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SUSTAINABILITY IN WINEMAKING: ROLE OF NON-*SACCHAROMYCES* YEASTS

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1. INTRODUCTION
1.1 Yeasts of oenological interest
1.1.1 Spontaneous fermentation
1.1.2 Inoculated fermentation
1.2 Non-*Saccharomyces* yeasts
1.2.1 Distinctive Features
1.2.2 Enhancement of Flavor and Aroma Complexity
1.2.3 Ethanol Reduction
1.2.4 Biocontrol action
2. THE AIM OF THE WORK

1. INTRODUCTION:

Grape juice is a non-sterile substrate with several species of microorganisms belonging from microbiota naturally present on grape berries. This microbial consortium is important to produce and characterize the final wine. Yeasts ferment the grape juice promoting the transformation of the sugars into ethanol, carbon dioxide (CO_2) and other metabolites through the alcoholic fermentation. Lactic acid bacteria (LAB) are responsible of malolactic fermentation, responsible of the decarboxylation of L-malic acid into L-lactic acid and CO_2.

The fermentation is carried out by different yeast species according to their metabolic aptitudes and alcohol tolerance: non-*Saccharomyces* yeasts are numerous at the beginning of alcoholic fermentation.

In fact during the first three days of fermentation several non-*Saccharomyces* yeasts such as *Hanseniaspora* spp., *Kloeckera* spp., *Candida* spp., *Pichia* spp., *Zygosaccharomyces* spp., *Schizosaccharomyces* spp., *Torulaspora* spp. *Kluyveromyces* spp. and *Metschnikowia* spp. have been isolated (Fleet 2003, 2008; Jolly et al. 2014). After the first phase of fermentation non-*Saccharomyces* yeasts are replaced by *Saccharomyces cerevisiae* (Pretorius 2000; Ciani et al. 2009). *S. cerevisiae* is considered the most important yeast for vinification process since it completes the fermentation of available sugars of the grape juice.

During the last few years, several works was focused on the microbial component of the grape juice for the characterization of the final wine (Bourdichon et al., 2012). Indeed, these studies highlighted that physiological characteristics of single species and the overall metabolic interactions of the microorganism is the key to control the safety, flavor, texture, and aroma of the final wines. Final wine is the result of the complex interactions among yeast, bacteria, and other fungi that origin in vineyards and continues with the fermentation process in cellar.

In recent years great attention of researchers was focused on the inoculation of non-*Saccharomyces* yeasts in combination with *S. cerevisiae* starter strains to improve the aromatic bouquet and flavor of wine, to reduce the ethanol content or to exploit their bio control effect (Rojas et al. 2003; Romano et al. 2003; Ciani et al. 2006; Jolly et al. 2006). From this point of view, the study of non-*Saccharomyces* biodiversity represents a subject of increasing interest in oenological field.

1.1 Yeasts of oenological interest

The yeasts characterize final wine through different mechanisms. Convert the compounds of grape juice producing ethanol and other metabolites that help to extract the aromatic components from the solid parts of the grapes. Indeed, producing enzymes and secondary metabolites active from aromatic point of view (eg

acids, alcohols, esters, polyols, aldehydes, ketones, volatile sulfur compounds) neutral compounds are transformed into aromatic compounds.

The uniqueness and individuality of the aromatic contribution of yeasts depends by the species and ecological strain of fermentation (Fleet G. H., 2001).

The main yeast species isolated from the grapes is represented by *Hanseniaspora uvarum* (and its anamorphic form *Kloeckera apiculata*), and to a lesser extent by *Candida* spp., *Cryptococcus* spp., *Hansenula* spp., *Kluyveromyces* spp., *Metschnikowia* spp., *Pichia* spp. and *Rhodotorula* spp.

S. cerevisiae is poorly represented on the grapes (10^2 ufc/ml). In fact many studies highlighted that *S, Cerevisiae* yeasts come mainly from cellar equipment as opposed to non-*Saccharomyces* yeasts which are found mainly on grapes (Folch-Mallol 2004).

The sequence of microbial activities during fermentation begin with the pre-fermentation phase with non-*Saccharomyces* yeasts. During fermentation phase *Saccharomyces* spp. are dominant because best adapted to metabolize glucose and fructose.

S. cerevisiae is an ascomycete fungus widely studied for its importance in the bakery and wine industry as well as for its ability to produce ethanol (Cocolin L. Et al 2004).

In fact, *S. cerevisiae* produces high quantity of ethanol by consuming the sugar content of the grape juice and lowering the pH inhibit the growth of non-*Saccharomyces* strains (Barata E., 2011). Fermentations are largely conducted by inoculations of a single commercial starter strain of *S. cerevisiae* to ensure greater control of fermentation process thus decreasing the risk of the development of spoilage microorganisms.

The selected yeasts have been used with excellent results in many countries, obtaining final products of more uniform quality than those produced with spontaneous fermentation (Mas A. Et al 2006).

When commercial yeasts are selected by winemakers their properties and the characteristics of the final wine must be considered. It is important to know the concentration of metabolites that they tolerate or need to successfully start fermentation or the optimal development temperature: most it does so between 12 and 36º C. Despite this, it is more effective to use pure yeasts cultures that originate from the vineyard area in which they are to be used, known as selected local yeasts, as the yeast strains found in a microzone are believed to be (Mas A, 2006): Specific to the area and fully adapted to the climatic conditions of the area.

Some strains produce a killer toxin which kills sensitive yeasts. These types of interactions define the evolution of different yeast populations during fermentation. Sometimes a killer strain of *S. cerevisiae*

predominates at the end of the fermentation process, suggesting that the toxin expression allowed it to lead part of the winemaking. This killer phenomenon can be an alternative method for the control of unwanted yeasts (Maqueda M. Et al, 2012).

1.1.1 Spontaneous fermentation

During the past years wine was being produced exclusively from the spontaneous fermentation of the natural microbiota (Pinto C., 2015). Several species of yeasts found on the grape and indigenous microorganisms associated with the cellar surfaces are involved in spontaneous fermentation. Non-*Saccharomyces* yeasts start alcoholic fermentation while the second phase of fermentation carried out by *S. cerevisiae* may not have a regular course. In fact, there could be different situations: complete alcoholic fermentation thanks to the presence of enological starter strains of *S. cerevisiae*; fermentation interrupted due to the low temperature or the lack of yeast strains with high fermentation power or uncontrolled fermentations due to the genera *Schizosaccharomyces, Brettanomyces, Zygosaccharomyces* and *Saccharomycodes* which produce unwanted aromatic substances.

Spontaneous fermentation is still widespread today, especially in Italy and in particular for the production of some wines. The supporters of spontaneous vinification attribute to the final wines a strong stylistic distinction, greater complexity of aroma taste and structure compared with wines obtained by inoculating commercial starter strains. In the early stages of spontaneous alcoholic fermentation yeasts characterized by limited fermentation activity, belonging to the genera *Candida, Hanseniaspora, Pichia, Rhodotorula, Issatchenkia* and *Kluyveromyces*.. Non-*Saccharomyces* yeasts contribute significantly to fermentation since they reach populations greater than 10^6-10^7 cells / ml. These high populations are thought to influence wine composition as well as the development of *S. cerevisiae*, since chemical changes in wine can affect both growth kinetics and metabolism of *S. cerevisiae*. With the increment of the alcoholic content the environmental conditions become progressively more restrictive for non-*Saccharomyces* yeasts. The most alcoholic yeasts are those sporogenic and among these, the wine strains of the *S. cerevisiae* species, which for the most part exhibit fermentation, power greater than 14% ethanol. In addition to *S. cerevisiae*, few other species have the possibility of intervening in the last stages of fermentation and in the central ones as they have a moderate alcoholic power; these are *Torulaspora delbrueckii* and *Zygosaccharomyces bailii* and various species belonging to the genus *Schizosaccharomyces* (G. Suzzi, 2018)

1.1.2. Inoculated fermentation

The practice of inoculating grape juice with selected starter strains began in 1890 with Muller-Thurgau (Petruzzi L.2017). In countries who produce wines, selected yeasts were used to correct fermentation defects or to activate refermentation operations in particular for sparkling wine. To arouse the interest of the microbial starter industry it is necessary to wait until the second half of the twentieth century, when under the pressure of the grain industry the production of yeasts for vinification in compressed form began. However, it had the drawback of being easily perishable due to the high humidity content (70%), which reduced its commercial diffusion. To repair this, in 1965 the first two wine starters were proposed and marketed in the form of active dry yeasts (ADY) (Fracassetti D. 2020).

ADY yeasts, thanks to their high vitality (50%), long shelf life due to the reduced humidity content (4-8%) and the vacuum packaging system, have allowed a wide diffusion of oenological starters. In Italy the rapid spread of the use of selected yeasts began in 1978 after the entry into force of the law that authorized its use (Ministerial Decree 10 October 1977). However, of all the yeasts marketed in the active dry form only a dozen are the most used in the world. The use of a few selected yeasts could lead to a standardization of the microbial agent with the result of obtaining the reduction of the biodiversity of the wine yeasts associated with the cellar environment and the consequent lower variability of the wines due to their activity. The selection of starter yeasts for enology takes place essentially within the genus *Saccharomyces* and in particular among the cultures belonging to the *S. cerevisiae* and *S. bayanus* species (Gerőcs A. 2022). The selection of wine yeasts aims to obtain yeast cultures capable of leading the fermentation process towards predetermined results. The first phase of a breeding program involves finding many crops by isolating them from various environments. The identification of the characteristics to be taken into consideration for wine starters is certainly an important phase of the selection process. In fact, the desirable characteristics for a starter culture for oenology are also different according to the different winemaking technologies to be adopted and the different types of products to be obtained. The *S. cerevisiae* yeast is the most vigorous yeast, more adaptable to the various winemaking conditions, with a high degree of variability for numerous characters, more alcoholic and with excellent fermentation purity. The term fermentation purity expresses the relationship between the volatile acidity and the ethyl alcohol produced. It varies from strain to strain and the more the ratio value is close to zero, the better the fermentation purity. The term alcoholic instead refers to the fermentation power that is the maximum production capacity of ethanol that the strain can form during fermentation in the presence of an excess of sugars. *S. cerevisiae* also has an excellent fermentation speed fermentation that is the ability to give rise to ready and rapid fermentations. This character is evaluated under standardized conditions of temperature and characteristics of the musts. The second phase of the inoculated fermentation is the addition of sulfur as an antimicrobial agent and inhibitor against non-*Saccharomyces* yeasts (Romano P.2019).

