g/L)	Ac. Lactic (g/L)
EC	13.38±0.43^{a}	2.29±0.05^{a}	0.60±0.14^{a}	1.50±0.00^{a}	9.6±0.04^{a}	3.35±0.01^{a}	1.92±0.07^{a}	0.9±0.00^{a}
LM 5-3	13.09±0.14^{a}	3.04±0.49^{a}	1.40±0.28^{a}	1.50±0.00^{ab}	8.5±0.01^{bc}	3.45±0.01^{c}	1.50±0.01^{bc}	0.9±0.00^{a}
Mpr 2-4	13.59±0.10^{ab}	2.53±0.12^{a}	1.15±0.07^{a}	1.40±0.14^{a}	8.5±0.11^{bc}	3.45±0.01^{ce}	1.31±0.10^{bd}	1.0±0.00^{ab}
TSB	13.61±0.18^{ab}	2.44±0.04^{a}	1.00±0.00^{a}	1.70±0.00^{abc}	8.8±0.23^{bd}	3.34±0.01^{a}	1.69±0.09^{acd}	0.8±0.07^{ac}
SIA 2	13.69±0.05^{ab}	7.89±2.47^{b}	5.75±2.05^{b}	2.00±0.14^{bc}	9.1±0.07^{be}	3.37±0.0^{ade}	1.55±0.04^{bc}	0.8±0.07^{ac}
ND1	12.43±0.12^{a}	3.41±0.19^{a}	2.00±0.14^{a}	1.55±0.07^{ad}	9.5±0.11^{ae}	3,41±0,01^{bce}	1.75±0.06^{ac}	0.8±0.07^{ac}
425	13.06±0.07^{ab}	1.84±0.10^{a}	0.95±0.07^{a}	1.70±0.00^{abd}	9.1±0.01^{be}	3.41±0.01^{bd}	1.57±0.01^{bc}	0.9±0.00^{a}
LC 2-1	12.77±0.62^{ab}	1.89±0.02^{a}	1.00±0.00^{a}	1.60±0.00^{a}	9.2±0.11^{ae}	3.40±0.0^{bed}	1.66±0.04^{bc}	0.9±0.00^{a}

Table 2.9 Main oenological parameters detected in wines obtained by the mixed starters (thesis 2- sequential inoculum) in comparison to single starter wine with EC1118. For each compound data are expressed as mean value ±SD 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

SAMPLE	Alcohol % (v/v%)	Glucose/ Fructose (g/L)	Fructose (g/L)	Glucose (g/L)	Total Acidity (g/L)	pH	Ac. Malic (g/L)	Ac. Lactic (g/L)
EC	12.87±0.17^{a}	1.62±0.06^{a}	0.85±0.07^{a}	1.55±0.07	9.74±0.06^{a}	3.39±0.01^{a}	1.87±0.02^{a}	1±0.00^{a}
LM 5-3	12.59±0.10^{a}	1.92±0.17^{ab}	1.15±0.21^{a}	1.30±0.00	9.39±0.01^{a}	3.46±0.01^{bc}	1.63±0.01^{ad}	0.85±0.07^{ab}
Mpr 2-4	13.25±0.18^{ab}	0.91±0.64^{a}	1.45±0.07^{ab}	2.15±0.49	9.68±0.65^{a}	3.38±0.03^{a}	1.29±0.11^{b}	0.95±0.07^{ac}
TSB	13.42±0.42^{ab}	2.00±0.19^{ab}	1.20±0.00^{a}	1.80±0.00	9.44±0.10^{a}	3.40±0.00^{ac}	1.78±0.06^{ad}	0.6±0.00^{b}
SIA 2	12.33±0.3^{a}	4,55±1.26^{ac}	3.30±0.85^{ab}	1.95±0.07	9.24±0.06^{ac}	3.41±0.01^{ac}	1.57±0.04^{bde}	0.7±0.00^{ab}
ND1	11.92±0.03^{ac}	7.95±4.12^{bc}	5.95±3.18^{b}	2.05±0.35	9.84±0.21^{a}	3.42±0.02^{ac}	1.76±0.11^{ae}	0.65±0.21^{ab}
425	12.65±0.61^{a}	2.56±0.19^{ac}	2.15±0.35^{ab}	2.00±0.14	10.33±0.04^{ab}	3.38±0.00^{a}	1.97±0.01^{ac}	0.8±0.00^{ab}
LC 2-1	13.15±0.32^{a}	1.35±0.10^{a}	1.05±0.07^{ab}	2.05±0.07	10.95±0.09^{b}	3.33±0.00^{ab}	2.36±0.00^{bde}	0.7±0.00^{ab}

Oenological parameters detected in the wines obtained by the simultaneous and the sequential inoculum are shown in the Tables 2.8 and 2.9, in which the data were reported as the average of the two replicates.

Some mixed starters, in both the inoculum modalities, produced wines with lower ethanol content than wine obtained by control fermentation. However, not all the starters consumed all the sugars contained in the natural must, leaving in the final wine different amounts of sugar residues. In particular, in both the inoculation conditions the mixed samples inoculated with SIA2 and ND1 in combination with EC1118 strain contained lower ethanol than control experimental wine, but the sugar content of wines obtained with sequential inoculum was higher than wine from control fermentation (4.55 and 7.95 g/L respectively). In co-inoculum condition, for the sample obtained with SIA2, a high sugar residue was found, while the mixed fermentation with *H. osmophila* (ND1) showed 3.41g/L of sugar content, confirming the ability of this mixed starter culture to complete the fermentative process.

A slight reduction of ethanol was found also in the samples inoculated with *T. delbrueckii*. Finally, the table 2.10 summarizes the variations of the main fermentation characteristics of the two theses respect to the control fermentation.

SAMPLE	ETHANOL(v/v%)	ACETIC ACID(g/L)	COMPETITION	TOTAL ACIDITY(g/L)	SECONDARY COMPOUNDS
ND1	*1) -* *2) -*	*1) + +* *2) + + +*	*1) +* *2) + +*	*1) -* *2) +*	*+ acetic acid*
LM 5-3	*1) ≈* *2) ≈*	*1) +* *2) + +*	*1) -* *2) + +*	*1) -* *2) ≈*	
SIA 2	*1) +* *2) -*	*1) + +* *2) + +*	*1) +* *2) + +*	*1) ≈* *2) ≈*	*+ acetic acid*
Mpr 2-4	*1) +* *2) +*	*1) -* *2) ≈*	*1) -* *2) +*	*1) -* *2)≈*	*+ isobutanol*
LC 2-1	*1) -* *2) ≈*	*1) ≈* *2) -*	*1) + +* *2) + +*	*1) ≈* *2) +*	*- acetic acid*
425	*1) ≈* *2) ≈*	*1) ≈* *2) -*	*1) + +* *2) + +*	*1) ≈* *2) +*	*- acetic acid*
TS-B	*1) +* *2) +*	*1) -* *2) -*	*1) +* *2) + +*	*1) -* *2) ≈*	*+ethyl acetate*

Table 2.10 Main fermentative parameters of mixed starters in the two tested inoculum modalities [simultaneous inoculum 1); and sequential inoculum 2)]

-) lower values than the control fermentation

≈) values like control fermentation

+) higher values than the control fermentation

2.9.5 Analysis of glycerol content

Glycerol can influence wine quality by increase of softness and body of the product. Generally, glycerol is produced by *S. cerevisiae* during glycolysis, reducing dihydroxyacetone phosphate to

glycerol 3- phosphate and later oxidize to glycerol. From biochemical point of view, the increase of glycerol content is related to the higher expression of glycerol-3-phospate dehydrogenase enzymatic activity in non-*Saccharomyces* species than *S. cerevisiae* (Benito et al., 2019)

The use of non-*Saccharomyces* yeasts in alcoholic fermentation can be a strategy to increase the glycerol content in wine from few decimals to 4 g/L compared to wine produced by *S. cerevisiae* only. However, recent studies suggest that the results depend on non-*Saccharomyces* yeast strains, on the genomic diversity and variability of this species.

In our study, a small increase of glycerol in wines from mixed cultures was found.

The best result was exhibited in mixed fermentation involving *M. pulcherrima* in co-inoculum condition, with an increase of 0.2 g/L of glycerol content respect the control fermentation. This increase of glycerol production can be explained by a possible overexpression of GDP1 gene (involve in dihydroxyacetone phosphate conversion in glycerol-3-phospate) in *S. cerevisiae*, probably over induced during the co-existence with *M. pulcherrima*.

The results showed in all cases an increment of glycerol only in thesis 1 (simultaneous inoculation), confirming the high influence of inoculum condition on the properties of wines.

In thesis 2 (sequential inoculum), wines produced with non-*Saccharomyces* yeasts had a lower glycerol content than control wine produced by *S. cerevisiae* monoculture, except wine produced by mixed starter including *T. delbrueckii* LC2-1 strain.

The lowest glycerol content in wines produced by non-*Saccharomyces* yeast was found also by Mendoza et al. (2010), whereas other authors reported contrary results (Benito et al., 2019).

Our results suggest that glycerol production is a trait closely related to the yeast strain and inoculum condition (Du Plessis et al., 2017- Du Plessis et al., 2019).

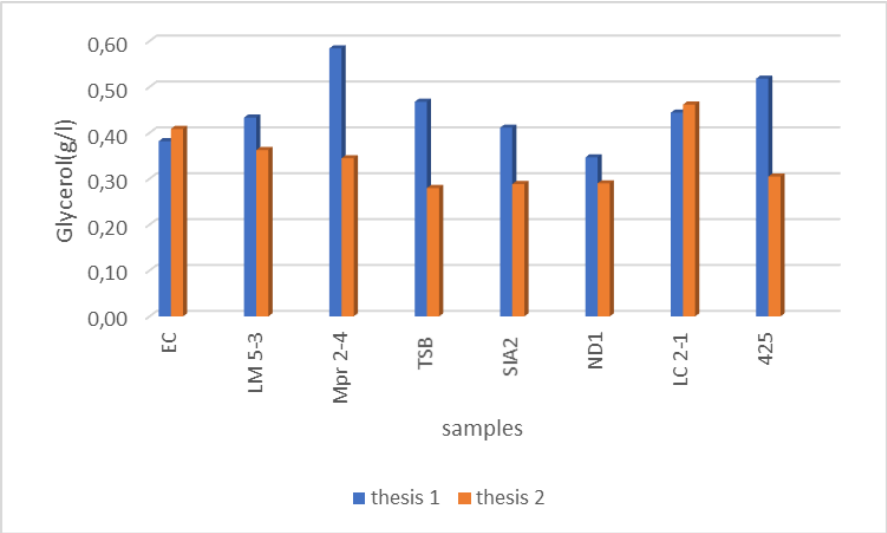


Figure 2.15 Glycerol content detected in wines obtained by the mixed starters (thesis 1-simultaneous inoculum; thesis 2- sequential inoculum) in comparison to single starter wine with EC1118. Data are expressed as means of two replicates.

All the parameters determined in experimental wines obtained by using mixed starter cultures in both the inoculation modalities, simultaneous (samples indicated with T1 code) and sequential (samples indicated with T2 code), and wine obtained by control fermentation (Control (EC)_T1 and Control (EC)_T2, 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 2.16.
The two principal components, PC1 and PC2, accounted for 50% of the total variance (29 and 21%, respectively).
As showed by the loading values of each variable (Figure 2.16), the PC1 was positively correlated mainly with acetaldehyde, acetoin, n-butanol, acetic acid, sugar residue, glycerol content, whereas the PC2 was mainly positively related to total acidity, pH, malic acid.
This analysis allowed to differentiate the experimental wines in function of inoculation modality, but also on the basis of yeast species.
In fact, almost all the samples obtained with non-*Saccharomyces* strains inoculated simultaneously with *S. cerevisiae* strain are located in upper part, while the sample obtained by sequential inoculum are grouped together in the lower part of the scatterplot.
The wines obtained by mixed starters including *H. osmophila, S'codes ludwigii* and *P. fermentans* strains are grouped very near, in the same quadrant of the graph both in sequential and simultaneous inoculum. The same behaviour was observed for the control wines EC1118.

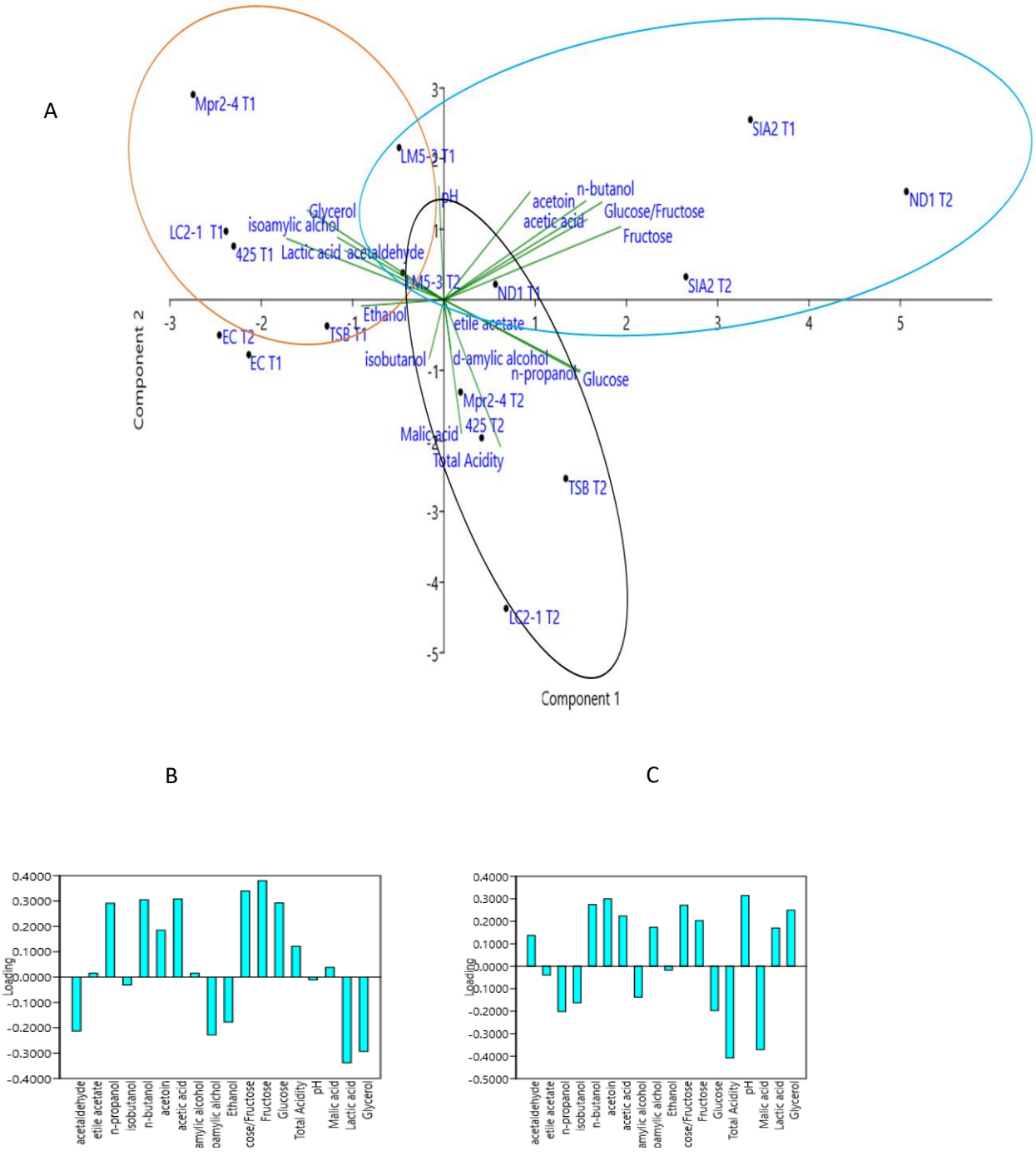


Figure 2.16 Scatter Plot (A) and loading plots of the first (B) and second (C) principal component corresponding to Principal component analysis (PCA) of the oenological parameters and main volatile compounds detected in experimental wines obtained by simultaneous (T1) and sequential (T2) inoculum of selected non-*Saccharomyces* strains.

2.10 Conclusion

The use of mixed starters containing selected non-*Saccharomyces* yeasts combined with *S. cerevisiae* represents a good strategy to produce wines with unique aromatic characteristics.

Their use in combination with *S. cerevisiae* can impact on wine final quality thanks to their capability to produce extracellular enzymes and modify the concentration of secondary metabolites.

In the present research phase, the possibility of using non-*Saccharomyces* yeasts in a mixed starter was evaluated with aim to reduce the alcohol content and improve the aromatic profile of wines. In this context, seven non-*Saccharomyces* strains (LM 5-3, Mpr2-4; TSB; SIA2, ND1, 425, LC 2-1) previously selected, were tested in simultaneous and sequential inoculation with *S. cerevisiae* EC1118 strain in natural pasteurized must. The obtained results display the influence of non-*Saccharomyces* yeast strains on wine composition.

The statistical elaboration by PCA of data obtained by gas-chromatographic analysis and main oenological parameters of the experimental wines showed the influence of inoculation modality on the quality of the experimental wines.

The samples appeared separated in function of inoculum modality, except for the wines obtained with ND1, SIA2, LM5-3, which appeared located near for both inoculum modalities.

Promising results were obtained by two yeast strains ND1 (*H. osmophila*) and 425 (*T. delbrueckii*), which showed a good competitiveness in mixed fermentations, probably linked to the strain ability to survive during the fermentative process together *S. cerevisiae* EC1118 and leading to a reduction of the alcohol content (particularly when used as co-inoculation).

The presence of these two strains during the fermentation improves the amounts of some secondary compounds, such as glycerol, with higher levels in wine samples obtained by mixed fermentation with simultaneous inoculum respect to the sequential fermentation.

However, the samples obtained with mixed fermentation with *H. osmophila* presented high value of acetic acid content, the main constituent of volatile acidity with negative influence on wine aroma. However, the value of this compound, mainly in thesis 1 (simultaneous inoculum) remains within the acceptable range for wine.

On the base of the data obtained in this step the strains ND1 and 425 were selected and tested in the subsequent research phase as different starter formulation (dry and fresh starter).

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Chapter III

Study of selected non-Saccharomyces yeasts in different starter formulation

Abstract

The use of starter cultures as cream formulation is consolidated in the oenological sector. The yeast paste is a ready-to-use product, which does not require the delicate rehydration phase, and this aspect represents the main advantage associated to this product. Unfortunately, one of the disadvantages of using yeast paste is the reduced shelf life of the product; in fact, it is necessary to use it as soon as possible in order to avoid product contamination. In order to cope with these drawbacks, the wine sector has developed techniques aimed to increase the conservation time of the product, preserving its properties, such as the use of Active Dry Yeasts (ADY) and immobilized yeast cells.

The advantages of the use of ADY include ease of storage, transport and use as well as the wide range of products available on the market, characterized by different oenological performances and specific for the type of wine to be developed. Otherwise, the application in oenology of immobilized and microencapsulated yeast cells is a relatively recent technology, mainly used in the second fermentation phase for production of sparkling wines, to improve the performances of the starter and preserve a high cellular vitality during the entire process.

This research phase was addressed to verify the applicability of both these technologies on the selected non-*Saccharomyces* yeasts (*H. osmophila* ND1 and *T. delbrueckii* 425). The effect of these treatments was assessed by evaluating the influence on both strain growth and fermentative performance.

3.1 Starter cultures formulation

Different methods are employed to preserve starter cultures for industrial application; the most common are cream yeasts; fluid yeasts; cells immobilized on solid supports or dried form (Active Dry Yeasts).

Dried preparations are one of the most utilized formulation of wine starters. Their wide diffusion is linked to some advantages, such as ease transport (it is not necessary to maintain the cold chain during storage); a prolonged shelf life (the low residual moisture guarantees good stability over time); reduction of production times and costs.

The production of active dry yeasts (LSA) is based on the ability of cells to arrest their metabolic activities in the absence of water and recover them as soon as optimal conditions are restored. Therefore, the main objective of the dried process is to maximize biomass production and ensure the recovery of the biological activities of the cells after the rehydration phase with aim to ensure a prompt start of alcoholic fermentation. (López-Martínez et al., 2013; Romano et al., 2015).

On the other hand, a promising strategy to preserve the integrity of microbial cells in industrial applications is represented by immobilization system, which represents an innovative technology to implement yeast performances in different food industrial processes. This methodology is based on physical confinement of cells in a limited space to protect the microorganisms to external environmental stress and preserve their viability (Benucci et al. 2021; Benucci et al., 2019; Canonico et al., 2016)

The applications tested until now in the oenological industry are various: bottle refermentations of sparkling wines in order to facilitate remuage operations; the deacidification of musts using *Schizosaccaromyces pombe* yeast, and finally the use for improving qualitative characteristics of the products.

Although this method allows to reduce process costs, it represents a flexible and simple technique, its use at industrial level is still extremely limited.

3.2 Active dry yeast production (ADY)

In the last twenty years the use of active dry yeasts (ADY) in oenology has become widespread, their use guarantees a reproducible result and better control of the fermentation process.

The first step consists in the production of starter culture biomass by "propagation" of the yeast cells in a suitable medium. The substrate generally used is molasses (the main waste product from the refining process of beet or sugar cane), easily available and quite cheap, rich source of carbon (50-60% of sucrose), but unfortunately poor in minerals such as nitrogen, phosphorus, and magnesium; as well as vitamins or other compounds that yeasts are unable to synthesize, so they need to be added in initial production phases.

Another important factor that determines the growth of yeast is the pH, to achieve optimal growth levels the pH of the molasses is corrected up to values between 4.5 and 5.

The first propagation phase of the selected yeast strain (adequately preserved to guarantee its purity and genetic stability), is carried out at laboratory scale, while the subsequent phases to favourite cell multiplication are carried out in fermenters of increasing volumes, working in fed-batch and adding sugar and nutrients.

During the process, good ventilation is necessary to ensure high availability of oxygen and promote the consumption of sugar by respiratory metabolism to activate the so-called Pasteur effect. At this purpose the most widely used control system for the gas flows entering and leaving the fermenters is the measurement of the respiratory quotient (ratio between the moles of CO_2 produced and O_2 consumed).

At the end of each fermentation the cells are collected, centrifuged, washed, and then stored at 2-4 ° C, the biomass is collected in the presence of limited concentrations of nitrogen, a gas that influences the entry of cells into the growth stationary phase, preventing them from completing the cycle of cell division and activating genes and response functions to external stress.

The cells must be able to implement mechanisms of response to stress by accumulation of reserves and protective substances, such as trehalose and glycogen and some sterols, mainly ergosterol, the major constituent of the plasma membrane, to protect the cell from the stress factors during the drying process and to have a good recovery of metabolic activities after the rehydration phase.

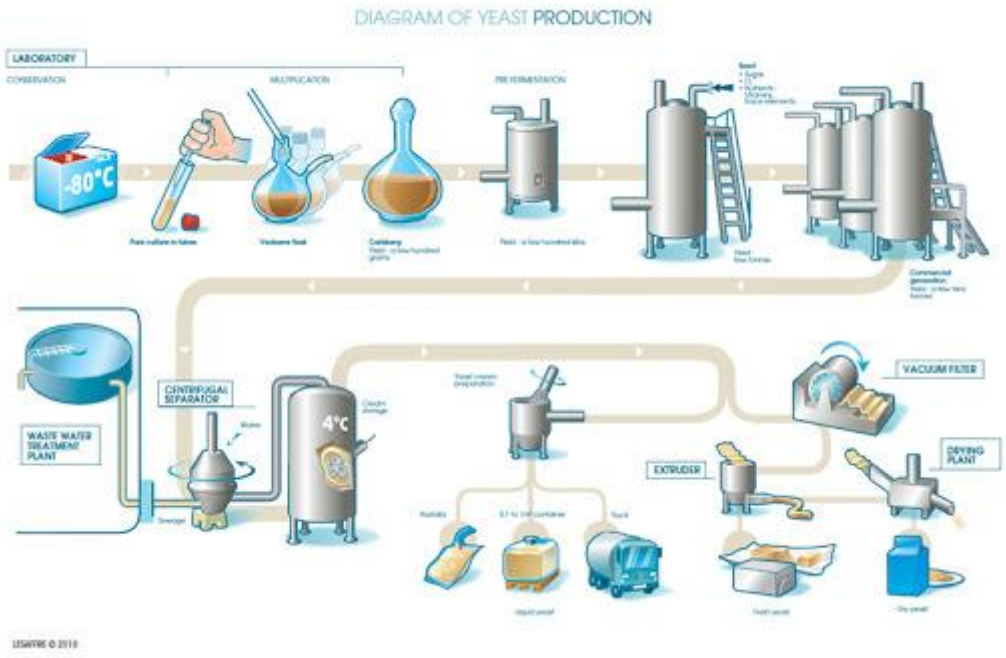


Figure 3.1 Yeast biomass production

3.3 Process and Storage condition

The washed and centrifuged biomass (pH 3.5) is stored at 4°C, in order to avoid microbial contamination.

The pasta obtained is filtered to reduce the dry substance to 30-35%, and subjected to drying by extrusion, using a jet of hot air generally using fluidized bed systems that maintain a constant temperature during the process (below 35-40°C).
The duration of the process is variable from 15 to 60 minutes in function of the volume of the biomass to be dried and the process conditions, the aim intent is reducing the humidity to about 8%.
The dry yeast obtained is vacuum packed or packaged under inert gas and can be stored at 4°C for about three years.

3.4 Rehydration of yeast biomass

The desiccation process caused a gradual water loss that arrests the viable functions of microbial cells. For this reason, the rehydration phase is necessary to establish metabolic functions and recovery the cell membrane structure before the use of microbial cells as starter culture.
The most followed protocol for rehydration involves the use of hot water (about 35-40° C), or a solution of sugar and water in a 1: 1 ratio (Novo et al., 2003).
The rehydration step has to be short (15-30 minutes) in order to avoid the loss of cellular vitality, particularly due to the osmotic shock (high concentration of glucose and fructose) (Novo et al., 2007). The rehydration can be divided into two stages, a first in static conditions (5-10 min) to reduce cell damages and the second one by keeping the solution stirred for another ten minutes.

3.5 Cell damage

The dehydration process is a stress condition that perturbs the cell viability and survival. This process causes some changes in the structures of macromolecules, the cytoplasmic space decreases and pH is altered.
The principal cellular component affected by extreme dehydration is plasma membrane because the hyperosmotic shock causes a movement of large quantity of water and increase the ratio between surface/volume of the cells and deforms the membrane structure by new structural arrangement of lipids component.
Most microorganisms present different mechanisms to adapt to the environmental conditions, such as the use of alternative metabolic pathways, active process such as the osmoregulatory pathways. In fact, the high-osmolarity glycerol-signalling system adjusts the internal osmotic pressure and control morphology, turgor and water content (Dupont et al., 2010).
Recent works have demonstrated that some microorganisms in water absence accumulate large amounts of carbohydrates such as trehalose and glucose, which cover an important role for the viability and physiological cell state. Trehalose preserves the integrity of membrane structure while glycogen metabolism is correlated to sterol production (Novo et al., 2003).

3.6 Cell Stress response

The cells subjected to dehydration processes are exposed to different stress conditions; therefore, over time they have developed molecular responses to protect themselves from the caused damages. The main response to stress is represented by the synthesis of a limited group of stress proteins that increase the level of tolerance to external factors and improve cells fit and survival. This response mechanism is very frequent in microbial cells often subject to changes in growth conditions, availability of nutrients and changes in temperature (Estruch et al., 2000).

The stress conditions to which cells may be subject during production of ADY can be divided into three groups: thermal, osmotic, and oxidative stresses.

- ***Response to thermal stress***

  Yeasts possess several transcription factors involved in the stress response, among of which there are the HEAT SHOCK PROTEIN (HSP), a family of proteins whose synthesis is induced in cells subjected to stress.

  These proteins are ones of the most abundant and conserved from a phylogenetic point of view, they are classified into different families based on molecular weight, and their main function is as molecular chaperons, addressed to the correct folding of proteins avoiding their aggregation and promoting the proteolysis of non-functional proteins (Gloves et al., 1998). The principal HSP in yeast cells are Hsp 100 and Hsp 70.

  In *S. cerevisiae* there are two regulation systems related to the induction of heat shock response genes: HSF (Heat shock Factor) and the transcription factor Msn2p and Msn4p, with the function of removing the protein aggregates formed under stress conditions and promoting degradation (Amoros et al., 2001).

- ***Hyperosmotic stress response***

  The vitality of the microorganisms is conditioned by some factors, such as osmotic pressure and temperature. In fact, some studies show that an increase in osmotic pressure, correlated to a decrease in temperature, causes loss of cellular turgor and, in extreme cases, can cause cell death.

  To recover turgor, the cell is able to implement a response system known as HOG (High Osmolarity Glycerol) by inducing the expression of some genes (*GPD1* and *GPD2*) involved in the synthesis of glycerol, which, accumulating in the cells, contrast the negative effects of dehydration (Holmann et al., 2002, Walker et al., 2006, Mager et al., 2002).

- ***Response to oxidative stress***

  Oxidative stress is caused by the presence of radicals (ROS), reactive compounds of the oxygen such as H_2O_2, OH^-, O_2^+, normally produced during cell metabolism, respiration, and oxidation of fatty acids. Their high concentration can cause damage to cell components, DNA, lipids, and proteins. Oxidative stress conditions occur when the presence of antioxidants is insufficient to maintain cellular redox balance. Cellular response systems to oxidative stress are divided into enzymatic systems (such as catalase, superoxide dismutase, which act by removing radicals) and non-

enzymatic systems (production of some substances with a protective function for cells, such as glutathione) (Jamienson et al., 1998; Walker et al., 2006)

3.7 Cell structural changes during dehydration and rehydration phases

The survival of the microorganism during drying is related to the ability of the cells to resist in conditions of anaerobiosis and to recover the metabolic and biological mechanisms once the optimal growth conditions have been restored. Cell structures are subject to changes during the dehydration and rehydration phases. The main is represented by the structure of the membrane, which during dehydration, in consequence of the loss of water molecules assumes a gel structure (the Wan der Walls bonds in the lipid bilayer increase), while it assumes the typical crystalline liquid state during rehydration. In this step two physical states can coexist; the membrane loses its barrier properties, with consequent loss of the cytoplasmic content and cell death. For this reason, dehydration is often carried out in the presence of carbohydrates (sucrose, trehalose) or amino acids, compounds capable of creating hydrogen bonds with lipid groups, thus inserting themselves into the structure of the membrane when the loss of water molecules occurs (Prestelski et al., 1993). Other authors report, among the causes of cell death at high temperatures (Beney et al., 2001), the formation of endovesicles, that increase the volume and cause cell lysis during rehydration (Simon et al., 2007).

In these conditions, the cells can use some compounds to increase defense mechanisms, such as ergosterol, glutathione and trehalose.

Ergosterol is the correspondent of cholesterol in animal cells, it is entrapped among the phospholipids of the membrane, establishing the structure.

Yeast cell can accumulate large quantities of trehalose (about 20% of its dry weight). This carbohydrate has the ability to form crystals, increases viscosity and decreases mobility, increases the protection of the membrane by limiting its movement and aggregation. The interaction keeps the membrane in a physical state similar to that formed in the presence of water (Shebor et al., 2000).

3.8 Spray drying process

At industrial level, as well as at laboratory scale, the spray-drying process is used in various sectors, such as chemical, agri-food, and pharmaceutical sectors.

Spray-drying process consists in reducing a liquid substrate into small droplets (0.5-0.02 mm) by contact with a hot stream flow to obtain the water evaporation. This preservation process is used to produce a very fine dehydrated powders (3-5% residual humidity) from food, chemical compounds, dry probiotic preparations and microbial starter culture. The main advantages and disadvantages of this technique are the following (Silva et al., 2011):

Advantages

- ✓ It is used in different applications
- ✓ It can be designed for any capacity required
- ✓ The process is fast and effective
- ✓ It allows the continuous monitoring of the process and the simultaneous check of several variables
- ✓ A wide range of models is available
- ✓ It can be used with both heat-resistant and heat sensitive products
- ✓ The product can be in solution, liquid, paste, gel, suspension
- ✓ Powder quality remains constant during the dryer process
- ✓ It can operate in open system

Disadvantages

- ✓ The equipment is very bulky and expensive
- ✓ Thermal efficiency is low
- ✓ Hot air treatment can lead to significant loss of vitality
- ✓ It can be dangerous for the fire or even explosion of the material being dried.

3.8.1 Stages of Spray Dryer process

The process can be divided into three phases: atomisation; vaporisation (reduction of the matrix humidity), and recovery of final product, each process steps are adapted to the physical and chemical characteristics of the product (figure 3.2).

The principal atomizer models are rotary atomiser; pressure nozzles; two-fluid or pneumatic nozzles; sonic nozzle, the choice of the model is connected to the product properties.

During the atomisation phase, the liquid substrate by air contact is break up into small droplets forming a spray, the spray-air contact is determined by the atomizer position respect the air inlet, that determine the size and form of dried particles of the product.

The heat is transferred by convention during the droplets-air contact, from air to droplets, and in the subsequent evaporation phase is converted into latent heat, consequently, the humidity is transported in air through the surface around the droplets by convention.

At this point the air-product separation could be obtained by use of cyclones, bag filters or electrostatic precipitators.

The final product (dried particles suspended into drying air) is collected in drying chamber and discharged (Silva et al., 2011).

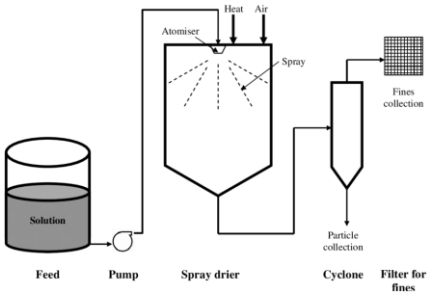


Figure 3.2 Schematic representation of SD process stages

3.9 Microorganism immobilization techniques: new trend of cells immobilization

In the last decades, the use of immobilized yeasts cells was proposed in sequential mixed fermentation during winemaking process to limit the ethanol production and improve the aromatic complexity.

The use of immobilized starter culture presents some advantages respect the conventional yeast inoculum as free cells; for example, it allows an easy separation of microbial cells, is extremely easy to use, presents a better control of contamination risks and reduces cost and time of process (Canonico et al., 2016; Garcia et al., 2018).

In general, many materials are employed as support surface for food and beverages: natural supports (commonly fruits pieces), organic and inorganic supports (alginate, ceramics), or membrane systems.

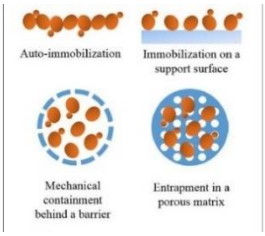


Figure 3.3 Basic methods of cell immobilization (from Garcia et al, 2018)

Various techniques have been investigated for this purpose: the adsorption on inert surface, which is a cheap and simple method based on the ability of cells to adhere on an inert support and form a biofilm of different thickness; the flocculation exploits the ability of some yeasts or mycelia to form cellular aggregates; and the encapsulation technique, based on coating or trapping cells in permeable polymeric material) (Abdel-Rahman et al., 2013; Genisheva et al., 2014).

The encapsulation of microbial cell allows to isolate, protect against mechanical and environmental stress and control the release of cells.

3.10 Microencapsulation of microbial cells

Microencapsulation technique represents a sort of evolution of traditional immobilization systems. It is based on a sophisticate technology to produce little microspheres or microcapsules. Microcapsule can be defined as a physical entrapment of an ingredient or core material (liquid, gaseous or solid) in a functional porous or semipermeable matrix, by use of various methodologies.

The product of microencapsulation process are little solid beads that, according to shape and size, are classified as nano capsule (< 1 µm); microcapsule (1-1000 µm); macrocapsule (< 1000 µm). (Wheleahan and Morrison 2011; Solanki et al., 2013).

According on the morphology there are five different structural forms of microcapsules used in food industry as showed the figure 3.4 (Wheleahan and Morrison 2011).

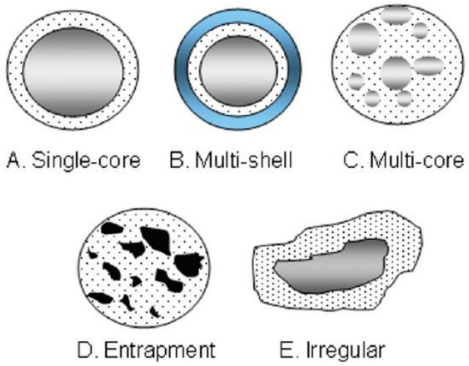


Figure 3.4 Principal morphologies and structural forms of microcapsules (Wheleahan and Morrison 2011).

- Type A: MONONUCLER, called also simple or single core; this is the simplest structure of microcapsules, formed by a liquid core material and an external surrounding outer layer. The volume of core material ranges from 10 to 90% of the structure volume.
- Type B: MULTI SHELLS (WALLS), a particular structure in which one or more shells are added to modify the permeability of the membrane.
- Type C: MULTICORE, this type contains one or more separated cores, and generally are obtained by emulsion technique.
- Type D: MICROSPHERES, the common use structure of microspheres, a particular capsule shape in which there is not physical separation between the encapsulated agent and the ingredient. A network is created between the two components, in this way the encapsulated agent is present over the entire microcapsule area. Furthermore, this type can be converted in type A by covering of a multilayer structure.
- Type E: IRREGULAR, characterized by a no spherical shape, and it is the most commonly used in industrial sector (Wheleahan and Morrison 2011).