1.2 Non-*Saccharomyces* yeasts

The presence of non-*Saccharomyces* yeasts in the past was often associated with stuck fermentation or anomalous analytical profiles of wines (Tufariello M. et al. 2021). Recently their role in wine fermentation has been re-evaluated to allow their peculiar characteristic to obtaining a wine with greater aromatic complexity (Capozzi V. 2015) The attention on the abilities of non-*Saccharomyces* yeasts to metabolize the sugars of the grape juice through alternative ways to alcoholic fermentation, diverting the metabolic pathways towards the production of secondary compounds (glycerol, volatile compounds, mannoproteins) other than ethanol, which positively influence the organoleptic characteristics of wine (Vincenzini M.,2016).

Many species of non-*Saccharomyces* yeasts coming from wine-related environments have limited fermentation potential, such as low fermentation power and rates, as well as low SO_2 resistance (Ciani and Maccarelli, 1998;Mendes Ferreira et al., 2001; Jolly et al., 2006). Moreover, the production of many compounds like acetic acid, ethyl acetate, acetaldehyde, and acetoin vinyl and ethylphenols at high concentrations generally prevents the use of these strains as starter cultures both in wine and beer industry. During the last few decades, many studies have been focused on the use of non-*Saccharomyces* yeasts during alcoholic fermentation. (Jolly et al., 2006; Domizio et al., 2007; Varela and Borneman, 2017).

In particular, the re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking is related to the use of controlled mixed fermentations using non-*Saccharomyces* / *S. cerevisiae* yeast species in sequential inoculation. Indeed, it was demonstrated that mixed fermentations using controlled inoculations of *S. cerevisiae* starter cultures and non-*Saccharomyces* yeasts represent a suitable strategy to improve the complexity of products enhancing particular and specific characteristics. Fermentations carried out using mixed inoculums can improve the quality of the final product, and can assure both a more standard fermentation process and an enhancement of the analytical composition of wine, by taking advantage of several metabolic pathways of non-*Saccharomyces* yeast strains.

However, non-*Saccharomyces* yeasts give their significant contribution during the early stages of fermentation.

With the increase of alcohol concentration, indigenous or commercial strains of *S. cerevisiae* take over and complete the fermentation process.

Some strains showed that can survive during the fermentation phase, highlighting the ability to produce metabolites that can contribute to the quality of the wine; for example, the enhancement of glycerol content by *Candida stellata* and *Starmerella* spp and the production of esters by *Candida pulcherrima* currently called *Metschnikowia pulcherrima*.

Other species, such as *Kloeckera apiculata*, are associated with the production of acetic acid, which can be detrimental to the quality of the wine.

Therefore, the initial activity of non-*Saccharomyces* yeasts in the grape juice is considered important for the final analytical and aromatic profile of the wines. Furthermore, the production of exo- and endonucleases by these yeasts plays a very important role, since pectinases have some applications in refinement, filtration and in the extraction of wine color. The use of pectolytic enzymes for steeping can also increase the juice content of terpenol. Other enzymes are esterases that form wine aroma compounds and lipases that degrade grape lipids (Esteve-Zarzoso et al, 1998).

1.2.1 Distinctive Features

The use of non-*Saccharomyces* yeasts in controlled mixed fermentation has been proposed and applied to take advantage of some their specific fermentative and metabolic features. For example, *S. pombe* and *Schizosaccharomyces japonicus* has been proposed as biological deacidification agent (Magyar and Panyik, 1989; Ciani, 1995) and could be profitable used since they have characteristics that are beneficial for winemaking. In addition, recent works showed that these yeasts in mixed fermentation determined and increased in the production of pigments and large amounts of polysaccharides (Domizio et al., 2017; Escott et al., 2018). The ability of grape juice/wine deacidification was also found in *Pichia kudriavzevii*, another non-conventional wine yeast. (Moreno et al., 2014). On the other hand, the characteristic to produce organic acids during the fermentation may be a desired feature in some winemaking environments and process conditions. In this regard, *L. thermotolerans* in sequential fermentation exhibited the ability to produce lactic acid determining an increase in total acidity of wine, desired feature in grape juices deficient in acidity generally coming from wines of warm climates (Kapsopoulou et al., 2007; Gobbi et al., 2013).
Osburn et al. (2018) found that other non-conventional species, such as *H. vineae*, *W. anomalus*, *S. japonicus*, and *Lachancea fermentati*, are able to produce lactic acid during fermentation.
Another positive trait desired by non-conventional yeast involvement is the reduction of volatile acidity. A low volatile acidity (mainly acetic acid) is one of the fundamental characters to select strain for the oenological use. Indeed, volatile acidity plays a significant role in wine aroma; an high concentration of acetic acid are highly detrimental to wine quality. The amount of volatile acidity produced by *S. cerevisiae* is usually low (up to 0.50 g/L) but may be higher during fermentation of high-sugar media. Indeed, *S. cerevisiae* produce acetic acid as response to osmotic due to the upregulation of genes encoding for aldehyde dehydrogenases (Blomberg and Adler, 1992). Some non-*Saccharomyces* species do not respond in the same way to osmotic stress and for these reasons researchers have been proposed to reduce the volatile acidity in wines with high initial sugar content. *T. delbrueckii* and *C. stellata* (now reclassified as *Starmerella bombicola*) exhibited a very low production of volatile acidity (Ciani and Maccarelli, 1998). In this regard, *T. delbrueckii*, show in mixed fermentation with *S. cerevisiae* a consistent reduction of volatile acidity in high sugar fermentation (Bely et al., 2008). Similarly, *C. stellata* in mixed and sequential fermentation with *S. cerevisiae* showed a

reduction of volatile acidity (Ciani and Ferraro, 1998). A reduction of acetic acid production was obtained in sweet wine fermentations using *C. zemplinina* (now reclassified as *Torulopsis bacillaris*) in simultaneous and sequential fermentation with *S. cerevisiae* (Rantsiou et al., 2012). Glycerol is a desired fermentation by product and is quantitatively the major end product other than ethanol and carbon dioxide. The amount of glycerol formed during fermentation by the yeast species *S. cerevisiae* is in the range of 7%–10% compared to that of ethanol.

Among nonconventional wine yeasts, the high glycerol producer species were used in mixed fermentation to enhance the glycerol content in wines. In this regard, immobilized cells of *C. stellata* showed an increase of glycerol content of about 100% in mixed fermentation (Ciani and Ferraro, 1998). In addition, *S. bacillaris* (formerly *C. stellata*) yeast in mixed fermentation with *S. cerevisiae* starter culture has been widely investigated and several studies demonstrated an increase in glycerol content in mixed wines, related with the mouth feel and complexity of wine flavor. An increase of glycerol content in wine was also found in mixed fermentation with *M. pulcherrima* (Comitini et al., 2011).

Polysaccharides production is another relevant and important feature that could be improved with the use of nonconventional yeasts in winemaking. Indeed, *S. cerevisiae* releases low amounts of polysaccharides, gen erally ranging from 50 to 150 mg/L (Giovani et al., 2010). Several studies have shown that nonconventional wine yeasts are generally characterized by the capacity to release a high quantity of polysaccharides (Comitini et al., 2011; Domizio et al.,2011; Gobbi et al., 2013). The possibility to increase the content of mannoproteins naturally using these yeasts could represent a valuable possibility to enhance the overall quality of wines. In this regard, *M. pulcherrima*, *Saccharomycodes ludwigii*, *L. thermotolerans*, *S. pombe*, *S. japonicus* showed high polysaccharides production and could be profitable used in mixed fermentation (Domizio et al., 2017).

1.2.2 Enhancement of Flavor and Aroma Complexity

Several investigations focused the attention on the enhancement of flavor and aroma complexity of wine using nonconventional yeasts in mixed fermentation. In this regard, *T. delbrueckii*, low frequently isolated on the surface of the grape, was one of the most studied species to increase flavor and aroma complexity in alcoholic beverages. Indeed, *T. delbrueckii* shows a positive effect on the taste and aroma of alcoholic beverages exhibiting a low production of acetaldehyde, acetoin, acetate, and ethyl acetate.

In winemaking, several investigations agree that *T. delbrueckii* impact on aromatic composition and sensory attributes of wines in both simultaneous and sequential fermentation. Indeed, these investigations found an increase acetate ester, (Cordero-Bueso et al., 2013), thiols (3-sulfanylhexan-1-ol and 3-sulfanylhexyl acetate) (Zott et al.,2011; Renault et al., 2016), terpenes (α terpineol and linalool) (Cus andJenko, 2013), 2 phenyl-ethanol (Comitini et al., 2011). Moreover, results of sensory evaluations of final wines revealed an impact on sensory attributes such as increased "aroma intensity," complexity, persistence, and "fruity" aroma, depending

on grape variety (Azzolini et al., 2015). Even in the brewing industry, *T. delbrueckii* was recently proposed to enhance and differentiate the aroma profile of final beer. Indeed, the success of craft beers induces brewers to look for new alternatives to impact on aroma and flavor and generate differentiated products (Basso et al., 2016). In this contest, mixed fermentation using *T. delbrueckii* and *S. cerevisiae* fully converted the fermentable sugars exhibiting distinctive analytical and aromatic profiles producing desirable fruity attributes (Canonico et al., 2016a, b, 2017; Michel et al., 2016). Also *H. uvarum* or *H. vineae* was proposed in mixed fermentation to enhance the aromatic profile of wines. Increasing the production of volatile compounds (acetals, terpens) Phenyl ethyl acetate fruity, floral notes.

Moreover, mixed fermentation trials in the presence of *H. uvarum* and *S. cerevisiae* starter cultures increased isoamyl acetate content, while the use of *Hanseniaspora osmophila* increased 2-phenylethyl acetate production (Moreira et al., 2010; Medina et al., 2013).

Another nonconventional yeast with interesting fermentation behavior is *M. pulcherrima*, species, generally recovered during the initial stages of alcoholic fermentation. *M. pulcherrima* is a high producer of β-glucosidase and increase the"fruity" characters (peach and pear). According to a report by Kurita (2008), mixed inoculations using *W. anomalus* (formerly *Pichia anomala*) resulted in positive enhancement of isoamyl acetate. Cañas et al. (2014) also studied the effect of mixed fermentations with *W. Anomalus* (banana aroma).