The choice of morphology is an important parameter affecting the microcapsule performance in the process.

3.11 Principal encapsulation technologies

Many different techniques are employed for microcapsules manufacturing; the principals are spray drying; spray cooling; spray chilling; air suspension; coating; extrusion; freeze-drying; coacervation; co-crystallization; interfacial polymerization; emulsion.

The most used microencapsulation process applied at industrial level are spray drying; extrusion; emulsion (Kavitake et al., 2018).

Spray drying is the simplest and commonly used technology to produces microcapsules, mainly for food industry, the only disadvantage of this methodology is the high process temperature that cause different cells damages, affecting consequently cell viability and resistance.

Emulsion, an extremely easy technique, it is high expensive in comparison to the other techniques, and different extra steps are required to eliminate the oil residues used in the process.

Microencapsulation by **extrusion** is based on the extrusion of a suspension (cells/polymer) by a nozzle (various diameters range), generating little droplets.

The vibrating jet technique (or vibrating nozzle) is based on the application of a vibrational force to modify the amplitude and break the jet. The sinusoidal force is applied at a previously establish frequency level to break the jet in different segments that then form the spherical uniform droplets. The process parameters setup is essential to obtain droplets of equal size and morphology. The characteristics of droplets depend on the nozzle diameter, flow rate of laminar jet, and on physical and chemical parameters of the extruded suspension (Heinzen et al., 2004).

The figure 3.5 shows the schematic representation of laminar break-up into droplets of equal size by controlled sinusoidal force application (Wheleahan and Morrison 2011).

Vibrating technology is recently applied in pharmaceutical, agricultural, medical field with good results.

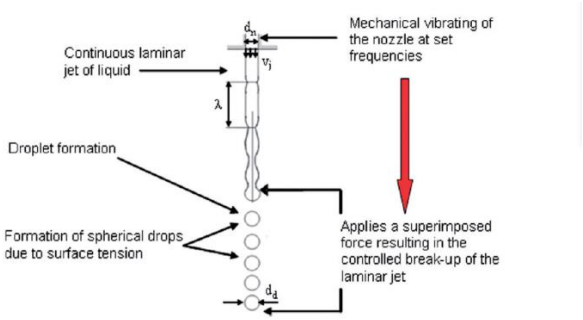


Figure 3.5 Application of sinusoidal force (Wheleahan and Morrison 2011).

Buchi company has recently developed a new encapsulation device optimized for microencapsulation of cells based on principle on laminar jet break up by vibrant force (Buchi encapsulator 395 pro). The device was equipped with an electrostatic dispersion unit to negatively charge the surface of the droplets after their extrusion. The repulsion forces generated by the droplets allow the particles to repel each other falling separately into the polymerization bath. This allows the recovery of the microspheres, avoiding the formation of agglomerates.

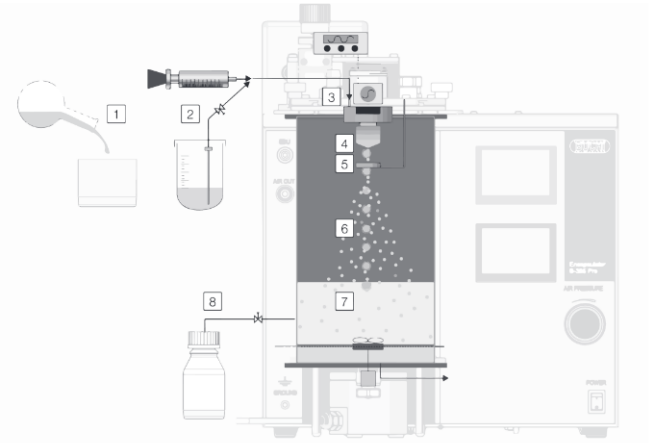


Figure 3.6 Principal steps of microcapsules production (www.buchi.com)

The main steps of the process are the following (figure 3.6):

1. Mixing of active ingredient and polymer.
2. Pumping mixture with syringe pump (0-60 mL/min) or air pressure (0.1-1 bar)
3. Setting the appropriate vibration frequency to break down the cell-suspension liquid jet into droplet of equal size.
4. Droplet formation through a nozzle (diameter range 80-1000 µm)
5. Electrostatic negative charge (0-250 V) introduced by an electrode, obtaining droplet repeal and dispersion.
6. Control of droplets formation by stroboscope lamp
7. Formation of microspheres by polymerization in a solution of $CaCl_2$ for alginate beads
8. Beads collection by drying system

The encapsulator Buchi 395 pro is also equipped by a double concentric nozzles system to produce core-shell microcapsules.

In consequence of its simplicity and adaptability, microencapsulation system is nowadays successfully used in various areas, such as pharmaceutical, agricultural, food industry.

3.12 Material used for microbial cells encapsulation

The first step to produce microspheres is a careful selection of the most suitable encapsulated material for use. In fact, the coating polymer has a considerable influence on the structural properties and performance of the obtained microspheres. Obviously, the materials used must be recognized as GRAS (Generally recognized as safe) and comply with all safety requirements.

Water-soluble materials with high permeability to nutrients and low molecular weight metabolites are preferred. The materials can be of synthetic or natural origin; the first type give to the structure

high mechanical resistance and a certain chemical stability, whereas the other type guarantee less cell damage.
The microspheres obtained can be covered by a layer to improve their mechanical resistance. Among the available materials, the most common used are alginate, chitosane and poli-L-lysine (Table 3.1).

3.12.1 Polymers used in microencapsulation

Starch

Starch is a reserve polysaccharide consisting of α-D-glucose units linked by $\alpha - 1 \rightarrow 4$ and $\alpha - 1 \rightarrow 6$ glycosidic bonds. It is composed by two different molecules, linear amylose (20-30%) and the highly branched amylopectin (70-80%). Adding starch in microencapsulation process improves bacterial survival compared to encapsulation in alginate alone.

Alginate

Alginate is a simple, biocompatible, low-cost, non-toxic and widely used biopolymer for encapsulation, derived from brown algae; it is a copolymer of β-D-mannuronic and α-L-guluronic acids, the alginate beads are formed by crosslinking Ca^{2+} gelling ions with the alginate, the ideal concentration of alginate to be employed in the process is between 0.5 and 4%.
It is also used in combination with other materials with the aim of improving the viability of encapsulated cells. Some applications are reported below.
As regards the probiotics, it was used to improve the viability respect free cells, *L. lactis* cells were encapsulated using alginate / pectin combination, while *L. plantarum and Staphylococcus xylosus* using a mixture of alginate-starch, *Lactobacillus rhamnosus* in a chitosan coating alginate bead, *L. plantarum* encapsulated with inulin-sodium alginate-skim milk by lyophilization.

Chitosan

Chitosan is a linear polysaccharide consisting of glucosamine units that polymerize by crosslinking in the presence of anions and polyanions. It is used as a coating material for alginate microcapsules, especially for probiotics in yogurt and milk, as well as for the encapsulation of *L. bulgaricus* viable cells to increase the survival rate in stressful conditions or at low temperatures.

Pectin

Natural pectin, widely present in the plant cell wall, finds great application in the food industry, for products such as ice cream, jam and fruit juices. The mechanism of gelatinization varies with different degrees of esterification of pectin and according to the characteristics of the product.

Protein

Proteins are generally characterized by good solubility, high resistance to oxidation, biocompatibility, and biodegradability.

One of innovative application is represents by microcapsules obtained using a combination of sodium caseinate and pea protein isolates.

Compared to animal proteins, they are less expensive and more environmentally friendly and meet the needs of vegetarians or people who wish to lose weight.

Yeast cells

Yeast cells, represent an economic and ecological material safe and non-toxic, they are characterized by high adaptability to the environment, they can be dispersed in aqueous solutions, and can be produced on a large scale, so they have been proposed as encapsulating material. The structure of eukaryotic cells represents a material potentially suitable for the encapsulation, it presents several advantages, such as the natural presence of the bilayer formed by the external cell wall and the internal cell membrane that could be exploited to avoid loss of aromatic substances by volatilization or to prevent oxidation of some compounds by exposure to environmental light. The release of aromatic substances or other active components is realized through contact with the mucous membrane of the tongue or nose, furthermore, β-glucan, the main constituent of the cell wall, protects the central substance from heating or freezing treatments (Yang et al., 2020).

Table 3.1 Polymers commonly used for microencapsulation (Rathore et al., 2013)

POLYMER	SOURCE	GELATION MECHANISM	REMARK
Agar	Red Algae	✓ Thermal gelation	✓ Hight cost ✓ Resistance to degradation ✓ Low mechanical strength
K-carrageenin	Red Algae	Thermal-ionotropic gelation in presence of Calcium or other ions ✓ Thermal gelation	✓ Toxicity to cells
Starch	Maize, potato, barley	✓ Thermal gelation	Decompose under acid condition.

			✓ Used for probiotic ✓ Is digest by amylases and can be fermented in the colon
Chitosan	Crustacean shell	Ionotropic gelation	✓ Used as coated agent for microsphere at low concentration
Alginate	Brown Algae	Ionotropic gelation	✓ No-toxicity ✓ Low cost Susceptible to chelating agents
Gellan/Gum	Sphingomonas elodea	Thermal ionotropic gelation	✓ Resistance to hight temperature ✓ Good mechanical strength
Gelatin	Collagene	✓ Thermal /cross linking chemicals or physical agents ✓ Irradiation	✓ Thermal-reversible gelling agent used alone or in combination with other polymers
Xantan gum	Xantomonas campestris	Ionotropic gelation	Highly resistance to pH variation ✓ Usually used in combination with Gellan gum
Milk proteins	Milk	✓ Acid induced gelation ✓ Heat induced gelation	✓ Usually used for probiotic encapsulation
Polyacrylamide	Chemical synthesis	Cross-linking using ammonium persulfate	✓ Synthetic polymer ✓ Acrylamide influence the gel hardness
Polyvynil Alcohol		Cross-linking using boric acid	Toxicity can be reduced replacing boric acid with sodium sulphate for the reaction

3.13 Applications of Microencapsulated cells in food industry

The versatility of this technique allowed the use of immobilized cells for various purposes in the industrial sector.

In fact, this technology is used to preserve the integrity of some ingredients to mechanical or thermal stress during process production, limit the evaporation of volatile compounds (aroma), masking unwanted flavors, such as the astringency of polyphenols, and sometimes is used to prevent unwanted reactions between the ingredient of interest and the surrounding food matrix (for example between essential oils and food water). The most successful application in the food sector has been the microencapsulation of probiotics to preserve the survival as bioactive food ingredient with functional effects for human health (Nedovik et al., 2011).

Microcapsules are widely used in baked goods, vegetables, or other plant foods; they are an effective tool to improve the sensory properties of food during storage especially in meat-based foods, in dairy products, and to enhance the antiseptic and antimicrobial properties of some compounds. For example, the addition of microencapsulated cress seed oil protected α-linolenic acid against oxidation in the biscuits and prolong their shelf life. Microencapsulated clove oil with antimicrobial effect could be used as an alternative preservative, the microencapsulated nisin shows antibacterial activity against *Listeria monocytogenes* in ready-to-use ham.

Nowadays, immobilized yeasts cell (*S. cerevisiae*) trapped in alginate matrix are often use as starter culture to improve fermentation productivity and wine yeasts performance, mainly in sparkling wine production (Bokkinm et al., 2018).

Although this technique is a little applied and still under study, many industries have already started the production of microcapsules; among them, the best microencapsulation companies involved in the food sector, especially in the fortified food market, are Capsulae (France), Microtek Laboratories, Inc. (United States), Aveka, Inc. (United States), Taste- Tech Ltd. (UK), LycoRed Ltd. (Israel) and Innobio Limited (China).

3.14 Materials and Methods (I)

During the spray dryer process the microorganisms are subjected to different stress factors that caused cell damages.

The damaged cells have difficulty to grow and presented a very long lag phase, in particular due to the high drying temperature.

For this reason, some aspects to improve the viability of microorganisms during SD and storage, such as the optimization of process parameters, the selection of most appropriate growth phase, the study of drying and rehydration conditions, the selection of some protective agents.

In this step, the response to drying process and rehydration phase of two non-*Saccharomyces* strains previously selected, *T. delbrueckii* 425 and *H. osmophila* ND1, was evaluated.

3.14.1 Preliminary study of yeasts growth phases

During the cellular growth, it is possible to distinguish several phases:

I. Lag phase: period of adaptation of yeast cells to the conditions of the specific growth substrate. During this phase, the number of cells remains constant.

II. Log phase: during the log phase, also known as the exponential phase, the cells begin to multiply rapidly (mitosis) and there is an intensification of metabolic processes until nutrient exhaustion, the cells number increases exponentially. The duration of cell multiplication depends on the environmental conditions and the yeast species.

III. Stationary phase: following the progressive exhaustion of resources, yeasts begin to adjust their metabolism to synthesize some secondary metabolites and accumulate some carbohydrates reserve. In this phase, a progressive decrease in the number of viable cells is observed. This step ends with cell lysis and death.

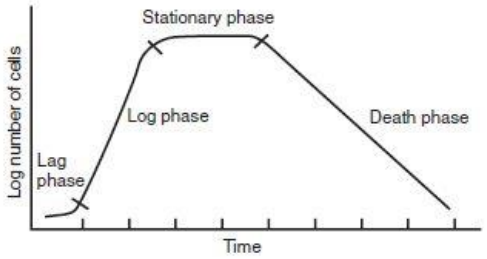


Figure 3.7 Phases of the growth curve

Yeasts cells in the stationary phase are more resistant to stress than cells in the exponential growth phase and probably more resistant to dehydration treatment.

For this reason, the selected strains were grown in flasks containing YPD medium in a rotatory shaker and monitored until the stationary phase was reached, both by optical density (OD) measure (600nm), determined with SPECTROSTAR ^{nano} BMGLABTECH, and by cell count on plate.

This phase is important to identify the time taken by the cells to enter the stationary phase.

3.14.2 Spray dryer (laboratory process)

Figure 3.8 Mini Spray dryer B-192, BUCHI

The yeast ability to survive to drying treatment is a highly sought-after feature in the selection of yeasts to be used as a starter.

Yeasts cells in the stationary phase have been shown to be much more resistant to stress conditions than cells in the exponential growth phase (Capece et al., 2016; Zambuto et al., 2017;).

For this reason, in the present step the ability to survive to the drying treatment of the two selected non-*Saccharomyces* strains was performed only on cells in stationary phase.

The growth of the strains in flasks containing liquid YPD was started from an initial optical density of 0.2 at 600 nm.

The growth parameters were the following:

- Temperature: 26°C
- Agitation: 180 rpm.

For each strain, after the time necessary to reach the exponential phase, the growth broth was taken in sterile conditions and centrifuged at 4700 rpm for 10 minutes, with the aim of recovering the biomass to be dried.

The biomass added of skim milk (as protective agent) in a volume equal to 10% was kept under constant stirring at a temperature of 26°C for 30 minutes to completely resuspend the pellet.

The test was carried out by mini spray dryer BUCHI B-191, the instrument was previously sterilized with deionized water at 220 °C for 20 minutes.

The inlet temperature was set at 120°C while the outlet temperature and the pump efficiency were established based on the characteristics of the cell suspensions used (around 50-56°C).

During the process, the pump injects the suspension into the drying chamber and into the cyclone, the particles move in a vortex towards the walls of the cyclone while the air rises towards the outlet at the top. At the end, dried yeast cells are collected in a sterile container.
After each application, the instrument was washed (using deionized water) to eliminate any residual sample inside.

3.14.3 Rehydration phases

The dry yeast produced was subjected to rehydration according to the following protocol:

- 0.5 grams of yeasts powder were resuspended in 4.5 mL of 5% glucose solution.
- the suspension is left at a constant temperature (26°C) for 10 minutes.
- the suspension was maintained under constant stirring for 20 minutes until it was completely dissolved.

3.14.4 Determination of cell vitality and survival rate % after Spray Drying

Serial dilutions have been prepared using both rehydrated yeast suspension and an aliquot of yeast biomass to compare the viability of the strain in fresh formulation versus dry formulation.
To determine the survival rate (%) the yeast powder was rehydrated with a glucose solution (5% v/v) and the suspension was shaken for 30 minutes at 180 rpm, the final viable cells number of dehydrated powder was evaluated by counting plate on YPD and expressed as colony forming unit (CFU)/g of dry powder.
Survival rate was calculated as ratio between the CFU present in the yeast cell suspension before drying (Ni) and CFU in spray dried powder (Nf), as below:
Survival%= (Nf/ Ni) *100

3.14.5 Mixed fermentation trials on laboratory scale with selected non-*Saccharomyces* yeast strains as fresh and dried cells

Figure 3.9 Mixed fermentations at laboratory scale with *T. delbrueckii 425* and *H. osmophila* ND1 as fresh and dried cells in combination with *S. cerevisiae* (EC1118).

The fermentative behaviour of the two non-*Saccharomyces* yeast strains both as fresh and dried cells were analysed in wine mixed fermentations at laboratory scale.
The strains in the fresh form were produced in a bioreactor using YPD as growth medium; the biomass was recovered by centrifugation after 24 hours of growth, while the starters in dried form were produced by drying the cells suspension in the stationary phase for each strain.
Fermentations were carried out in duplicate in 400 mL of natural grape must (Primitivo variety) for both starter culture formulations (fresh and dried).
The non-*Saccharomyces* strains 425 and ND1 were inoculated at $2*10^7$ cell/mL in combination with *S. cerevisiae* ($2*10^7$ cell/mL and $2*10^3$ cell/mL respectively).
As control, it was used a fermentation inoculated with only *S. cerevisiae* cells at concentration of $2*10^7$ cell/mL.
The fermentations were carried out at 26°C and were monitored by measuring the °Brix degree until the end of the process (constant °Brix for three consecutive days).

3.14.6 Microbiological control of fermentation

Samples of fermenting must and wine were diluted in saline solution and plated on WL Nutrient Agar (Oxoid, Hampshire, UK) as growth medium to monitor the evolution of the two species during alcoholic fermentation. To differentiate *Saccharomyces* from non-*Saccharomyces* strains the selective medium Lysine agar (Oxoid, Hampshire, UK) was used.
For the mixed fermentations with *T. delbrueckii*, non-*Saccharomyces* cells were counted using a specific agar medium, YPDA (1% yeast extract, 1% peptone, 2% dextrose, 2% agar; pH 4.8) supplemented with 1 µg/mL cycloheximide to promote only the growth of *T. delbrueckii*. Plates were incubated at 24 °C for 4 days.
The count of *S. cerevisiae* colonies was calculated as the difference between the total plate count and the plate count using non-*Saccharomyces* selective medium (Bely et al., 2015).
At the end of fermentation process, some principal chemical parameters were detected using OenoFoss™ instrument, whereas the main secondary compounds were analysed by GC analyses.

3.14.7 Statistical Analysis

Analysis of variance (ANOVA) was applied to data related to the principal chemical parameters of the wines. Data are expressed as means of two independent experiments. The significant differences were determined using Tukey test, the data were considered significant if the p-value were < 0.05.

3.15 Results (I)

With the aim to identify the time required by each strain to reach the stationary phase, cell density (cfu/mL) and the optical density value were monitored over time.

The trend of cell density (cfu/mL) over time and optical density (OD) over time has been reported in the graph for each stain.

The figure 3.10 shows the values of the OD over time of the reference strain EC1118 (*S. cerevisiae*). The latency phase was short, and the cells enter in the exponential phase after 7 hours, in the following time the growth rate increases up to reach a cell concentration of 10^7 cfu/mL. Subsequently, the growth rate slows down, and the number of cells stabilizes over time up to 10^8 cfu/mL). At this point the cells can be considered in the stationary phase.

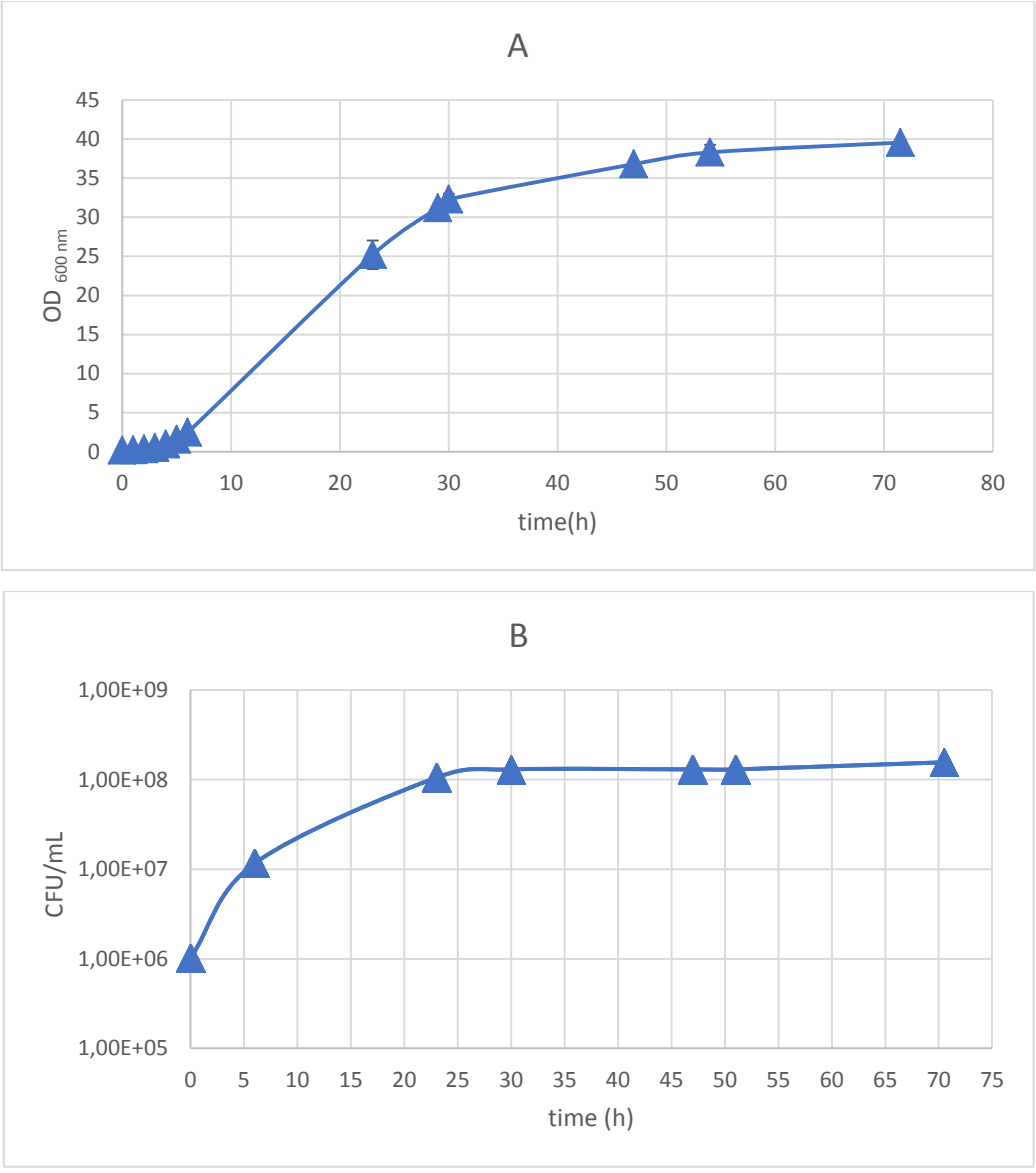


Figure 3.10 Growth curve expressed as OD /t (A) and CFU/mL (B) of the strain EC1118.

The graph 3.11 shows OD values for the strain *T. delbrueckii* 425. The strain enters in exponential phase and remains in this phase until about 24 hours, after that it starts the stationary phase (10^8 cfu/mL).

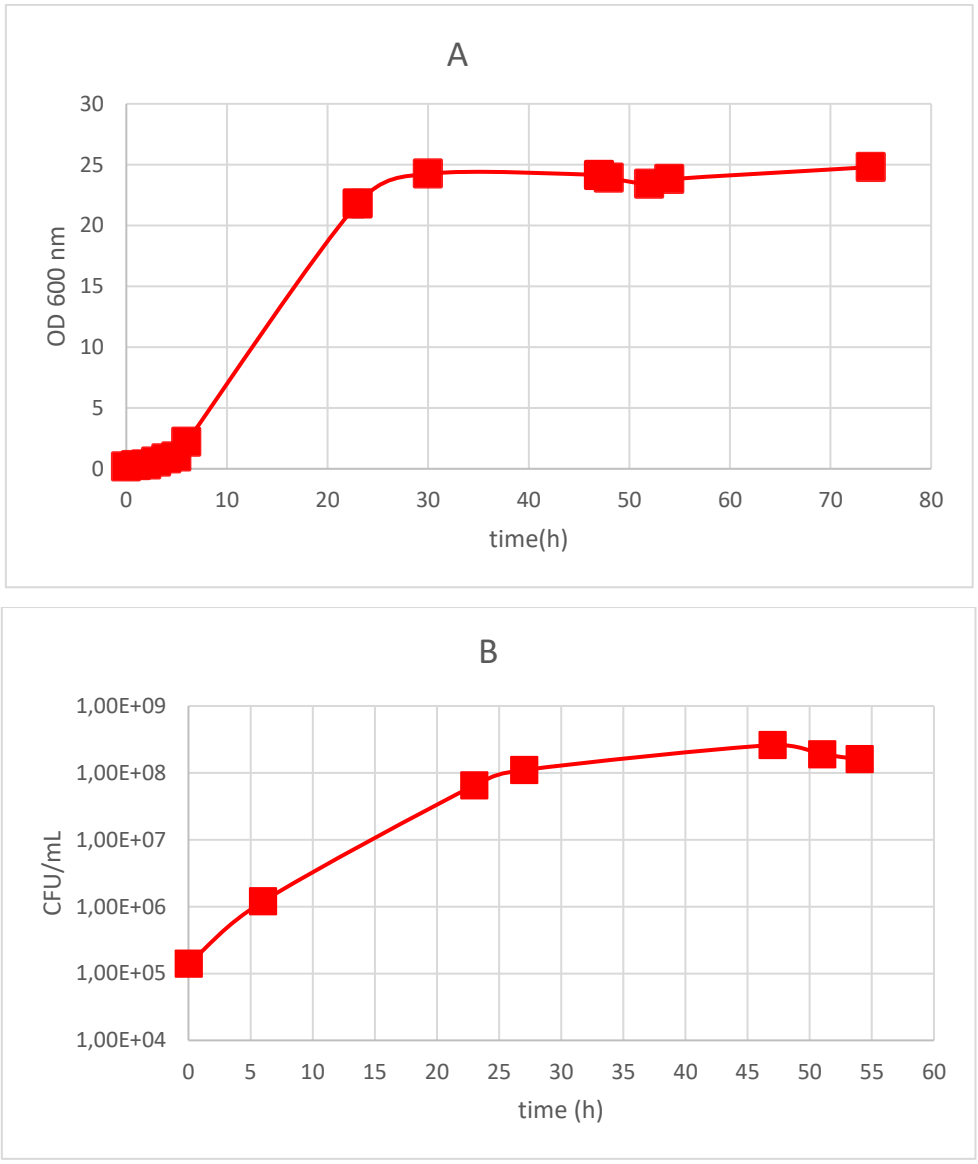


Figure 3.11 Growth curve expressed as OD /t (A) and CFU/mL (B) of the strain 425.

The graph 3.12 shows the results of growth curves for the strain ND1 (*H. osmophila*). In this case, growth curve was different from those observed in the other two strains; in fact, the cells remain in the exponential phase for more time and enter in stationary phase after about 60 hours. This value has been considered indicative for the drying phase.

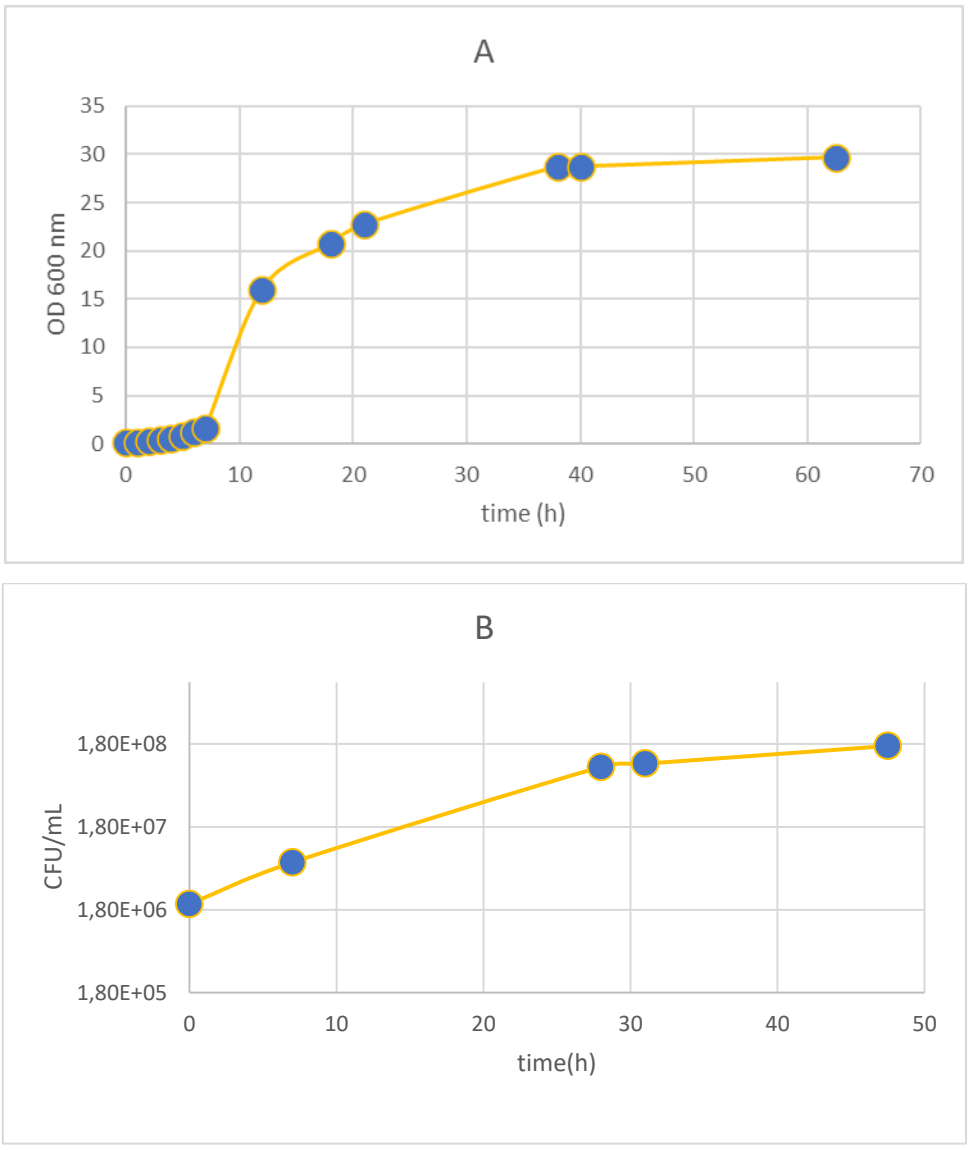


Figure 3.12 Growth curve expressed as OD /t (A) and CFU/mL (B) of the strain ND1.

3.15.1 Strains cellular viability

Cell viability defines the yeast's ability to reproduce and depends on various factors, such as yeast species, growth conditions (temperature, osmotic pressure and other), process production conditions and grape must composition.

The figure 3.13 shows the results obtained from the evaluation of cell viability in the two formulations of starter produced at the laboratory scale (dried and fresh formulation). In both strains, the drying process has determined a reduction of cell viability, although the number of viable cells present for one gram of dried yeast is enough for their use as starter in wine fermentations. As regards the survival rate, *H. osmophila* exhibited higher survival rate (%) than *T.debrueckii* strain, as reported in the table 3.2.

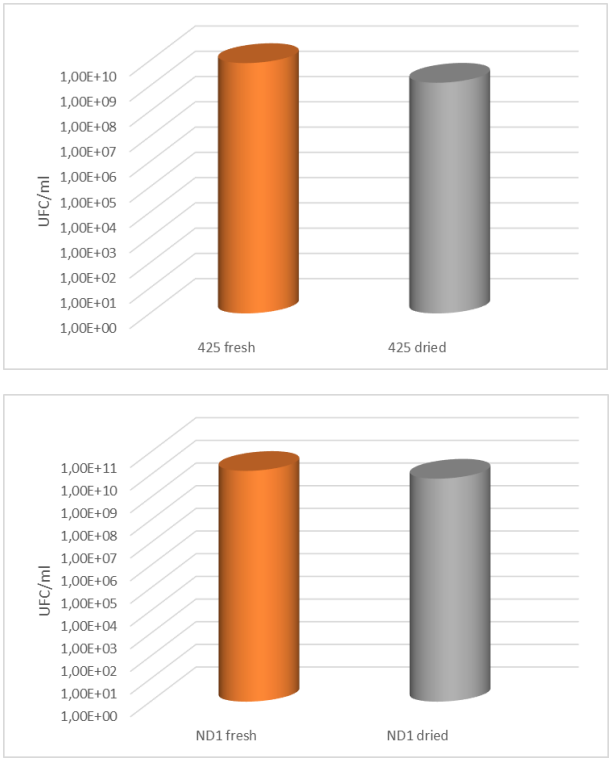


Figure 3.13 Cell viability exhibited by the two starter formulations

For each strain, the survival rate % values are show in the table. 3.2

Strain	**Survival (%)**
T. delbrueckii **425**	11
H. osmophila **ND1**	**29**

Table 3.2 Survival rate % of the two non-*Saccharomyces* strains to spray drying process

3.15.2 Fermentative performance

Laboratory fermentations were performed in pasteurized natural must, using both the different starter formulations with the aim to evaluate the influence of the drying treatment on the yeast fermentative activity. As showed in the figures 3.14, the fermentations were monitored by evaluating sugar consumption (°Brix value) during the process.

Only mixed fermentation with *H. osmophila* strain exhibited some differences in fermentation activity in function of starter formulation. In fact, in this case the starter formulation influenced the sugar consumption profile as the use of dried formulation of starter culture reduced the duration of fermentation in comparison to fresh culture, while for *T. delbrueckii* the curves of sugar consumption have a similar behaviour in both formulations.

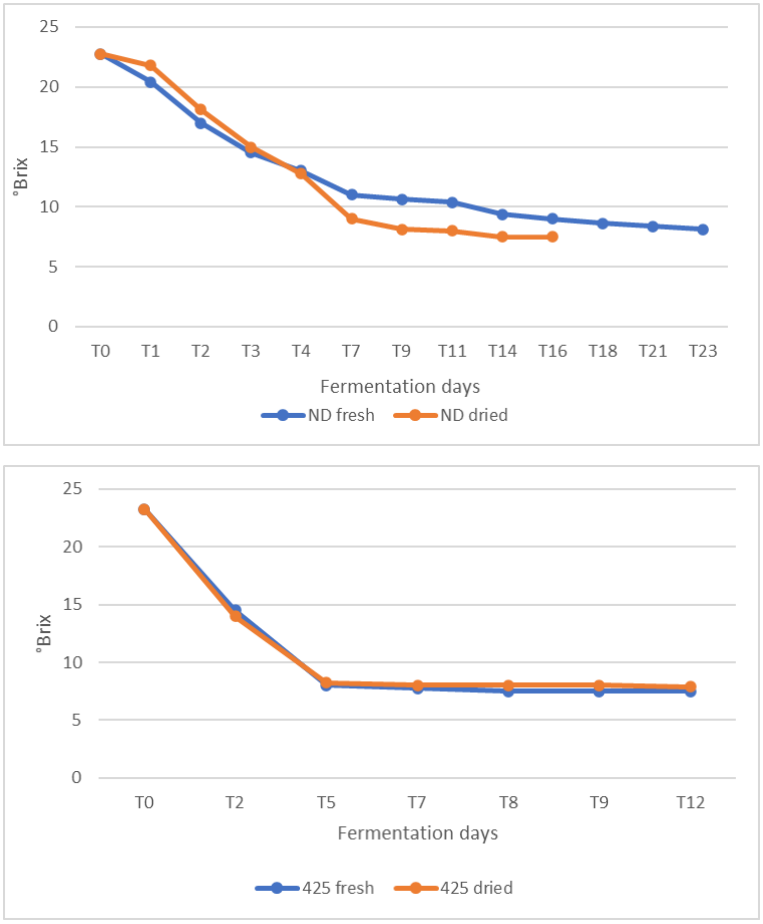


Figure 3.14 Sugar consumption in mixed fermentations using dried and fresh culture of selected non-*Saccharomyces* yeasts.

3.15.3 Analysis of cell viable population in mixed fermentation with *H. osmophila* as fresh and dried cells

The concentration of viable cells during micro fermentations with fresh and dried *H. osmophila* cells was monitored by cell count at different steps of mixed fermentations (Figure 3.15).

At T2 (2nd day after inoculum), figure3.15-a, the highest number of viable cells was found for *H. osmophila* strain as fresh cells.

As regards *S. cerevisiae* cells in mixed fermentations, the number of viable cells was higher in mixed fermentation with *H. osmophila* as fresh form than in sample with *H. osmophila* in dried form. However, after four days (figure 3.15-b) in all the fermentations an increase of cell number of *H. osmophila* strain was observed respect to inoculation level both in dried and fresh formulations ($4.8*10^7$ cfu/mL and $2.7*10^7$ cfu/mL as fresh and dried, respectively), while the viable cells number of *S. cerevisiae* remain constant in all cases.

At T7 (7th day after inoculum), figure 3.15 (c), the number of *S. cerevisiae* viable cells increases in both mixed fermentation while the number of ND1 viable cells decreased most rapidly in mixed fermentation with *H. osmophila* dried cells ($1,4*10^6$ cfu/mL and $1,5*10^5$ cfu /mL in fresh and dried form, respectively).

A similar trend was observed after 11 days of fermentation (figure 3.15-d), the cell number of ND1 strain decreased in the mixed fermentation inoculated with ND1 fresh cells ($2*10^3$cfu/mL) and completely disappeared in the sample inoculated with ND1 dried cells.

In the last isolation step (figure 3.15-e), for the sample inoculated with *H. osmophila* fresh cells only *S. cerevisiae* cells were found ($1,4*10^5$ cfu/mL), probably as a consequence of increase in ethanol concentration.

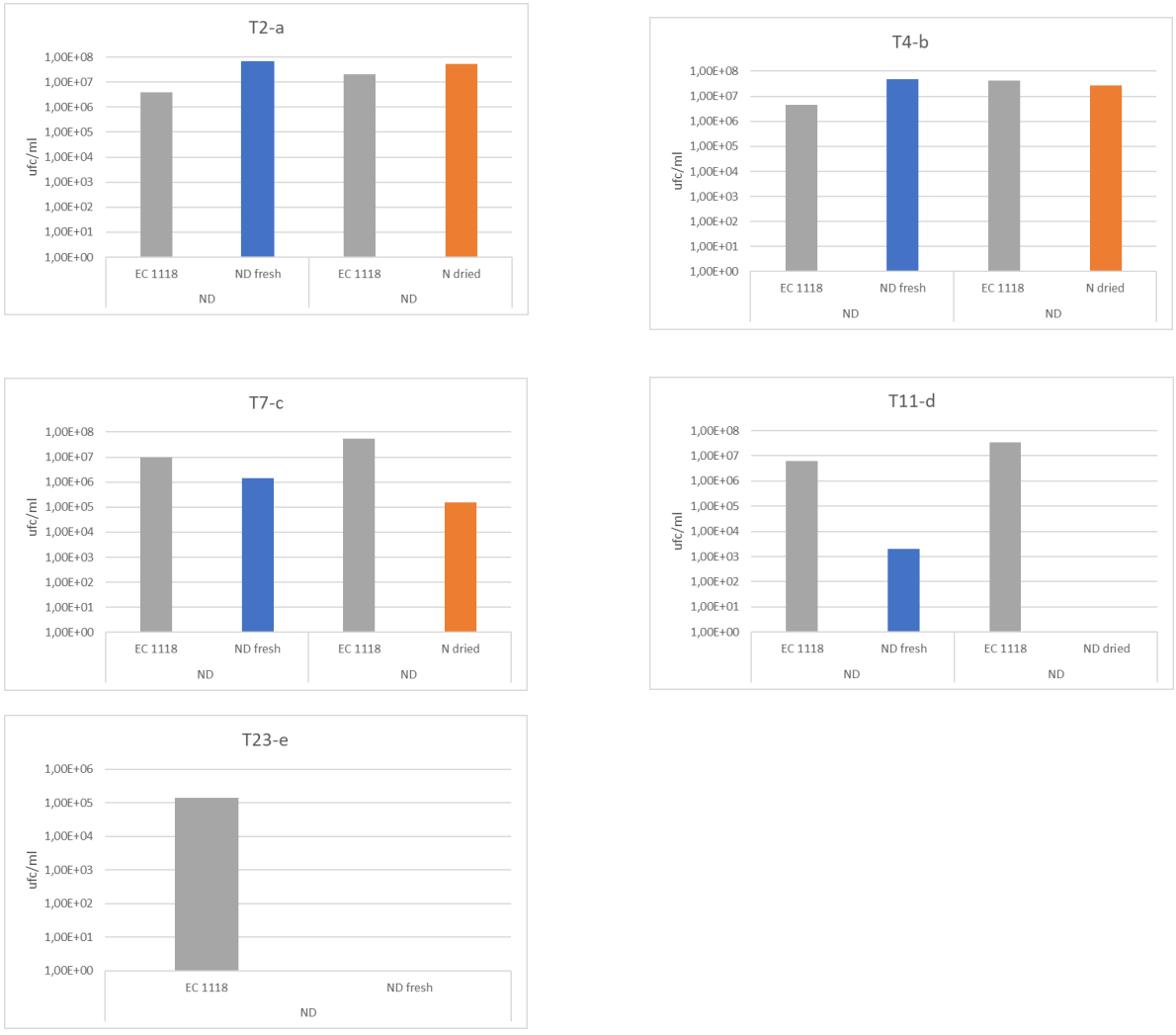


Figure 3.15 Yeast cell count detected at different times of fermentations inoculated with S. cerevisiae EC1118 in mixed culture with H. osmophila ND1 strain as fresh cells and dried cells. Data are the means of two independent experiments.

3.15.4 Analysis of cell viable population in mixed fermentation with *T. delbrueckii* as fresh and dried cells

The concentration of viable cells during micro fermentations with fresh and dried *T. delbrueckii* cells was monitored by yeast isolation at different steps of mixed fermentations (Figure 3.16).

At T2 (2nd day after inoculum), figure 3.16-a, it was found the highest number of viable cells of *T. delbrueckii* in mixed fermentation with the strain as fresh formulation ($9.5*10^8$cfu/mL), also the sample with 425 as dried cells presented a high level of viable cells (about $1.4*10^7$ cfu/mL).

As regards *S. cerevisiae* cells in mixed fermentation, the number of viable cells was high in all samples both with *T. delbrueckii* as dried form (EC1118 =$2.12*10^8$ cfu/mL) and fresh formulation (EC1118 =$3.07*10^8$ cfu/mL).

At T5 (5nd day after inoculum), figure 3.16-b, the number of *S. cerevisiae* viable cells increases in both mixed fermentations, while the number of 425 viable cells decrease most rapidly in mixed fermentation with *T. delbrueckii* dried cells ($1.4*10^6$ cfu/mL and $1.5*10^5$ cfu /mL as fresh and dried cells, respectively).

In the last isolation steps (figure 3.16-c), in both the samples only *S. cerevisiae* cells were found. At the end of fermentative process, the highest number of *S. cerevisiae* viable was found in mixed fermentation with 425 fresh cell (1.0^7*10^8 cfu/mL) respect to the mixed fermentation with 425 dried cells ($6.15 *10^6$ cfu/mL).

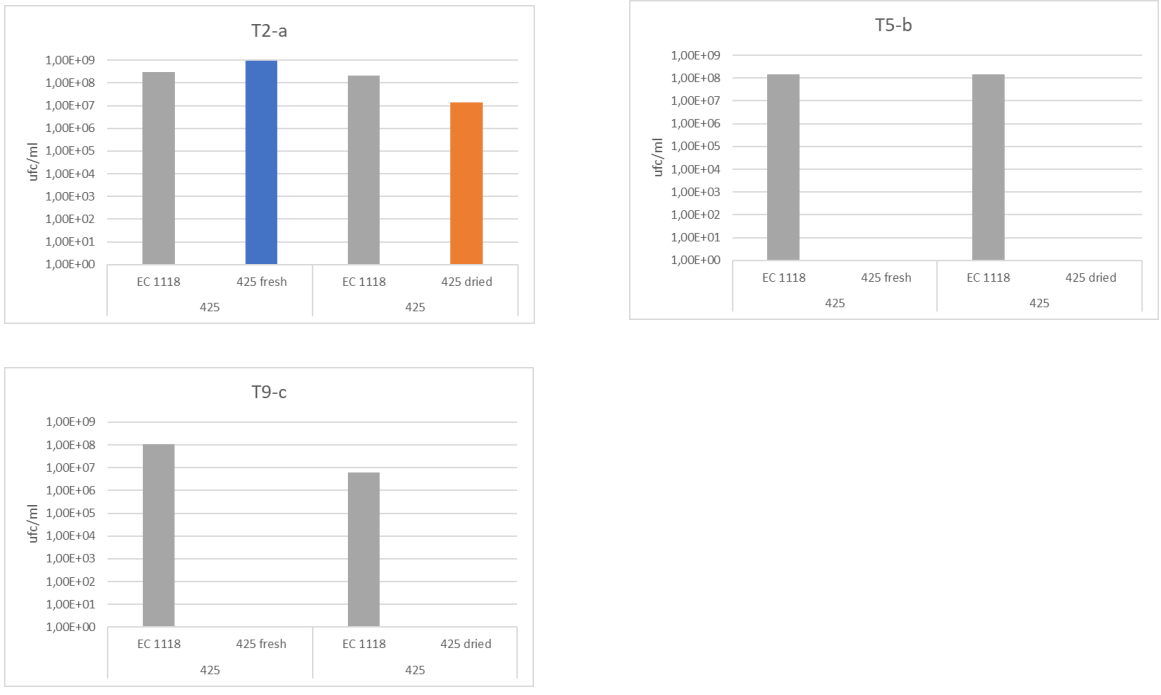


Figure 3.16 Yeast cell count detected at different times of fermentations inoculated with S. cerevisiae EC1118 in mixed culture with T. delbrueckii strain as fresh cells and dried cells. Data are the means of two independent experiments.

3.15.5 Conclusion

The data show that the *H. osmophila* strain in mixed fermentations with *S. cerevisiae* is more competitive than the T. *delbrueckii* strain. In fact, viable cells of ND1 were found up to T11 (eleventh day of fermentation) for the sample inoculated with ND1 as fresh cells, while until T5 for the sample inoculated with dried cells of ND1.

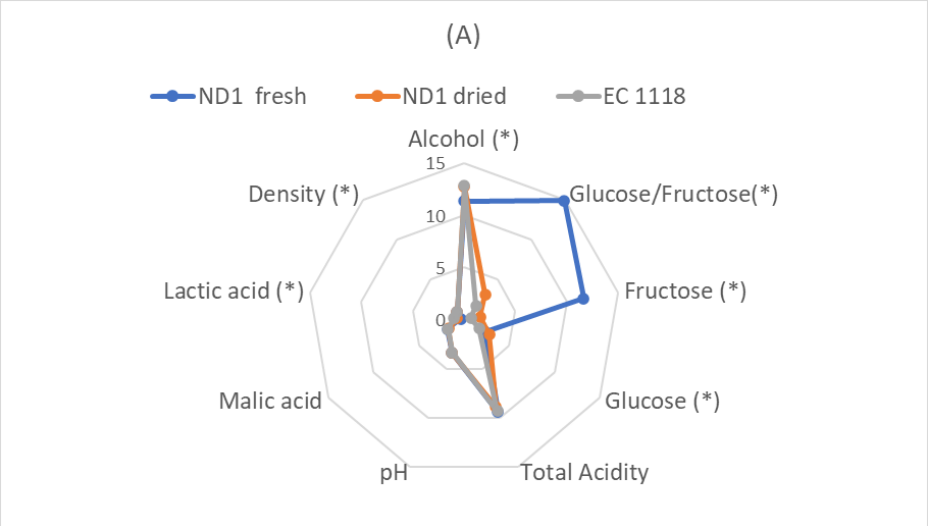
As regards the fermentations with the 425 strain, in both the tested formulations (fresh/dried) the *T. delbrueckii* strain was not found after the 5th day of fermentation. The fermentation is completed in a shorter time when the dried starter is used, in which the number of viable cells of EC1118 increases considerably earlier than in the corresponding mixed fermentations inoculated with the starter in fresh form. In all cases EC 1118 dominated the fermentation, completing the process.

3.15.6 Analysis of experimental wines for main oenological parameters

The wines obtained by the mixed fermentations with EC1118 and *H. osmophila* (ND1) or *T. delbrueckii* (425), as dried and fresh formulations, in comparison to single starter wine, were analysed for parameters of oenological interest. The data highlighted that statistically significant differences were found for some parameters.

As showed in the radar chart (figure 3.17), the profiles of the wines obtained with ND1 dried, and the control (EC 1118) are more similar than the profile obtained by wine fermented with fresh cells of ND1. Significant differences were found for the lower ethanol values of wine from ND1 fresh cells compared with control wine and the sample fermented with dried cells, but it also exhibited significantly higher levels of residual sugar at the end of fermentation.

On the contrary, in the case of mixed fermentations carried out with strain 425 (*T. delbrueckii*) the profiles obtained from the three samples are very similar among them, they almost tend to overlap and only the sample (wine fermented with 425 dried cells) differed from the others for the highest residual sugar at the end of fermentation.



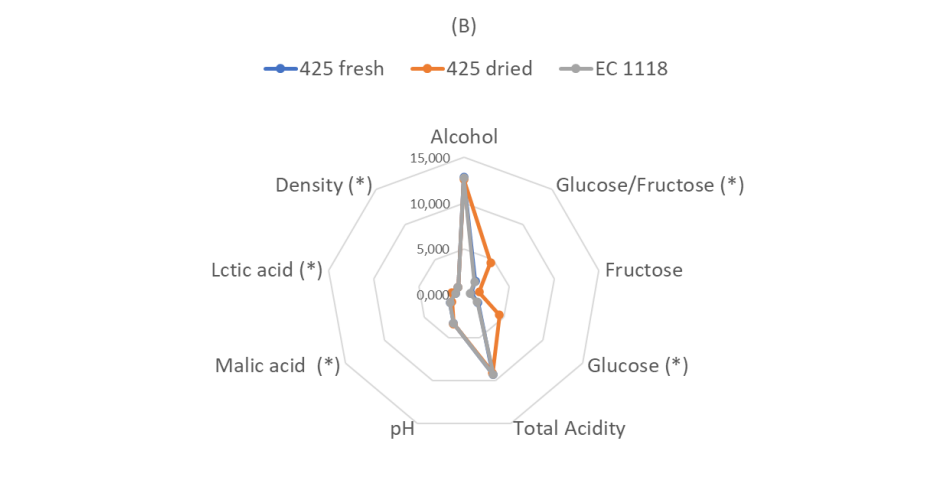


Figure 3.17 Main oenological parameters detected in wines obtained by the mixed starters with *S. cerevisiae* EC1118, *H. osmophila* (A) or *T. delbrueckii* (B) as dried and fresh formulation in comparison to single starter wine with EC1118. Significant differences at $p<0.05$ among wines produced in the different conditions were highlighted with (*) in the graph for each compound.

3.15.7 Evaluation of aromatic profile of experimental wines by the mixed starters with *S. cerevisiae* (EC1118) and *T. delbrueckii* (425) strains

The experimental wines were analysed also for the content of some secondary compounds as reported in Table 3.3.

Table 3.3 Main by-products content (mg/L) detected in wines obtained by the mixed starters with *S. cerevisiae* (EC1118) and *T. delbrueckii* (425) in two different formulations (dried and fresh) in comparison to single starter wine with EC1118. For each compound, superscript letters mean significant differences (Tukey's test, $p \leq 0.05$) among wines produced in the different conditions. Data are expressed as mean value ± SD of two independent experiments.

Secondary compounds (mg/L)	**Control EC1118**	**425 fresh**	**425 dried**
Acetaldehyde	47,71 ± 2,25	42,88 ± 1,14	45,39 ± 1,80
Ethyl acetate	13,65 ^{a} ± 0,29	16,04 ^{b} ± 0,14	14,69 ^{ab} ± 0,50
n-propanol	12,39 ^{a} ± 0,05	13,63 ^{b} ± 0,01	13,60 ^{b} ± 0,31
Isobutanol	35,85 ^{a} ± 1,14	32,27 ^{b} ± 0,04	35,01 ^{ab} ± 0,53
n-butanol	13,74 ^{a} ± 0,18	13,89 ^{a} ± 0,24	24,02 ^{b} ±0,36
Acetoin	4,80 ± 0,50	7,71 ± 1,31	7,15 ± 0,66
Acetic acid	372,45 ± 33,30	357,95 ± 16,28	397,53 ± 24,08
D-Amyl alcohol	67,70 ± 5,29	60,58 ± 1,82	57,52 ± 1,07
Isoamyl alcohol	183,25 ± 11,35	170,69 ± 0,99	166,83 ± 3,20

Differences statistically significant between control fermentation and wines from mixed starters were found only for four secondary compounds: ethyl acetate, n-propanol, isobutanol, n-butanol.

The wine obtained with dried cells exhibited the highest value of two alcohols (isobutanol and n-butanol), while the wines obtained by mixed starters in both the two formulations presented similar values of ethyl acetate and n-propanol respect to the control.

The level of acetaldehyde, acetoin and acetic acid were similar in all samples.

3.15.8 Evaluation of aromatic profile of experimental wines obtained by the mixed starters with *S. cerevisiae* (EC1118) and *H. osmophila* (ND1)

The experimental wines were analysed also for the content of some secondary compounds as reported in Table 3.4. Differences statistically significant between control and wines from mixed starters were found for the majority of detected aromatic compounds.

The sample fermented with fresh ND1 cells contained higher level of n-butanol and D-amyl alcohol than the other two experimental wines, while the acetoin content was significantly higher in wines from mixed starters than control, mainly in sample fermented by fresh formulation. Mixed starters increased the production of ethyl acetate in both the formulations respect to the single starter.

Table 3.4 Main by-products content (mg/L) detected in wines obtained by the mixed starters with *S. cerevisiae* EC1118 and *H. osmophila* ND1 in two different formulations (dried and fresh) in comparison to single starter wine with EC1118. For each compound, superscript letters mean significant differences (Tukey's test, $p \leq 0.05$) among wines produced in the different conditions. Data are expressed as mean value ± SD of two independent experiments.

Secondary compounds (mg/L)	**Control EC1118**	**ND1 fresh**	**ND1 dried**
Acetaldehyde	47,71 ± 2,25	42,20 ± 0,04	53,28 ± 3,87
Ethyl acetate	13,645 [a] ± 0,29	15,89 [b] ± 0,74	24,29 [c] ± 0,07
n-propanol	12,39 [a] ± 0,05	15,19 [b] ± 0,67	19,75 [c] ± 0,36
Isobutanol	35,85 ± 1,14	26,79 ± 8,44	26,86 ± 0,18
n-butanol	13,74 [a] ± 0,18	86,40 [b] ± 12,42	21,30 [a] ± 1,29
Acetoin	4,80 [a] ± 0,50	19,65 [b] ± 1,91	12,56 [c] ± 1,07
Acetic acid	372,45 [a] ± 33,30	1260,90 [b] ± 165,66	706,37 [a] ± 191,06
D-Amyl alcohol	67,70 [a] ± 5,29	82,72 [a] ± 2,99	50,35 [b] ± 7,72
Isoamyl alcohol	183,25 ± 11,35	150,27 ± 17,78	150,02 ± 35,82

For easily visualization of contribution of each mixed starter culture in the production of aromatic compounds, the figures 3.18/ 3.19/3.20 report all secondary compounds for which significant different values were found between mixed and control fermentations.

As showed in the figures, the contribution of *H. osmophila* (Figures 3.19 and 3.20) in the production of secondary fermentation metabolites was greater than *T. delbrueckii* (Figure 3.18).

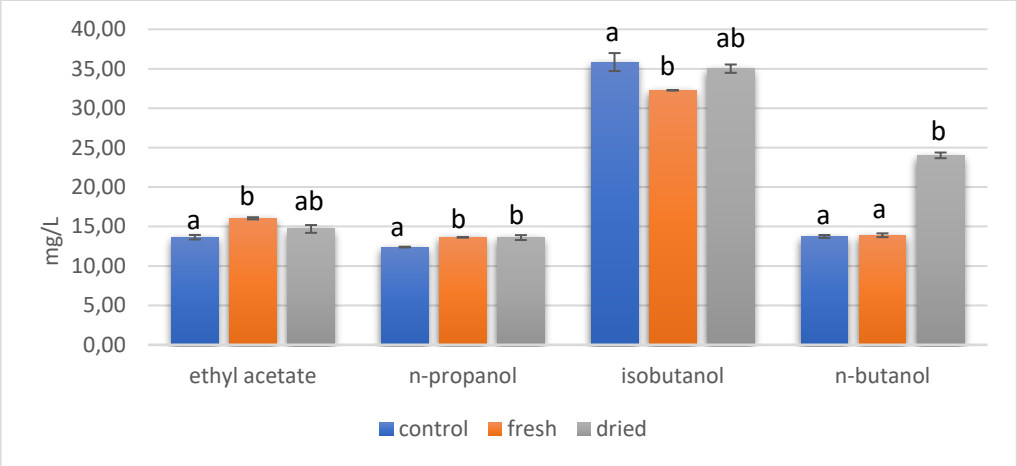


Figure 3.18 By-products (mg/L) with differences statistically significant in function of starter, which were mixed starters with *S. cerevisiae* EC1118 and *T. delbrueckii* 425 (fresh and dried), EC1118 (control).

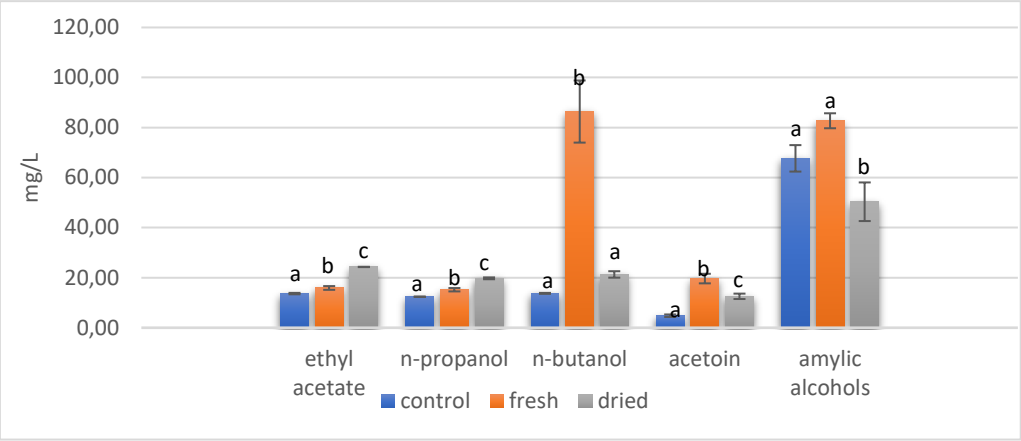


Figure 3.19 By-products content (mg/L), with differences statistically significant in function of starter, which were mixed starters with S. cerevisiae EC1118 and *H. osmophila* ND1 (fresh and dried), EC1118 (control).

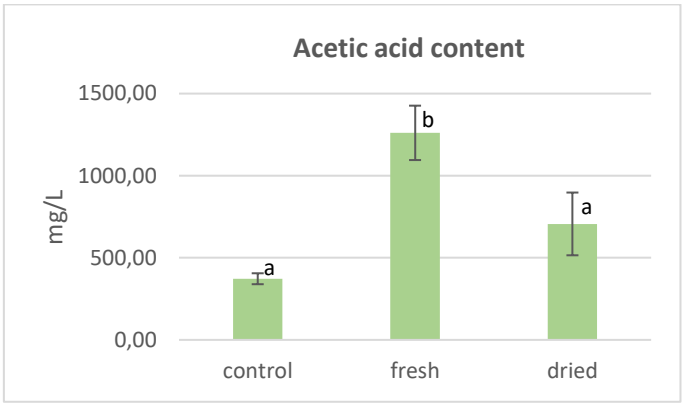


Figure 3.20 Acetic acid content in mixed fermentations with *H. osmophila* (as fresh and dried form) ND1 respect to control fermentation.

Hanseniaspora osmophila strain in mixed fermentations produced a higher content of secondary compounds in comparison to the single starter culture EC1118; furthermore, it was detected an influence of the starter formulation on the production of some compounds, especially for acetic acid production, with a lower content in mixed fermentation with ND1 as dried cells, whereas the wine produced by inoculating ND1 fresh cells contained acetic acid level exceeding the acceptability level of this compound in wines.

3.15.9 Conclusion

In conclusion, the use of mixed starter containing *H. osmophila* increased the content of some by-products in the experimental wines, mainly when used as fresh cells, although this formulation yielded too high levels of volatile acidity, that negatively affect the wine aroma.

In consequence of different behaviour exhibited by *H. osmophila* ND1 strain in function of cell formulations, this strain was chosen for further investigation as microencapsulated yeasts in fermentation trials.

3.16 Material and method (II)

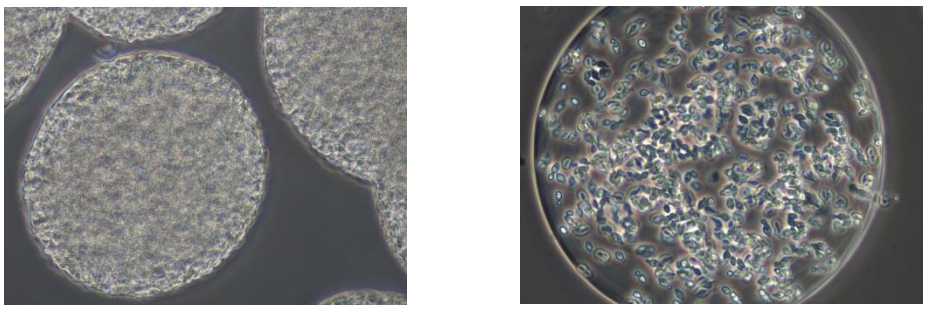
3.16.1 Yeast strains culture and biomass production

Yeast strain ND1 *H. osmophila* and EC1118 *S. cerevisiae* were refreshed on YPD for 24h at 26°C.
For biomass production, an aliquot of each strain was inoculated in 300 mL of YPD broth and incubated at 26°C for 72 h in a rotatory shaker at 180 rpm, the microbial biomass was harvest by centrifugation at 4700 rpm for 10 minutes and stored for limited time at 4°C until the use.
The biomass of *H. osmophila* was divided in two parts, one used for microcapsules production and the other part was used as inoculum for mixed fermentation.

3.16.2 Microencapsulation of ND1 strain

Microencapsulation of *H. osmophila* strain cells was carried out utilizing the Encapsulator B-95 Pro (BUCHI, Flawil, Switzerland). The solution to be encapsulated was obtained by suspending yeasts pellet in 500 mL of 15 g/L alginic acid solution salt (Sigma, Milan, Italy). The feeding solution was loaded by syringe and extruded trough the nozzle (80µm). The microencapsulation process conditions were the following: flow rate 3.5mL/min, vibrating frequency 25000Hz, electrode voltage of 950 V. Alginate droplets containing yeast cells were formed in 500 mL of 0.5 mol/L sterile $CaCl_2$ solution in stirring (at 300 rpm). To obtain microcapsules sedimentation the suspension was left 30 min at room temperature, after that it the solution was discarded, and microspheres were harvest and stored at 4 °C until the use. Microencapsulation process was carried out in collaboration with the University of Naples “Federico II”, (Portici, Italy).

Figure 3.21 Optical microscopy images at x 400 magnification of ND1 strain after microencapsulation



3.16.3 Entrapment efficiency of microencapsulation process

The microencapsulation efficiency was calculated as ratio between the viable count of disrupted microcapsules and cell load of feeding solution.

According to De Prisco et al. (2005) and Benucci et al. (2019), microcapsules were disrupted by serial dilution in 0.5mL/l of phosphate buffer solution (pH 7) and the viable counting was determined on YPD Agar at 26°C for 24h.

3.16.4 Mixed inoculated fermentations at laboratory scale

The *H. osmophila* strain was tested in mixed fermentation with *S. cerevisiae* at laboratory scale, the strain ND1 was used as free and microencapsulated form, whereas *S. cerevisiae* only as free formulation**.**

Two fermentation experiments were performed, by using the same process conditions but in different volumes (100 mL and 2 L). As fermentation media, natural grape must, previously pasteurized (T=90°C for 20 minutes) was used.

The two yeast strains were simultaneously added in grape must by using different inoculation ratio. *S. cerevisiae* was inoculated in all the samples as free cells at concentration of $2*10^3$ cell/mL, whereas *H. osmophila* was inoculated as free and microencapsulated cells at concentration of $2*10^7$ cell/mL.

In the control fermentation, only *S. cerevisiae* cells at concentration of $2*10^7$ cell/mL was inoculated.

All the experiments were monitored until the end of process by weight loss and sugar consumption and were stopped when these parameters were constant for three consecutive days.

Figure 3.22 Mixed fermentation at laboratory scale

3.16.5 Evolution of cell viability during fermentation process

Aliquots of must samples were analysed at different intervals to monitor the evolution of the two species during alcoholic fermentation.

The samples were diluted in saline solution and plated on WL Nutrient Agar (Oxoid, Hampshire, UK), a differential yeast growth medium in which *H. osmophila* (ND1) develops small and intense brilliant green colonies, whereas EC1118 strain grows as cream-green colonies.

The *H. osmophila* population was also analysed by using the selective medium Lysine agar (Oxoid, Hampshire, UK).

During the process, aliquots of must were observed under microscope to verify the integrity of microcapsules structure.

To verify the total number of viable encapsulated cells, microcapsules were disrupted by serial dilution in citrate buffer solution (citric acid 0.2 M, sodium citrate 0.2 M, pH 4.3, Sigma, St. Louis, MO 63304, USA) to dissolve alginate matrix and promote the release of cells. Viable cells count was performed on WL Nutrient Agar at 26°C for 5 days.

3.16.6 Evaluation of analytical parameters and analysis of volatile compounds by gas-chromatography of the experimental wines

The experimental wines were analysed by Wine Scan instrument (FOSS, Hillerød, Denmark) for determination of some chemical parameters, such as residual sugar concentration, ethanol content, total and volatile acidity, malic acid content.

The amounts of principal secondary compounds involved in the wine aroma formation (acetaldehyde, ethyl acetate, acetoin, n-propanol, isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol) were detected by gas-chromatographic analysis, following the procedures previously described (Capece et al., 2013).

The compounds present in lowest concentration (1-hexanol, methionol, isoamyl acetate, 2-phenylethyl acetate, ethyl hexanoate, ethyl lactate, ethyl octanoate and diethyl succinate) were quantified using a gas chromatograph (GC) 7890B with triple quadrupole mass detector (MS/MS) 7000C, following the protocol described by Rubio-Bretón et. al. (2018). This analysis was carried out in collaboration with the University de La Rioja, Istituto de Ciencias de la Vid y del Vino (Logrono, Spain).

Figure 3.23 Experimental wines obtained at laboratory scale

3.16.7 Supplementary analysis of the experimental wines obtained from fermentations performed in 2 L of grape must.

3.16.7.1 Evaluation of the total polyphenol content in experimental wines

The experimental wines were analyzed for the total polyphenol content, by using Folin Ciocalteu colorimetric method. Red wine samples were diluted (1/10), an aliquot of 500 µl of Folin Ciocalteu reagent and 2 mL of Sodium Carbonate (10%) were added to stop the reaction.

The mixture was stored in the dark for 60 minutes and the measurement was performed by reading the absorbance value at 765 nm. Gallic acid at different concentrations (from 100 mg/L to 500 mg/L) was used to obtain the calibration curve. Results were expressed as gallic acid equivalent (mg GAE/l).

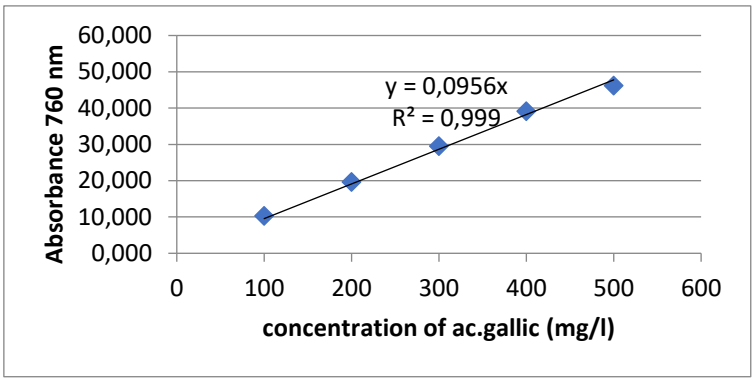


Figure 3.24 Gallic acid calibration line

3.16.7.2 Determination of Antioxidant activity by DPPH (2,2 diphenyl-1-picryhydrazyl) assay

This test determines the radical scavenging activity by electron and hydrogen transfer. Red wine samples were diluted (1:10,1:50;1:100;1:200) with distilled water, and the antioxidant activity of wine samples was evaluated spectrophotometrically using a solution of 0.6mM of 1,1diphenyl-1-picryhydrazyl (DPPH) dissolved in ethanol.

At this purpose, 990 µl of DPPH were added to 10 µl of each sample and stored in the dark for 30 minutes, while a blank sample was prepared using ethanol instead wine. The measure of the absorbance at 517 nm was performing in microplates.

Standard solutions of TROLOX (from 0.5µM to 5 µM) were used to obtain the calibration line and the results were expressed % radical scavenging activity as reported:

$$\% \text{ RSA} = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} * 100$$

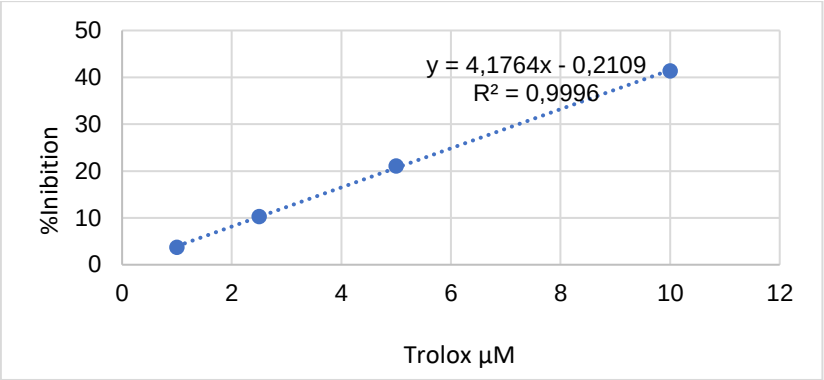


Figure 3.25 DPPH calibration line

The DPPH radical scavenging activity was expressed as Trolox Equivalent Antioxidant Capacity (TEAC Mm).

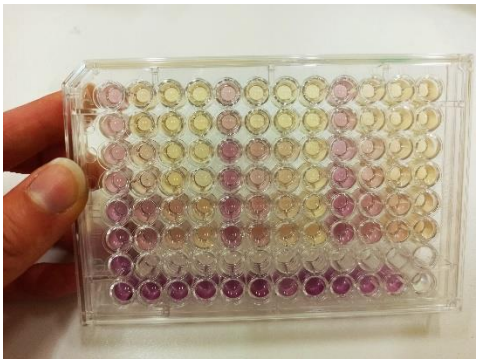


Figure 3.26 Determination of Antioxidant activity by DPPH assay in microplate

3.16.7.3 Analysis of non-phenolic anthocyanin Compounds

The isolation of the non-anthocyanin phenolic compounds was carried out by an extraction on PCX solid-phase extraction (SPE) cartridges (500 mg, 6 mL; Bond Elut Plexa, Agilent, Palo Alto, CA) containing a mixture of reverse-phase and cation-exchanger materials.

Cartridges were placed in the extraction system (Vac Elut 20 station from Varian, Palo Alto, CA).

Wine samples were diluted with 3 mL of 0.1 N HCl, were passed through the PCX SPE cartridges (previously conditioned with 5 mL of methanol and 5 ml of water) and washed with 5 mL of 0.1 N HCl and 5 mL of water. The non-anthocyanin phenolic compound fraction was eluted with 3 × 5 mL of methanol. Adsorbed anthocyanins were removed by passing 2 × 5 mL of 2% ammonia in 80% methanol, then 3 × 5 mL of 2% hydrochloric acid in 80% methanol and finally 5 mL of water.

At this point the non-anthocyanin phenolic fraction was dried in a rotary evaporator (35 °C) and resolved in 1.5 mL of 20% (v/v) aqueous methanol solution. The anthocyanin-free fraction was used to analyze some compounds such as: stilbenes, hydroxycinnamic and hydroxybenzoic acids, flavonols (Portu et al., 2015).

3.16.7.4 Analysis of phenolic compounds

Phenolic compounds were analyzed by reverse-phase HPLC on an Agilent 1260 Infinity chromatograph, equipped with a diode-array detector (DAD) coupled to an Agilent Chem Station.

In the case of anthocyanin analysis, 10 µL of wine sample, previously filtered was injected, while for the analysis of non-anthocyanin phenolic compound fractions, the injection volume was 20 µL.

Identification of phenolic compounds was carried out according to retention times of pure compounds and UV−vis data obtained from authentic standards.

Phenolic compounds were identified according to the DAD chromatograms obtained at 520 nm (anthocyanins), 360 nm (flavonols), 320 nm (hydroxybenzoic and hydroxycinnamic acids and stilbenes), and 280 nm (flavanols) and calibration graphs of the respective standards.

Were used: malvidin 3-o-glucoside for anthocyanins, quercetin 3- o-glucoside for flavonols, trans-caftaric acid for free hydroxycinnamic acids, catechin for procyanidins B1 and B2, epicatechin for epigallocatechin, and trans-piceid and trans-resveratrol for their cis isomers. concentrations in wines were expressed as milligrams per liter of wine (mg/L) (Portu et al., 2015).

3.16.7.5 Influence of acetic acid addition on *S. cerevisiae* EC1118 fermentative performance

The influence of acetic acid on commercial strain EC1118 (C) was evaluated by determining the effect of acetic acid addition on fermentative performance of this strain.

The strain was grown overnight in YPD broth at 26°C with agitation, and a volume of biomass containing about $1x10^6$ cells/mL, detected by measuring optical density at 600nm, was used to inoculate 100 mL of natural red grape must, thermally treated at 90°C for 20 minutes.