Debaryomyces vanrijiae also determined an increase in esters and fatty acids (Maturano et al., 2015). Dashko et al. (2015) using *Kazachstania gamospora* and *Zygosaccharomyces kombuchaensis* in sequential fermentations of Ribolla gialla grape juice, found an increase in "flavor persistence," "flavor intensity," and several fruity attributes. *Z. bailii* in simultaneous fermentation positively influence the aroma profile in chardonnay wine enhancing the ethyl esters production (Garavaglia et al., 2015) while *Zygotorulaspora florentina* increased fruity and floral notes in Sangiovese grape juice (Lencioni et al., 2016). Finally, *Pichia kluyveri* was proposed in both wine and beer fermentation to enhance the aroma profile of the final product (Benito et al., 2015; Saerens et al., 2017). In particular, in wine mixed fermentation with *S. cerevisiae*, *P. kluyveri* increased varietal thiols concentrations in Sauvignon Blanc and overall impression and peach-apricot characters (Benito et al., 2015).

1.2.3 Ethanol Reduction

Over the last few decades, there has been a progressive increase in the ethanol content in wines due to global climate change and to the new wine styles that are associated with increased grape maturity. Consequently, there is a rising interest in ethanol reduction in wine. In this context, microbiological approach for decreasing ethanol concentrations appears a promising way since it takes advantage of the differences in energy metabolism among the wine yeast species. There is a growing interest to investigate on nonconventional wine

yeast. Indeed, they show a wide variability in ethanol yield that could be a potential tool for the reduction of alcohol content in wine. Recent works investigated on interspecies and/or intraspecies variability in ethanol yield among nonconventional wine yeasts (Magyar and Tóth, 2011; Contreras et al.,2014; Gobbi et al., 2013; Contreras et al., 2015a,). Low ethanol yield was found in some strains of *C. zemplinina* (Magyar and Tóth, 2011) and in strains belong to *Hanseniaspora*, and *Zygosaccharomyces genera* (Gobbi et al., 2013). Ethanol yield like other fermentation features is a species-related trait but, similarly to other fermentation parameters, a pronounced intraspecies variability was also evident (Ciani and Maccarelli, 1998; Comitini et al., 2011; Domizio et al., 2011).

A low ethanol yield was also found in strains of *M. pulcherrima*, *Schizosaccharomyces malidevorans*, and *C. stellata*) (Contreras et al., 2014). The regulatory respiro-fermentative metabolism in yeasts might be used as strategy to reduce the ethanol concentration in wine. In addition to a low ethanol yield, among non-*Saccharomyces* wine yeasts some strains/species showed and sugar consumption by respiration (Crabtree negative). Both these approaches have indicated the promising use of nonconventional wine yeast to limit ethanol production. Since most non-conventional yeasts are unable to complete alcoholic fermentation *S. cerevisiae* wine strain should be added in simultaneous or sequentially. In this regard several works recently investigated on the combination of selected nonconventional yeasts such as *M. pulcherrima*, *T. bacillars*, *T. bombicola*, *Z. rouxii*, *T. delbrueckii*, and *P. kudriavzevii*. These yeast species can divert the carbon flux toward multiple metabolites rather than ethanol, with the high fermentative ability of *S. cerevisiae* strains (Englezos et al., 2015; Canonico et al., 2016a, b; Varela, 2016).

The different respiro-fermentative regulatory mechanisms of some nonconventional yeasts compared to *S. cerevisiae* was evaluated to reduce the ethanol content through partial and controlled aeration of the grape juice in simultaneous and sequential fermentation (Contreras et al., 2015a, b; Quirós et al., 2014; Rodrigues et al., 2016). Results in terms of ethanol reduction are promising and ranging from 0.3% to 2.2% *v*/*v* depending on the strain and the fermentation conditions.

However, in simultaneous fermentation aeration condition showed consistent increase of volatile acidity since *S. cerevisiae* in this condition has the tendency to produce large amount of acetic acid

(Morales et al., 2015). On the other hand, these nonconventional yeast species produce very little volatile acidity even under oxygenated conditions (Quirós et al., 2014; Rodrigues et al., 2016). For these reasons, sequential fermentation using before the nonconventional yeast with moderate aeration condition followed by the inoculum of *S. cerevisiae* in strict anaerobiosis condition could be a suitable strategy to avoid increase in acetic acid content and obtain at the same time a reduction in ethanol content in wine.

In a recent work carried out at pilot scale level using *T. delbrueckii* or *M. pulcherrima* in sequential fermentation and in aerated conditions a consistent reduction of ethanol content in the final wines was obtained. However, sensory and aroma analysis revealed that the quality of mixed fermentations was affected

by the high levels of some yeast amino acid related by-products and further investigations and set up of fermentation conditions needed (Tronchoni et al., 2017).

1.2.4 Biocontrol action

Another possible applicative feature of non-*Saccharomyces* yeasts in winemaking is regard to the control of undesired microorganisms. During the various stages of fermentation, a punctual and timely control of potential spoilage microorganisms is needed. Indeed, in winemaking and brewing processes, a wide number of yeasts can participate during the various production phases determining, sometimes, undesired organoleptic features of the final product. Moreover, nowadays there is an increasing interest in the use of natural antimicrobial agents in foods and beverages to control spoilage microflora, thus reducing the chemical additives. In this context, killer yeasts and their secreted toxins appear to represent an interesting solution as antimicrobial agents, for the partial or complete substitution of the use of synthetic agents. Indeed, one of the topical subjects in winemaking is the reduction in the use of SO_2 and its partial or complete substitution with natural antimicrobials, which would be more compatible with the requests of consumers for safe and unspoiled food products. Killer toxins are proteins or glycoproteins naturally produced by yeasts that kill sensitive cells and some of them were purified, characterized. The mode of action of most of the killer toxins were well studied even if the modalities to kill the sensitive cells in some of the newly discovered killer toxins are still unknown (Liu et al., 2015). Studies on the killer phenomenon in yeasts have provided valuable insights into a number of fundamental applicative aspects, particularly in winemaking. In relation to the ecological aspect, during the years, killer strains were isolated from various oenological sources, including grape berries, grape musts, and wines. Afterwards several studies have been carried out evaluate the possible application in winemaking (Santos et al., 2011; Comitini et al., 2011). At present, the control of wild spoilage yeast at the pre-fermentative stage is generally achieved by the addition of SO_2 to freshly pressed must. At this stage, apiculate yeasts and in particular *H. uvarum* are widely present that needed to be controlled. About this, *Tetrapisispora phaffii* represents an interesting application killer phenomenon since its killer toxin is able to control the proliferation of apiculate yeasts during the prefermentation phase (Comitini and Ciani, 2010).
During fermentation and mainly in the aging stages, another undesirable yeast is *B. bruxellensis* that is responsible for undesired odors in wine and considered the current major concern for winemakers, since an effective method to control their growth has not yet been developed.
To reduce the *Brettanomyces* proliferation high doses of sulfur dioxide were commonly employed but the efficiency of this chemical compound is subject to wine composition and physicochemical characteristics. Recently, Mehlomakulu et al. (2017) focused on the identification and characterization of killer toxins from *Candida pyralidae* that show a potential antimicrobial effect against *B. bruxellensis*in wine. They were active and stable with winemaking conditions and the activity of these killer toxins was not affected by the ethanol

and sugar concentrations typically found in grape juice and wine. Also, Belda et al. (2017) studied and characterized two killer toxins from *Pichia membranifaciens* (PMKT1 and PMKT2), which is able to inhibit *B. bruxellensis* while *S. cerevisiae* was fully resistant. In addition, Kwkt and Pikt, two killer toxins produced by *Kluyveromyces wickerhamii* and *W. anomalus*, respectively, showed an antimicrobial activity against *Brettanomyces*/*Dekkera* wine-spoilage yeast (Oro et al., 2016). Villalba et al. (2016) addressed their study on the identification and partial characterization of a new killer toxin from *T. delbrueckii* with potential biocontrol activity of *B. bruxellensis* and also other spoilage non-*Saccharomyces* yeasts such as *Pichia guilliermondii*, *Pichia manshurica*, and *P. membranifaciens*.

Finally, Nissen et al. (2003) revealed another interesting modality in the control of undesirable microflora during fermentation. These authors showed that early death of *L. thermotolerans* and *T. delbrueckii* in mixed-culture fermentations with *S. cerevisiae* was not induced by ethanol or any other toxic compound but rather by a cell-to-cell contact mediated mechanism. Subsequent studies (Renault et al., 2013) supported the previous assumption that death of *T. delbrueckii* is mediated by a cell-to-cell contact mechanism.

On the other hand, Albergaria and Arneborg (2016) well described how *S. cerevisiae* establishes antagonistic interactions against several wine-related microbial species (both yeasts and bacteria), mediated by the secretion of antimicrobial peptides that play an important role in its dominance within high-sugar ecosystems.

2. THE AIM OF THE WORK

During the last few years, many researchers have focused their studies on the use of non-*Saccharomyces* yeasts highlighting their metabolic impact and abilities. The results have shown that non-*Saccharomyces* yeasts are useful for their role in bio control as a substitute of chemicals like sulfur dioxide, to enhance aromatic complexity of the final wine and to decrease the ethanol content in wine. For these reasons, this research was focused on the use of non-*Saccharomyces* yeasts to produce evaluated to produce high quality organic wine with a particular aromatic bouquet, to test their bio control capacity and to reduce the alcohol content of the wine. Different non-*Saccharomyces* strains, previously selected on the basis of the main oenological characteristics have been used in vineyard, during pre-fermentation phase and in mixed fermentation with *S. cerevisiae* selected starter strain. In fact in this study the uses of non-*Saccharomyces* yeasts spp. at multiple levels were evaluated. In particular, the analysis of microbial population on the grapes, the fermentation and growth kinetics, the analytical profile and the main volatile compounds were evaluated.

The study involved several species of non-*Saccharomyces.* With the aim to evaluate the bio control effect *Metschnikowia pulcherrima* selected strain was used directly in vineyard and during the pre-fermentation phase in comparison with a selected strain of *Torulaspora delbrueckii*. The same strains were tested to enhance

the aromatic bouquet the final wine and the capacity of *Starmerella bombicola* selected strain to reduce the alcohol content in wine.

The first part of the thesis was focused on verifying the bio control effect of *M. pulcherrima* sprayed on Verdicchio and Montepulciano grapes directly in vineyard. After that was tested during the pre-fermentative phase (at lab and industrial scale). Moreover, verify the ability of this selected strain to increase the aromatic complexity of final organic wine in combination with *S. cerevisiae* selected starter strain and in comparison with a selected strain of *Torulaspora delbrueckii*.

The last part of the research was focused on the evaluation of the metabolic capacity of *S. bombicola* to reduce the alcohol content in wine under aeration condition during the first three days of fermentation and followed by the commercial starter strain.

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CHAPTER 1

USE OF *METSCHNIKOWIA PULCHERRIMA* FOR THE BIOCONTROL AND TO ENHANCED AROMA COMPLEXITY IN THE PRODUCTION OF WINES WITH LOW SULPHITES

1. **INTRODUCTION**

1.1 Alternative vineyard treatments against fungal decay to reduce the use of chemical treatments

1.2 Spoilage microorganisms in winemaking

1.3 Sulfur dioxide as antimicrobial agent

1.4 European regulations on the use of SO_2

1.5 Vineyard biocontrol to reduce the use of SO_2

1.6 *M. pulcherrima* and its features to enhance the aromatic bouquet in winemaking process

1 INTRODUCTION

A wide variety of non-*Saccharomyces* yeasts are present on the grape surface and are involved in the spontaneous fermentation process. These yeasts are often associated with sluggish or stuck fermentations and are considered undesirable yeasts. In modern oenology, sulfur dioxide (SO_2) is suitable for its antimicrobial agent and for other characteristics such as antioxidant properties, moreover it's low cost and certified effectiveness make it's the best solution to control microbial growth (Esparza et al., 2020).

During the last few years, the attention of winemakers was focused on the research of new strategies to reduce the use of SO_2 because affect the human health at different stages of wine making process (pre-fermentative phases, during fermentation and post fermentation phases).

In this regard, in addition to conventional chemical and physical strategies the use of non-*Saccharomyces* yeasts could be a suitable and innovative strategy to reduce the use of SO_2 and at the same time improve the

aroma profile of wine. Several studies have reported the activity and the efficacy of bio control microorganisms which were found to be effective against spoilages. In this regard *Metschnikowia pulcherrima*, *Metschnikowia fructicola* and *Metschnikowia viticola* are the most species found in wine environments (Brysch-Herzberg et al., 2015; Belda et al., 2016; Vicente et al., 2020). *M. pulcherrima* is a yeast characterized by several positive features in winemaking (Morata et al., 2019). *M. pulcherrima* showed a broad-spectrum bioactivity against spoilage microorganism.

This antimicrobial activity would not be linked to a protein, as for the killer factor, but to the presence of an acid precursor of the pigment responsible of coloration of the colonies. This compound is an insoluble red pigment with antimicrobial activity which gives the yeast its typical red color.

Several studies highlighted that pulcherrimin cause the the precipitation of iron (III) ions in the medium caused by the interaction with pulcherriminic acid, a precursor of pulcherrimin secreted by *M. pulcherrima* Some studies highlighted that this activity did not affect the growth of *S. cerevisiae* but showed an inhibition against spoilage yeasts such as *Brettanomyces* / *Dekkera* spp., *Hanseniaspora* spp., and *Pichia* spp . and different postharvest fungal pathogens on grape, apple, or tomatoes, such as *Botrytis cinerea*, *Penicillium* spp., *Monilia* spp. and *Alternaria* spp. (Oro et al., 2014, Kuchen et al., 2019). Indeed, *M. pulcherrima* it is considered a versatile yeast specie able to act as biocontrol agent but also modulate the synthesis of secondary metabolites thus improving the sensorial profile of wine, representing a valid tool in winemaking industry.Furthermore, its metabolic capacity could producers are interested to this non-*Saccharomyces* yeast to replace the use of SO_2 to take advantages of market opportunities in the "natural" wine movement by following organic certification guidelines. Nowadays, different products based on yeasts reached advanced stages of development and commercialization such as Shemer^{TM} based on *Metschnikowia pulcherrima* Pitt & Mill, however, the widespread use of postharvest biocontrol products remain limited for their instable performance under commercial conditions as well as the limited market and small size companies involved in their development and commercialization.

In this context, the present research is placed, with the dual purpose of studying the antimicrobial and aromatic enhancement characteristics of *M. pulcherrima* in winemaking and obtaining preliminary results that can pave the way for large-scale application.

1.1 Alternative vineyard treatments against fungal decay to reduce the use of chemical treatments

Fungal decay in postharvest is mainly due to ubiquitous fungus *B. cinerea* that can grow and spread even at low temperatures. This fungus can develop in different steps of winemanking process such as in the field, during transportation of the grapes in the cellar, during storage and marketing. The principal symptoms of infections start with small necrosis on the skin of the grapes. In this contest only agronomic practices cannot

prevent the disease in many vineyards, so chemical treatments were used in the world(Jacometti, M.A et al., 2010).

During postharvest storage, many countries have banned chemical fungicides that can affect the human health. Therefore, the use of SO_2 is the most commercial method to control the development of grey mold. The use of SO_2 has been successful worldwide because it is efficiency, easy to use, cheap, and low health risk in comparison with industrial fungicides. However, several studies highlighted that an excessive use of SO_2 can damage the grape, causing early browning of the rachis and allergies in consumers (Ahmed, S. et al., 1992). For these reasons, new strategies have been proposed in the postharvest management of *B. cinerea* including chemical (Romanazzi, G. et al., 2017), biological (Romanazzi, G. et al., 2012) and physical instruments (Candir, E et al2018),.

In line with sustainable winemaking, ‘organic viticulture’ arouses great interest from winemakers. It is defined by the International Federation of Organic Agriculture Movement (IFOAM) as a system which promotes agro-ecosystem health, including biodiversity, biological cycles, and soil biological activity. It emphasizes the use of management practices in particular highlighting the use of biological and alternative chemical method in vineyard (Beardsley, P.M et al., 2014)

1.2 Spoilage microorganism in winemaking

Spoilage microorganisms can cause deterioration of wine reducing its quality and commercial value. Deterioration of wine can cause the increase of acetic acid content and volatile acidity, viscosity and the formation of unpleasant smells due to volatile compounds such as ethyl acetate and volatile phenols. In addition, during fermentations can develop compounds that affect human health, such as biogenic amines, acrolein, and ethyl carbamate (Ryu, et. al 2015).

About 40 different species of yeasts are the most frequent contaminants associated with wine, but the species that can potentially cause the real deterioration are much less. As reviewed by Loureiro and Malfeito-Ferreira (2003) the latter can be grouped as follows: fermenting strains (*S. cerevisiae*) when refermenting wines bottled with residual sugar, *Zygosaccharomyces bailii* which form sediments or turbidity in bottled wines; film-forming yeasts and ester producers like *Hansenula* spp*., Kloeckera* spp*., Pichia* spp*., Metchnikowia* spp. and *Debaryomyces* spp. and off-flavor producing yeasts (*Brettanomyces* spp., *Schizosaccharomyces pombe*, and *Saccharomycodes ludwigii*).

However, only *Brettanomyces bruxellensis*, *Z. bailii* and *S. cerevisiae* (in the case of wines bottled with residual sugars) are generally considered spoilage yeasts while some species that could cause defects in

uncontrolled conditions, can be used in co-inoculation with *S. cerevisiae* in controlled fermentations (De Filippis, F. et al 2018).

Lactic acid bacteria (LAB) and acetic acid bacteria (AAB) are the only families of bacteria present in grape juice. These include four genera of LAB (*Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus*) and three genera of AAB (*Acetobacter, Gluconobacter* and *Gluconacetobacter*). While some LAB species find technological applications in winemaking as *Oenococcus oeni* and *Lactobacillus plantarum* involved in malolactic fermentation, all AAB species are considered spoilage bacteria. The growth of *Lactobacillus, Pediococcus*, and *Oenococcus* strains in wine gives rise to numerous spoilage scenarios, as they can form unwanted aroma and flavor compounds, as well as biogenic amines, acrolein and carbamate (Bartowsky, EJ 2009).

However, the deterioration of wine depends by various parameters, including the chemical-physical characteristics (ethanol content, concentration of residual sugar, pH, amount and composition in main acids, i.e., malic acid and oxygen), the species bacteria present and their initial population, the type and intensity of stabilization treatments and the level and type of chemical preservative added.

The microbiological stability of wine is essential to preserve its quality. Effectively, the increasing incidence of the spoilage microbes could be responsible for considerable economic losses in this sector and biocontrol provides alternatives to chemical preservatives, such as SO_2, which is associated with adverse reactions in humans.

1.3 Sulfur dioxide as antimicrobial agent

The most common, economic, and effective chemical compound in wine is sulfur dioxide (SO_2). Its antimicrobial properties have been exploited against LAB, AAB, *Brettanomyces* spp., myco-dermal yeasts, and various wine spoilage bacteria.

Vineyard geography and environment, cellar practices, time of harvest, grape juice processing and fermentation conditions can affect yeast population dynamics during wine fermentation (Epifanio S.I., et al. 1999; Jemec, K.P. et al. 2005; Albertin, W. Et al. 2014; Maturano, Y.P. et al. 2016). Fermentation conditions are modulated by winemakers and the addition of sulphur dioxide represents the main available intervention Several studies have hown that species of wine yeasts are affect differently to the application of SO_2, with commercial starter strains of *S. cerevisiae* showing different but higher tolerance to SO_2 (Nardi, T. et al.,2010), while non-*Saccharomyces* yeasts display lower tolerances (Morgan, S.C. et al.,2017; Henick-Kling, T et al., 1998). Since SO_2 has been used in winemaking, most of the microbiological alterations have disappeared from

the cellars and today among the authorized agents, SO_2 is the only one that exerts a well-proven efficacy for the microbiological stabilization of wine along with other advantages such as low treatment costs, ease of use and with a broad-spectrum efficacy (Ribéreau G. Et al 2006).

Molecular sulfur dioxide is the most effective form for the antimicrobial activity. Once it enters the cell, SO_2 reacts with enzyme and coenzymes (NAD^+, FAD, FMN) systems, cofactors, nucleic acids and vitamins (thiamine). Due to the higher intracellular pH (about 6.5) the molecular SO_2 is largely converted into HSO_3 ions after diffusion into the cytoplasm. These ions reduce the intracellular concentration of SO_2, thus allowing further diffusion within the organism until the SO_2 concentration is equal on both sides of the plasma membrane (Stratford, M., & Rose AH 1986).