Each microfermentation trial was carried out in duplicate under static conditions at 26°C, for about 13 days. At different time intervals (C-0h=at the beginning; C-48= 48h after inoculum and C-72=72h after inoculums) 1.5 g/L di acetic acid (Sigma, St. Louis, MO 63304, USA) was supplemented.

The evolution of the fermentations was evaluated by measuring daily the sugar consumption with a bench-top refractometer. Fermentations were considered to be finished when the °Brix was constant for 2-3 consecutive days. At the end of the process, the acetic acid content in the experimental wines were detected by GC analysis and the residual sugars by OenoFOSS.

3.17 Results (I)

This section reports the results obtained in the mixed fermentations performed in 100 mL of grape must.

3.17.1 Fermentation Kinetic

The consumption of sugars was monitored during the mixed fermentations by comparing the °Brix degree values obtained from the mixed fermentation samples with *H. osmophila* as free (C + NF)

and microencapsulated cells (C + NM) respect to *S. cerevisiae* single fermentation (C). As showed by the figure (3.27), some differences in the metabolism of sugars were observed in the early stages of the process. The samples of mixed fermentations C + NF and C + NM consumed the sugars more slowly than sample C, which completed the fermentation in about ten days. Mixed fermentations show a similar trend until the sixth day of fermentation (T3), after that the C + NM sample showed higher decrease in the °Brix degree compared to the C + NF sample.

At the end of fermentation, the sample with *H. osmophila* strain as free cell showed a very high sugar residue compared to the other samples, on the contrary the sample with *H. osmophila* strain as immobilized form presents a similar sugar residue to "C" fermentation, completing the fermentation.

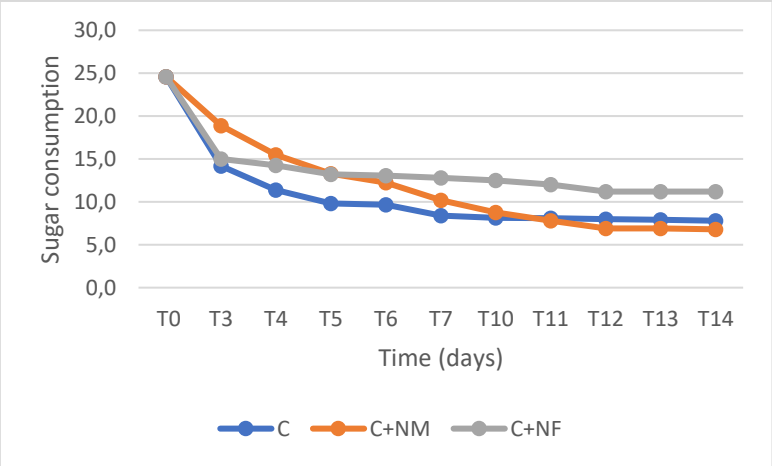


Figure 3.27 Kinetics of sugar consumption (g/L) in the three fermentation conditions. C= control fermentation, inoculated with EC1118; C+NF= mixed starter EC1118+ND1 as free cells; C+NM=mixed starter EC1118+ND1 as microencapsulated cells.

3.17.2 Evaluation of yeasts viable population

The viable cells concentration during fermentation process was evaluated for each strain by isolation at different time intervals.

At T3 (3rd day after inoculum, fig 3.28-a) for both mixed fermentation (C+NM; C+NF) a high number of viable cells for *H. osmophila* was found. The highest number of viable cells was found for *H. osmophila* strain as free cell (C+NF), whereas *S. cerevisiae* presented a higher cell number in sample with microencapsulated *H. osmophila* strain.

At six day of fermentation the number of ND1 viable cells increased in both mixed fermentations samples, while at 10 days decreased, probably as a consequence of increase in ethanol content (Pietrafesa et al., 2020).

In the last isolation, *S. cerevisiae* population remain stable in C+NM sample while decrease in C+NF, *H. osmophila* cells were found only in the sample with ND1 cells as free form.

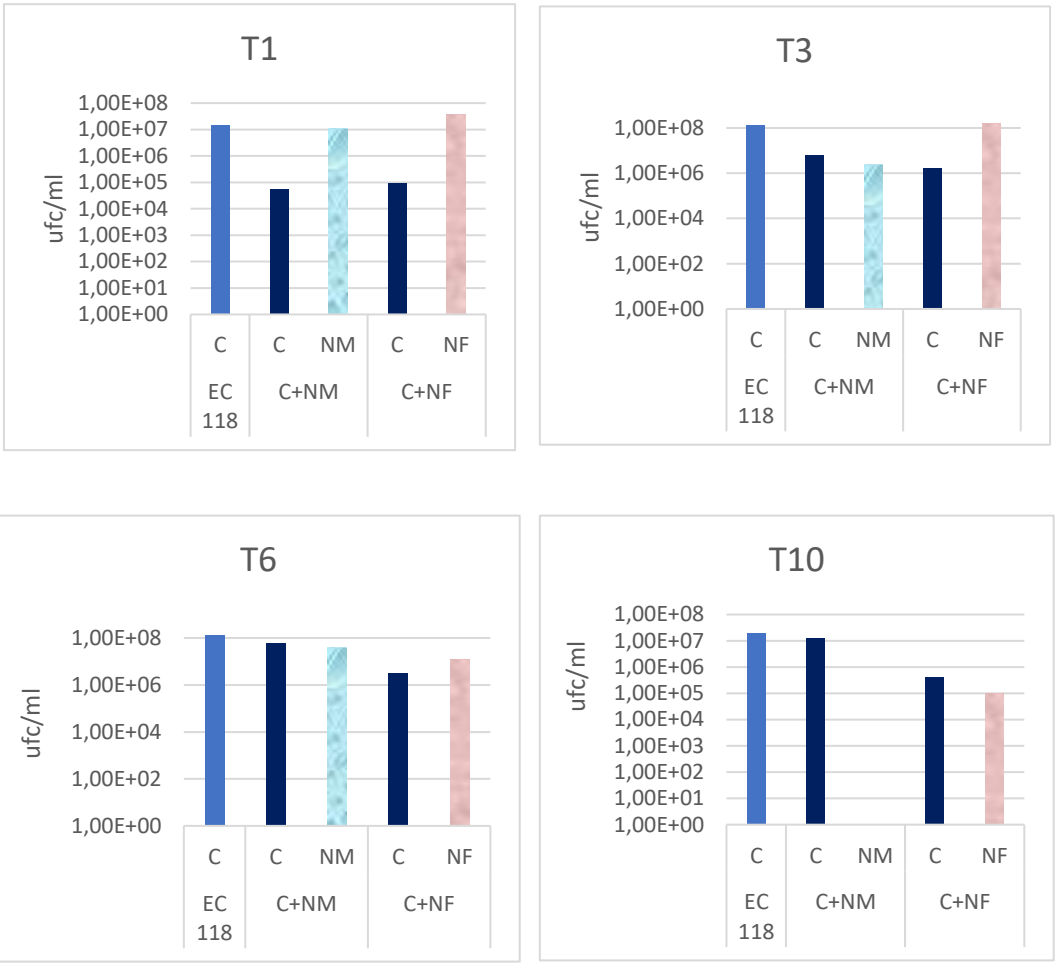


Figure 3.28 Yeast cell count detected at different times (T1=1 days; T3= 3 days; T6= 6 days; T10 = 10 days of fermentations) inoculated with *S. cerevisiae* EC1118 in mixed culture with *H. osmophila* ND1 strain as free (NF) and microencapsulated (NM) cells. Data are the means of triplicate experiments.

3.17.3 Analysis of the main oenological parameters of experimental wines

The wines obtained from mixed fermentations with *H. osmophila* strain as free and microencapsulated cells were analyzed for some main oenological parameters and compared with fermentation with EC1118 single strain. The data showed significant differences for most of the parameters analyzed. As shown in the table 3.5, the results indicate a significant reduction of the ethanol content in both samples with *H. osmophila* strain (C + NM; C + NF) compared to the control fermentation. However, as regards the consumption of sugars at the end of the fermentation process, the values obtained for the C + NM sample are similar to those obtained in the control fermentation, on the contrary the glucose and fructose residue in the C+NF sample is significantly higher than for the other fermentations.

Table 3.5 Main oenological parameters analyzed in wines obtained by the mixed starters with *S. cerevisiae* EC1118 and *H. osmophila* (ND1) as free (C+NF) and microencapsulated (C+NM) cells in comparison to single starter wine with EC1118 (C). Data are expressed as mean value ±SD of three independent experiments.

Parameters	C	C+NM	C+NF
Ethanol	13.63[a]± 0.15	12.12[b]± 0,37	11.01[c]±0.01
Residual sugar	1.09[a] ± 0.15	0.66[b]± 0.03	20.0[c]±0.00
Glucose	0.45[a]±0.21	0.75[a]± 0.07	10.00[b]±0.00
Fructose	0.00[a]±0.00	0.00[a]±0.00	6.40[b]±0.71
Total acidity	7.96[a]± 0.04	5.82[b]± 0.07	8.35[c]± 0.11
Malic acid	1.06[a] ± 0.06	0.35[b]± 0.01	0.92[a]± 0.17
Lactic acid	0.60[a] ±0.00	0.00[b]±0.00	0.65[a]±0.07

The wine obtained with the mixed starter as microencapsulated cells has a significantly lower residue of malic and acid lactic content compared to the other wines.

Some studies reported a moderate acid malic metabolism by non-*Saccharomyces* yeasts, such us *H. uvarum*; *T. delbrueckii*, *M. pulcherrima* and *Schyzosaccharomyces pombe* (Du Plessis et al., 2017; Benito et al., 2019; Ruiz et al., 2018).

3.17.4 Analysis of volatile compounds

The experimental wines were analyzed for the content of the main secondary compounds (Table 3.6). Statistical differences between control fermentation and wines obtained from mixed fermentations were highlighted for most of the compounds analyzed by gas chromatography. As expected, higher values of acetaldehyde and ethyl acetate were found in the samples with NF and NM compared to sample C. As regards the content in higher alcohols, the samples of fermentation with *H. osmophila* (NF and NM) showed a lower content of these metabolites.

Table 3.6 Main volatile compounds content (mg/L) detected in wines obtained by the mixed starters with *S. cerevisiae* EC1118 (C) and *H. osmophila* ND1 in two different formulations (NF= free cells; NM= microencapsulated cells) in comparison to single starter (C). Data are expressed as mean value ±SD of three independent experiments.

Volatile Compounds	C	C+NM	C+NF
Acetaldehyde	38.90± 1.46[a]	45.90± 1.26[b]	48.04±1.02[b]
Ethyl acetate	7.73± 0.54a	13.89± 0.54b	16.02±0.91b
n-propanol	11.76±0.18	12.78± 0.79	12.76± 0.66
Isobutanol	25.41± 1.18	21.60± 1.99	22.14± 1.66
m-butanol	11.92±0.03	9.61±0.10	8.89± 1.70
Acetoin	6.71±0.02	7.44±6.37	4.76±0.80
Acetic acid	329.08± 75.64	454.03±72.69	402.63±34.51
D-Amylic Alcohol	61.01±5.86[a]	37.08±1.13[b]	37.02±1.22[b]
Isoamylic Alcohols	188.56±7.28[a]	115.95±0.08[b]	118.28±4.51[b]

As shown in the figure the volatile acidity content, measured as acetic acid (mg/L), in wines from mixed fermentations was higher than in single fermentation wine, however the use of *H. osmophila*

as free cells allowed to obtain a lower level of acidity (Figure 3.29); however, all the detected values were included in the usual level for wine the samples.

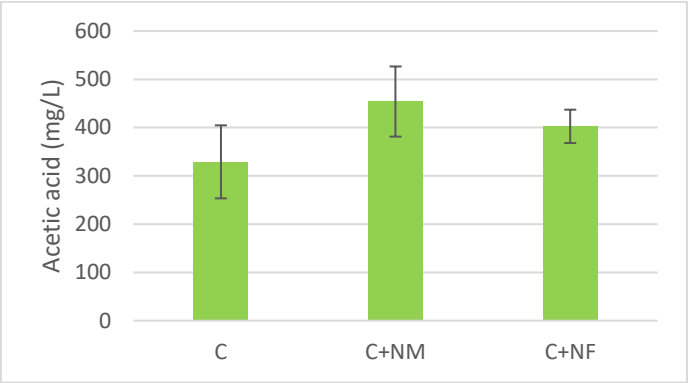


Figure 3.29 Acetic acid content in wines obtained by the mixed starters with *S. cerevisiae* EC1118 and *H. osmophila* (ND1) as free (C+NF) and microencapsulated (C+NM) cells in comparison to single starter wine with EC1118 (C). Data are expressed as mean value ±SD of three independent experiments.

The principal component analysis was applied for all the data determined in experimental wine samples. The first principal component (PC1) explained 69.3 % of variability, and it was principally correlated with acetaldehyde, isobutanol, n-butanol, D-amylic alcohol and isoamyl alcohol, while the second component (PC2) explained only 30.6 % of data variability and it was correlated with and ethanol, malic acid, acetoin and residual sugars.

The scatter plot (Figure 3.30) highlight that the wines were divided in function of starter formulation in different quadrants and the two experimental wines obtained with *H. osmophila* as free and microencapsulated cells were far from wine obtained with single starter fermentation (C).

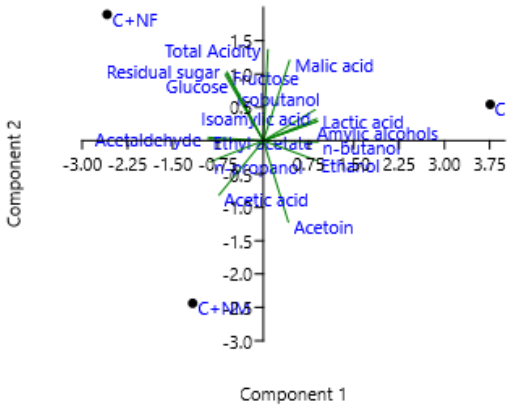


Figure 3.30 Principal component analysis (PCA) biplot of volatile compounds and main oenological parameters determined in wines obtained by the mixed starters with *S. cerevisiae* EC1118 (C) and *H. osmophila* ND1 in two different formulations (C+NF, C+NM) in comparison to monoculture wine with EC1118 (C)

3.17.5 Conclusion

The results obtained in this step indicated the promising use of the selected *H. osmophila* strain in mixed fermentation and the influence of non-*Saccharomyces* strain formulation on fermentative behaviour.

Particularly interesting were the values obtained for mixed fermentation with *H. osmophila* as microencapsulated form (C+NM). In fact, the use of *H. osmophila* in immobilized form resulted in reduction of ethanol.

Also, the sample with *H. osmophila* as free cells determined an ethanol reduction, but in this case the sugar residue at the end of fermentation is significant higher respect to the values of two other starter formulations.

In order to confirm the promising results obtained by the use of *H. osmophila* as microencapsulated form, the same starter formulations were tested in subsequent research phase by using a higher volume of fermentation medium.

3.18 Results (II)

The results of obtained in mixed fermentation performed in 2 L of grape must were published in the Article: “Influence of Microencapsulation on Fermentative Behavior of *Hanseniaspora osmophila* in Wine Mixed Starter Fermentation” reported below.

fermentation

MDPI

Article

Influence of Microencapsulation on Fermentative Behavior of *Hanseniaspora osmophila* in Wine Mixed Starter Fermentation

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Abstract: In recent years, as a consequence of the re-evaluation of the role of non-*Saccharomyces* yeasts, several studies have been conducted on the use of controlled mixed fermentations with *Saccharomyces* and different non-*Saccharomyces* yeast species from the winemaking environment. To benefit from the metabolic particularities of some non-*Saccharomyces* yeasts, the management of a non-*Saccharomyces* strain in mixed fermentation is a crucial step, in particular the use of procedures addressed to increase the persistence of non-*Saccharomyces* strains during the fermentative process. The use of microencapsulation for cell immobilization might represent a strategy for enhancing the competitiveness of non-*Saccharomyces* yeasts during mixed fermentation. This study was aimed to assess the fermentative performance of a mixed starter culture, composed by a wild *Hanseniaspora osmophila* strain (ND1) and a commercial *Saccharomyces cerevisiae* strain (EC1118). For this purpose, free and microencapsulated cells of ND1 strain were tested in co-culture with EC1118 during mixed fermentations in order to evaluate the effect of the microencapsulation on fermentative behavior of mixed starter and final wine composition. The data have shown that *H. osmophila* cell formulation affects the persistence of both ND1 and EC1118 strains during fermentations and microencapsulation resulted in a suitable system to increase the fermentative efficiency of ND1 strain during mixed starter fermentation.

Keywords: non-*Saccharomyces* yeasts; mixed starter cultures; *Hanseniaspora osmophila*; *Saccharomyces cerevisiae*; microencapsulated cells

Citation: Alberico, G.; Capece, A.; Mauriello, G.; Pietrafesa, R.; Siesto, G.; Garde-Cerdán, T.; Maresca, D.; Romano, R.; Romano, P. Influence of Microencapsulation on Fermentative Behavior of *Hanseniaspora osmophila* in Wine Mixed Starter Fermentation. *Fermentation* **2021**, *7*, 112. https://doi.org/10.3390/fermentation7030112

Academic Editors: Francesco Grieco, Vittorio Capozzi and Carmen Berbegal

Received: 22 June 2021
Accepted: 9 July 2021
Published: 13 July 2021

1. Introduction

Although *Saccharomyces cerevisiae* represents the main microorganism involved in the alcoholic fermentation of grape must, many other species of yeasts belonging to various non-*Saccharomyces* genera occur in grape juice and contribute to the fermentation and to the organoleptic characteristics of the final wine [1]. Although in the past these yeasts were considered as undesirable agents, in recent years the role of non-*Saccharomyces* yeasts has been revalued and several studies reported the use of controlled mixed fermentations with different indigenous non-*Saccharomyces* yeast species and *Saccharomyces* [2–4].

The use of non-*Saccharomyces* selected yeasts for winemaking is becoming a promising tool to improve the complexity and enhance specific characteristics of wine, satisfying winemakers' requests for product differentiation. Therefore, 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 [5,6]. In fact, non-*Saccharomyces* yeasts, which are naturally present in un-inoculated spontaneous fermentations, can contribute to improving the final wine flavor due to their ability to produce favorable metabolites and the activity of certain enzymes that interact with the precursors of aromatic compounds [7,8], such as glycerol, 2-3 butanediol, polyols, alcohols, mannoproteins and esters [1,9]. A further application of non-*Saccharomyces* yeasts relies on the use of these yeasts for producing wine with reduced alcohol content [10,11]. In recent decades, climate changes have determined the production of grapes with high sugar content and consequently an increase of the alcohol content in wine, representing a healthy and economic problem [12]. A promising strategy for producing wine with reduced alcohol content, as alternative approaches focused on vineyard management and winemaking practices [13,14], take advantage of non-*Saccharomyces* wine yeasts, which metabolize sugar with low efficiency in ethanol conversion [15]. Different studies reported a reduction in ethanol concentration using different non-*Saccharomyces* species in co-cultures or in sequential inoculation with *S. cerevisiae* wine strain [16,17]. The various metabolic traits typical of the different non-*Saccharomyces* species, such as ethanol yield, fermentation efficiency, biomass production, by-product formation and respiro-fermentative metabolism, can be exploited to reduce the ethanol concentration in wine.

To benefit from the metabolic particularities of some non-*Saccharomyces* yeasts in mixed fermentation, the management of non-*Saccharomyces* strains in mixed fermentation is a crucial step. In particular, the use of a procedure aimed to increase the persistence of non-*Saccharomyces* strains during the fermentative process. One of the strategies proposed to increase the competitiveness of non-*Saccharomyces* yeasts during mixed fermentation is cell immobilization, which was also reported as an instrument to increase the expression of some metabolic activities [18,19]. Various techniques have been investigated for microbial cell immobilization, such as adsorption or attachment of cells to an inert substrate, self-aggregation by flocculation and entrapment or encapsulation using polymers [20,21]. In the microencapsulation technique, the polymeric matrix isolates the cells from the external environment and protects the microorganism from the principal stress agent, it is permeable to low weight molecular nutrient, metabolites and oxygen and maintains a hospitable internal condition. This structure protects the microbial cells from environmental stress, preserves some metabolic activities, enhances the production of secondary compounds and removes some odors [22,23].

In this study, the fermentative performance of a mixed starter culture, composed by a selected *Hanseniaspora osmophila* strain and a commercial *S. cerevisiae* strain was evaluated. The non-*Saccharomyces* strain was tested as free and microencapsulated cells, in order to evaluate the effect of the microencapsulation on the fermentative behavior of the mixed starter and chemical characteristics of the final wines.

2. Materials and Methods

2.1. Yeast Strains

The yeast strains tested in this study were *H. osmophila* ND1, belonging to the Yeast Collection of University of Basilicata (UBYC), and the commercial strain, *S. cerevisiae* EC1118, purchased from Lallemand Inc. (Toulose, France). The presumptive identification of the ND1 strain was performed by PCR amplification of the internal transcribed spacers between the 18S and 26S rDNA genes (ITS1-5.8S-ITS2) and subsequent restriction analysis, by following the protocol reported by Esteve-Zarzoso et al. [24]. The identification as *H. osmophila* was confirmed by sequencing of the ITS region, performed at Eurofins Genomics Srl (Vimodrone, Italy). The sequences were compared with those deposited in the GenBank database (National Center for Biotechnology Information, Bethesda, Maryland, USA) using the Basic Local Alignment Search Tool (BLAST).

The ND1 strain was selected on the basis of some desirable oenological criteria, such as the ability to tolerate high concentration of sugar and ethanol, very high β-glucosidase activity and good resistance to osmotic stress [25].

The yeast strains were stored at −20 °C in YPD broth (1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone; 2% (*w*/*v*) glucose; Oxoid, Hampshire, UK) supplemented with 50% glycerol (Sigma, St. Louis, MO 63304, USA) as protective agent, until further analysis.

2.2. Biomass Production

S. cerevisiae (C) and *H. osmophila* (ND1) strains were refreshed on YPD plates and incubated at 26 °C for 24 h. A loopful of 24 h culture of each strain was inoculated in 300 mL of YPD broth and incubated at 26 °C for 24 h in a rotary shaker at 180 rpm. The biomass of each strain was produced by the BioFlo/CelliGen 110 bioreactor (Eppendorf, Hamburg, Germany) in a vessel containing 2 L of YPD liquid. The growth parameters used were: controlled temperature at 26 °C; stirring at 200 rpm; oxygen at 2 vvm. After overnight growth in the bioreactor, the yeast biomass was collected and centrifuged at 4700 rpm for 10 min. The recovered biomass was stored for limited times at 4 °C until use. For the ND1 strain, the biomass obtained was divided in two parts, one was used for the production of microencapsulated cells, whereas the other was used as inoculum for mixed fermentation.

2.3. Microencapsulation of ND1 Strain

The microencapsulation of ND1 strain was performed by using the Encapsulator B-395 Pro (BÜCHI, Flawil, Switzerland). A scheme of the BÜCHI Encapsulator is reported in De Prisco et al. [26]. Overnight yeast culture was centrifuged (4700 rpm for 10 min at 4 °C) and the solution to be encapsulated was prepared by suspending the cell pellet in 500 mL of 15 g/L alginic acid sodium salt (Sigma, Milan, Italy). The feeding solution was loaded in the syringe, forced into the pulsation chamber and finally extruded through the nozzle (80 µ) [27]. The microencapsulation conditions used were: flow rate 3.5 mL/min, vibration frequency 2500 Hz and electrode voltage of 950 V. Droplets containing yeast cells were hardened in 500 mL of a sterile 0.5 mol/L $CaCl_2$ solution, continuously stirred at 300 rpm. After sedimentation (30 min at room temperature) and discarding of hardening solution, microcapsules were stored at 4 °C for further experiments.

2.4. Efficiency of Microencapsulation Process

According to De Prisco et al. [26] the microencapsulation efficiency was calculated by dividing the viable count of disrupted microcapsules by the cell load of the feeding solution. Microcapsules were disrupted by serially dilution in 0.5 mol/L citrate buffer solution pH 7.0 and viable counting of the yeast cells was performed on YPD Agar (YPD medium with addition of 7.5 g/L agar) at 26 °C for 24 h.

2.5. Mixed Inoculated Fermentations at Laboratory Scale with ND1 Strain in Different Formulations

The *H. osmophila* ND1 strain was tested in mixed fermentations with commercial *S. cerevisiae* strain EC1118. The *H. osmophila* strain was used in free (NF) and microencapsulated (NM) form, whereas the *S. cerevisiae* strain was used onlyin free form. Fermentation with free cells of *S. cerevisiae*(C) was used as control.

The fermentations were performed in natural grape must, Aglianico del Vulture, a red grape variety cultivated in Basilicata (Southern Italy). The main analytical parameters of the grape juice were the following: total acidity 5.37 g/L; pH 3.6; °TSS 22.2; density, 1.097 g/L; yeast assimilable nitrogen 211.9 mg N/L and malic acid 1.66 g/L.

The fermentations at laboratory scale were conducted in triplicate at 26 °C in 2 L of pasteurized (T = 90 °C; t = 20 min) grape must; the absence of viable cells in grape must before inoculation was checked by plate counting on Wallerstein Laboratory Nutrient Agar medium (WL, Oxoid, Hampshire, UK). The two yeast strains were simultaneously inoculated in the grape must, by using different inoculation ratio. In each mixed fermentation, *S. cerevisiae* strain was inoculated as free cells at concentration of 2×10^3 cell/mL, whereas 2×10^7 cells/mL of *H. osmophila* were inoculated as free and microencapsulated cells. In the control fermentation, *S. cerevisiae* strain was inoculated at 2×10^7 cells/mL.

All the experiments were monitored until the end of fermentation by determination of weight loss due to CO_2 production and sugar consumption. The fermentation process was stopped when weight and °Brix reductions were constant for three consecutive days.

2.6. Evaluation of Yeast Viable Population during the Fermentations

At different time intervals, a fermenting must aliquot was taken from each sample to analyze the evolution of the two inoculated species during the mixed fermentations by plate counting. The sample was diluted in saline solution, plated on WL Nutrient Agar (Oxoid, Hampshire, UK), a differential yeast growth medium in which *H. osmophila* (ND1) develops small and intense brilliant green colonies, whereas EC1118 strain grows as cream-green colonies. Furthermore, the *H. osmophila* population was also analyzed by using LA (Lysine agar, Oxoid, Hampshire, UK) medium, a substrate inhibiting *S. cerevisiae* growth.

In order to verify the integrity of microcapsules, aliquots of must during the fermentation were periodically sampled and observed under microscope. Furthermore, the total number of viable encapsulated cells during the fermentation process was also determined, by diluting the must sample 1:10 in citrate buffer solution (citric acid 0.2 M, sodium citrate 0.2 M, pH 4.3, Sigma, St. Louis, MO 63304, USA) to dissolve the alginate matrix and promote cell release.

After incubation at 26 °C for 5 d, the dilution plates statistically representative were counted; from each fermentation sample, around 30 colonies (showing the two different morphologies of the two inoculated strains) were randomly selected and purified on YPD for yeast identification, by restriction analysis of amplified ITS region [24].

2.7. Analytical Determination of Experimental Wines

The experimental wines obtained from fermentations were analyzed by a Fourier transfer infrared WineScan instrument (FOSS, Hillerød, Denmark) for determination of conventional chemical parameters, such as residual sugar concentration, ethanol content, total and volatile acidities and malic acid. Furthermore, the content of the main secondary compounds affecting wine aroma were analyzed by direct injection of 1 µL of sample into a glass column packed with 80/120 Carbopak B/5% Carbowax 20 M (Supelco, Sigma-Aldrich, Milano, Italy) by an Agilent 7890A gas-chromatograph, equipped with an integrated flame ionization detector (GC-FID), following the procedures previously described [28]. The secondary compounds detected were acetaldehyde, ethyl acetate, acetoin, *n*-propanol, isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol. Other volatile compounds, usually present in lower concentrations, such as 1-hexanol, methionol, isoamyl acetate, 2-phenylethyl acetate, ethyl hexanoate, ethyl lactate, ethyl octanoate and diethyl succinate, were identified by gas chromatographic analysis performed using a gas chromatograph (GC) 7890B with triple quadrupole mass detector (MS/MS) 7000C, following the protocol described by Rubio-Bretón et al. [29].

2.8. Influence of Acetic Acid Addition on S. cerevisiae EC1118 Fermentative Performance

The influence of acetic acid on commercial strain EC1118 was evaluated by determining the effect of acetic acid addition on fermentative performance of this strain.

The strain was grown overnight in YPD broth at 26 °C with agitation, and an aliquot of biomass containing about 1×10^6 cells/mL, detected by measuring optical density at 600 nm, was used to inoculate 100 mL of Aglianico del Vulture grape must, thermally treated at 90 °C for 20 min.

Each microfermentation trial was carried out in duplicate under static conditions at 26 °C, for about 13 d. At different time intervals (C-0 h = at the beginning; C-48 = 48 h after inoculum and C-72 = 72 h after inoculum) 1.5 g/L of acetic acid (Sigma, St. Louis, MO 63304, USA) was supplemented.

The evolution of the fermentations was evaluated by measuring the sugar consumption daily with a bench-top refractometer. Fermentations were considered to be finished when the °Brix was constant for 2–3 consecutive days. At the end of the process, the

experimental wines were analyzed for residual sugars by OenoFOSS and the acetic acid content by GC analysis, as described in Section 2.7.

2.9. Data Analysis

The experimental data were analyzed by one-way ANOVA and the homogeneity of variance was verified applying the Levene's test.

In order to compare the main oenological parameters and the volatile compounds content in wines obtained by the mixed starters with *S. cerevisiae* EC1118 (C) and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells and single starter wine with EC1118 (C), the Tukey's test was used. The differences were considered significant when the associated *p*-value was ≤ 0.05. Principal component analysis (PCA) was performed on all the parameters detected in the experimental wines.

Data regarding the volatile compounds of the experimental wines were analyzed by a heatmap, a method used to reduce the dimensionality of the data. The amount of each analyzed compound was converted to Z-scores, to easily visualize which starter is relevant aroma producers in relation to average production, which was calculated as follows: Z-score = $(X - \mu)/\sigma$, where, for each parameter, X is the concentration, μ is the mean value and σ is the standard deviation among all the starters.

Paleontological Statistics Software (PAST) ver. 3.26 (Natural History Museum– University of Oslo, Norway) [30] was used for all statistical analyses.

3. Results

In order to evaluate the feasibility of using the *H. osmophila* ND1 strain as mixed starter culture for wine fermentation, we evaluated the fermentative behavior of this strain as free and microencapsulated cells (Figure 1) in co-culture with the commercial *S. cerevisiae* strain EC1118.

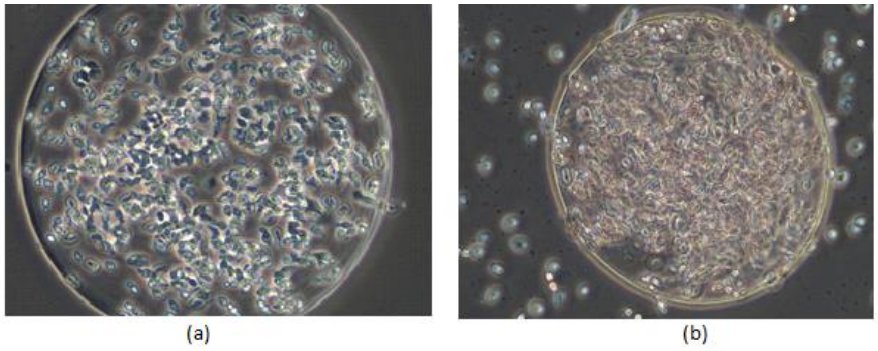

Figure 1. Optical microscopy images at 400× magnification of ND1 strain after microencapsulation treatment in alginate (**a**) and during the fermentation process (**b**).

3.1. Efficiency of Microencapsulation Process

Results of cell counting on alginate cell suspension (8.2×10^8 CFU/g) and disrupted microcapsules (7.7×10^7 CFU/g) showed an encapsulation efficiency of 94%.

3.2. Fermentation Kinetics

The sugar depletion during the mixed fermentations, using *H.osmophila* strain as free and microencapsulated cells (NF and NM, respectively), in comparison to EC1118 single fermentation (C) is shown in Figure 2. High differences in sugar consumption between the control and the mixed starter cultures were found from the first step of the process till the end of fermentation and the fermentation dynamic was faster for the control fermentation, as expected, which consumed the majority of sugars within seven days. As regards the mixed fermentations, in the first days of fermentation, the sugar consumption was very similar among them, whereas after T4 the sugar depletion in C + NM was faster than in the mixed fermentation with free cells of ND strain. At the end of the process, the residual

sugars detected in NM mixed fermentation was similar to the sugar content detected in the control fermentation, whereas the mixed fermentation including ND1 strain as free cells (C + NF) contained the highest residual sugar.

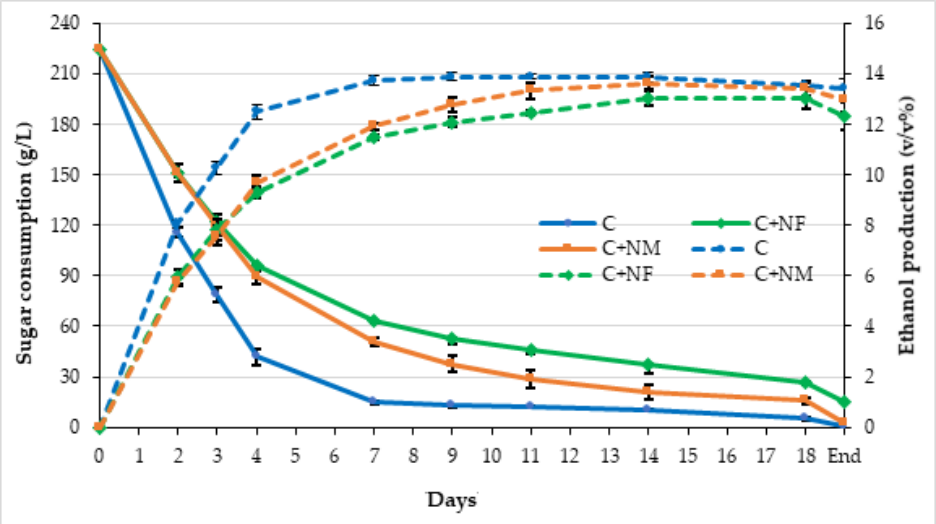

Figure 2. Kinetics of sugar consumption (solid lines) and ethanol production (dashed lines) in the three fermentation conditions. C—control fermentation, inoculated with EC1118; C + NF—mixed starter EC1118 + ND1 as free cells; C + NM—mixed starter EC1118 + ND1 as microencapsulated cells; End—last fermentation day, variable for each starter. Data are the means of triplicate experiments ± standard deviation.

As expected, the kinetics of ethanol increase during the time reflects the same trend of sugar consumption (Figure 2). The control fermentation yielded the highest final content of ethanol and also the ethanol production during the time was faster than the ethanol increase observed in the mixed fermentations. The slowest rate of ethanol production over the time was observed in the process driven by ND1 as free cells (C + NF), such as the lowest final content of ethanol, corresponding to the highest residual sugar.

3.3. Evaluation of Yeast Viable Population during the Fermentations

The concentration of viable cells during micro fermentations for each yeast population was monitored by yeast isolation at different steps of mixed and control fermentations (Figure 3A–F).

At T2 (2nd day after inoculum, Figure 3A), when the sugar content was reduced by about 50% for the control fermentation and 35% for both the mixed starters (Figure 2), the highest number of viable cells was found for the *H. osmophila* strain as microencapsulated cells (C + NM mixed fermentation), whereas the number of viable cells for ND1 in free form (NF) was very similar to *S. cerevisiae* viable cells, present in the control fermentation (C). Concerning *S. cerevisiae* cells in mixed fermentation, the number of viable cells was significantly higher in C + NM mixed fermentation than in the C + NF trial. However, at this time, in all the fermentations an increase of cell number was observed with respect to inoculation level, mainly for *S. cerevisiae* cells in both the mixed fermentations (6.9×10^6 and 1.57×10^6 CFU/mL in C + NM and C + NF, respectively).