Wine yeasts show a different resistance to SO_2; many studies highlighted that non-*Saccharomyces* are more sensible than *Saccharomyces* spp., even if a high intraspecific variability has been found (Vincenzini M. et al. 2005).

Many strains of *S. cerevisiae* are resistant up to 1-2 mg / l of molecular SO_2, some strains of *Z. bailii* and *S. pombe* show a similar response while *Saccharomycodes ludwigii* can grow at higher molecular SO_2 concentrations (up to 3 mg / L).

Regarding the free form of SO_2 *Oenococcus oeni* strains are more sensitive to SO_2 than *Lactobacillus* and *Pediococcus*. Combined SO_2 have a weak antibacterial activity, while it does not have an antimicrobial action on yeasts. Combined SO_2 appears to have five to ten times less antibacterial activity than free SO_2. The different sensitivities of microorganisms to the various forms of SO_2 cause a selective antimicrobial action (bacteria> non-*Saccharomyces* yeasts> *Saccharomyces* yeasts) (Ribéreau-Gayon et al, 2006). This determines a selection on the microflora of the grape juice towards the *Saccharomyces* yeasts, favoring a correct performance of the alcoholic fermentation and the control of fermentation.

In final wine, typical targets for preventing microbial spoilage are at least 0.6 mg / l of molecular SO_2 for dry wines and at least 0.8 mg / l of molecular SO_2 for sweet wines (Waterhouse et al., 2016). However, the molecular SO_2 level must be kept below the sensory threshold (2 mg /L).

In addition to that given, SO_2 is produced by *S. cerevisiae* during alcoholic fermentation, as an intermediate during the assimilatory reduction of sulphate to sulfur, which is essential for the biosynthesis of sulfur containing amino acids methionine and cysteina. SO_2 can derive from the metabolic product of yeast in different quantities (up to 100 mg / l, but rarely more than 10 mg / l), depending on the strain and the composition of the must (Rauhut, D. 2009). *S. cerevisiae* also produces SO_2 combined with carbonyl compounds (SO_2-binding carbonyl compounds), such as acetaldehyde, pyruvic acid, and α-ketoglutarate, in a wide range of concentrations; therefore, the choice of the yeast strain can have a significant impact on the SO_2

binding power of a wine and therefore on the quantity of SO_2 to add to maintain an adequate free SO_2 level (Wells, A. Et al 2011).

In recent decades, the trend in yeast selection has been to lower the production of both SO_2 and carbonyl compounds.

1.4 European regulations on SO_2 application

Sulfur dioxide (SO_2) is one of the most used additives in the food industry, thanks to its antimicrobial and antioxidative properties. Moreover, in the wine industry, SO_2 addition to the must, prior to alcoholic fermentation, shortens the fermentation time by repressing non-*Saccharomyces* yeasts and promoting the growth of sulfite-tolerant *Saccharomyces* yeasts (microbial selection).

According to the EU Regulation, SO_2 and other forms of sulphites, must be labeled with codes (E followed by a number) in the range E 220-228 (EU Regulation no. 1129/2011).

Regarding to the wines the European legislation establishes the limit concentration of total SO_2 reaches up to 150 mg / l in red wines and 200 mg / l in white and rosé wines with a maximum of 5 g / l of reducing sugars (EU regulation no. 606/2009 and modifications).

These limits increase by 50 mg /l if the reducing sugar concentrations are equal or higher than 5 g / l (EU regulation no. 606/2009) and are reduced from 30 to 50 mg / l in organic wines (EU regulation no. 203/2012).

During the last few decades, the use of SO_2 in the food industry has raised some consumer safety concerns. In fact, SO_2 has clearly been shown to strongly contribute to the appearance of undesirable effects of wine in a small population (about 1%) of "sulphite-sensitive" individuals (Fazio, T., & Warner, CR 1990). Reactions observed include bronchospasm, bradycardia, gastrointestinal symptoms, urticaria, angioedema, hypotension, shock and, in rare cases, anaphylactic reactions (EFSA, 2014). Furthermore, SO_2 and its derivatives can be systemic toxic agents as it has been found that they are able to induce an increase in the frequencies of chromosomal aberrations, sister chromatid exchanges and micronuclei in mammalian cells, they can also cause oxidative damage in mammalian cells. (Fazio, T., & Warner, CR 1990).

Based on this evidence, the World Health Organization (WHO) has estimated a maximum allowable daily intake (ADI) of 0.7 mg SO_2 per kg body weight (WHO, 2009).

Considering the SO_2 dose limits, imposed by the European Community for the different food categories (EU regulation 1129/2011), the ADI for SO_2 is hardly exceeded due to the normal human intake of each individual food category; however, concerns may arise after excessive cumulative intake (WHO, 2009).

In the 2009, the World Health Organization (WHO) estimated a maximum acceptable daily intake (ADI) of 0.7 mg SO_2 per kg body weight (WHO, 2009), thus encouraging research on alternative storage methods aimed at reducing its use. It has been estimated that wine is one of the main foods contributing to SO_2 intake in adults, at least in countries where it is consumed regularly (WHO, 2009).

Moreover, the obligation of labelling with the phrase “containing sulfites” the food products, including wine, in which the concentration of SO_2 is higher than 10 mg/L or 10 mg/kg (Directive 2003/89/EC) has raised worries among consumers who are generally more and more oriented toward “healthy” products free of chemical preservatives (Costanigro, Appleby, & Menke, 2014). In winemaking there is a general trend in the reduction of SO_2, and in recent years the oenological research has been strongly oriented towards the study of techniques and additives as alternatives to SO_2.

1.5 Vineyard biocontrol to reduce the use of SO_2

In wine industry, the impact of bunch rot is well established, because all cultivars are susceptible to this infection. Recently, it was introduced that a class of synthetic fungicides belongs to the succinate dehydrogenase inhibitors (SDHIs). Other chemicals, such as salts solutions recognized as safe (iron sulphate, ammonium bicarbonate, sodium silicate, sodium bicarbonate and sodium carbonate), are widely used to sanitise grapes surface. Ethanol vapours and other gas such as chlorine dioxide and ozone fumigation are also used, even if the sulphur dioxide remains the main method that is used (De simone N. et al 2020).

These conventional anti-*Botrytis* treatments are considered unsustainable In this context, the development of complementary methods to synthetic agents, such as biological control agents (BCAs), could be considered an alternative approach to reducing gray mold (Parafati L. et al. 2015; Lemos Junior, W.J.F. et al 2015; Linares-Morales, J.R. et al. 2018; Raveau, R. et al. 2020). The use of yeast as a BCA take some advantages, including the easy colonisation of dry surfaces for extended periods, simple nutrition requirements, rapid growth and potential antagonistic effects against pathogens (Dukare, A.S. et al. 2019). Among the different antagonistic yeasts, *Metschnikowia pulcherrima,* naturally present on the grapes, is a relevant yeast species that has been successfully applied to control pathogens of fruits and vegetables. The competition of *M. pulcherrima* for nutrients and the iron depletion capacity are the dominant mechanisms during the biocontrol of *B. cinerea* (Oro, L. et al. 2018).

Yeasts that are naturally present and apparently endemic on the grape surfaces represent the major group of yeasts utilized to manage postharvest diseases. However, antagonists have also been isolated from other sources, such as the phyllosphere, roots and soil. For example, the phyllosphere yeast *M. pulcherrima* was isolates from noble-rotted grapes. *Metschnikowia pulcherrima* is a ubiquitous species of yeast, with numerous strains found on grapes from all over the world, cherries, and flowers. The strains of *M. pulcherrima* show

strong biocontrol activity against various microorganisms and it is used for all step of winemaking. The synergistic activity between strains of *Metschnikowia* spp. naturally present on grapes with selected strains enhances the biocontrol activity against spoilage microorganisms. Infact, *M. pulcherrima* was also sprayed directly in vineyard on the grapes before the harvest, as an alternative treatment with the aim of containing the spoilage microbiota normally present on grapes, to suppress the develop of gray mold**.**

1.6 *M. pulcherrima* and its features to enhance the aromatic bouquet in winemaking process

Most wine aroma compounds, including the varietal fraction, are produced, or released during wine production and derived from microbial activity. Some aromas such as terpenes and thiols, have been described as derived from their non-volatile precursors, released during wine fermentation by different yeast hydrolytic enzymes. The perception of these minority aroma compounds depends on the chemical matrix of the wine, especially on the presence of majority aroma compounds, such as esters or higher alcohols. Strategies aiming to reduce the production of these masking flavors are on the spotlight of enology research as a way to better define varietal standard profiles for the global market. Regarding to its contribution in aroma complexity of wine, *M. pulcherrima* in mixed fermentation showed a reduction of ethyl acetate production favoring the formation of 2-phenylethyl acetate, an enhancement of acetate esters, β-damascenone and higher alcohols, particularly isobutanol and 2-phenyl ethanol (Varela et al., 2016; Zhang et al., 2018). Furthermore, adding *M. pulcherrima* before *S. cerevisiae* changes the profile of fermentative compounds and aromas produced during winemaking. During *M. pulcherrima* / *S. cerevisiae* fermentation, a higher concentration of glycerol and a lower concentration of acetate have been obtained (Comitini et al., 2011; Sadoudi et al., 2012; González-Royo et al., 2015). Among these compounds, thiols play a central role, particularly in white wines, as they possess sought-after aromas of box tree, citrus and passion fruit. These molecules include 3-mercaptohexan-1-ol (3MH), 3-mercaptohexylacetate (3MHA), and 4-mercapto-4-methylpentan-2-one (4MMP). Regarding their formation, 3MH and 4MMP are released during fermentation by the action of hydrolytic enzymes, the β-lyases, on non-odorous precursors present in grape must (Roncoroni et al., 2011). Glutathione and cysteine S-conjugates as well as cysteinyl-glycine S-conjugates and γ-glutamyl-cysteine *S*-conjugates have been identified as thiol precursors in various grape varieties. Furthermore, 3MH may be formed by sulfur addition to E-(2)-hexenal, but these pathways account for a limited part of total thiol formation. 3MHA is synthetized by the acetylation of 3MH by Atf1p (Roland et al., 2011).

Finally, combining *M. pulcherrima* with *S. cerevisiae* results in changes in the formation of aromas, with an increase in the final concentration of higher alcohols (Rodríguez et al., 2010; Sadoudi et al., 2012) and variations in the production of ethyl esters and acetate esters depending on the fermentation conditions (Rodríguez et al., 2010; Comitini et al., 2011; Varela et al., 2016; Hranilovic et al., 2018). Overall, wines fermented with *M. pulcherrima* are perceived as more floral, with smoky aromas (González-Royo et al., 2015).

Many studies highlighted that wines obtained by a selected strain of *Metschnikowia pulcherrima*, in combination with different *Saccharomyces cerevisiae* starter strains showed an increase in the levels of the thiol 4-MSP (4-methyl-4-sulfanylpentan-2-one) over its sensory threshold, together with a decrease in higher alcohol production. This has an important impact on these wines, making them fruitier and fresher

Some non-*Saccharomyces* strains are already available in the market as active dried yeasts. non-*Saccharomyces* strains have been successfully used for the diversification of the aromatic profile of the product, increasing both the formation of fermentative aromas and the release of varietal aromas due to their capacity to excrete hydrolytic enzymes (Egli et al., 2002; Rodríguez et al., 2010; Zott et al., 2011; Belda et al., 2017).

Another important feature of this non-*Saccharomyces* yeast is the wide possess among the strains of the enzymatic activities such as pectinase, protease, glucanase, lichenase, β-glucosidase, cellulase, xylanase, amylase, sulphite reductase, lipase and β-lyase activity (Barbosa et al., 2018; Reid et al., 2012). In particular, the proteolytic activity it is important in mixed fermentation to release amino acids as nutrient for *S. cerevisiae*. In addition, the glucosidase activity promotes the release of varietal aromas from the grape (Fernández et al., 2000; Mendes Ferreira et al., 2001).

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FIRST PART

VINEYARD TREATMENTS AND *METSCHNIKOWIA PULCHERRIMA* COLONIZATION OF GRAPE SURFACE

Evaluation of microbial population growth with ozonated water treatments and with the use of selected strains of *Metschnikowia* spp. in vineyard

1. **INTRODUCTION**
2. **THE AIM OF THE WORK**
3. **METHERIALS AND METHODS**

3.1 Ozonated water treatment

3.2 *M. pulcherrima* treatments

3.2.1 Yeast strain

3.2.2 Biomass Production and Microbial Growth

4. RESULTS

4.1 Ozonated water treatment

4.2 *M. pulcherrima* DISVA 269 treatment on Verdicchio vineyard

4.3 *M. pulcherrima* DiSVA 269 and *M. fructicola GAIA®* treatment on Montepulciano vineyard

5. **GENERAL CONCLUSIONS**

1. INTRODUCTION

Because the properties of SO_2 allow many different winemaking applications, the reduction or completely removing of SO_2 from winemaking requires an investigation of each processing step, beginning with grape harvesting and transport.

In this regard in recent years, one of the most investigated vineyard treatments is the use of ozonated water, with the aim to replace (Modesti M. et al.2019)-the chemical treatments.

Ozone is an unstable gas with a high oxidizing power, it can suppress microorganisms such as bacteria and fungi in the moment in which contact occurs.

For these reasons its biocide power is arousing great interest looking for an alternative method to conventional techniques for safeguarding the quality of the grapes. The great advantage of ozone is a natural gas, which is found in the atmosphere and does not leave any type of residue on the crops. But alongside these positive aspects there are some negative points that make it a tool still not usable by farms.

Ozone is a natural gas composed of 3 oxygen molecules that occurs spontaneously in nature but the instability of O_3 simultaneously represents the advantage and disadvantage of this technique. Ozone is highly unstable and for this it must be produced just before use. There are portable machines (expensive and heavy) for on-site production. In summary, atmospheric oxygen is separated from nitrogen and transformed into ozone through electrical discharges. The gas is then mixed with the water, which is sprayed on the crop or even injected into the soil to sanitize it.

The positive consequence of this treatment is its effectiveness: ozone can sanitize crops killing microorganisms, being an unstable gas does not leave residues and it is possible to proceed with harvesting immediately after application. It has no environmental impact, since it is a gas already present in nature.

Furthermore, acting by oxidation, it cannot generate resistance in the pathogens.

Along side these great qualities, there are negative aspects that makes it a tool that is still not easily usable by farms. The cost: ozone requires much more complex and expensive equipment and in a crop like grapevine, many interventions are required to protect the plants. (Raio A. et al. 2015)

In addition, to the use of ozonated water many agronomists seek to reduce SO_2 with other new bio-strategies to take advantage of market opportunities in the “natural” wine movement by following organic certification guidelines and to utilize natural products known to have similar efficacy to synthetic products for targeted applications.

In this regard the attention was focused on the use of non-*Saccharomyces* yeasts directly sprayed on the grapes in vineyard against spoilage microorganisms that can deteriorate wine. Bio protection is a relatively new term and emerging concept in several food industries. In recent years, many studies have focused on the use of non-*Saccharomyces* yeasts with the aim of exploiting their properties as bio protection agents against spoilage microorganisms to reduce the use of SO_2 during the first stages of wine making.

Several species of non-*Saccharomyces* yeasts were involved in different studies directly on the grapes above all *Torulaspora* spp. and *Metschnikowia* spp.

The use of *M. pulcherrima* as a biological control agent is possible thanks to its ability to produce the natural compound pulcherrimin that by subtracting iron from the environment makes it more competitive. Several microorganisms present on the grapes exhibit inhibitory effects from pulcherrimin, including *Candida tropicalis*, *Candida albicans*, *Brettanomyces/Dekkera*, *Hanseniaspora*, *Pichia*, and *Botrytis cinerea.* In addition, some strains of *M. pulcherrima* produce a killer factor to suppress growth of killer-sensitive organisms. *M. pulcherrima* is also described as a biofungicide agent capable of reducing *B. cinerea* on postharvest fruits via nutrient competition.

2. THE AIM OF THE WORK

During the last few years, great attention by winemakers was focused on the use of natural alternative treatments in vineyard like use of ozonated water and non-*Saccharomyces* yeasts.

The replacement of chemical treatments with ozonated water could represent an alternative to reduce the environmental impact due the massive use of phytosanitary chemical products in the vineyards. The ozonated water do not leave residues and it seems that they also have an effect of inducing the immune defenses of the plant.

On the other hand, the knowledge and application on field of non_*Saccharomyces* yeasts were deeply investigated.

With the aim to control the diffusion of spoilage microorganisms that can affect the fermentation process and organoleptic characteristics of the wine it was evaluated the use of ozonated water in field, collaborating with Terre Cortesi Moncaro s.r.l.the larger cellar of the Marche region, located in Montecarotto (AN) which is in the DOC production area of the classic Verdicchio of Castelli di Jesi. This company have more than of 1000 Ha of vineyards, harvesting more than of 70’000 quintals of grapes and producing 6’321’587 bottles of wine every years.

In this study, the bio-protection term refers to a natural agent that controls the growth of spoilage organisms through ecological processes such as competition. During experimental plan, in comparison with the use of ozonated water in vineyards it has been tested a biological treatment with *M. pulcherrima* spp. that were sprayed on the grapes of Verdicchio and Montepulciano varieties.

In the first trials it was evaluated the efficacy of a selected strain of *M. pulcherrima* directly sprayed with a blaster on Verdicchio grapes of a vineyard.

In the seconds trials the settlement and permanence and the biocontrol effect of *M. pulcherrima* DiSVA 708, also in comparison with *M. fructicola* GAIA® commercial strain, was evaluated in a Montepulciano vineyard

3. METHERIALS AND METHODS

3.1 *Ozonated water treatment*

The efficiency of the treatment carried out directly on the grapes was evaluated. The ozonated water was sprayed at sunset with an atomizer and at the sunrise of the following day the grapes were collected.

The following image shows the sampling scheme followed for the study conducted on the grapes from the Terre Cortesi Moncaro s.r.l. vineyard. The vineyard area examined is one hectare divided in two parts, in green portion the unthread vineyard (control) and in the red portion the treated vineyard. The samples were collected from both parts before and after treatment.

Figure 1. The vineyard areas analyzed; in green portion the untreated portion and in red portion the treated portion. In both areas are highlighted the parcels.

Each parcel is identified by a number (1-10) and were collected 3 samples which have been taken following an equilateral triangle pattern. The 3 samples relating to the same parcel will then be combined into a single sample in the laboratory in a sterile environment.

Each sample (vertex of a triangle) is made up of a cluster of grapes considered by the operator to have consistent dimensions and an adequate state of ripeness and health. Each cluster was collected from a selected vine in such a way as to make the sampling as representative as possible, therefore at distances that covered the entire surface evenly.

Indeed the grapes were collected in sterile bags and then transported as quickly as possible to the laboratory and quickly analyzed.

The growth kinetics of the yeast strains were monitored during the fermentation at established intervals using WL nutrient Agar medium(Oxoid, Hampshire, U.K.), which allows the growth of all yeasts and differentiating the various genera in based on the color and morphology of the colonies, Rose Bengal agar (Oxoid,

Hampshire, U.K.) for the differentiation of the mold and MRS agar (Oxoid, Hampshire, U.K.) for the isolation and cultivation of *Lactobacillus* spp.

3.2 *M. pulcherrima* treatments

3.2.1 Yeast strain

M. pulcherrima DiSVA 269 was selected on the bases of previous works on biocontrol ability (Oro et al., 2014; Oro et la., 2018). The yeast strain was maintained on YPD agar medium (yeast extract 1%, peptone 2%, dextrose 2% and agar 1.8%) at 4 ◦C for short-term storage and in YPD broth supplemented with 80% (w/v) glycerol at −80 ◦C for long-term storage. All strains were pre-cultured in modified YPD (0.5% w/v yeast extract, 2% w/v glucose, and 0.1% w/v peptone) for 1 day at 25 °C in an orbital shaker (rotation, 150 rpm). *M. fructicola* GAIA® commercial strain was selected for its capable to control unwanted yeasts but it is devoid of fermentation power. This Dry Active Yeast was rehydrated and used as industrial protocol.

The biocontrol effect of *M. pulcherrima* DiSVA 269 and *M. fructicola* GAIA® and their colonization of grape surfaces were evaluated.