After three days of fermentation (Figure 3B), the number of *S. cerevisiae* viable cells remains similar to the first isolation for the control fermentation (C). In mixed fermentations, the number of ND1 viable cells was similar to the levels found after 48 h both in C + NM (1.12×10^8 CFU/mL) and C + NF (4.43×10^7 CFU/mL) trials, whereas the number of *S. cerevisiae* cells increased in both the mixed fermentations. However, the number of EC1118 cells was significantly higher in the fermentation inoculated with ND1 microencapsulated cells (9.18×10^6 CFU/mL) than in the trial inoculated with free cells of

H. osmophila (3×10^6 CFU/mL). A similar trend was also observed after 4 fermentation days (Figure 3C).

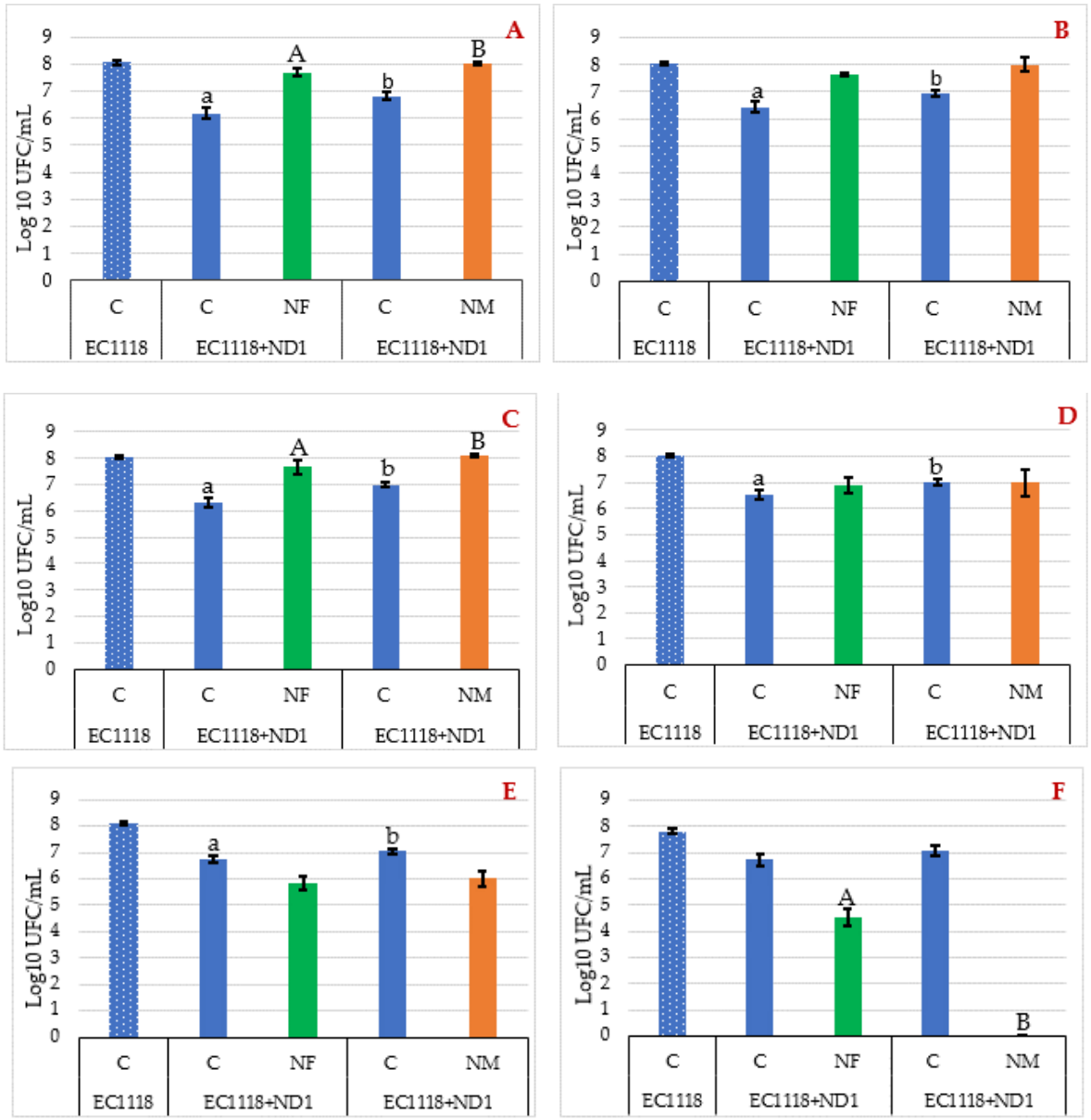

Figure 3. Yeast cell count detected at different times: (**A**) 2 days; (**B**) 3 days; (**C**) 4 days; (**D**) 7 days; (**E**) 9 days; (**F**) 11 days of fermentations inoculated with *S. cerevisiae* EC1118 in mixed culture with *H. osmophila* ND1 strain as free (NF) and microencapsulated (NM) cells. As control, pure fermentation with EC1118 was used (C). Data are the means of triplicate experiments ± standard deviation. Letters on plot bars indicate significant differences ($p < 0.05$) in cell count within EC1118 (**a**, **b**) and ND1 (**A**, **B**) in the two mixed starter fermentations.

After 7 days of fermentation (Figure 3D), the cell number of the ND1 strain decreased in both the mixed fermentations (1.37×10^7 and 8.67×10^6 CFU/mL for C + NM and C + NF, respectively), probably as a consequence of the increase in ethanol concentration, which at this time reached values of about 9% *v*/*v* in the two mixed fermentations (Figure 2). The *S. cerevisiae* population remained stable for C + NM, whereas it increases for C + NF. In this step, the cell populations of *S. cerevisiae* and *H. osmophila* were very similar in both C + NM and C + NF mixed fermentations. A similar trend was also observed after 9 days of fermentation (Figure 3E), with a reduction of *H. osmophila* cells for both the mixed fermentations and the dominance of *S. cerevisiae* strain.

In the last isolation steps, *H. osmophila* cells were found only at the eleventh day of fermentation in the trial inoculated with ND1 cells in free form (Figure 3F), whereas in the mixed fermentation with ND1 microencapsulated cells only *S. cerevisiae* cells were found. In C + NF fermentation, *H. osmophila* cells were detected until the 14th day of the process, after that only *S. cerevisiae* cells were found. At the end of fermentations, the number of *S. cerevisiae* cells isolated from C + NF (4.23×10^6 CFU/mL) was lower than *S. cerevisiae* cells isolated from C + NM (8.28×10^6) and from the control fermentation (6.8×10^7). This could be related to the highest residual sugar found at the end of C + NF fermentation (about 15 g/L, Figure 2).

3.4. Analysis of Experimental Wines for Main Oenological Parameters

The wines obtained by the mixed starters with EC1118 and *H. osmophila* ND1 as free (NF) and microencapsulated cells (NM), in comparison to the single starter wine (C), were analyzed for parameters of oenological interest, such as ethanol, volatile acidity, residual sugar and sugars, and malic acid, which are presented in Table 1. The data highlighted that statistically significant differences were found for almost all the parameters, with the exception of ethanol. About the alcohol concentration obtained at the end of the fermentation, the values ranged between 12.32 ± 0.55 and 13.45 ± 0.35 (v/v%), for C + NF and C, respectively. Although the differences were not statistically significant, these results indicated a reduction of ethanol content in wines obtained by the mixed starters in comparison to the single starter fermentation. However, the wine from the C + NF mixed starter contained too high residual sugars (15.16 ± 1.00 g/L), in particular, the wine obtained by using this starter showed the highest glucose and fructose content (11.30 ± 1.97 and 2.77 ± 0.90 g/L), with values significantly different from both the wines obtained by single starter inoculum and microencapsulated cells of ND1.

Table 1. Main oenological parameters analyzed in wines obtained by the mixed starters with *S. cerevisiae* EC1118 (C) and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells in comparison to the single starter wine with EC1118 (C). The ethanol was expressed as v/v%, while the other parameters were expressed as g/L. For each compound, different superscript letters mean significant differences at $p < 0.05$ (*) and 0.001 (**) among the wines produced in the different conditions. n.s.: not significant. Data are expressed as mean value ± SD of three independent experiments.

Parameters.	C	C + NF	C + NM	*Sign.*
Ethanol	13.45 ± 0.35	12.32 ± 0.55	12.98 ± 0.50	n.s.
Residual sugar	0.95 ± 0.03^{a}	15.16 ± 1.00^{b}	2.51 ± 0.46^{a}	**
Glucose	0.33 ± 0.15^{a}	11.30 ± 1.97^{b}	1.60 ± 0.10^{a}	**
Fructose	0.51 ± 0.01^{a}	2.77 ± 0.90^{b}	1.43 ± 0.15^{a}	*
Volatile acidity	0.40 ± 0.09^{a}	1.56 ± 0.20^{b}	1.05 ± 0.09^{c}	**
Malic acid	1.60 ± 0.09^{a}	1.29 ± 0.07^{b}	1.30 ± 0.04^{b}	*

The volatile acidity was included in the acceptable level (1.2 g/L) [31], except for the wine obtained by the C + NF starter, in which the detected level was 1.56 ± 0.20 g/L (Table 1). Furthermore, both the wines obtained with the mixed starters exhibited a level of volatile acidity statistically different from that of the single starter wine, but also different among them, with the highest level in wine from free ND1 cells (NF).

Finally, significantly lower values of malic acid were detected in samples fermented with the starters containing ND1 as free and microencapsulated cells (1.29 ± 0.07 and 1.30 ± 0.04 g/L, respectively) in comparison to the single starter wine (1.60 ± 0.09 g/L).

3.5. Aromatic Compounds of Experimental Wines

The experimental wines were also analyzed for the content of some secondary compounds affecting wine aroma, reported in Table 2.

Table 2. Main volatile compounds content (mg/L) detected in wines obtained by the mixed starters with *S. cerevisiae* EC1118 (C) and *H. osmophila* ND1 in two different formulations (NF—free cells; NM—microencapsulated cells) in comparison with the single starter wine with EC1118 (C). For each compound, different superscript letters mean significant differences (Tukey's test, $p \leq 0.05$) among wines produced in the different conditions. Data are expressed as mean value ± SD of three independent experiments. * compounds identified by GC/MS.

Volatile Compounds (mg/L)	C	C + NF	C + NM
Acetaldehyde	32.59 ± 2.82 ^{a}	45.85 ± 3.88 ^{b}	41.80 ± 2.56 ^{b}
Acetoin	3.16 ± 0.52 ^{a}	58.22 ± 8.67 ^{b}	8.32 ± 1.28 ^{a}
n-Propanol	11.91 ± 1.00 ^{a}	19.44 ± 1.59 ^{b}	19.06 ± 1.23 ^{b}
Isobutanol	33.68 ± 1.43	36.07 ± 2.44	27.97 ± 2.28
n-Butanol	8.75 ± 0.78 ^{a}	56.43 ± 2.52 ^{c}	13.93 ± 1.97 ^{b}
2-Methyl-1-butanol	71.13 ± 9.68 ^{a}	47.31 ± 3.01 ^{b}	45.31 ± 3.74 ^{b}
3-Methyl-1-butanol	235.26 ± 10.26 ^{a}	171.76 ± 9.66 ^{b}	150.32 ± 12.25 ^{b}
2-Phenylethanol	62.54 ± 4.53 ^{a}	46.05 ± 1.81 ^{b}	39.32 ± 1.84 ^{b}
1-Hexanol *	0.22 ± 0.02 ^{a}	0.26 ± 0.01 ^{ab}	0.28 ± 0.03 ^{b}
Methionol *	0.17 ± 0.01 ^{a}	0.04 ± 0.01 ^{b}	0.05 ± 0.01 ^{b}
Ethyl acetate	29.44 ± 2.84 ^{a}	89.58 ± 7.88 ^{b}	87.94 ± 7.79 ^{b}
Isoamyl acetate *	1.45 ± 0.20 ^{a}	0.63 ± 0.06 ^{b}	0.51 ± 0.06 ^{b}
2-Phenylethyl acetate *	1.20 ± 0.14 ^{a}	40.90 ± 0.87 ^{b}	30.57 ± 2.42 ^{c}
Ethyl hexanoate *	0.16 ± 0.03 ^{a}	0.05 ± 0.01 ^{b}	0.06 ± 0.01 ^{b}
Ethyl lactate *	0.18 ± 0.02 ^{a}	0.08 ± 0.01 ^{b}	0.17 ± 0.01 ^{a}
Ethyl octanoate *	0.09 ± 0.01 ^{a}	0.01 ± 0.01 ^{b}	0.01 ± 0.01 ^{b}
Diethyl succinate *	0.20 ± 0.03 ^{a}	0.05 ± 0.01 ^{b}	0.11 ± 0.01 ^{c}

Statistically significant differences were found between the control and the wines from mixed starters for almost all the compounds, with the exception of isobutanol. Conversely, for the majority of detected aromatic compounds no differences were recorded between the wines fermented with free (NF) and microencapsulated (NM) ND1 cells.

The acetaldehyde content was significantly higher in wines from the mixed starters than in the control wine, although the detected values were included in the usual level for all the samples (10–75 mg/L) [32].

The sample fermented with free ND1 cells contained a much higher level of acetoin (60 mg/L) than the other two experimental wines, in which the values were below 10 mg/L. The highest levels of higher alcohols were found in the wine fermented with single starter, with the exception of *n*-butanol. Mixed starters significantly improved the production of this higher alcohol, mainly the inoculation with free cells of *H. osmophila*. In this fermentation (NF), the *n*-butanol content was 8-fold higher than the level detected in monoculture wine (C).

The mixed starters inoculation increased the content of ethyl acetate and 2-phenylethyl acetate (Table 2). High content of ethyl acetate was found in the wines from mixed starters including free and microencapsulated cells of *H. osmophila* strain, with the concentration of ethyl acetate approximately 3-fold greater than that produced by *S. cerevisiae* pure culture. Furthermore, the level of 2-phenylethyl acetate was highly increased by co-inoculation with the ND1 strain; the content of this ester was also influenced by the ND1 formulation, with levels significantly higher in wines fermented with free cells of *H. osmophila* strain (NF) than the sample fermented with microencapsulated ND1 cells (NM).

With regard to the esters present at lower concentrations, such as ethyl hexanoate, ethyl lactate, ethyl octanoate and diethyl succinate, higher levels were found in the control wine than in the samples from mixed fermentations (Table 2). In particular, the use of microencapsulated cells of ND1 (NM) increased the level of these esters in comparison to free ND1 cells (NF), mainly of ethyl lactate and diethyl succinate.

For easy visualization of contributions of each starter to the production of aromatic compounds, all these data were converted to Z-scores and used for the heatmap reported

in Figure 4. As already reported, the single *S. cerevisiae* EC1118 starter produced wine characterized by the highest content of some higher alcohols (2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethanol and methionol) and some esters, such as ethyl hexanoate, ethyl lactate, ethyl octanoate and diethyl succinate. Conversely, both the mixed starter cultures were characterized as producing the highest amount of acetaldehyde, *n*-propanol, 1-hexanol, ethyl acetate and 2-phenylethyl acetate. Furthermore, the wine produced with free ND1 cells contained the highest level of acetoin and *n*-butanol.

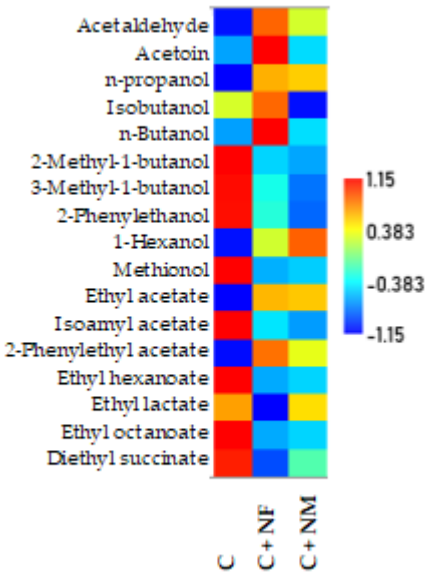

Figure 4. Heatmap based on all the volatile compounds detected in the experimental wines obtained by the mixed starters with *S. cerevisiae* EC1118 (C) and *H. osmophila* ND1 in two different formulations (NF—free cells; NM—microencapsulated cells) in comparison to the single starter wine with EC1118 (C). Colors represent the range of *Z*-scores (calculated over the rows), with blue indicating lower than average production, light green indicating average production and red indicating higher than average production of each compound.

Principal component analysis (PCA) was applied to all the parameters determined in the experimental wines obtained by the mixed starters with *S. cerevisiae* EC1118 and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells in comparison with the single starter EC1118 (C). The first principal component (PC1) explained 77.98% of data variability and it was mainly correlated with acetaldehyde, ethyl acetate and 2-phenylethyl acetate, while acetoin, *n*-butanol, 3-methyl-1-butanol, 2-phenylethanol and ethyl hexanoate contributed more strongly to the second principal component (PC2), which explained 22.02% of data variability. The scatter plot of the three wines on the plane defined by these first two components is shown in Figure 5. The PCA showed that the wines were located in three different quadrants in function of the starter formulation. In fact, the wines from mixed starters obtained by ND1 in the two different formulations (free (NF) and microencapsulated cells (NM)) were located far from each other and also in different quadrants in comparison to the monoculture wine (C).

3.6. Influence of Acetic Acid Addition on S. cerevisiae EC1118 Fermentative Performance

This assay was carried out in order to evaluate the existence of a potential correlation between the lowest number of *S. cerevisiae* cells in NF fermentation and the higher acetic acid level in the produced wine. Therefore, single fermentation trials with EC1118 were performed in grape must with 1.5 g/L acetic acid added. Acetic acid was added at different time intervals, i.e., at the start of the process (time 0), after 24 and 48 h of fermentation. The monitoring of fermentative performance of EC1118 in different conditions, evaluated by sugar depletion, is reported in Figure 6. High differences in sugar consumption were

found between the control (C without acetic acid addition) and C-48 h and C-72 h (addition of acetic acid after 48 and 72 h, respectively). At day six of the process, the residual sugar was lower in the control C (about 20 g/L) than in C-48 h andC-72 h, with values of 41 and 52 g/L, respectively. This trend was maintained until the end of the micro fermentation process. No differences were detected between the control and the fermentation with addition of acetic acid at time 0 (C-0 h) during the overall process.

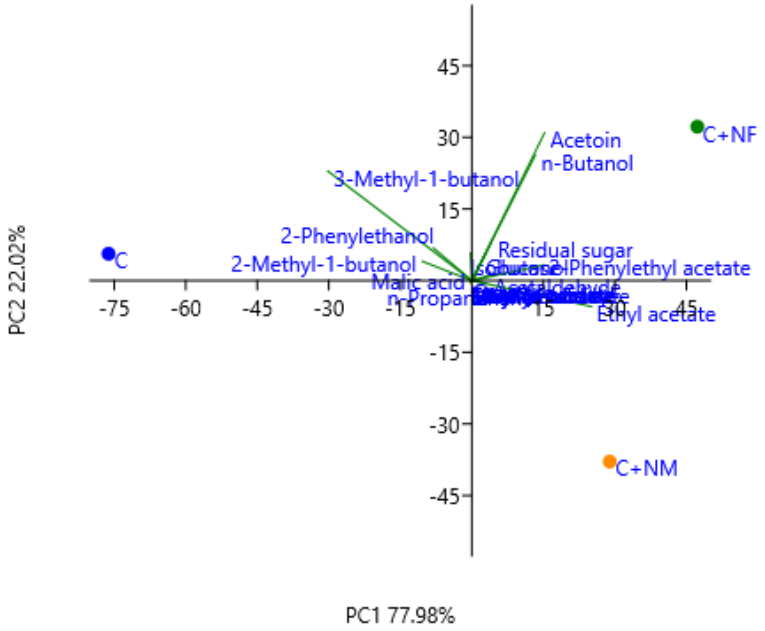

Figure 5. Principal component analysis (PCA) biplot of volatile compounds and main oenological parameters, determined in wines obtained by the mixed starters with *S. cerevisiae* EC1118 (C) and *H. osmophila* ND1 in two different formulations (NF—free cells; NM—microencapsulated cells) in comparison to single starter wine with EC1118 (C).

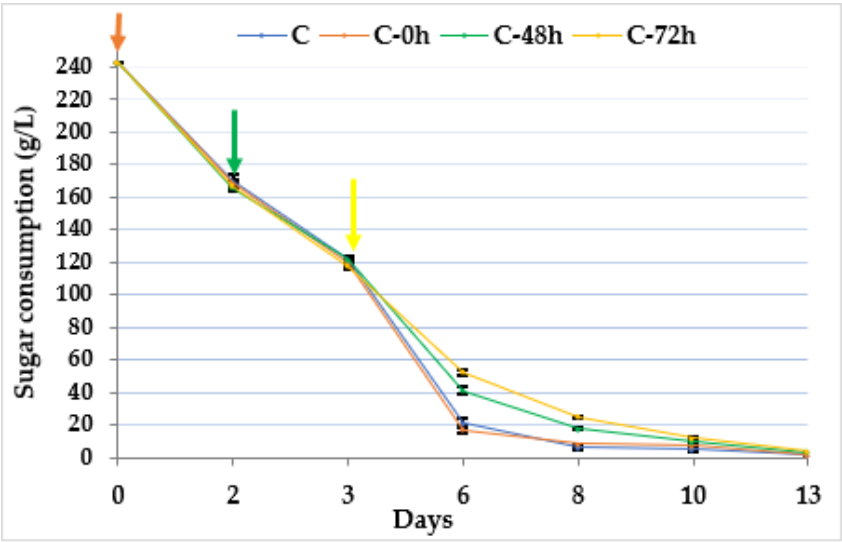

Figure 6. Kinetics of sugar consumption in single starter fermentation with EC1118 strain in grape must added with acetic acid at different times. C—control fermentation, without acetic acid addition; C-0 h—addition at the beginning of the fermentation process; C-48 h—addition 48 h after inoculum and C-72 h—addition 72 h after inoculum. The time of acetic acid addition is shown by the arrow in the graph. Data are expressed as mean value ± SD of three independent experiments.

The data regarding the amount the residual sugar and the acetic acid detected at the end of the fermentative process are shown in the Table 3. Significant statistical differences were found for both parameters analyzed in the function of the addition time of acetic acid. Regarding the residual sugar, the highest values (3.7 ± 0.15 and 3.1 ± 0.14 g/L) were recorded in C-72 h and C-48 h samples, whereas values very similar were found in the control and in the experimental wine C-0 h (acetic acid added at the beginning of the process). The same trend was also recorded for acetic acid content. These data demonstrated a significant influence of acetic acid on the fermentative behavior of EC1118, when added after 24 and 48 h, whereas no influence was recorded when acetic acid was added at the beginning of the process.

Table 3. Residual sugar and acetic acid content (g/L) in wines obtained by single strain EC1118 in fermentations added with acetic acid. The acetic acid was added at different times, at the beginning (C-0 h), after 48 h (C-48 h) and after 72 h (C-72 h) in comparison with samples without acetic acid addition (C). For each compound, different superscript letters mean significant differences (Tukey's test, $p \leq 0.05$) among wines produced by single strain EC1118 in fermentations with and without acetic acid addition. Data are expressed as mean ± standard deviation of two replicates.

	C	C-0 h	C-48 h	C-72 h
Residual sugar	1.45 ± 0.02 [a]	1.81 ± 0.01 [a]	3.1 ± 0.14 [b]	3.7 ± 0.15 [c]
Acetic acid	0.36 ± 0.04 [a]	0.94 ± 0.08 [b]	1.44 ± 0.03 [c]	1.68 ± 0.09 [c]

The later addition of acetic acid reduced the fermentative performance of this strain, which was not able to consume all the sugar present in the grape must. This result might be due to the potential ability of this *S. cerevisiae* strain to reduce the acetic acid, when the compound was added at time 0. This ability was reduced when the compound was added later and this is confirmed by the acetic acid content detected in the final wines, which was higher in the samples obtained by C-72 h and C-48 h than the level detected in C and C-0 h.

4. Discussion

The management of non-*Saccharomyces* yeasts in the mixed fermentation is one of the tools useful to increase the limited persistence of these species during mixed fermentation trials. In this context, the use of the microencapsulation for the cell immobilization increased the resistance against toxic compounds, such as ethanol formed during wine fermentation. Although different studies reported the use of immobilization of non-*Saccharomyces* strains for wine fermentation, in particular for sequential inoculations [18,19], as far as we know this is the first time that *H. osmophila* was used as calcium alginate microcapsules for wine fermentation in comparison to inoculum of free cells.

Although the slow fermentation rate observed in the first fermentation days for the mixed starter in comparison to *S. cerevisiae* strain (Figure 2), the *H. osmophila* strain tested in this study, mainly as microencapsulated cells, confirmed the good fermentative performance already reported for *H. osmophila* (and *H. vineae*), compared with other *Hanseniaspora* species. In general, *Hanseniaspora* species are considered poor fermenters, but it was demonstrated that the different species of this genus exhibit different fermentative behaviors [33,34]. Valera et al. [35] demonstrated the existence of a high degree of similarity between glycolytic and alcoholic fermentation enzymes of *H. vineae* and *H. osmophila* with *S. cerevisiae.* In consequence of the presence in these species of active genes typically related to wine fermentation capacities, such as sulfite tolerance (SSU1) and sucrose hydrolyzing invertase (SUC2), these two species should be classified as fermenters, while the remaining *Hanseniaspora* species were included in the fruit group.

In our study, the use of the microencapsulated cells increased the fermentative efficiency of *H. osmophila* cells in comparison to free formulation. These results were in contrast with previous work performed on microencapsulated cells of *S. cerevisiae* strain [36], in which it was found that encapsulated yeast consumed the fermentable brewing sugars

slowly with respect to the free form. It is demonstrated that cell immobilization affects cell growth, physiology and metabolic activity, but the induced effects are hard to predict [37].

However, our study tested a mixed starter culture, in which the interaction mechanisms between different strains play a fundamental role in fermentative kinetics and the confinement of *H. osmophila* cells in microspheres might offer protection against environmental stresses [22], such as the presence of toxic compounds, usually ethanol, or contact with other yeast cells.

The evaluation of cell population evolution during the mixed fermentations showed a different persistence for both ND1 and EC1118 strains in the function of *H. osmophila* cell formulation. When inoculated as microencapsulated cells, the ND1 strain was able to persist until the 9th day of fermentation, whereas a longer persistence was found for free *H. osmophila* cells (Figure 3). With regards *S. cerevisiae* cells, during the entire process the number of *S. cerevisiae* cells isolated from the mixed fermentation with free ND1 cells was lower than that of *S. cerevisiae* cells isolated from the process inoculated with microencapsulated cells of ND1.

These results seem to indicate an inhibition by the ND1 strain on *S. cerevisiae* populations when it was inoculated as free cells, also justifying the higher presence of residual sugar in the experimental wine obtained with free ND1 cells in comparison to wine from ND1 microencapsulated cells (Table 1). Some studies reported that the growth of apiculate yeast may inhibit *S. cerevisiae* growth, resulting in sluggish fermentation [38]. In our case, the inhibition could be related to cell-to-cell contact, in the case of free cells of *H. osmophila*, whereas the presence of encapsulation layer material might reduce this phenomenon in microencapsulated cells. Alternatively, this different behavior might be related to the presence of some compounds produced by yeasts during alcoholic fermentation that may become inhibitory to other yeast species or strains. Although in mixed fermentations ethanol is considered the main factor responsible of inhibitory interactions mediated by metabolites with toxic effects [39], other metabolites, such as short- to medium-chain fatty acids (e.g., acetic, hexanoic, octanoic and decanoic acids) can reach concentrations leading to cell death of different yeast species, including some *S. cerevisiae* strains [40,41]. In our study, the highest level of acetic acid (the main component of volatile acidity) was found in both the wines from mixed starter, as expected for apiculate yeasts, but the use of *H. osmophila* strain in different cell formulations influenced the volatile acidity level, with a significant higher content in wine from free cells (Table 1). This higher content of acetic acid might be responsible fora reduced number of *S. cerevisiae* cells in the fermentation inoculated with free ND1 cells.

The influence of high acetic acid content on EC1118 strain was confirmed by the trials performed by adding acetic acid (1.5 mg/L) to grape must at different times in the fermentation process (Figure 6). The influence of this compound was evident when it was added after 48 and 72 h, whereas no effect was observed when this compound was added at the start of the fermentation. This might be correlated with the ability of *S. cerevisiae* to remove acetic acid, a practical approach used to eliminate excessive amounts of this compound during alcoholic fermentation [42,43], and investigated by different scientific papers, either by mutants of *S. cerevisiae* [44] or by wild strains [45]. In our study, this hypothesis was corroborated by the lower level of acetic acid detected in the wine obtained by the addition at time 0 compared to the acetic acid level found in the wine obtained from musts with this compound added after 48 and 72 h of fermentation (Table 3).

Other than volatile acidity, the use of mixed starter also affected the ethanol content of the experimental wine, with a reduction of alcohol content by both the mixed starters, although in wine fermented with free *H. osmophila* cells this result was correlated with too high residual sugar (Table 1). The use of microencapsulated cells of the ND1 strain reduced the ethanol content by about 0.5 with respect to the single starter fermentation. This reduction level was lower than the ethanol reduction reported by other authors using cell immobilization of different non-*Saccharomyces* strains. Canonico et al. [18] found that the use of immobilized strains of *Starmerella bombicola*, *Metschnikowia pulcherrima*, *H. osmophila*

and *H. uvarum* in sequential fermentation with *S. cerevisiae* determined a variable ethanol reduction in the functioning of the non-*Saccharomyces* strain, with the lowest ability for *H. osmophila* (reduction from 0.78 to 1.33% *v*/*v*).

With regards the aromatic characteristics of the final wines, the use of mixed starter affected the content of almost all the aromatic compounds detected in this study, while less influence was found in the function of cell formulation of the ND1 strain (Table 2).

The use of mixed starter including *H. osmophila* strain increased the content of ethyl acetate and 2-phenylethyl acetate. This result was expected as it is well known that all *Hanseniaspora* species increase the concentration of almost all the acetate esters [34,46,47]. The content of the main ester of wine, ethyl acetate, was increased using the mixed starter, independently from cell formulation. However, the values were below the acceptable level in all the wines, considering that wines are defective when this compound is present at levels of 150–200 mg/L [48].

The inclusion of *H. osmophila* strain also significantly increased the level of 2-phenylethyl acetate, which imparts honey, fruity, flowery, rosé-like aromas, and the amounts detected in wines from the mixed starters were higher than the level usually detected in the wine, ranging from 0 to 18.5 mg/L [32]. The increased level found in our study (from 30- to 40-fold greater than level found in the *S. cerevisiae* monoculture, Table 2) was higher than values previously reported for mixed starter cultures including *H. osmophila* strains [49] and for mutants derived from the commercial wine strain AWRI796 [50]. It was discovered that the amount of 2-phenylethyl acetate found in wines obtained by the mixed starter including *H. osmophila* was significantly different from that formed by the *S. cerevisiae* monoculture if the percentage of *H. osmophila* in the culture was at least 50% [46].

The *H. osmophila* strain did not affect the content of the other acetate ester found in this study, isoamyl acetate, which was present in higher concentration in the wine obtained by *S. cerevisiae* fermentation, suggesting that *S. cerevisiae* metabolism is mainly responsible for isoamyl acetate formation [46], whereas *H. uvarum*, whether in pure or mixed cultures, yielded higher levels of isoamyl acetate in comparison to those produced by *S. cerevisiae* monoculture [51].

5. Conclusions

The fermentative performance of the *H. osmophila* ND1 strain in mixed starter fermentation is widely affected by cell formulation. Microencapsulation resulted in a suitable system to increase the fermentative efficiency of this strain in comparison with the use of free cells during mixed starter fermentation. Furthermore, this formulation was also promising for a reduction of ethanol content in wine, a very attractive trend for winemaking. However, our results confirmed the key role of interaction mechanisms between strains in mixed starter fermentation [52], in our case most probably correlated to the strong selective pressure exerted by the high content of acetic acid. These mechanisms have to be accurately investigated before the use of mixed starter cultures at industrial level in order to allow the expression of the particular metabolic footprint of each strain included in the mixed starter and to take all the advantages correlated with the use of this innovative starter.

Author Contributions: Conceptualization, all the authors; methodology, all the authors; software, R.P., G.S. and G.A.; investigation, G.A., R.P., D.M. and R.R.; resources, P.R. and A.C.; writing—original draft preparation, all the authors; writing—review and editing, A.C., G.M., T.G.-C. and P.R.; funding acquisition, P.R. and A.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the project PSR Regione Basilicata 2014–2020, sottomisura 16.1 GO Vites&Vino PROduttività e Sostenibilità in vITivinicoltura (PROSIT), N. 54250365779 and sottomisura 16.2 IN.VINI.VE.RI.TA.S (Innovare la viti-VINIcolturalucana: VErso la RIgenerazionevarieTAle, la Selezione di vitigni locali e proprietà antiossidanti dei vini), N. 976. G.A.'s fellowship was supported by PON RI 2014/2020, Azione I.1– "Dottorati Innovativi con caratterizzazione industriale" (Grant No. DOT1338191).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article.

Acknowledgments: The JRU MIRRI-IT (http://www.mirri-it.it/, accessed on 13 July 2021) is greatly acknowledged for scientific support.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript, or in the decision to publish the results.

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3.19 Results (III) -Supplementary analysis of the experimental wine fermentations performed in 2 l of grape must.

3.19.1 Polyphenol content and antioxidant activity

The polyphenol content and the antioxidant power of wine principally depend on the type of maceration technology. However, the concentration of phenolic compounds also can depend also by yeast strain used in the vinification (Gutierrez-Escobar et al., 2021).

There are three mechanisms of interaction between yeast cells and phenolic compounds:

- Absorption of anthocyanins on the cell wall
- Extraction of phenols
- Release of polysaccharides (mannoproteins)

Therefore, even if starting from the same must and the same process conditions were applied, the starter culture could influence colour and phenolic content in wine (Topić Božič et al. 2019).

In fact, during the fermentation, some mechanisms could decrease or increase the wine color, such as the adsorption of anthocyanins by yeasts cell wall and the activity of β-glucosidase, which releases the corresponding glycosylated anthocyanidin, exposing it to oxidation phenomena which transforming it into colorless compounds (Gutierrez-Escobar et al., 2021). Moreover, it has been shown that some non-*Saccharomyces* species could also be involved in the stabilization of wine color thanks to the production of reactive precursors in the formation of new stable pigments, such as pyruvic acid and acetaldehyde (Topić Božič et al. 2019).

The antioxidant activity of all experimental wines was evaluated by radical scavenging activity assay DPPH (Mitrevska et al., 2020).

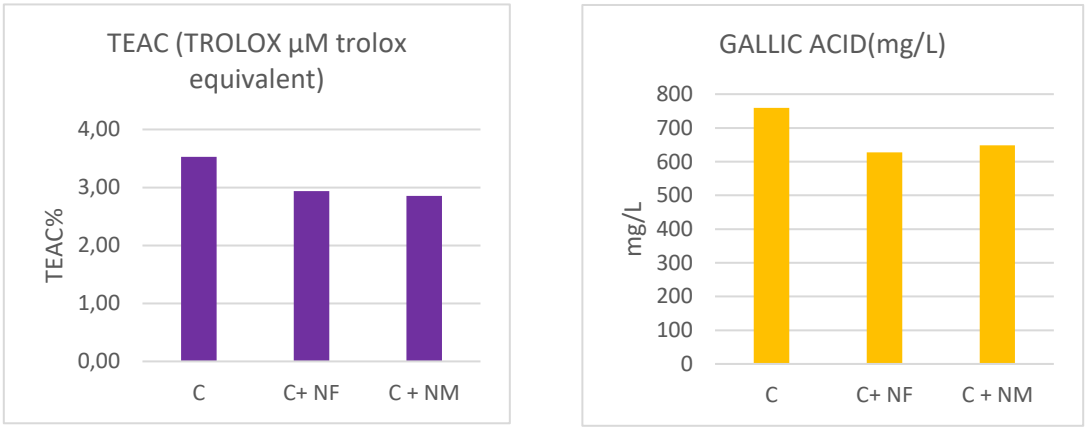

Figure 3.31 Total polyphenol content (Gallic acid mg/L) and TEAC % value in experimental wines obtained by the mixed starters with *S. cerevisiae* EC1118 (C) and *H. osmophila* ND1 in two different formulations (NF= free cells; NM= microencapsulated cells) in comparison to single starter wine with EC1118 (C).

As regard the radical scavenging activity, the results obtained by wines of two mixed starter fermentations demonstrated a similar trend in the antioxidant activity while, as showed in the figure 3.31, the highest value was observed for wines of control fermentation (C).

Similar trend was observed also for polyphenols content, the highest value was found in the monoculture fermentation (C), while for the fermentations with *H. osmophila* in the two formulations (NM, NF) slightly higher values were found in the sample with H. *osmophila* microencapsulated cells.

The results obtained in this step highlight the correlation between antioxidant activity and polyphenols content and demonstrated the importance of phenols as principal antioxidant components of the wine.

3.19.2 Anthocyanins

The color especially for red wines has particular importance in the evaluation of the wine by consumers. Nowadays among the new methods to improve the color of wine the microbiological approach is based on a careful selection of yeast species that could be useful to promote the synthesis of stable pigments, such as vinyl phenol, pyranoanthocyanins and vitisins.

Phenolic compounds contribute to organoleptic characteristics of the wine, such as color, astringency, and bitterness; these compounds are divided into two groups, not flavonoid and flavonoids compounds. The first group includes several subgroups, represented by flavones, flavanols, flavanones and anthocyanins. Among these, the most abundant are the anthocyanins, their name derives from the Greek "*anthos"* which means flower and "*kyanos"* which means blue, and represent the natural pigments responsible for the red, blue, and purple colors of musts and wines. They are mainly localized in the grape skin and the quantity of extracted anthocyanins depends on various factors, such as fermentation and maceration temperature, variety, maturity, seasonal conditions, extraction conditions, pH and addition of enzymes. Further differences between wine / grape anthocyanins derive from glucose acylation by hydroxycinnamic acids (caffeic acid, p-coumaric acid) or aliphatic acids, such as acetic acid. Anthocyanins in grapes and wines can be divided into six types, including cyanidin (red-orange color), peonidin, pelargonidin (orange), delphinidin, petunidin and malvidin (red, blue). The main ones of the *Vitis vinifera* grapes are the monomeric anthocyanins, such as -3-O-monoglucosides of delphinidin, cyanidin, petunidin, peonidin e malvidina.