3.2.2 Biomass Production and Microbial Growth

All the yeast strains were pre-cultured in modified YPD medium (0.5% yeast extract, 0.1% peptone and 2% glucose) for 48 h at 25 °C in an orbital shaker (150 rpm). After this period, the pre cultures were used to inoculate 30L Bench-top bioreactor (Biostat® C; B. Braun Biotech Int., Goettingen, Germany) containing 25 L of 1% di yeast extract, 0.5% peptone and 5% invert sugar for *M. pulcherrima* DiSVA 269 strain under agitation condition (400 rpm/min) and with air flow of 1 vvm (L/L/min). The biomass production was carried out using a feed batch procedure and at the end of the process, it was collected by centrifugation, washed three times with sterile distilled water and inoculated into the grape juice to obtain an initial concentration of approximately 1×10^6 cell/mL. The growth kinetics of the yeast strains were monitored during the fermentation at established time.

In the Verdicchio vineyard it was used a suspension of *M. pulcherrima* DiSVA 269 (10^6 cells/ml) in bidistilled water that then was sprayed on the grapes with a blaster. Before and after the treatment 30 samples (15 samples treated and 15 samples untreated) were collected in sterile bags, transported as quickly as possible to the laboratory, and stored at a low temperature (4°C) and quickly analyzed.

In the Montepulciano vineyard was used a suspension of *M. pulcherrima* DiSVA 269 (10^7 cells / ml) and a *M. fructicola*® in dry form (200 gr/ha) in a suspension of bidistilled water (10^7 cells / ml). The suspensions were sprayed on the grapes with an atomizer. The vineyard was divided in three portions: untreated area,

treated area with *M. pulcherrima* DiSVA 269 and treated area with *M. fructicola* GAIA®. In Montepulciano vineyard 30 samples were collected in sterile bags (10 samples from treated portion with *M. fructicola,* 10 samples collected from treated portion with *M. pulcherrima* DiSVA 269 and 10 samples collected from untreated portion), transported as quickly as possible to the laboratory, stored at a low temperature (4°C) and quickly analyzed.

In the pre-harvest treatment carried out on the Verdicchio and Montepulciano vineyards, the bio effect of *M. pulcherrima* DiSVA 269 and *M. fructicola* GAIA® commercial strain were evaluated.

Biomass evolution was analyzed by viable cells counts that were made using differential and selective medium with the aim to obtain more detailed and more easily interpretable results.

WL-agar that allows the growth of all yeasts and differentiating the various genera in based on the color and morphology assumed by the colony, Lysine Agar (Oxoid, Hampshire, UK), a selective medium that does not support the growth of *S. cerevisiae* and Rose Bengal agar for the differentiation of the mold.

4. RESULTS AND DISCUSSION

4.1 Ozonated water treatment

The growth of microbial biomass pre and post treatment phases with ozonated water is shown in Fig 2.

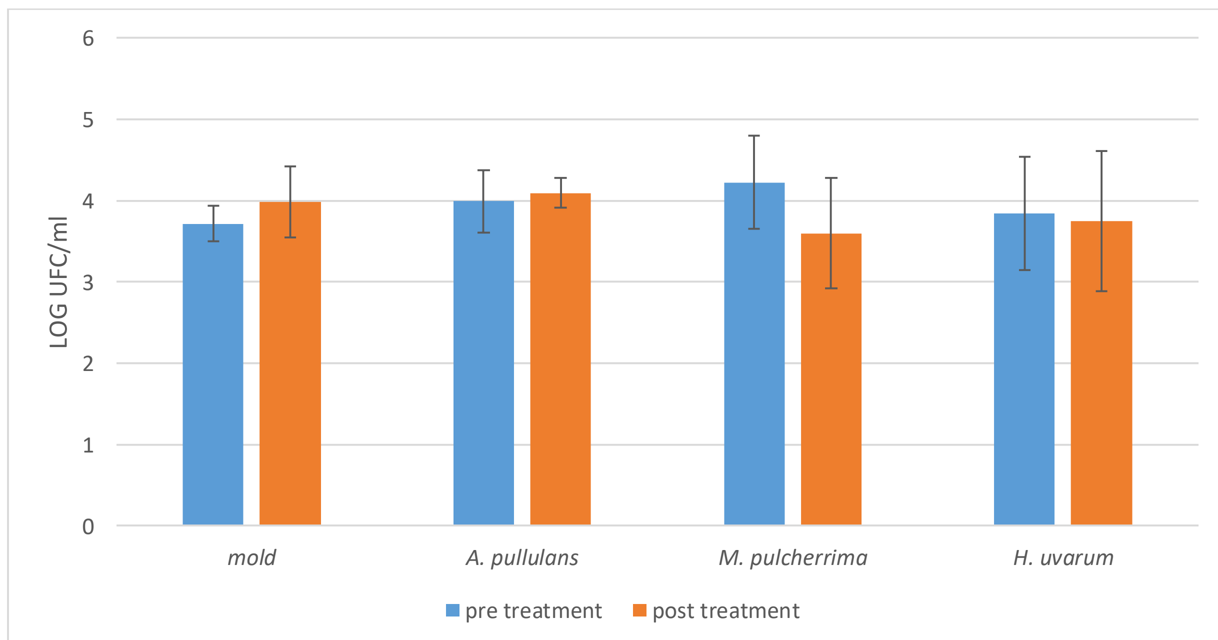

Figure 2. The effect of ozonated water treatment on wild yeasts and molds pre-treatment and after one night of treatment

The results showed that the concentration of the population of *M. pulcherrima* decreased (from 10^4 UFC / ml to 10^3 UFC / ml) after the time of action of ozonated water (one night).

The treatment highlighted a control on the development of population of *H. uvarum* that showed the same concentration pre and after treatment (10^4 CFU / ml).

Aerobasidium pullulans which is a yeast naturally present on grapes and molds seem not to be affected by the treatment carried out, showing a cellular concentration comparable to that shown in the pre-treatment (10^4 CFU / ml). All the variations in populations of the wild non-*Saccharomyces* yeasts (WNS) present on the grapes that occur between pre and post treatment are not statistically significant.

The results did not show relevant effects of the treatment and further treatments in different environmental conditions (rainy weather conditions, injury or sick grapes) , and the distribution of useful microorganisms on the soil to prevent new pathogens are still ongoing.

4.2 *Metschnikowia* DiSVA 269 treatment on Verdicchio vineyard

The results of the treatments with *M. pulcherrima* DiSVA 269 on Verdicchio grapes were showed in Figure 3.

In Figure 3a is reported the yeast and mold population after the treatment with *M. pulcherrima* (initial time).

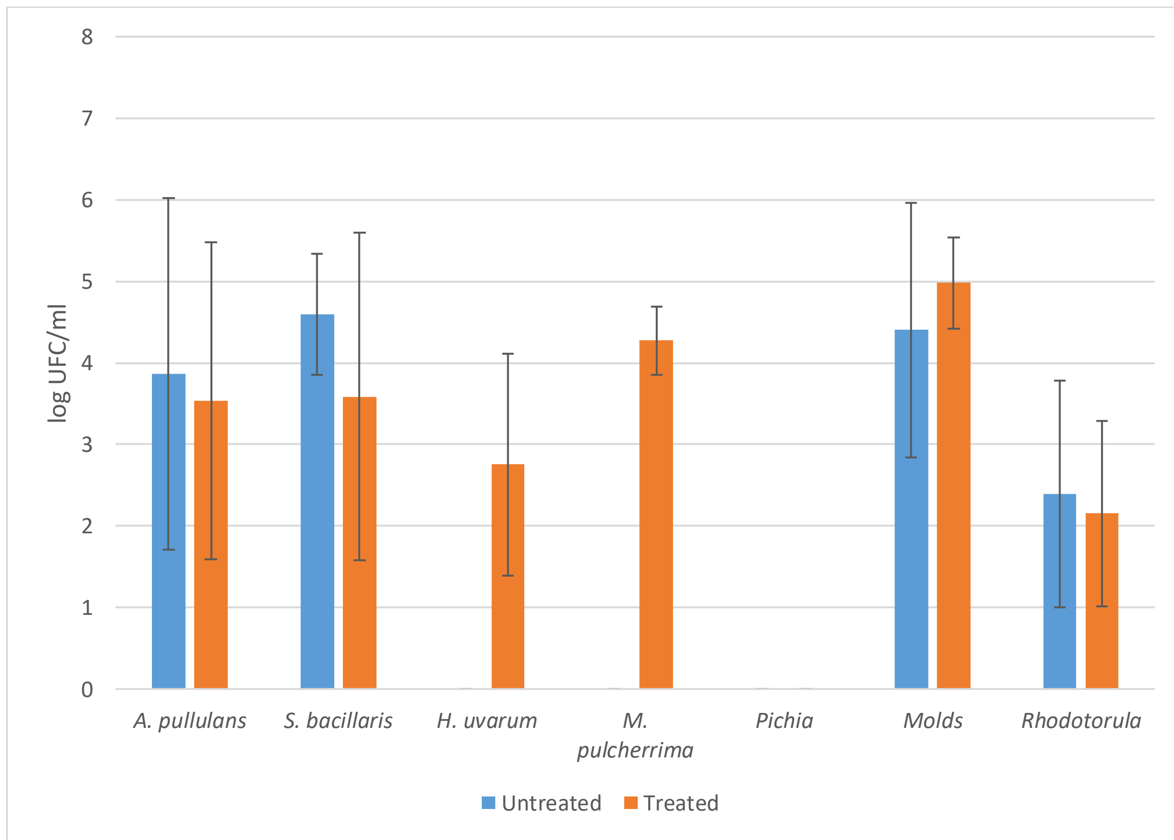

Figure 3a Grapes treated with *M. pulcherrima* DiSVA 269 at initial time

The figure 3b shows the results after 15 days from the treatment, highlighting the biocontrol effect of *M. pulcherrima* DiSVA 269 over time and its ability to colonize the grape surface.