Among the anthocyanins, malvidina is the most involved in the formation of various forms of pigments, both during storage and the aging of the wine. Anthocyanins are not particularly stable biomolecules; they are highly reactive and their color changes according to the pH of the medium and the presence of sulfur dioxide, which causes the loss of its characteristic red color. Phenomena of self-association and co-pigmentation with other phenols present in wine causes the stabilization of the anthocyanins and improves the color of the wine.

As shown in the figure 3.32, the highest content of total anthocyanins was found in experimental wines obtained with *S. cerevisiae* in monoculture and mixed fermentation with microencapsulated

ND cells (27.43 ± 0.92 and 26.34 ± 0.86 mg/L, respectively) in comparison to sample obtained by *H. osmophila* free cells (23.88 ± 1.51 mg/L).

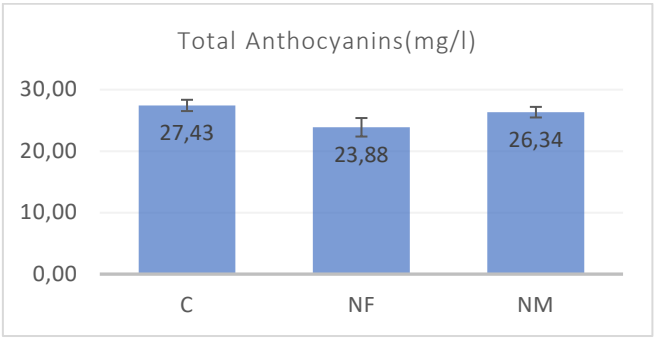

Figure 3.32 Total Anthocyanins content (mg/L) detected in experimental wines by the mixed starters with *S. cerevisiae* EC1118 (EC) and *H. osmophila* ND1 in two different formulations (NF- free cells; NM-microcapsules) in comparison to monoculture wine with EC1118 (C).

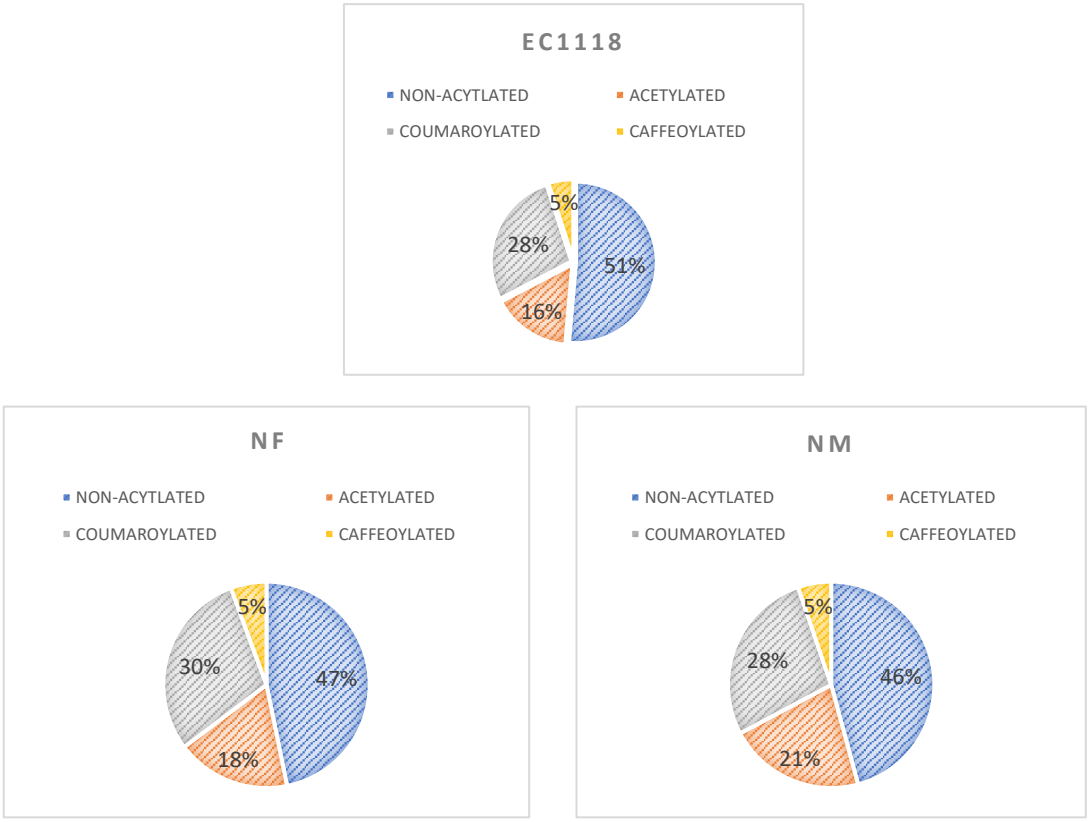

Figure 3.33 Percentage of the different chemical compounds categories in the in experimental wines by the mixed starters with *S. cerevisiae* EC1118 (C) and *H. osmophila* ND1 in two different formulations (NF- free cells; NM-microcapsules) in comparison to monoculture wine with EC1118 (EC).

Although the mixed fermentation with “NF” presents lower levels of all detected compounds, the distribution in percentage of the different chemical compounds categories is very similar in all the wine samples as shown in the graphs.

In all samples the most representative categories are the non-acylated anthocyanins and the coumaroylated anthocyanins.

Among the non-acetylated compounds, the highest concentration was exhibited by the Malvidin-3-glc**.**

Between the acetylated compounds, only Petunidin-3-acglc, showed significantly higher values in mixed fermentations than in control fermentation, while Delphinidin-3-acglc was detected only in fermentations with *H. osmophila* as microencapsulated cells.

In the group of coumaroylated compounds no significant differences were found for all compounds except for Malvidin-3-trans-cmglc and Peonidin-3-cmglc which showed higher values in the control fermentation.

3.19.3 Visitins

Vitisins are more stable and resistant compounds in confront to anthocyanins, less subject to degradation in common wine fermentation conditions.

In wine the most common are Vitisin A (malvidin-3-O-glucoside-pyruvic acid) and Vitisin B (malvidin-3-O-glucoside-vinyl-adduct). Vitisins are formed during fermentation by condensation of the anthocyanins present in the must with some metabolites produced by yeasts during glycolysis such as pyruvic acid or acetaldehyde. They are formed starting from the main anthocyanin present in grape must, malvidin-3-O-glucoside, which, reacting with pyruvic acid forms Vitisine A, while reacting with acetaldehyde forms Vitisin B, these compounds are more resistant to oxidation, and they are less sensitive to bleaching by sulfur dioxide. During fermentation, glucose is transformed into pyruvate which is further metabolized into acetaldehyde thus providing precursor molecules for the formation of vitisins. The amount of pyruvic acid e the acetaldehyde released during fermentation varies depending on the yeast strain.

The results obtained do not show any differences in the production of these compounds in the three types of analyzed wines.

Table 3.7 Anthocyanin content (mg/L) of the wines.

CLASS	COMPOUND	C	C+NF	C+NM
NON-ACYLATED	**Delphinidin-3-glc**	1.69 ± 0.05	1.55 ± 0.08	1.64 ± 0.08
	Cyanidin-3-glc	1.35 ± 0.01^{a}	1.34 ± 0.01^{a}	1.37 ± 0.00^{b}
	Petunidin-3-glc	1.96 ± 0.07^{a}	1.73 ± 0.12^{b}	1.76 ± 0.07^{ab}
	Peonidin-3-glc	1.68 ± 0.04	1.55 ± 0.08	1.63 ± 0.03
	Malvidin-3-glc	7.43 ± 0.54^{a}	5.01 ± 0.97^{b}	5.66 ± 0.54^{ab}
ACETYLATED	**Delphinidin-3-acglc**	n.d.^{a}	n.d.^{a}	1.34 ± 0.01^{b}
	Cyanidin-3-acglc	1.34 ± 0.01^{a}	1.35 ± 0.01^{ab}	1.37 ± 0.01^{b}
	Petunidin-3-acglc	1.36 ± 0.02^{a}	1.40 ± 0.02^{b}	1.42 ± 0.00^{b}
	Malvidin-3-acglc	1.66 ± 0.03^{a}	1.52 ± 0.07^{b}	1.52 ± 0.02^{b}
COUMAROYLATED	**Delphinidin-3-cmglc**	1.34 ± 0.01	1.34 ± 0.01	1.34 ± 0.01
	Petunidin-3-cmglc	1.36 ± 0.02	1.36 ± 0.01	1.37 ± 0.01
	Peonidin-3-cmglc	1.40 ± 0.02^{a}	1.34 ± 0.01^{b}	1.36 ± 0.00^{b}
	Malvidin-3-*cis*-cmglc	1.36 ± 0.01	1.35 ± 0.01	1.36 ± 0.01
	Malvidin-3-*trans*-cmglc	2.14 ± 0.09^{a}	1.70 ± 0.12^{b}	1.83 ± 0.09^{b}
CAFFEOYLATED	**Malvidin-3-cfglc**	1.37 ± 0.02	1.35 ± 0.01	1.36 ± 0.01
	Total anthocyanins	**27.43 ± 0.92^{a}**	**23.88 ± 1.51^{b}**	**26.34 ± 0.86^{ab}**
	Vitisin A	1.43 ± 0.16	1.44 ± 0.04	1.43 ± 0.03
	Vitisin B	1.46 ± 0.12	1.45 ± 0.18	1.50 ± 0.05

All parameters are given as average values ± the standard deviations (n = 3). n.d.: not detected.

Nomenclature abbreviations: glc, glucoside; acglc, acetylglucoside; cmglc, *trans*-*p*-coumaroylglucoside; cfglc, caffeoylglucoside

3.19.4 Flavonol

Flavonols are an important subclass of flavonoids, principally detected in white wine and characterized by important antioxidant properties.

Are yellow pigments present in the skin of berries, and such as anthocyanidins, they are usually present in glycosidic forms, linked to a sugar (glucose or rhamnose, galactose, arabinose, xylose). The main flavonols described in grapes and wine are myricetin, quercetin, laricitrin, kaempferol, isorhamnetin and syringatin, they are present in both white and red wines in which the high presence of anthocyanins (red-violet pigments) masks the yellow color, but at the same time the occurrence of co-pigmentation phenomena between flavonols (mainly quercetin and kaempferol glucosides) and anthocyanins greatly enhances its color. The presence of these compounds is associated with the sensory perception of astringency and bitterness. In red wine the maximum content described is 60 mg / L.

The results obtained show that wines produced with single starter (*S.cerevisiae*) and mixed fermentation "C + NM" are significantly different from fermentation with "NF" for almost all the detected compounds. In fact, as the figure shows, the flavanol concentration is significantly lower in the C-NF fermentation. The flavonols: Quercetin-3-glcU, Quercetin-3-glc, Syringetin-3-glc exhibited a high content in all three wines.

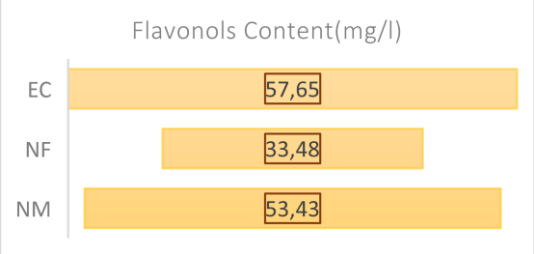

Figure 3.34 Flavonols content (mg/L) detected in experimental wine by the mixed starters with *S. cerevisiae* EC1118 (EC) and *H. osmophila* ND1 in two different formulations (NF- free cells; NM-microcapsules) in comparison to monoculture wine with EC1118 (EC).

Table 3.8 Flavonol content (mg/L) of the wines.

	C	C+NF	C+NM
Myricetin-3-gal	3.73 ± 0.14^{a}	3.26 ± 0.07^{b}	3.55 ± 0.05^{a}
Myricetin-3-glcU	3.69 ± 0.10^{a}	n.d.^{b}	3.58 ± 0.05^{a}
Myricetin-3-glc	9.29 ± 0.72^{a}	4.24 ± 1.02^{b}	8.50 ± 0.13^{a}
Quercetin-3-glcU	13.09 ± 1.56^{a}	5.67 ± 1.19^{b}	10.78 ± 0.44^{a}
Quercetin-3-glc	8.41 ± 0.67^{a}	4.73 ± 0.23^{b}	7.76 ± 0.58^{a}
Laricitrin-3-glc	4.70 ± 0.19^{a}	3.54 ± 0.09^{b}	4.47 ± 0.04^{a}
Kaempferol-3-gal	0.22 ± 0.02^{a}	n.d.^{b}	0.20 ± 0.01^{a}
Kaempferol-3-glc+Kaempferol-3-glcU	0.38 ± 0.03^{a}	0.19 ± 0.01^{b}	0.33 ± 0.02^{a}
Isorhamnetin-3-glc	1.11 ± 0.09^{a}	0.39 ± 0.06^{b}	0.98 ± 0.14^{a}
Syringetin-3-glc	5.39 ± 0.11^{a}	3.93 ± 0.18^{b}	5.14 ± 0.17^{a}
Free-Myricetin	3.64 ± 0.43	4.29 ± 1.69	3.80 ± 0.38
Free-Quercetin	3.98 ± 0.76	3.24 ± 0.07	4.32 ± 0.54
Free-Kaempferol	0.01 ± 0.00	n.d.	0.03 ± 0.02
Total flavonols	**57.65 ± 4.66^{a}**	**33.48 ± 4.18^{b}**	**53.43 ± 1.84^{a}**

All parameters are given as average values ± the standard deviations (n = 3). n.d.: not detected.

Nomenclature abbreviations: gal, galactoside; glcU, glucuronide; glc, glucoside.

3.19.5 Flavanol

Flavanols represent another subgroup of flavonoids also present both in grapes and wine, the main ones are: catechin, epicatechin, and their derivatives (gallocatechin, epigallocatechin).

The results show that among the flavanols catechin exhibited the greatest concentration in the control wine and in the mixed fermentation with "NM", the values are sixteen and eleven times higher respectively in comparison to "NF" sample.

Procyanidin B2 exhibited the highest value in control fermentation while the Procyanidin B1 was found only in fermentation with *S.cerevisiae* and in mixed fermentation with "NM".

In conclusion in the sample "NF" lower values were highlighted for all compounds compared to the other samples, as shown by the total value of the favanols detected, significantly lower in NF.

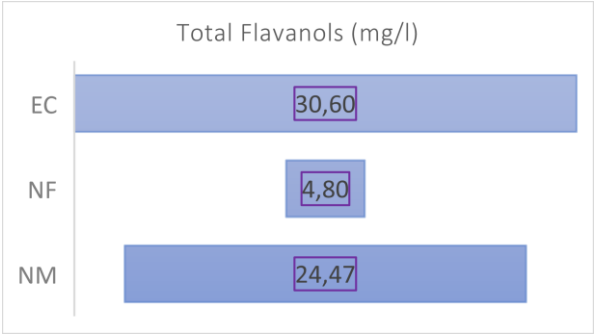

Figure 3.35 Flavanols content (mg/L) detected in experimental wine by the mixed starters with *S. cerevisiae* EC1118 (EC) and *H. osmophila* ND1 in two different formulations (NF- free cells; NM-microcapsules) in comparison to monoculture wine with EC1118 (EC)

Table 3.9 Flavanol content (mg/L) of the wines

	C	C+NF	C+NF
Epigallocatechin	4.02 ± 0.11^{a}	2.87 ± 0.36^{b}	4.66 ± 0.49^{a}
Catechin	21.95 ± 2.69^{a}	1.32 ± 0.54^{b}	15.16 ± 2.91^{c}
Epicatechin	1.31 ± 0.20^{a}	0.35 ± 0.02^{ab}	2.41 ± 0.98^{a}
Procyanidin B1	0.61 ± 0.23^{a}	n.d.^{b}	0.31 ± 0.09^{ab}
Procyanidin B2	2.71 ± 0.43^{a}	0.26 ± 0.10^{b}	1.92 ± 0.29^{c}
Total flavanols	**30.60 ± 3.63^{a}**	**4.80 ± 0.71^{b}**	**24.47 ± 3.92^{a}**

All parameters are given as average values ± the standard deviations (n = 3). n.d.: not detected.

3.19.6 Phenolic acids

Phenolic acids in grapes and wine are divided into two groups: hydroxycinnamic acids and hydroxybenzoic acids.

The group of hydroxycinnamic acids includes coumaric acid, ferulic acid, caffeic acid while that of hydroxybenzoic acids mainly includes vanillic acid, salicylic acid, gallic acid and protocatecuic acid. Some hydroxycinnamic acids, such as coumaric acid and caffeic acid, can combine with tartaric acid to form hydroxycinnamoyltartaric acids, such as caftaric acid, p-coutaric acid and fertaric acid. This

group is the third most abundant in polyphenols component in grapes and must, unfortunately they are easily oxidizable and are often associated with wine browning phenomena. Their concentration varies between 100 and 30 mg / L in red and white wines, respectively, although some authors have found higher concentrations: 130 mg / L in white wines and 60 mg / L in red wines. (Gutiérrez-Escobar et al., 2021).

Phenolic acids also influence the sensory properties of wines, they help to increase the sensation of astringency and bitterness. (Zang et al., 2021).

Gallic Acid is the major representant of the Hydroxybenzoic acids group, as show the table the samples do not exhibited differences for this compound. The value detected are 7.65 mg/L, 6,94 mg/L, and 7,12 mg/L for C, NF, and NM wines respectively.

Also, for this group of phenols the profile founded in the three sample is similar to the trend detected for the other compounds, in fact, the control sample presented similar value to the NM fermentation while the concentrations founded in the NF fermentation were much lower.

As regards the group of Hydroxycinnamic acids (HCA) the highest value was showed by trans-Caftaric acid, with 25.83 ± 0.64 and 22.23 ± 0.97 in C and NM samples respectively.

3.19.7 Stilbenes

Stilbenes naturally present in the skin and leaves of grapes, are bioactive compounds consisting of two connected aromatic rings from an ethyl bridge (Zang et al., 2021) among the stilbenes the main are trans and cis forms of resveratrol and its glycoside piceid. Resveratrol is a polyphenol characterized by interesting health properties. The trans-resveratrol form is found in higher concentration in red wine than in white, the different concentration of these compounds is due both to the oenological techniques and to the microorganisms present in the fermentation process. In fact, yeasts endowed with β-glucosidase activity can increase the concentration of free resveratrol in the wine whose presence is related to a lower incidence of some diseases and a greater antioxidant capacity of wine, therefore, in the selection of a wine starter should also consider these capacities which could potentially be exploited to improve the color, the content of polyphenols and the healthiness of the wine produced. (Viana et al., 2019).

As shown in the table 3.10, the higher concentrations of these compounds were observed in the C and NM samples, on the contrary much lower values were found in the NF sample.

The content of trans piced and cis piceid is respectively 4.5 and 3 times greater in the NM sample than that found in NF, while the trans and cis forms of resveratrol, are respectively 8 and 1.5 times higher.

As expected the concentration of total stilbenes is significantly higher in the wine fermented by *S. cerevisiae* and in the mixed fermentation with *H.osmophila* as microencapsulated form.

Table 3.10 non-Flavonoid content (mg/L) of the wines.

	ECC118	ECND1	ECCAP
Hydroxybenzoic acid			
Gallic acid	7.65 ± 0.45	6.94 ± 0.98	7.12 ± 0.28
Hydroxycinnamic acids (HCA)			
***trans*-Caftaric acid**	25.83 ± 0.64^{a}	7.99 ± 2.00^{b}	22.23 ± 0.97^{c}
***trans+cis*-Coutaric acids**	4.20 ± 0.15^{a}	1.52 ± 0.25^{b}	3.57 ± 0.13^{c}
Caffeic acid	1.34 ± 0.10^{a}	0.33 ± 0.08^{b}	1.35 ± 0.07^{a}
***trans*-Fertaric acid**	1.66 ± 0.04^{a}	0.59 ± 0.15^{b}	1.46 ± 0.04^{a}
p-Coumaric acid	0.36 ± 0.02^{a}	0.20 ± 0.04^{b}	0.47 ± 0.03^{c}
Ferulic acid	0.31 ± 0.01^{a}	0.15 ± 0.03^{b}	0.26 ± 0.04^{a}
Total HCA	**33.70 ± 0.94^{a}**	**10.78 ± 2.51^{b}**	**29.34 ± 1.14^{c}**
Stilbenes			
***trans*-Piceid**	1.43 ± 0.12^{a}	0.30 ± 0.03^{b}	1.36 ± 0.04^{a}
***cis*-Piceid**	0.20 ± 0.03^{a}	0.06 ± 0.01^{b}	0.20 ± 0.02^{a}
***trans*-Resveratrol**	0.20 ± 0.00^{a}	0.02 ± 0.00^{b}	0.16 ± 0.04^{a}
***cis*-Resveratrol**	0.07 ± 0.01^{a}	0.04 ± 0.00^{b}	0.06 ± 0.00^{a}
Total stilbenes	**1.90 ± 0.15^{a}**	**0.41 ± 0.04^{b}**	**1.77 ± 0.09^{a}**

All parameters are given as average values ± the standard deviations (n = 3).

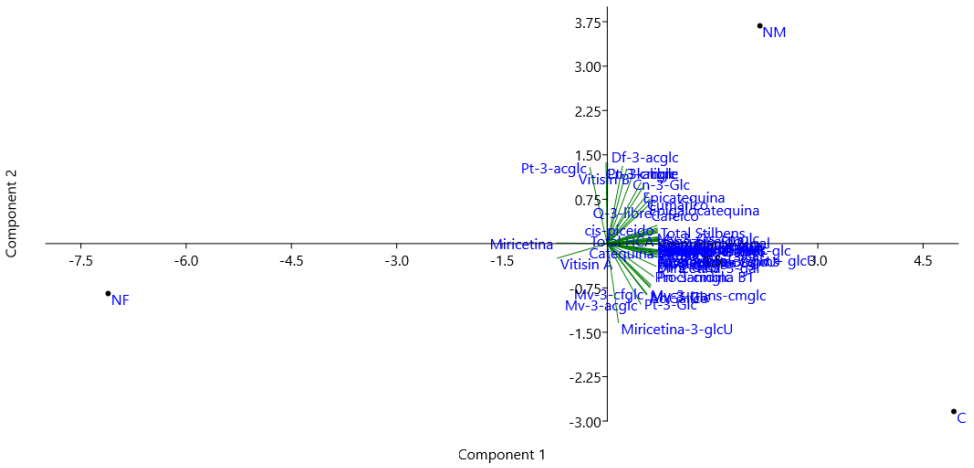

Figure 3.36 Principal component analysis (PCA) biplot of phenol compounds determined in wines obtained by the mixed starters with *S. cerevisiae* EC1118 (C) and *H. osmophila* ND1 in two different formulations (C+NF, C+NM) in comparison to monoculture wine with EC1118 (C).

As reported in the figure 3.36, the analysis of the main principal component (PCA) of all detected phenolic compounds shows the wine samples in different quadrants depending on the starter formulation. The wine samples fermented with *S. cerevisiae*(C) and mixed inoculation of *S.cerevisiae* and *H.osmophila* as microcapsules, are more characterized by the presence of phenolic compounds compared to the sample fermented with S*. cerevisiae* and *H.osmophila* as free cells. The results confirm that the concentration of these compounds can be modulated by using specific starter cultures. The evaluation of phenolic composition of experimental wines was carried out in collaboration with the University de La Rioja, Istituto de Ciencias de la Vid y del Vino (Logrono, Spain).

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Chapter IV

Study of non-Saccharomyces selected strain in winemaking condition

Abstract

In this research phase, the selected non-*Saccharomyces* strain ND1 was tested at pilot scale during vinification in the winery "Cantine del Notaio" located in “Rionero in Vulture” (PZ), Basilicata.

In the cellar three trials were performed in parallel, a pure culture fermentation with the commercial starter *S. cerevisiae* used in this cellar (control fermentation), and mixed culture fermentations with the starter selected in lab-scale (*H. osmophila*-ND1 and *S. cerevisiae*), inoculated as free (NF) and microencapsulated (NM) form, whereas the *S. cerevisiae* strain was used just in free form.

The aim of this step was to validate the results obtained at laboratory scale by the use of the selected mixed starter both on volatile composition and ethanol content of wine.

4.1 Materials and methods

4.1.1 ND1 production

H. osmophila (ND1) strain both as fresh and microencapsulated cells was produced by following the same procedure already reported (paragraphs 3.16.1).

4.1.2 Mixed inoculated fermentations with ND1 strain in different formulations

During pilot-scale vinification, the same inoculation procedure used in laboratory fermentations was applied. The *H. osmophila* ND1 strain was tested in mixed fermentations as free (NF) and microencapsulated form (NM), with the commercial *S. cerevisiae* strain used by the cellar used in free form. The control fermentation was inoculated with a pure culture of the commercial starter *S. cerevisiae* with an initial cell population of 2×10^6 cells/mL (C).

The fermentations were performed in 9hL of natural grape must Aglianico del Vulture, a grape variety cultivated in Basilicata.

The two yeast strains were simultaneously inoculated in the grape must, by using different inoculation ratio. In each mixed fermentation, *S. cerevisiae* strain was inoculated as free cells at concentration of 10^6 cell/mL, whereas *H. osmophila* strain was inoculated with an initial cell population of 10^6 cells/mL as free and 10^5 cell/mL as microencapsulated cells respectively (Table 4.1).

Before inoculation in steel tanks, the yeasts cream was homogenized in a must aliquot to facilitate the adaptation of the cells to the substrate.

Figure 4.1 Fermentation trials at cellar scale

Table 4.1 Mixed starter inoculation levels used during pilot-scale fermentations

Control fermentation **(C)**	**C** (10^6 cell/mL)
Mixed fermentation with *H.osmophila* as free cells **(C+ NF)**	**NF** (10^6 cell/mL) + **C** (10^6 cell/mL)
Mixed fermentation with *H.osmophila* as microencapsulated form **(C+ NM)**	**NM** (10^5 cell/mL) + **C** (10^6 cell/mL)

All the fermentations were performed in steel tanks at the constant temperature of 26°C and were monitored until the end by determination of sugar consumption.
The images detected by microscopy analysis reports the three different starter formulations in the experimental fermentation trials.

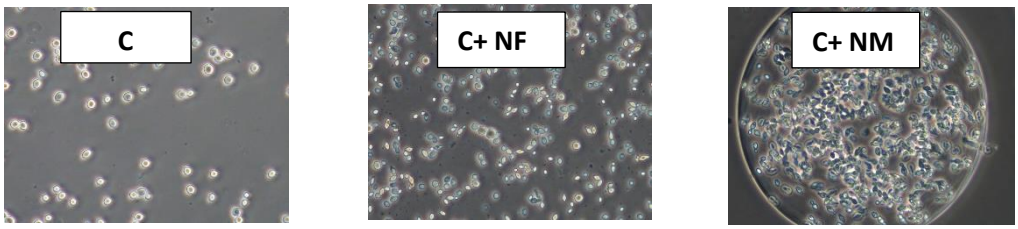


Figure 4.2 Optical microscopy images at x 400 magnification of ND1 strain and *S. cerevisiae* during the fermentation process.

4.1.3 Evaluation of yeast viable population during the fermentations

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), a fermenting "Aglianico" of must/wine samples were collected and submitted to plate count on WL medium, as already reported.
After incubation at 26°C for 5 days, the dilution plates statistically representative were counted; from each fermentation sample, some colonies showing different morphologies were randomly purified on YPD for yeast identification by restriction analysis of amplified ITS region (Esteve-Zarzoso et al., 1999).

4.1.4 Analytical determination of experimental wines

The experimental wines obtained from cellar fermentations were monitored by using WineScan instrument for determination of residual sugar concentration, ethanol content, total and volatile acidities, glycerol, malic acid, and other oenological parameters.
To understand the influence of the different starter formulation on the wine aroma composition the samples at the end of the vinifications were analyzed for content of main volatile compounds

(acetaldehyde, ethyl acetate, n-propanol, isobutanol, n-butanol, acetoin, D-amyl and isoamyl alcohols) by direct injection gas chromatography, as already described.

4.1.5 Data analysis

The experimental data were submitted to PCA, by using PAST3 software ver. 3, to compare the main oenological parameters and the volatile compounds content detected on wines obtained in the cellar by the mixed starters with *S. cerevisiae* (C) and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells and single starter wine with *S. cerevisiae* (C).

4.2 Results and discussion

4.2.1 Fermentation kinetics

The fermentative behavior of the *H. osmophila* ND1 strain both as free and microencapsulated cells in combination with *S. cerevisiae* was analyzed at pilot vinifications, in order to evaluate the feasibility of using this strain as mixed starter culture for wine fermentation at industrial cellar scale. The consumption of sugars present in the must was evaluated in the two samples of mixed fermentations (C + NM and C + NF) in comparison to the monoculture fermentation conducted by *S. cerevisiae* starter (C).

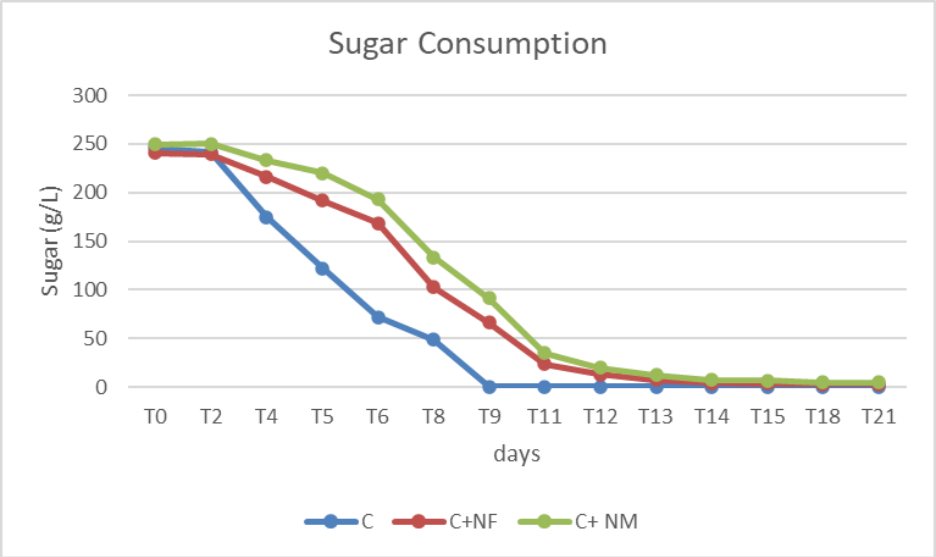


Figure 4.3: Sugar utilisation (g/L) during pilot scale fermentations in Aglianico del Vulture in three fermentation conditions. C= control fermentation, inoculated with *S. cerevisiae*; C+NF= mixed starter *S. cerevisiae* +ND1 as free cells; C+NM=mixed starter *S. cerevisiae* +ND1 as microencapsulated cells.

As shown in the figure 4.3, *S. cerevisiae* pure fermentation exhibited a faster sugar consumption in comparison to the two mixed fermentations (C + NF, C + NM). In these samples sugar depletion

during the fermentation process did not display great differences, only in the first days the sample C + NM showed a slower fermentation kinetics than the sample C + NF.
However, after T11 (eleventh day of fermentation) the consumption of sugars followed a similar trend in both mixed fermentations, which were completed after about 20 days. As regards the single fermentation (C), the process was completed in about ten days.
At the end of the process, the residual sugars detected in NM mixed fermentation were similar to the residue detected in NF sample, whereas lowest value was found in control fermentation (C).

4.2.2 Evaluation of yeast viable population during the fermentations

To evaluate the presence of inoculated strains during the fermentative process, in each vinification trial yeasts isolation on WL medium was performed at different fermentation times and the yeast population dynamics during pilot scale fermentations is reported in figure 4.4.
As expected, high number of yeast cells was found at the beginning step, in fact at T0 the number of *H. osmophila* cells was higher in fermentation inoculated with free cells than the number found in trial inoculated with microencapsulated cells (about $1x10^6$ and 10^4 cfu/mL, respectively).
In our experiments, as expected for process at pilot scale, other yeasts, different from inoculated starters participated at the fermentative process.
The results of analysis of the colonies isolated from the three fermentations indicate the presence of high number of yeast species; in the early fermentation stages, other than *H. osmophila,* also of *H. uvarum* and *Candida* yeasts were found in all the fermentation trials.
At T2 (2^{nd} day after inoculum, figure 4.4) the highest number of viable cells for *Hanseniaspora spp* (which includes both autochthons *Hanseniaspora* present in must and *H. osmophila* inoculated strain) was found in mixed fermentation C+NM, whereas in C+NF high number of *S. cerevisiae* cells were found.
At the beginning of fermentation, the control fermentation (C) presented a high cells number of *S. cerevisiae* ($1.24*10^7$cfu/mL) and *Hanseniaspora spp* ($1.8*10^6$ cfu/mL).
After 4 days from inoculation, both mixed fermentations showed a similar dynamic. In fact, the C + NM and C + NF samples presented a high cellular concentration of *S. cerevisiae* cells, higher in fermentation with free cells of *H. osmophila* (respectively $1.7 * 10^6$ cfu/ mL in C + NM and $2.67 *10^7$ cfu/mL in C + NF).
The evolution during the fermentative processes of indigenous non-*Saccharomyces* yeasts showed similar levels in both mixed fermentations, both for *Candida* species ($2 * 10^5$ cfu/mL in C+NM; $2 * 10^6$ cfu/mL in C+NF) and *Hanseniaspora spp.* ($1.4 * 10^6$ cfu/mL in C + NM and $2.2 * 10^6$ cfu /mL in C + NF.
A similar trend was found for mixed fermentation samples on the 6th day of process, in which the number of viable *S. cerevisiae* cells increased, mainly in the fermentation sample with *H. osmophila* as microencapsulated cells ($4.6 * 10^7$ cfu/mL). In this fermentation step in the control C a high

number of viable *S. cerevisiae* cells was found (4.8 * 10^7 cfu/mL), accompanied also in this case by the presence of *Hanseniaspora spp* (1 * 10^7 cfu/mL) and *Candida* (2 * 10^6 cfu /mL).

In the subsequent isolation steps, in pure culture of *S. cerevisiae* (C), *S. cerevisiae* cells were found. As regards *S. cerevisiae* cells, during overall the process the number of cells isolated from mixed fermentation with free ND1 cells was higher than that of *S. cerevisiae* cells isolated from the sample inoculated with microencapsulated cells of ND1, probably correlated to the strong protection exerted by the confinement of the cells in microcapsules structure and some mechanisms of interactions/inhibition between the two species. In the last phase of isolation, in mixed fermentation only *S. cerevisiae* cells were detected (Canonico et al.,2016).

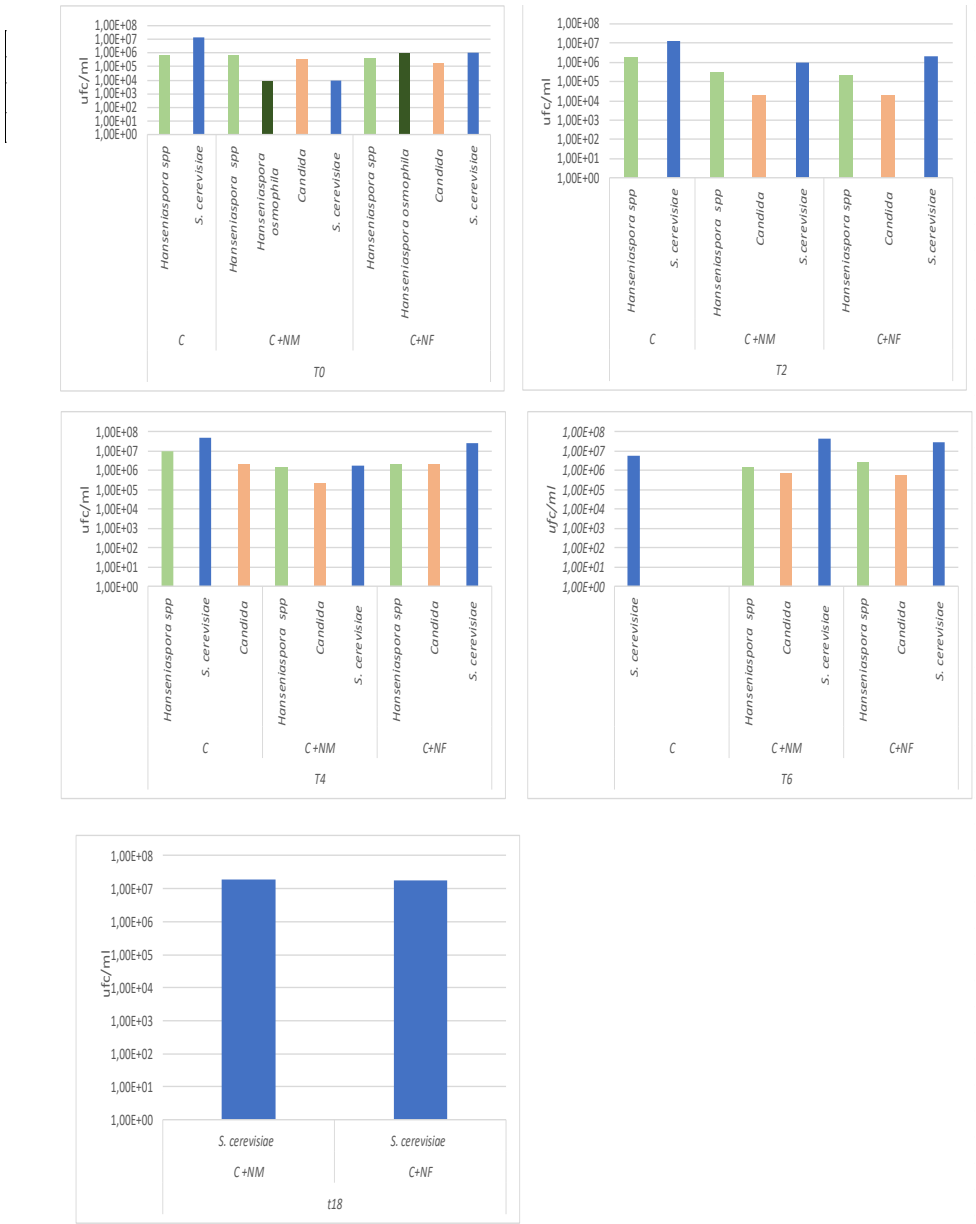
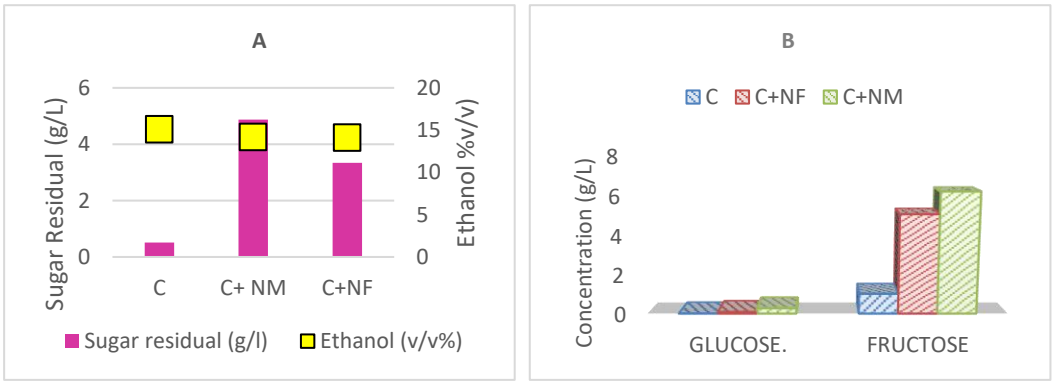


Figure 4.4 Yeast cell count detected at different times of fermentations inoculated with *S. cerevisiae* in mixed culture with *H. osmophila* ND1 strain as free (NF) and microencapsulated (NM). As control, pure fermentation with *S. cerevisiae* was used *(C)*.

4.2.3 Analysis of experimental wines for main oenological parameters

The wines obtained by pilot-scale vinifications were analyzed for chemical parameters of oenological interest.

As showed in the figure 4.5 (A), the alcohol content of the three wines were different. An ethanol reduction of about 1 v/v % was obtained by using mixed starter cultures; in fact, the ethanol content found in C+NF and C+NM wines (14.16 and 14.09 %v/v, respectively) was lower in comparison to wine from single fermentation C (15.06 v/v%). However, as reports the figure 4.5 (B), also the residue of glucose and fructose (g/L) in the three wines was different. The samples from mixed fermentation (mainly C+NM) exhibited a high sugar residual, especially for the fructose, confirming the preference of this strain for glucose than fructose consumption.



Figures 4.5 (A) Sugar residual (g/L) and ethanol production (v/v%) at the end of the fermentations; (B) Glucose and Fructose residuals (g/L) in wines obtained by the mixed starters with *S. cerevisiae* (C) and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells in comparison to single starter wine with *S. cerevisiae* (C).

Organic acids after sugars are the most abundant solids present in grape juice and in wine; these compounds are responsible for the sour taste, and also influence the color and pH (Vilela et al.2019). Total acidity is the result of the sum of all the individual organic acids that are present in wine, the predominant acids are tartaric and malic, which can represent over 90% of the total acidity in the grape, both contribute to the pH of the must and of the wine during vinification and aging. Malic acid has a sour metallic taste related to green apple, while the taste attributed to tartaric acid is often associated with attributes, such as "mineral" or “citrus”. Citric acid is a weak organic acid and an important intermediate in the tricarboxylic acid cycle, characterized by a citrusy flavor, with antimicrobial activity against molds and bacteria, it can prevent browning by chelating metal ions. Considering the great influence of these compounds on the quality of the wine during the winemaking process, it is advisable to monitor the concentration of organic acids present in the grapes, such as tartaric, malic, and citric, and those originating during fermentation, such as lactic acid (perceived as acid and spicy, and mainly produced by lactic bacteria) and succinic (sour, salty and bitter taste, main acid produced by yeast). The specific concentration of each acid in the wine, could influence taste and aroma.

As the figure 4.6 shows, all the wines contained a similar level of volatile acidity, higher value was found for the wines obtained by mixed fermentations containing ND1 as free and microencapsulated cells (0.42 and 0.47 g/L, respectively), in comparison to single starter wine (0.3 g/L); however, the value of volatile acidity falls within the maximum acceptable limit for volatile acidity (OIV- 1.2 g/L of acetic acid) in all experimental wines.

Similar trend was observed for the total acidity content; in particular, the highest value was found for the mixed fermentation with *H. osmophila* as microencapsulated cells.

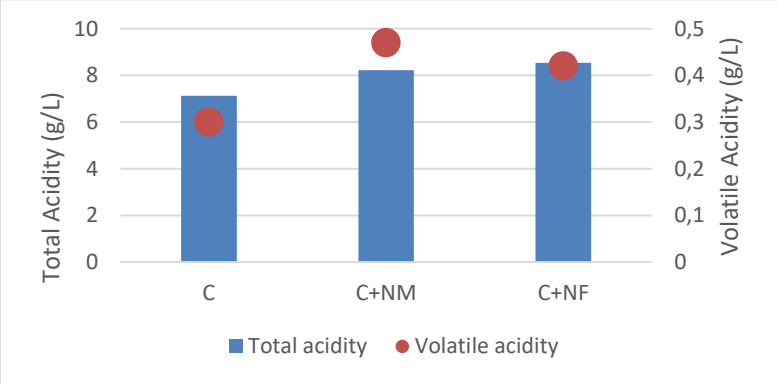


Figure 4.6 Total and volatile acidity content in wines obtained by the mixed starters with *S. cerevisiae* (C) and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells in comparison to single starter wine with *S. cerevisiae* (C).

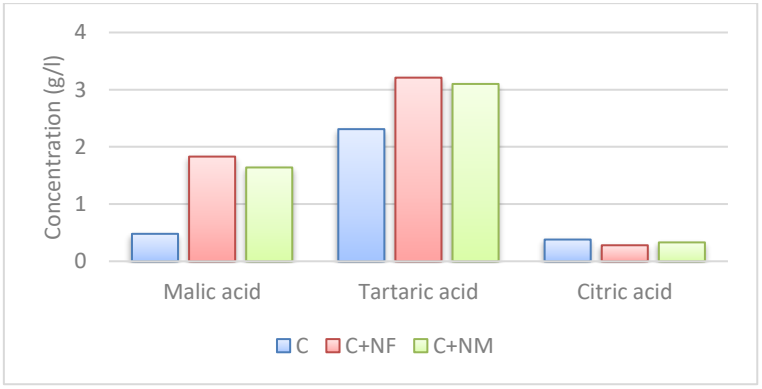


Figure 4.7 Organic acids content in wines obtained by the mixed starters with *S. cerevisiae* (C) and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells in comparison to single starter wine with *S.cerevisiae* (C).

The figure 4.7 reports the content of principal organic acids of experimental wines; as expected, the samples of mixed fermentations presented the highest values for the content of all analysed acids. The acidity is a characteristic highly affecting the quality of the wine; in this regard, the use of non-*Saccharomyces* yeasts represents a biological tool capable of modulating the acidity of the wine, both by increasing its content (biological acidification) and decreasing it (biological deacidification) without the addition of oenological products (Vilela et al., 2019).

4.2.4 Glycerol content

Glycerol after ethanol and carbon dioxide is one of the main products of alcoholic fermentation, its quantity is influenced by the grape variety, the process temperature and the type of yeast used. The production of glycerol is one of the desirable characteristics as it contributes to the aroma and sweet taste of the wine produced. The results obtained from pilot scale fermentations indicates that the glycerol content was quite similar among all the wines, well above the threshold value established for this compound (5.2 g/L) (Ciani et al., 1996), particularly for the control fermentation and the mixed starter fermentation with *H. osmophila* as microencapsulated cells.

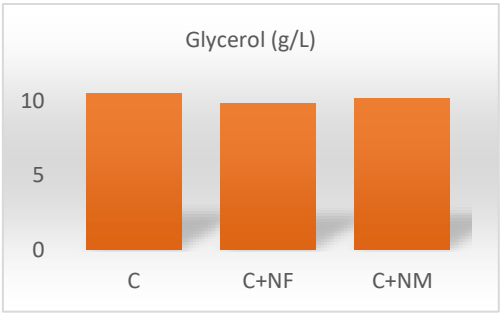


Figure 4.8 Glycerol content in wines obtained by the mixed starters with *S. cerevisiae* (C) and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells in comparison to single starter wine with *S.cerevisiae* (C).

4.2.5 Polyphenolic content and antioxidant activity of experimental wines

The concentration of phenol compounds in grapes is influenced by several variables: the vineyard; winemaking techniques; the storage of wines. Various strategies have been evaluated to modulate the concentration of polyphenols, both for the pre-fermentation phase and during the maceration. Recently, the possibility of using mixed cultures including non-*Saccharomyces* with *S. cerevisiae* has also been evaluated as a useful approach to modify the content of phenolic compounds as the enzymatic activities potentially correlated to the phenolic composition are expressed at higher level in non-*Saccharomyces* yeasts than in *S. cerevisiae* (Gutiérrez-Escobar et al., 2021).

Our results confirmed this theory. In fact, as showed in the figure 4.9, the value of total phenols and anthocyanins content is higher in wines from mixed fermentations than values found in the single starter wine (C).

The wine obtained with *S. cerevisiae* single strains showed an anthocyanins content of 561 mg/L, while the vinification performed by *H. osmophila* reached an anthocyanin content of 463 mg/L (C+NF) and 763 mg/L (C+NM), values higher than that observed for *S. cerevisiae*. The same trend was observed for total polyphenol content.

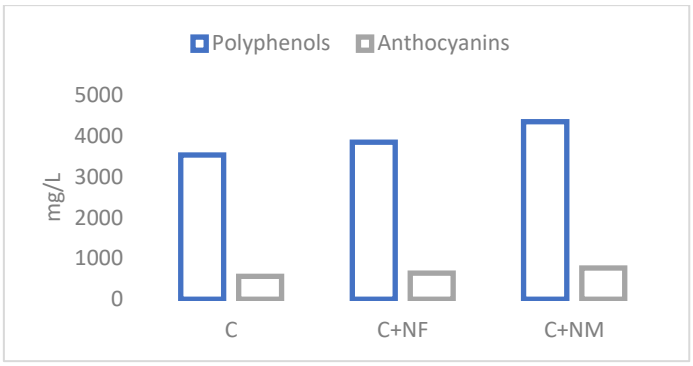


Figure 4.9 Polyphenols and anthocyanins contents in wines obtained by the mixed starters with *S. cerevisiae* (C) and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells in comparison to single starter wine with *S. cerevisiae* (C).

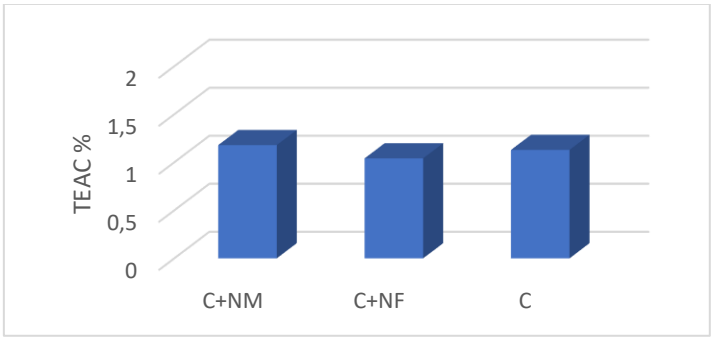


Figure 4.10 TROLOX µM TROLOX EQUIVALENT in wines obtained by the mixed starters with *S. cerevisiae* (C) and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells in comparison to single starter wine with *S. cerevisiae* (C).

The antioxidant activity of all the experimental wines was evaluated by measuring the radical scavenging activity (DPPH). No difference among the three samples were found; the wines obtained with *H. osmophila* microencapsulated and the control fermentation showed a slightly higher value in confront to the sample with *H.osmophila* as free cells.

4.2.6 Aromatic compounds of experimental wines

The experimental wines obtained by the mixed starters with *S. cerevisiae* and *H. osmophila* ND1 as free (NF) and microencapsulated cells (NM), in comparison to single starter wine (C), were analyzed also for the content of some secondary compounds affecting wine aroma.

As reported in Table 4.2 differences were found between the control (C) and the wines from mixed starters for almost all the analyzed volatile compounds.

Table 4.2 Main volatile compounds of wines obtained during pilot-scale vinifications in Aglianico del Vulture grape must by the mixed starters with *S. cerevisiae* (C) and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells in comparison to single starter wine with *S. cerevisiae* (C).

Volatile compounds (mg/l)	C+NF	C+NM	C
Acetaldehyde	28.24	32.19	30.07
Ethyl acetate	49.42	42.75	29.64
n-propanol	26.47	26.07	16.69
Isobutanol	26.87	26.22	30.31
n-butanol	26.80	28.43	16.89
Acetoin	15.34	6.72	12.63
Acetic acid	273.84	321.80	330.35
d-amyl alcohol	63.89	67.60	64.74
Iso-amyl alcohol	170.07	174.10	172.23

As regards ethyl acetate, the content detected in the wines was in the usual range (10-75 mg/L); as expected, an increase of ethyl acetate content was found in samples inoculated with mixed starter (49.42 and 42.75 g/L in C+NF and C+NM respectively) than wine from single culture fermentation (29.64g/L).

Higher alcohols represent the largest group of volatile metabolites, synthesised by yeast during alcoholic fermentation. Mixed starters fermentations determined higher levels of these higher alcohols (mainly in the inoculation with free cells of *H. osmophila*) in which the highest levels of n-butanol, n-propanol, isobutanol were found, while similar levels of d-amyl alcohol and iso-amyl alcohol were found in wine fermented with mixed and single starters.

The sample fermented with microencapsulated ND1 cells contained much lower level of acetoin (6.72 mg/L) respect the other two experimental wines, in which the values were 15.34 and 12.63 (mg /L) for C and C+NF, respectively.

Acetoin is a sub-product of the glycerol pyruvic fermentation; at the beginning of fermentation the yeasts produce diacetyl which is then reduced in the following phases to acetoin and 2,3 butanediol during contact of the wine with the biomass of the yeast. Both diacetyl and acetoin give to wine an

aroma of butter. Therefore, limited concentrations of these compounds positively increase the sensorial complexity of the wine, on the contrary, excessive levels can lead to a decline in the organoleptic quality of the product.

As regards acetaldehyde, low levels of this compound give a desirable fruity aroma, whereas an excessive content produces an apple-like off-flavor in the wine, and levels more than 200 mg/L cause wine flatness. The wine inoculated with mixed and single starters contained very similar levels of acetaldehyde and below the threshold value (100 mg/L).

The principal component analysis (PCA) was applied to all the parameters determined in the experimental wines obtained by the mixed starters with *S. cerevisiae* and *H. osmophila* (ND1) as free (NF) and microencapsulated (NM) cells in comparison to the single starter *S. cerevisiae* (C) to better visualize the differences in the chemical profiles of wines produced. The first principal component (PC1) explains 68.84% of data variability, while the (PC2) explain 31.16%; the plot of the three wines on the plane defined by these first two components is shown in Figure 4.11.

As explain the loadings values, the first principal component (PC1) was mainly correlated with ethyl acetate, n-propanol, butanol, total acidity and organics acids, phenols, whereas acetaldehyde, acetoin, acetic acid contributed more strongly to the second principal component (PC2).

The scatter plot of the three wines on the plane (Figure 4.11) displays that the wines were located in different quadrants in function of the starter formulation, indicating differences in the chemical composition of the obtained wines in function of starter used for inoculation of grape must.

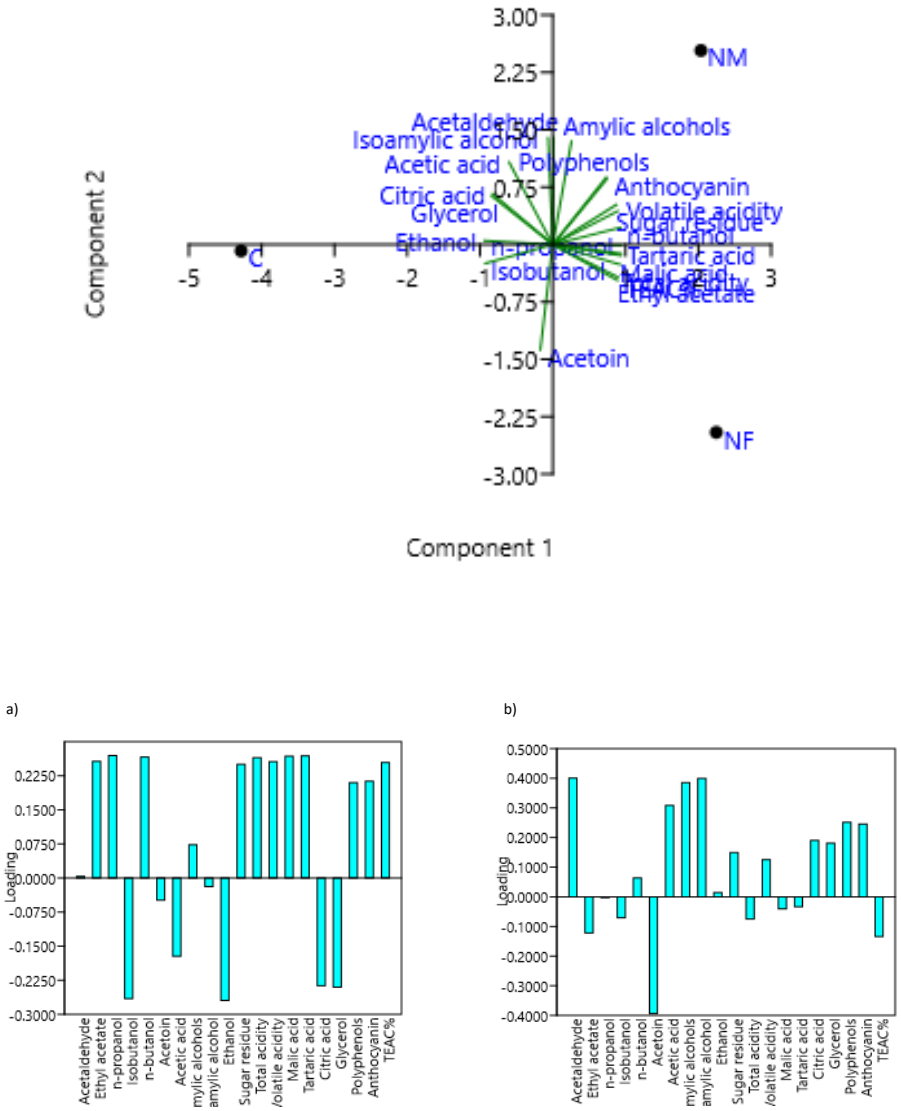


Figure 4.11 Principal component analysis (PCA) biplot of volatile compounds and main oenological parameters determined in wines obtained by the mixed starters with *S. cerevisiae* (C) and *H. osmophila* ND1 in two different formulations (NF= free cells; NM=microencapsulated cells), loading scores related to the PC1(a) and PC2 (b).

4.3 Conclusion

The management of multi-starter fermentation is more complex than monoculture fermentation, for this reason the application of this modern approach multi-species must be accompanied by a rigorous selection of yeast species for oenological and technological characteristics to identify the species will show desirable characteristics that can help producers to keep the production process under control and avoid the development of unpleasant aromas.

The knowledge by the wine producers of the characteristics of the "mono-species" and "multispecies" starters, will allow them to produce a wide range of different styles of wine by selecting a market segment defined by the characteristics of the product.

The biodiversity of non-*Saccharomyces* yeasts represents a potential instrument for the wine industry that is still partially unused. Nowadays, one of the most promising application of microbiological approaches in wine industry, most appreciated by consumers and producers, is the reduction of ethanol content in wine based on the selection of species for their low yield in ethanol and the formation of the by-products of alcoholic fermentation.

In the present research phase, the fermentative abilities of *H. osmophila* ND1 strain were analysed in mixed starter fermentation performed at cellar scale to evaluate the behaviour of selected multi-starter at industrial level and the feasibility of microencapsulation system in cellar application.

Our results demonstrate that the use of the mixed starter affected the wine final quality and display also promising for a reduction of ethanol content respect the control fermentation conducted by monoculture of *S. cerevisiae*.

In both mixed fermentations a good fermentative efficiency was highlight by *Hanseniaspora* both as free both as microencapsulated cells.

The evaluation of the viable population in the early stages of the mixed fermentation showed a good persistence of *Hanseniaspora* cells in both tested mixed starter formulation, demonstrating that the microencapsulation system plays an important role in the fermentative performance of the *H. osmophila* strain as a mechanism of protection against the stress agents, particularly ethanol produced during the fermentation, or cell to cell contact with other yeast specie in the grape juice.

As regards the aromatic characteristics of the final wines, a very similar profile of secondary compounds was found between the samples of mixed fermentations with ND1 (free/microcapsule), on the contrary the use of mixed starter affected the content of some aromatic compounds, which were found at different levels respect to the levels detected in wine obtained in the control fermentation. The presence of *H. osmophila* in fermentation increased the concentration of ethyl acetate, higher alcohol, n-propanol and butanol.

Only the acetoin production results influenced by cells formulation of ND1 strain, showing less content in the sample with *H. osmophila* as immobilized form.

In conclusion, the use of the ND1 strain (in both formulations) in simultaneous fermentation tests at cellar scale has shown promising both to reduce the content of ethanol in wine and to modify some aromatic component.

However, further investigations are necessary for the industrial use of this mixed starter, in particular it has to be evaluated the potential increase in process costs due to the use of microencapsulated cells.

The use of immobilized cells offers many other advantages compared to free cell systems, for example it facilitates the separation and recovery of biomass, minimizes the risk of contamination,

reduces process times. However, it also has some disadvantages mainly related to the costs and complexity of the production process. In fact, this technique, although well established in the production of beer and sparkling wine, has many problems in its application at industrial scale. For this reason, future research will have to be directed to large-scale applications of this technology, as well as to the study of conservation and storage methodologies that could be easily adopted at industrial level (Nedovik et al., 2011).

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Chapter V

Gene expression analysis in mixed starter fermentations involving Saccharomyces cerevisiae and Hanseniaspora osmophila

Abstract

In order to investigate the influence of *Hanseniaspora osmophila* on *Saccharomyces cerevisiae* during mixed starter fermentations, the gene expression of some key enzymes of the alcoholic fermentation pathway were studied. Therefore, the expression levels of some genes were analyzed in the *S. cerevisiae* transcriptome during the first hours of mixed fermentations (3 and 12 hours) in comparison to single starter fermentation. This research phase was conducted in collaboration with the Instituto de Ciencias de la Vid y del Vino (CSIC-Universidad de La Rioja-Gobierno de La Rioja), Logrono, under the supervision of Dr. Jordi Tronchoni, and in collaboration with the Cogentech laboratory (Milan, Italy).

5.1 Introduction

The study of microbial interactions in winemaking is generally based on the transcriptomic approach, for example, studies have shown that *S. cerevisiae* reduces its global transcription activity in mixed fermentations with *Hanseniaspora guilliermondii* (Curiel et al., 2017), by altering gene expression, through the up-regulation of genes correlated with the biosynthesis of vitamins and down-regulation of the genes involved in the absorption and biosynthesis of amino acids. Other studies have also shown significant transcriptional reprogramming in mixed fermentations of *S. cerevisiae* in combination with *Torulaspora delbrueckii* by simultaneous inoculation, for both strains as soon as 2 hours after being brought into contact. Furthermore, several studies have shown that the co-cultivation of *S. cerevisiae* with different non-*Saccharomyces* yeast species corresponds to changes in the *S. cerevisiae* transcriptome over short periods of time during fermentation, i.e. after 3 and 12 hours from the start of the process (Tronchoni et al., 2017, Curiel et al., 2017, Mencher et al., 2021). Therefore, on the basis of these studies, it was decided to verify whether *S. cerevisiae* EC1118 strain in co-culture with *H. osmophila* ND1, shows transcriptional changes in a set of genes. The chosen genes were selected on the basis of results obtained in previous works, in which their expression was significantly increased or decreased in co-cultivation of *S. cerevisiae* with different non-*Saccharomyces* yeasts, such as *T. delbrueckii*, *Candida sake*, *H. uvarum* or *Metschnikowia pulcherrima*.

The genes analyzed are the following: *PUT1*, *ALD6*, *OLE1*, *GAP1*, *GPH1*, *PUT2*, *HXT4*, *INA1* and *OAC1*.

5.2 Role of the selected genes

GAP1

The availability of nitrogen is one of the main parameters influencing alcoholic fermentation, the speed of the process and its completion. In fact, the yeast growth is correlated with both the quantity and the quality of the available nitrogen sources. A common practice in the wine industry is the addition of inorganic nitrogen, such as ammonium salts, to grape must before the start of fermentation. The presence of nitrogen compounds is linked to the grape variety and harvesting practices, the main sources are ammonium ions (about 40% assimilable nitrogen) and some amino acids, such as arginine and proline in the juice (30-65% of the content total amino acids). *Saccharomyces cerevisiae* selects the nitrogen sources that allow for greater growth through a mechanism called repression of nitrogen catabolites (NCR). Therefore, the *GAP1* gene, encodes a permease that transports all amino acids across the plasma membrane, this is transcriptionally regulated by *GLN*3 and *URE2* and is inactivated by dephosphorylation in the presence of good nitrogen sources, such as glutamate and glutamine. In these conditions the transport of amino acids is reduced, while in the presence of a poor nitrogen source, such as proline or arginine, a higher permease activity is induced. The *GAP1* gene is regulated in response to the available nitrogen

source. The absorption of nitrogen can affect the production of aroma and unpleasant compounds, such as hydrogen sulfide and urea (the main precursor of ethyl carbamate). In conclusion, the GAP1 gene transcription is a good indicator of nitrogen availability during wine fermentations (Chiva et al., 2008).

OLE 1

Stopped fermentation is among the most feared problems by winemakers and is related to the composition of the musts and to the availability of nitrogen and oxygen.

Recently it has been shown that this phenomenon can also depend on the lipid content of grape must. In fact, fatty acids and sterols play a fundamental role in the vitality of yeast, they influence the fermentation capacity and adaptation to changes in temperature and the presence of ethanol. The *OLE1* gene encodes the unique Delta-9 fatty acid desaturase of *S. cerevisiae*, a membrane protein for the production of monounsaturated fatty acids. Since these fatty acids are critical components of cell membranes, the *OLE1* gene is essential. In the alcoholic fermentation, the prolonged anaerobic conditions underregulate the synthesis pathways of fatty acids and sterols limiting the growth of yeasts. In these conditions, cell development is only influenced by the lipid composition of the grapes. In fact, it has been observed that must clarification practices can reduce the production of unsaturated fatty acids and sterols, in these cases it is advisable to add oxygen in the early stages to promote the consumption of sugars and the biosynthesis of lipids (Zara et al., 2007).

PUT 1* and *PUT 2

Proline is an amino acid necessary for protein synthesis and is a good source of nitrogen in grapes. When nitrogen sources are no longer available, proline is converted by *S. cerevisiae* into glutamate in the mitochondria by proline oxidase (product of the *PUT1* gene) and D1-pyrroline-5-carboxylate dehydrogenase (product of the *PUT2* gene). The expression of *PUT* genes is regulated by the activator protein *PUT3*. Transcriptional upregulation occurs only in the presence of proline and in the absence of a yeast-preferred nitrogen source. The regulation of these genes also occurs through an effect known as nitrogen catabolite repression (NCR), this mechanism prevents the use of proline as a nitrogen source if better nitrogen compounds such as ammonia, asparagine or glutamine are present. The presence of proline in the medium increases the transcription of the *PUT1* and *PUT2* genes (Salmon et al., 1998).

GPH1

In the yeast cell the activity of glycogen phosphorylase is regulated by the cyclic phosphorylation of the enzyme mediated by AMP. *GPH1* is not an essential gene in yeast cells, in fact the absence of *GPH1* does not prevent the cells from synthesizing and storing trehalose.

ALD

Aldehyde dehydrogenases play an important role in the formation of acetylCoA starting from acetaldehyde. Acetaldehyde is formed during the metabolism of pyruvate to acetate by the cytoplasmic bypass pathway of pyruvate dehydrogenase (PDH), involving the enzyme pyruvate decarboxylase (*PDC6*, *PDC5*, *PDC1*), acetaldehyde dehydrogenase (*ALD6*) and acetyl-CoA synthetase (*ACS1*). Alternatively, pyruvate is first decarboxylated to acetaldehyde in the cytosol by pyruvate decarboxylase and then converted into acetate by mitochondrial acetaldehyde dehydrogenases (*ALD4* and *ALD5*). Ald2p and Ald3p are cytosolic enzymes that only use NAD^+ as a cofactor. Both genes are induced in response to ethanol or stress and repressed by glucose. Ald4p and Ald5p are instead mitochondrial and use NAD and NADP as cofactors and are K+ dependent. *ALD6* encodes the activated cytosolic enzyme Mg^{2+}, which uses $NADP^+$ as a cofactor and is constitutively expressed. Aldehyde dehydrogenases are conserved in many species and are key enzymes in metabolic pathways, some of which function to detoxify harmful chemical intermediates.

HXT

Saccharomyces cerevisiae has a large family of transporter genes that allow the passage of hexose sugars across the plasma membrane, this group of genes is called "*HXT*" or HeXose Transport. They include genes *HXT1* to *HXT17*, *GAL2*, and the *SNF3* and *RGT2* glucose sensors. During fermentation, the concentration of sugars decreases and at the same time the ethanol content increases, this situation represents a stressful condition for the cells and can have negative effects on the activity of membrane proteins. Furthermore, the rate of consumption of sugars is different, glucose is consumed faster than fructose, which means that the relative concentrations of glucose and fructose will change from approximately equimolar to pure fructose concentrations in the next stage of fermentation. Hexose's transporters have the inherent ability to transport glucose or one of the other hexoses (Karpel et al., 2008).

IZH1

IZH1, *IZH2*, *IZH3* and *IZH4* genes encode a family of membrane proteins which influence the content of membrane sterols and can alter cellular Zinc levels. This family of proteins is characterized by the presence of at least seven transmembrane domains and four highly conserved motifs rich in metal-binding amino acids. *IZH* genes show high expression in zinc-deficient cells. *IZH1* and *IZH2* are direct targets of the transcription factor Zap1p which detects zinc deficiency, while *IZH4* is induced by excess zinc. *IZH1*, *IZH2* and *IZH4* are also induced by fatty acids via the Oaf1p / Pip2p complex which binds to the oiled response elements (*ORE*). In situations of zinc deficiency, the induction of *IZH1* and *IZH2* by Zap1p leads to the overexpression of the Izh proteins, this suggesting a connection between these genes, sterols and zinc metabolism. Therefore, the elimination of the *IZH1* or *IZH2* genes leads to an increase in zinc sensitivity, on the contrary it decreases by the elimination of *IZH3*

or *IZH4*. Possible functions for Izh proteins include sterol metabolism, influence on plasma membrane permeability, homeostasis of cations such as zinc, zinc transporters (this would explain their regulation by Zap1p and their effect on the activity of Zap1p).

OAC1

OAC catalyzes both one-way transport and substrate exchange. In *S. cerevisiae*, it is found in the internal mitochondrial membranes and deletion of its gene reduces the transport of oxaloacetate sulfate, thiosulfate and malonate. The main physiological role of the *OAC* gene seems to be to use the proton-motive force to absorb the oxaloacetate produced by pyruvate in the mitochondria thanks to cytoplasmic pyruvate carboxylase. (Palmieri et al., 1999).

5.3 Technique useful for the analysis of gene expression

Molecular methods are increasingly used to study, detect, and quantify microbial populations in food products. They allow to analyze entire microbial communities, their composition, their dynamics. Among these, quantitative real-time PCR (qPCR) is one of the main techniques used for the detection and quantification of microbial populations. The qPCR and the RT-qPCR are very fast and highly sensitive methods, specific and allow to simultaneously detect different microorganisms; furthermore, these are accurate and reliable techniques for the quantitative analysis of gene expression. In fact, real time-PCR represents one of the main technologies for detecting, and quantifying traces of mRNA and the level of expression of specific genes, a combined technique that involves a retro-transcription (rt) reaction followed by a polymerase chain reaction of quantitative type (qPCR). For this reason, before carrying out the PCR in real time, it is necessary to reverse-transcription in cDNA. In general, the quantitative PCR (qPCR), compared to the traditional "end-point" PCR, is a quantitative and not qualitative technique, it does not require the final analysis of the amplified product on agarose gel, but the quantification takes place in real time during the exponential phase of amplification kinetics. The real time PCR allows to determine the quantity of initial template (genomic DNA or cDNA) by monitoring the amplification reaction using fluorophores or fluorescent probes. Among the intercalating agents used in the Real-Time quantitative PCR technology, the main ones are the dyes intercalating in the DNA, able of emitting fluorescence if suitably excited, or hybridization probes. For Real-Time PCR the most widely used intercalating fluorophore is Syber green, a cyanine which, if combined in the double helix, produces a fluorescence signal 200 times greater than when it is free in solution. This fluorescence is directly proportional to the amount of DNA produced. The Syber green does not have a specificity of sequences like the probes, therefore also non-specific DNA amplifications are detected. On the contrary, the probes are designed for hybridization in a specific DNA region. An example of a probe is the Taqman, an oligonucleotide complementary to a specific DNA sequence identified within the sequence amplified by the primers, which has a Reporter molecule at the 5 'end and a Quencer

molecule at the 3', which prevents the fluorescence emission by the reporter. During the extension phase, the polymerase cuts the 5 'end of the probe, removing the reporter from the template. In this way, the reporter molecule passes into solution, increasing the intensity of the fluorescence which will be directly proportional to the concentration of amplification product. The equipment used for the Real-Time PCR analysis is a thermal cycler, equipped with a laser excitation system (as light source) and a detector, that collects the fluorescence emitted at each cycle at the specific emission wavelength. The DNA template is initially single-stranded and therefore no signal is detected by the instrument, subsequently the double-stranded is synthesized, and its fluorescence begins to be detected by the instrument. After a certain number of cycles, the fluorescence level will increase, and the number of cycles required for the formation of a measurable quantity of the product can be determined. This value, known as “Ct”, is related to the initial DNA quantity, larger quantities of the initial DNA will give rise to lower Ct values. The detected signal is transmitted to a computer and analyzed by a software, which gives the results as a graph, called "Amplification plot". This graph correlates the fluorescence signal with the number of cycles, and it can be divided into different phases (baseline, exponential, linear, plateau). The baseline represents the non-specific fluorescence emitted during the initial cycles; when a significant amount of the target amplicon begins to accumulate, the exponential phase starts. Above the baseline, the "threshold" line (with a fluorescence level greater than that of the baseline) is fixed, which intersects all the amplification curves of the samples within the exponential region. The Ct (threshold cycle) is identified by the number of cycles at which the accumulation of an amplicon crosses the line associated with the threshold. Subsequently, the reaction enters the linear phase, and the amplification decreases, thus entering the plateau phase (constant fluorescence). At the end of the trial (about 30 -40 cycles), the software collects and normalizes the data, on the basis of internal or passive reference. Normalization is obtained by dividing the emission intensity of the fluorophore by that of the internal reference (ROX), a fluorophore already presents in the master mix. The ratio obtained is thus referred to as rn (normalized reporter). The software allows to view the results in terms of fluorescence (rn) or number of cycles or of the two combined measurements. The quantitative analysis of fluorescence can be absolute or relative. The absolute approach is based on the interpolation of the data obtained on a calibration line (log c-DNA vs cycles) to quantify the unknown concentration of a DNA sample. The relative approach, on the other hand, allows to make a comparison between different samples. The data obtained are normalized with respect to a certain target, an endogenous control, that is a gene present in the same concentration and with the same level of expression in all the samples examined to eliminate the effect of the variability in the different reactions. The genes generally used as a reference are called "housekeeping" genes, which code for molecules that act as basic components of cell organelles or that participate in the basic biochemical metabolism of the organism and probably not regulated or influenced by the environment. Among housekeeping genes, the main ones are β-actin, rRNA 18s or glyceraldehyde-

3-phosphate dehydrogenase. A calibrator is also identified, that is the control sample with respect to which the comparison is to be made. The data on the amount of target gene present in the sample are normalized with respect to the amount present in the reference. The most used quantification method is the comparison of Ct.

5.4 Materials and Methods

5.4.1 Wine fermentations

The strains were produced in flasks using YPD as growth medium; the biomass was recovered by centrifugation after 24 hours of growth.

Fermentations were carried out in 100 mL of natural grape must (Aglianico variety) previously pasteurized (T=90°C for 20 minutes).

The two yeast strains were simultaneously added in grape must by using different inoculation ratio. *S. cerevisiae* was inoculated in all mixed fermentation samples at concentration of $2*10^3$ cell/mL, whereas *H. osmophila* was inoculated at concentration of $2*10^7$ cell/mL. In the control fermentations, only *S. cerevisiae* cells at concentration of $2*10^7$ cell/mL was inoculated.

All the experiments were monitored, and after three and twelve hours the biomass was recovered by centrifugation, washed three times using EDTA solution (50mM) and stored at -80°C util RNA extraction.

5.4.2 RNA extraction

RNA extraction was performed by using the “Aurum ™ RNA Mini Kit” (BIO-RAD). For RNA extraction, an aliquot of yeast culture with OD value at 600 nm equal to 3 was transferred in 2 mL tubes and was centrifugated at maximum speed for 1 minute. The supernatant was eliminated, and the obtained pellet was added with 1 mL of Lyticase (50 units / mL), previously diluted in buffer and equilibrated to 30 °C. The pellet was completely resuspended in the buffer and incubated for 10 minutes. After this time, the tubes were centrifuged at 5000 rpm for 5 minutes, the supernatant was removed and 350 µL of lysis solution were added to the pellet. After this step, 350 µL of ethanol (70%) was added to the cell lysate and was transferred to the column provided by the RNA binding kit, placed inside 2 mL tubes. The tube was centrifuged for 30 seconds, the eluate was removed, and the column was washed with 700 µL of “low stringency solution”, then it was centrifuged at 13000 rpm for 30 seconds. The eluate was removed, and the digestion with DNase was carried out directly on the column by pipetting 80 µL of a solution consisting of 5 µL of DNase I (reconstituted in 10 mM TRIS buffer, pH 7.5) diluted in 75µL of DNase dilution solution. It was left to act for 15 minutes at room temperature, subsequently, the column was washed with 700 µL of "high stringency solution” and centrifuged for 30 seconds. The eluate was removed, and the column washed with 700 µL of

low stringency solution, centrifuged for 1 minute at 1300 rpm, after that the eluate was removed and centrifuged again for 2 minutes. At this point, it was transferred in a new 1.5 mL Eppendorf in which was added with 40 µL of elution solution and left to act for 1 minute to saturate the RNA-binding membrane column, then it was centrifuged for 2 minutes at 13000 rpm to elute the RNA extract. The RNA was used immediately in the reverse transcription reaction or stored at -80 ° C until its use.

5.4.3 RNA dosage and purity grade evaluation

The RNA extracted was measured by spectrophotometry using the Nanodrop instrument. This technique is based on the ability of nucleic acid molecules to absorb UV light at 260nm. The instrument allows to obtain the nucleic acid concentration value (ηg/µL) and also some parameters correlated to the degree of purity of the analyzed samples, such as the ratio between the absorbance values measured respectively at the wavelengths of 260 and 280nm. The A260 / A280 ratio is an indicator of contamination from proteins, a value comprised between 1.9 and 2.1 indicates a good degree of purity of the sample, while higher values indicate contamination by proteins, whereas lower values indicate a contamination by DNA. The A260 / A230 ratio indicates contamination by phenols, aromatic compounds and carbohydrates and must be between 2-2.2.

5.4.4 Qualitative analysis by electrophoresis

In order to evaluate the structural integrity of the extracted RNA, the samples were analyzed by electrophoresis on agarose gel (2%) in 1X TBE buffer (Tris-borate-EDTA). After loading of 2 µL of RNA mixed with 8 µL of H_2O-DEPC (RNase free) and 1 µL of loading dye into the gel, the electric field is activated by applying a potential difference of 90 V for 30 minutes. Finally, the image of the agarose gel is analyzed by UV transilluminator (BIO RAD Gel Doc TM XR) for visualization of the RNA bands. Two bands, corresponding to 28S rRNA and 18S rRNA, should be highlighted on the gel.

5.4.5 qPCR Analysis

The qPCR analysis was performed in collaboration whit COGENTECH laboratory (Milan, Italy).

5.5 Results

5.5.1 Evaluation of RNA purity

The quantification and purity analysis of RNA extracted were reported in the table 5.1. different amounts of RNA were isolated by the different samples, whereas the quality and purity of isolated RNA fall in the desirable values for almost all the analyzed samples.

SAMPLE	Abs 260/280	Abs 260/230	ng/μL
EC 1118 biomass	2.27	2.05	82.77
EC 1118 T3	2.21	0.62	35.87
EC 1118 T12	2.25	1.57	48.6
EC 1118 + ND1 T3	2.24	1.76	99.93
EC 1118 + ND1 T12	2.40	0.54	25.2

Table 5.1 Evaluation of purity and quantity (ng/μL) of RNA isolated from EC1118 biomass, single fermentation with EC1118 after 3 and 12 hours (EC1118 T3 and EC1118 T12, respectively) and mixed fermentation with EC1118 and ND1 after 3 and 12 hours (EC 1118 + ND1 T3 and EC 1118 + ND1 T12, respectively).

5.5.2 Evaluation of genes expression at different fermentation times

Based on previous work by Tronchoni and co-workers (Tronchoni et al., 2017, Curiel et al., 2017, Mencher et al., 2021), a set of genes was selected for analysis by qPCR. In these works, co-cultivation of *S. cerevisiae* with different non-*Saccharomyces* yeast species identified different set of genes that respond to co-cultivation modifying the transcriptome of *S. cerevisiae* at short time periods, specifically at 3 and 12 hours. Based on these works, it was decided to check whether in EC1118 in co-culture with *Hanseniaspora osmophila*, transcriptional changes were observed in several genes that had shown significant differences in expression between mixed cultures and simple cultures in the previous mentioned works. This work was done in collaboration with Dr. Jordi Tronchoni.

Time points for the qPCR assay were establish at 3 and 12 hours under co-cultivation in grape must. Although these are preliminary results and in some cases the samples in co-culture were not amplified correctly (*INA1*, *OAC1* and *IZH1* at time point 12 hours), in some cases differences between samples in co-cultivation and *S. cerevisiae* single fermentation were found. For instance, the general amino acid permease *GAP1* appears up-regulated at 3 and 12 hours in the single fermentation, in contrast with the co-culture where it is down-regulated at both time points (figure 5.1).

Figure 5.1 GAP1 expression in EC 1118 biomass (Ec0); in monoculture fermentation with EC1118 and in mixed fermentation samples by EC1118 and ND1, after three (Ec_t3; Ec+ND_t3;) and twelve hours (Ec_t12; Ec+ND_t12).

Figure 5.2 ALD6 expression in EC 1118 biomass (Ec0); in monoculture fermentation with EC1118 and in mixed fermentation samples by EC1118 and ND1, after three (Ec_t3; Ec+ND_t3;) and twelve hours (Ec_t12; Ec+ND_t12).

For the rest of the genes assayed, results seem to follow the same trend of *GAP1* gene among conditions with little differences in quantification, this is true for *GPH1*, *HXT4*, *OLE1*, *PUT1* and *PUT2*. The use of a set of reporter genes would simplify the assessment of whether the strains chosen for co-cultivation influence the *S. cerevisiae* transcriptome in a way that could jeopardize fermentation.

Figure 5.3 GPH1 expression in EC 1118 biomass (Ec0); in monoculture fermentation with EC1118 and in mixed fermentation samples by EC1118 and ND1, after three (Ec_t3; Ec+ND_t3;) and twelve hours (Ec_t12; Ec+ND_t12).

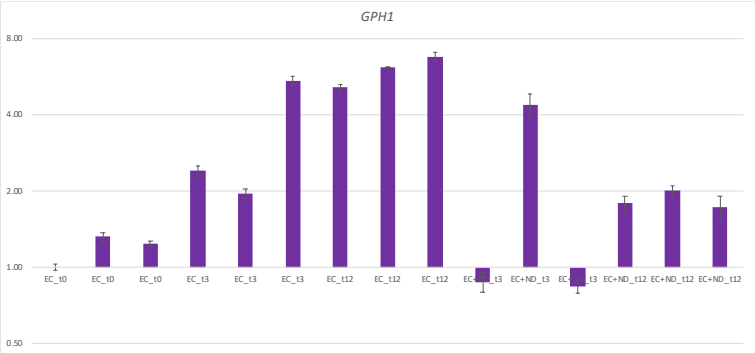


Figure 5.4 HXT4 expression in EC 1118 biomass (Ec0); in monoculture fermentation with EC1118 and in mixed fermentation samples by EC1118 and ND1, after three (Ec_t3; Ec+ND_t3;) and twelve hours (Ec_t12; Ec+ND_t12).

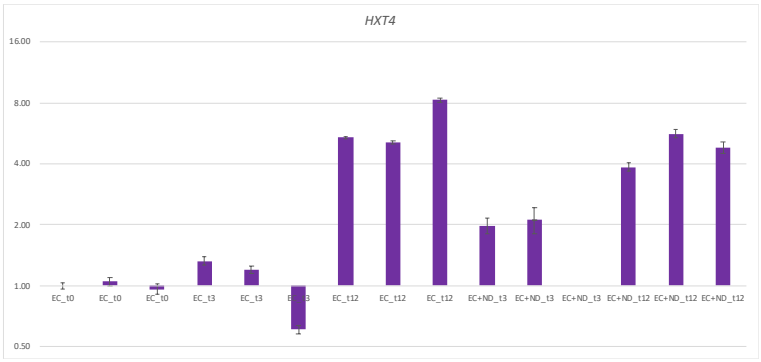


Figure 5.5 OLE1 expression in EC 1118 biomass (Ec0); in monoculture fermentation with EC1118 and in mixed fermentation samples by EC1118 and ND1, after three (Ec_t3; Ec+ND_t3;) and twelve hours (Ec_t12; Ec+ND_t12

Figure 5.6 PUT1 expression in EC 1118 biomass (Ec0); in monoculture fermentation with EC1118 and in mixed fermentation samples by EC1118 and ND1, after three (Ec_t3; Ec+ND_t3;) and twelve hours (Ec_t12; Ec+ND_t12).

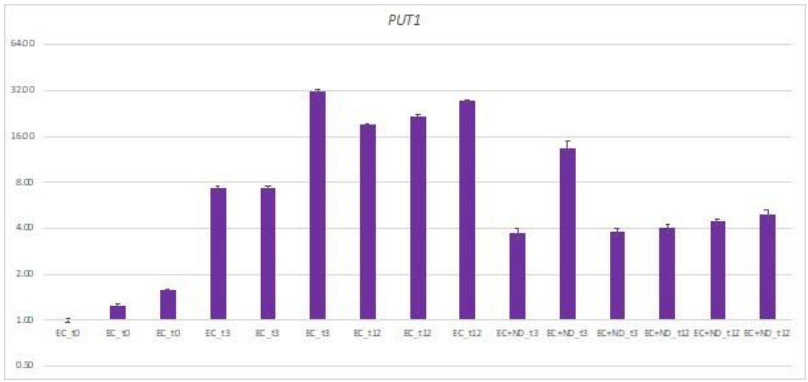
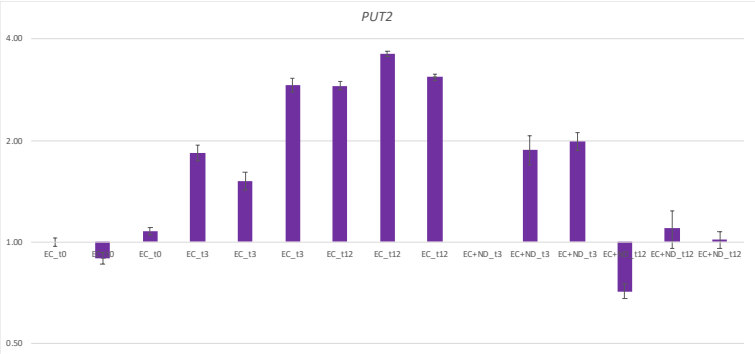


Figure 5.7 PUT2 expression in EC 1118 biomass (Ec0); in monoculture fermentation with EC1118 and in mixed fermentation samples by EC1118 and ND1, after three (Ec_t3; Ec+ND_t3;) and twelve hours (Ec_t12; Ec+ND_t12).



5.6 Conclusion

This research phase was addressed to the analysis of yeast-yeast metabolic interaction by the analysis of the different gene expression in *S. cerevisiae* during mixed fermentation. The environmental modifications of the must induced by the presence of *H. osmophila* might induce the alteration of the expression of some genes, such as *GAP1* and *ALD6,* in *S. cerevisiae* at the different time intervals analyzed. Further investigations are needed to better define the nature of the interactions that develop during mixed fermentations at the transcriptomic level.

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General conclusion

Warm climate and long ripening periods can lead to grapes with high concentrations of sugars, and this, in turn, leads to wines with high concentrations of ethanol which can compromise the quality of the wine, also increasing the perception of spiciness, viscosity and can lead to a decrease in aroma and intensity of flavor. In this context, although several technological applications have been proposed for the reduction of the alcohol content of wine, a promising alternative is represented by the biotechnological approach based on the use of mixed starter cultures, represented by selected associations of non-*Saccharomyces* yeasts and *S. cerevisiae*. In recent years, several studies have re-evaluated the use of non-*Saccharomyces* yeasts in controlled mixed fermentations with *S. cerevisiae*, indicating that the rational selection of this yeasts could allow the recovery of some positive qualitative characteristics, present in natural fermentations to produce wines characterized by a lower concentration of ethanol, compared to the levels reached with the single inoculum of *S. cerevisiae*. The general objective of this research project was the selection of non-*Saccharomyces* yeasts which, in mixed cultures with *S. cerevisiae*, can positively influence the aromatic quality of wines and produce a lower alcohol content. To this end, an initial screening phase was carried out on 29 non-*Saccharomyces* yeast strains of oenological interest, belonging to the species *T. delbrueckii*, *H. guilliermondii* / *osmophila*, *M. pulcherrima*, *P. fermentans*, *S'codes loudwigii*, based on the evaluation of some technological characteristics such as H_2S production level, resistance to ethanol, SO_2 and copper, evaluation of enzymatic activities with oenological impact (β - glucosidase activity), ability to tolerate high concentrations of sugars and ethanol, in order to select the most suitable strains to be part of a mixed starter culture with *S. cerevisiae*. The technological screening used in the present study confirmed that non-*Saccharomyces* yeasts represent a source of unexplored biodiversity that could be of great value to the wine industry. Based on the results obtained in the first screening on 29 non-*Saccharomyces* yeasts, we selected 12 strains that showed interesting and desirable properties to improve the sensory profile of wine, such as the presence of β-glucosidase activity, high tolerance to osmotic and oxidative stress as well as a good survival ability in the presence of ethanol. The strains possessing the best combination of these parameters belonging to the species *T. delbrueckii* (425), *P. fermentans* (LM5-3), *M. pulcherrima* (Mpr2-4), *H. guilliermondii* / *osmophila* (TSB / ND1), *S'codes ludwigii* (SIA2) have been selected to be used in combination with *S cerevisiae* to evaluate the behavior of the strains during the fermentation process and their oenological aptitude. The selected strains were tested as mixed starters with a commercial *S. cerevisiae* strain (EC1118) during laboratory-scale fermentation using different inoculation modalities (sequential and co-inoculation). Strain growth and fermentative kinetic were analyzed during fermentation to identify the conditions that allow the best yield of the selected

strains. The determination of the analytical and aromatic parameters of the wines (ethanol, volatile acidity, acetaldehyde, glycerol, and higher alcohols) allowed to evaluate the role played by non-*Saccharomyces* yeasts on the aromatic characteristics of wines. The data obtained in this phase showed that non-*Saccharomyces* yeast strains influence the ethanol content and the chemical and aromatic profile of the wines. Promising results were obtained from two yeast strains ND1 (*H. osmophila*) and 425 (*T.delbrueckii*), which showed good competitiveness in mixed fermentations, probably linked to the ability of this strains to survive during the fermentation process together with *S. cerevisiae* EC1118 and alcohol reduction, mainly if used in the co-inoculation modality. Their presence during fermentation increases the quantities of some secondary compounds, but also the acetic acid production in the samples obtained with mixed fermentation with *H. osmophila*, although the content remains in the acceptable range for wine. Based on the data obtained, the subsequent research phase was directed to an extensive investigation on a mixed starter culture, composed of the two non-*Saccharomyces* strains (ND1 and 425) and EC1118, to obtain a mixed starter, potentially useful at cellar level. With the aim to develop a mixed starter in dry form, in this research phase the selected non-*Saccharomyces* yeasts were subjected to spray-drying treatment at laboratory scale to identify the best experimental conditions to guarantee the maximum survival of the non-*Saccharomyces* yeasts. The results obtained highlighted the ability of both species to survive to drying process. The evaluation of the fermentation capacity of dried non-*Saccharomyces* yeasts during fermentations showed that the treatment did not affect the performance of 425 strain, whereas for the ND1 strain, the use as dried yeast has shown a faster consumption of sugars and a lower residue at the end of the process compared to the performance of the starter culture in fresh form. Furthermore, the use of dried cells in mixed fermentations has led to a reduction of volatile acidity in the wine produced by using *H.osmophila* strain. Considering the results obtained, the starter “ND1 + EC1118” was chosen for the use in fermentation as starter composed by non-*Saccharomyces* cells immobilized in microcapsules together with free cells of *S. cerevisiae*. Our results showed the good fermentative performance of *H. osmophila* strain used as microencapsulated cells in the production of wine characterized by a lower ethanol percentage than the wine obtained by pure culture of EC1118 and confirmed that the inclusion of the *H. osmophila* strain in the starter culture influenced the content of almost all aromatic compounds, with a significant increase in the level of 2-phenylethyl acetate.

Based on the results obtained in the laboratory screening, the final validation of the mixed starter culture, composed of ND1 (microcapsules / free cells) and EC1118, was performed during pilot-scale vinification at cellar level in Aglianico del Vulture must. The use of the mixed starter in both formulations (free and microencapsulated cells) in simultaneous fermentation tests at cellar scale has influenced the final quality of the wine, both in ethanol reduction and the aromatic properties of the wine produced, furthermore, the content of volatile acidity was not negatively influenced using the mixed culture. All the results obtained in this research confirmed that the combination in

mixed starter cultures of strains with different oenological characteristics represents an innovative fermentation strategy in commercial winemaking and its success depends on the selection of suitable combinations of yeast strains. Currently, the selection of mixed yeast cultures is one of the main challenges for the wine industry for identifying the most promising starter cultures to be used at the winery level to offer to winemakers the opportunity to obtain innovative and high-quality products.

Publications

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 ~~wine's~~ 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
H. osmophila	ND 1
Torulaspora delbrueckii	425, 365, LC 2-1
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). ~~The~~ Major variability was recorded within the species *H. guilliermondii*, with strain resistance ranging from 100

Table 2. Adimensional values assigned to technological parameters.

Parameters	Values			
	0	**1**	**2**	**3**
SO_2 resistance[a]	50	100–125	150–175	200
$CuSO_4$ resistance[b]	<100	100	200	300
H_2 S 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.

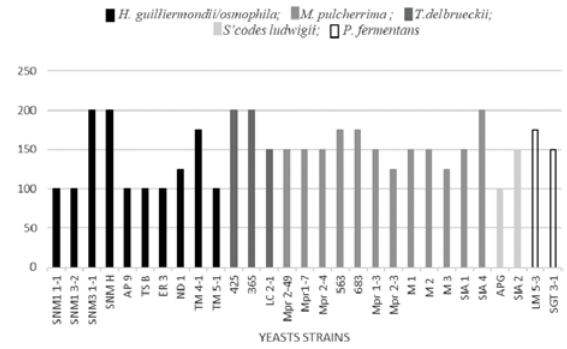

Figure 1. Tolerance level to SO_2.

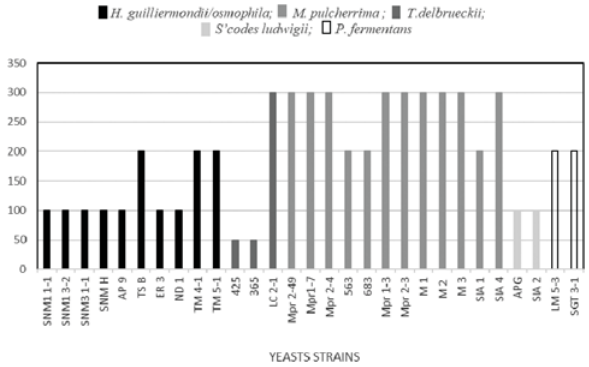

Figure 2. Tolerance level to $CuSO_4$.

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

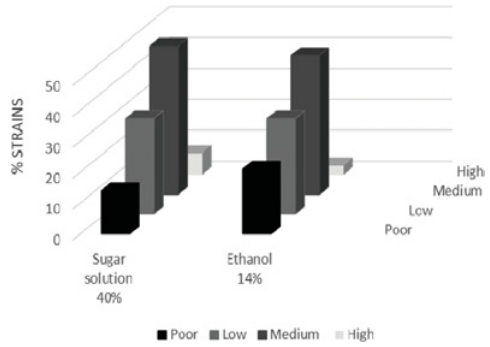

Figure 3. Strain growth in high sugar (40%) and ethanol (14%) concentrations.

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.

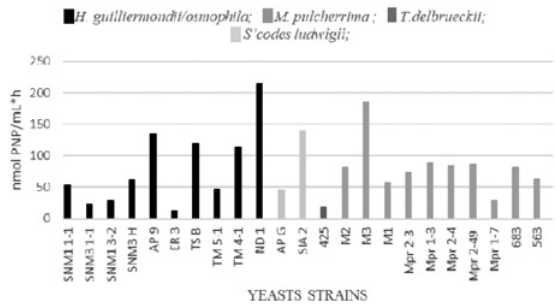

Figure 4. β-glucosidase activity exhibited by 23 strains.

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 chararacterization

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,

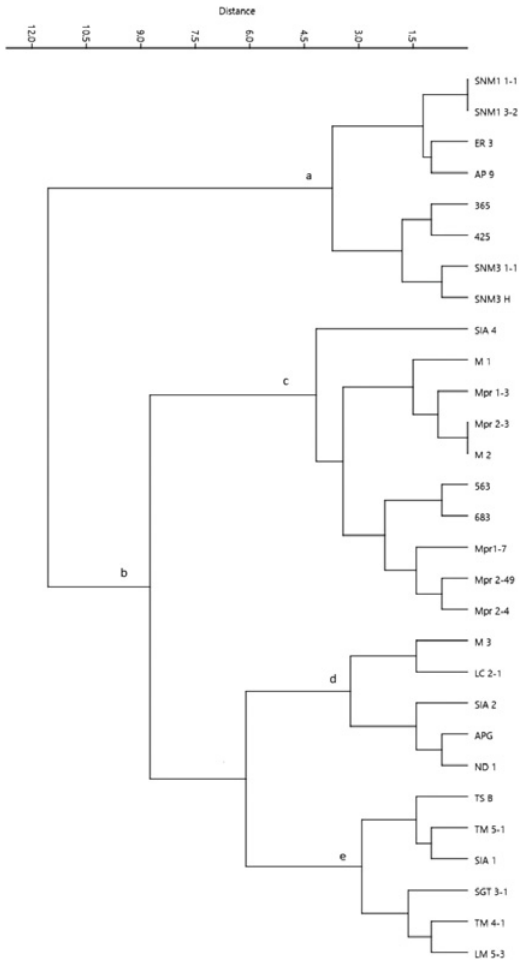

Figure 5. Dendrogram obtained after cluster analysis on data of technological characterization of 23 non-*Saccharomyces* strains.

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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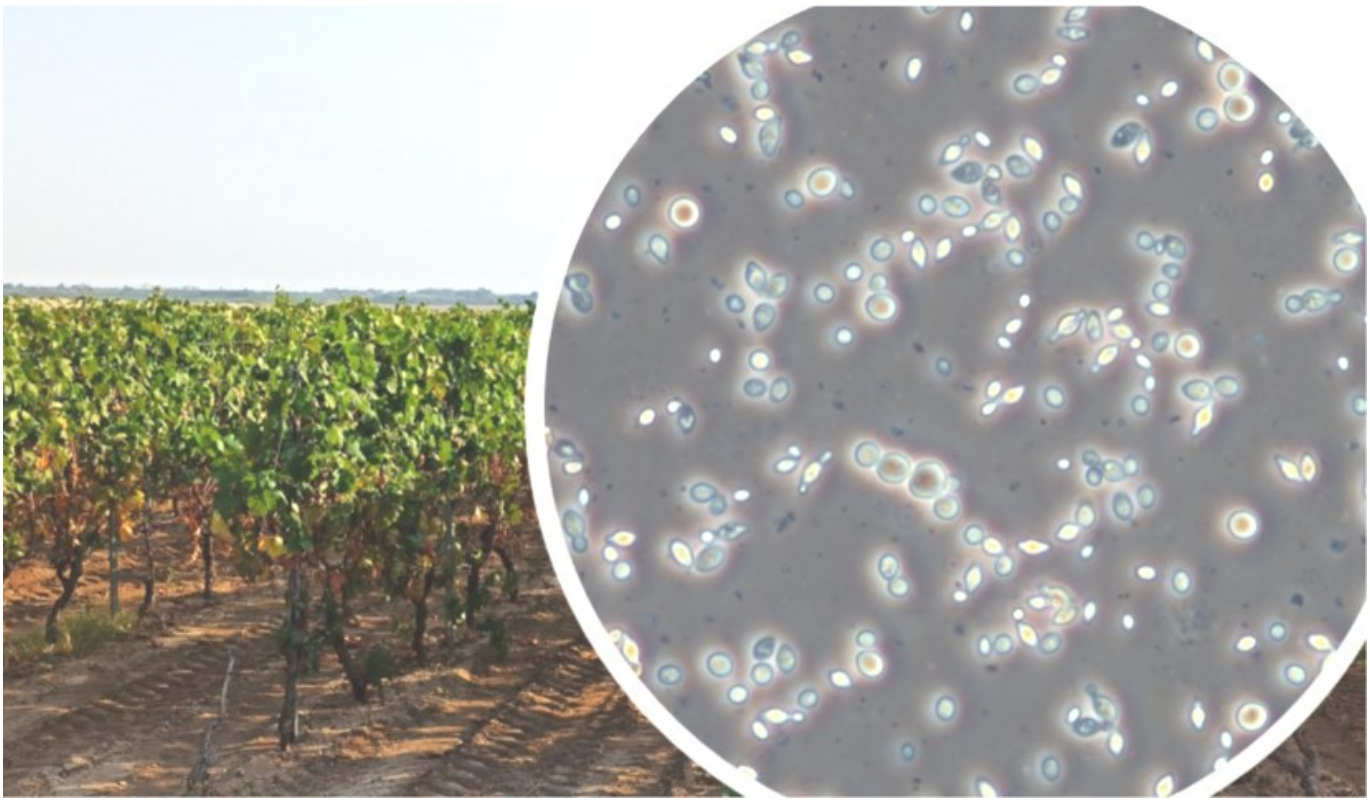
Focus

Lieviti non-Saccharomyces per ridurre il tenore alcolico del vino

Sebbene caratterizzati da scarso potere fermentativo, posseggono attività metaboliche che possono contribuire a formare un prodotto con maggiore complessità aromatica



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Data:*11 set 2020*

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L'articolo viene pubblicato nell'ambito della collaborazione avviata tra la rivista AGRIFOGLIO e la Scuola di Scienze Agrarie, Forestali, Alimentari e dell'Ambiente dell'Università della Basilicata.

Negli ultimi decenni nel settore enologico si osservano due tendenze contrastanti: da un lato un incremento del contenuto alcolico del vino, a seguito dell'aumento del contenuto di zuccheri nelle uve provocato dal riscaldamento globale, dall'altro la richiesta del mercato di bevande a ridotto tenore alcolico. Questa richiesta emerge sia dal crescente interesse dei consumatori verso il ruolo esercitato dall'alimentazione sulla salute sia in seguito all'emanazione di direttive, comunitarie ed extracomunitarie, volte a tassare bevande che superano una determinata percentuale di etanolo.

Pertanto, i viticoltori sono alla ricerca di soluzioni che permettano la produzione di “vini di nuova generazione”, caratterizzati da ridotto contenuto alcolico e caratteristiche aromatiche peculiari.

Negli ultimi anni sono state proposte diverse applicazioni di tipo tecnologico per la riduzione del tenore alcolico del vino, come osmosi inversa, nanofiltrazione, distillazione, che pur determinando una riduzione del tenore alcolico, non hanno alcuna influenza sul miglioramento del profilo organolettico dei vini prodotti.

Un’alternativa promettente è rappresentata dall’approccio biotecnologico, basato sull’impiego di colture starter specifiche. Questi starter possono essere rappresentati da lieviti ottenuti mediante tecniche di ingegneria genetica (lieviti OGM) o da starter detti “non convenzionali”, appartenenti al gruppo dei lieviti indicati come non-*Saccharomyces*, per distinguerli dal lievito solitamente usato come starter, ovvero *Saccharomyces cerevisiae*.

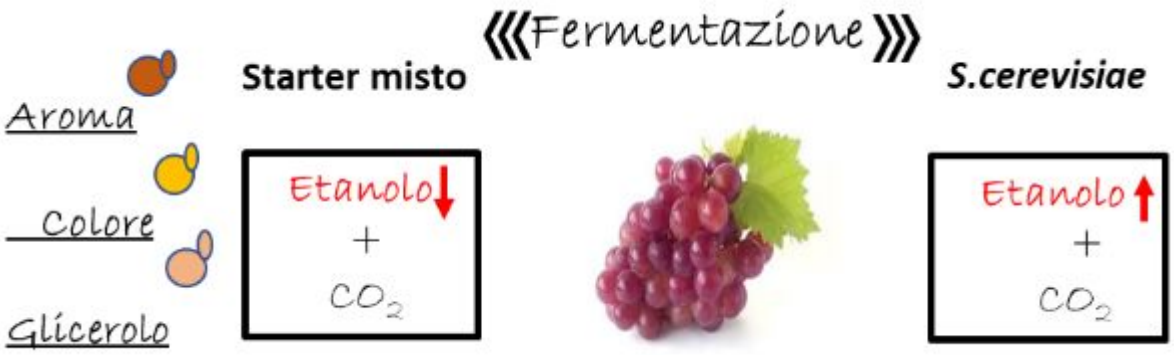
I lieviti non-*Saccharomyces* sono la risposta alla problematica dell’aumento del grado alcolico del vino?

La presenza dei lieviti non-*Saccharomyces* in passato era spesso associata ad arresti di fermentazione o a profili analitici dei vini anomali. Recentemente, il loro ruolo nelle fermentazioni vinarie è stato rivalutato, poiché, seppure caratterizzati da scarso potere fermentativo, posseggono attività metaboliche particolari, diverse da quelle espresse da *Saccharomyces cerevisiae*, che possono contribuire all’ottenimento di un prodotto con maggiore complessità aromatica, che richiami l’originalità delle fermentazioni spontanee.

Di conseguenza, negli ultimi decenni, l’attenzione è stata puntata sulla individuazione di ceppi di lievito non-convenzionali, da impiegare in associazione con *S. cerevisiae* (al fine di garantire il completamento del processo fermentativo) come strumento biotecnologico innovativo, per l’ottenimento di un vino a ridotto contenuto alcolico, ma con caratteristiche aromatiche peculiari.

Principio base di questo approccio è la capacità dei lieviti non-*Saccharomyces* di metabolizzare gli zuccheri del mosto d’uva mediante vie alternative alla fermentazione alcolica, deviando i percorsi metabolici verso la produzione di composti secondari (glicerolo, composti volatili, mannoproteine) diversi dall’etanolo, che influenzano positivamente le caratteristiche organolettiche del vino.

Figura 1. Confronto tra starter misto e singolo



Purtroppo, a volte i vini prodotti utilizzando “inoculi misti”, oltre a un minor contenuto in etanolo, contengono livelli elevati di alcuni composti, ad esempio acetaldeide e acido acetico, che, se presenti in alte concentrazioni, hanno ripercussioni negative sulla qualità sensoriale del vino.

Con l’obiettivo di ridurre il contenuto alcolico del vino, esaltandone alcune caratteristiche aromatiche varietali, presso il “Laboratorio di Lieviti Fermentativi” dell’Università degli Studi della Basilicata sono state allestite prove di fermentazioni multistarter su piccola scala, impiegando lieviti non-*Saccharomyces* (indicati con Ho e Td), precedentemente selezionati per caratteri di interesse enologico, in combinazione con un ceppo commerciale di *S. cerevisiae*. Come controllo, è stata condotta una prova con il ceppo starter commerciale di *S. cerevisiae* (indicato con Sc). I vini sperimentali ottenuti sono stati analizzati per il contenuto di etanolo (Figura 2) e di altri composti che influenzano le caratteristiche qualitative del vino, come l’acido acetico (Figura 3).

La Figura 2 mostra che entrambi i vini ottenuti dagli starter misti (Ho e Td) presentano un contenuto di etanolo inferiore rispetto a quello ritrovato nel vino ottenuto con il solo ceppo di *S. cerevisiae* (Sc). In particolare, la massima riduzione di etanolo è stata ritrovata nel vino prodotto con lo starter misto contenente Ho.

Questo stesso vino, però, presentava il più alto contenuto di acido acetico (Figura 3), sebbene il livello ritrovato era inferiore a 1 g/L, considerato il livello al di sopra del quale l’acido acetico conferisce caratteristiche organolettiche indesiderabili.

Figura 2. Contenuto di etanolo di vini ottenuti da colture starter miste (Ho e Td) e singola (Sc)

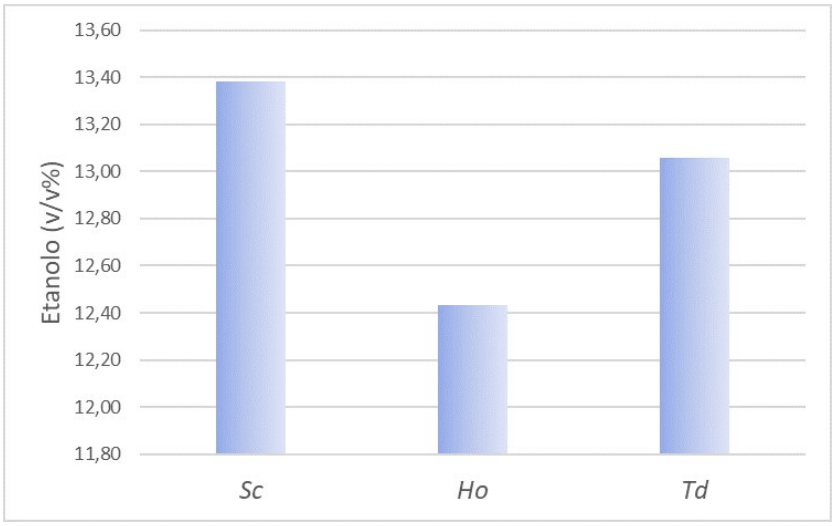
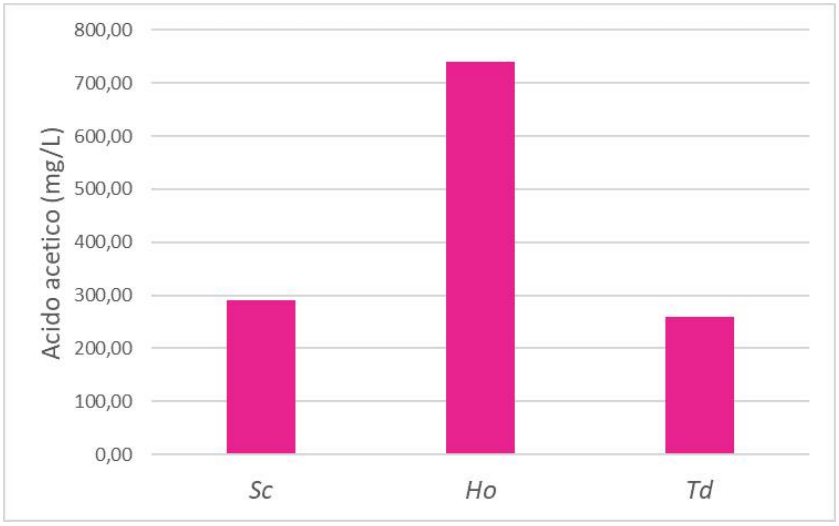


Figura 3. Contenuto di acido acetico di vini ottenuti da colture starter miste (Ho e Td) e singola (Sc)



Conclusioni

L'impiego di "starter misti" composti da ceppi selezionati di lieviti *non-Saccharomyces* e *Saccharomyces* potrebbe rappresentare uno strumento utile per la produzione di vini a ridotto contenuto alcolico, ma è necessaria l'accurata selezione della combinazione di ceppi da utilizzare al fine di salvaguardare anche le caratteristiche qualitative dei vini ottenuti.

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AGRIFOGLIO
Periodico dell’ALSIA

Direttore Responsabile: Sergio Gallo
Reg. Tribunale di Matera n. 222 del 24-26/03/2004
ISSN 2421- 3268
ALSIA - Via Annunziatella, 64 - 75100 Matera
www.alsia.it - urp@alsia.it

Thanksgivings

Thanks to Prof.ssa Angela Capece and Prof.ssa Patrizia Romano,

for guiding me in the realization of the thesis project, thanks for your support, for your availability, for your fundamental teachings, and advice.

Thanks to Rocchina, Gabriella, Rossana,

for the many experiences shared in the laboratory and for always supporting me!

Thanks to Professor Gianluigi Mauriello, Dr Jordi Tronchoni and Dr. Teresa Garde-Cerdàn, for their fundamental contribution in the research activities.

Thanks to the winery "Cantine del Notaio",

for giving me the opportunity to work with his team in the cellar.

Thanks, Grazia

La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005),
Fondo Sociale Europeo, Azione I.1 “Dottorati Innovativi con caratterizzazione Industriale”

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