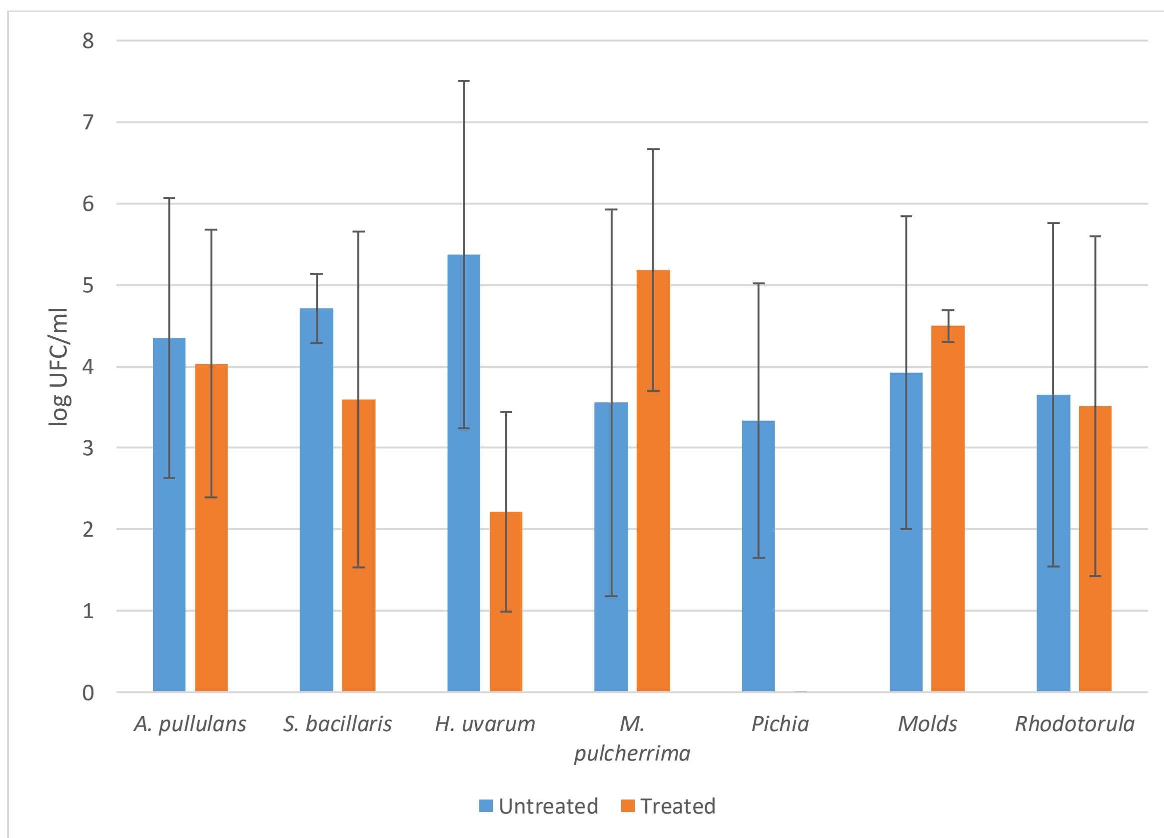

Figure 3b Grapes treated with *M. pulcherrima* after 15 days

The results of the bio treatment showed that the indigenous microbiota present on the grapes has undergone a slight modification after treatment. A significant reduction in the concentration of *H. uvarum* (from 10^5 UFC / ml to 10^2 UFC / ml) in the in detection conducted after 15 gg. The molds are not affected by the presence of *M. pulcherrima* DiSVA 269. Furthermore, the presence of *M. pulcherrima* prevents the development of *Pichia* spp. The other species of non-*Saccharomyces* yeasts present on the grapes before and after the treatment were not affected by the presence of *M. pulcherrima*.

Regarding to the settlement and permanence on grape surface, *M. pulcherrima* DiSVA 269 has been shown to colonize grape berries and persist for all the duration of the treatment without a decreasing during the days.

4.3 *Metschnikowia* DiSVA 269 and *M. fructicola* GAIA® treatment on Montepulciano vineyard

The results of the treatments with *M. pulcherrima* 48 and *M. fructicola* on Montepulciano grapes were showed in Figure 4.

In figure 4a it was reported the microbial population of the control and after the treatments at initial times.

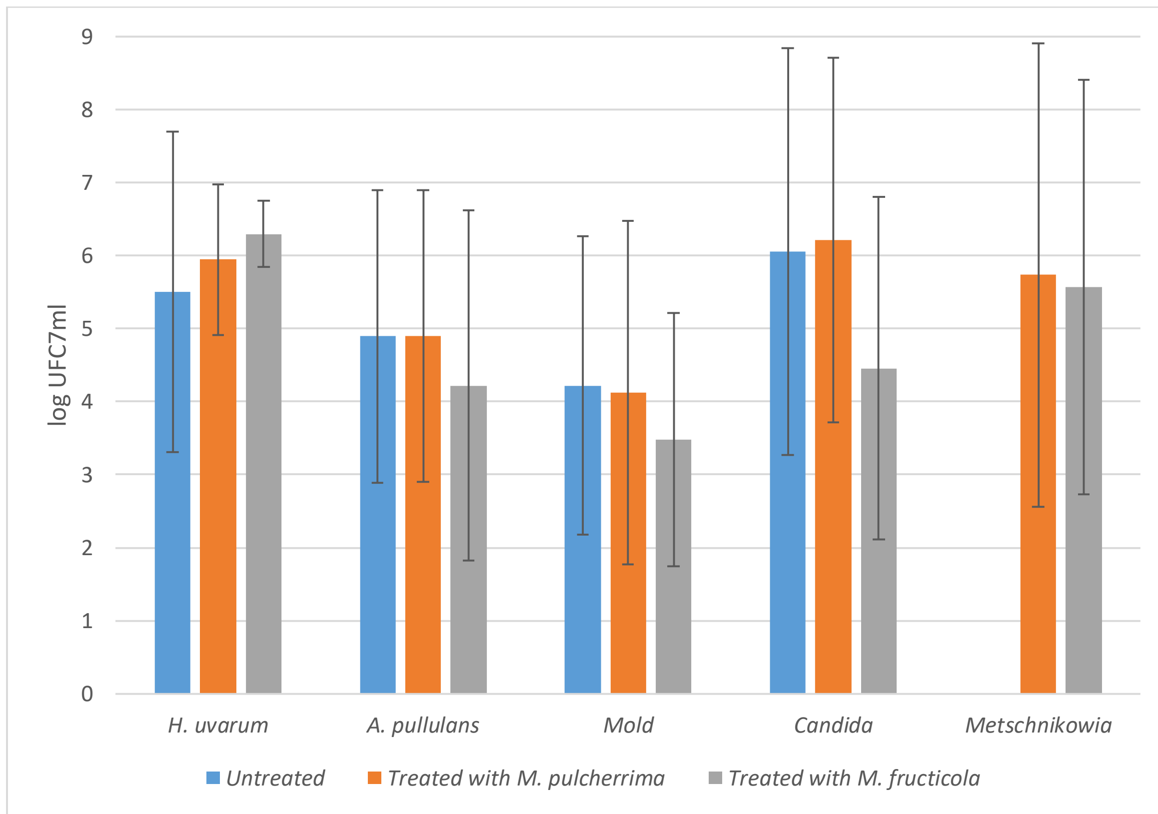

Figure 4a Grapes treated with *Metschnikowia* spp. at initial time

The Figure 4b shows the fungal population after fifteen days

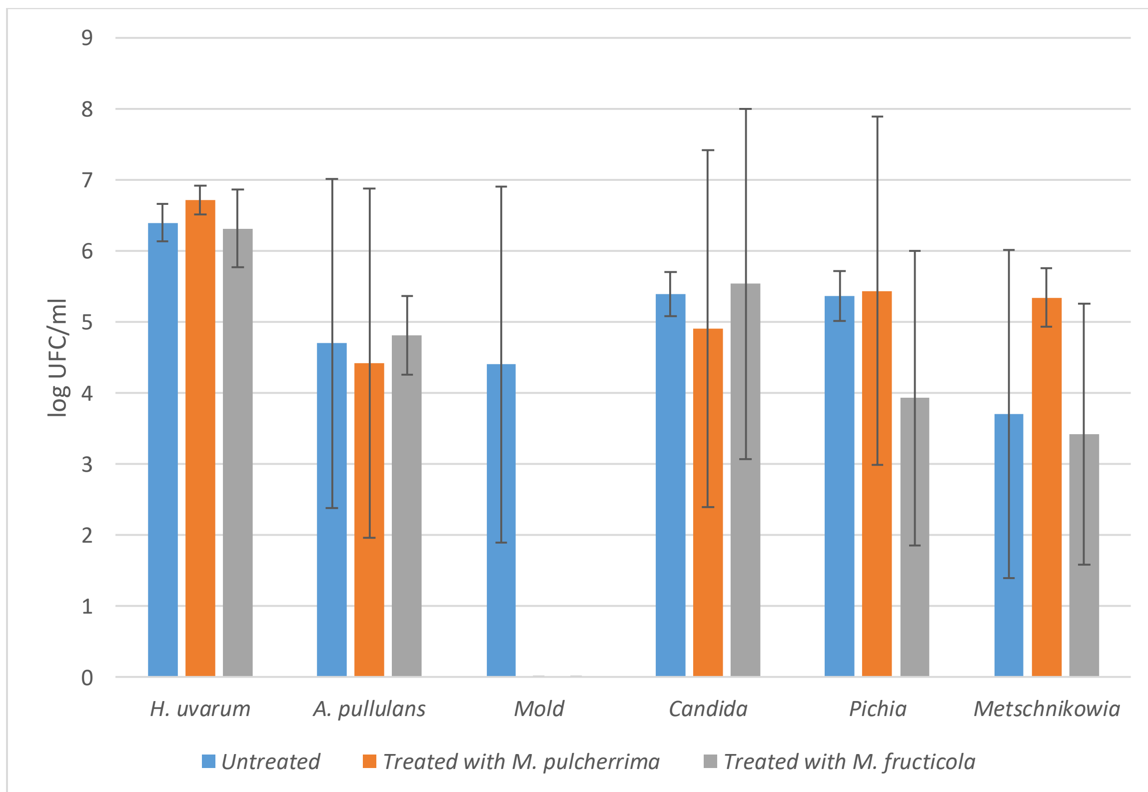

Figure 4b Grapes treated with *Metschnikowia* spp. after 15 days

The results obtained showed that both *Metschnikowia* strains exhibited a biocontrol action especially against molds that disappeared from the grapes unlike to the untreated trial. The microbial concentration has undergone a slight modification after *Metschnikowia* spp. Treatments. *A. pullulans* and *Candida* spp. (from 10^6 UFC / ml to 10^5 UFC / ml) were affected by the presence of *M. pulcherrima* DiSVA 269 (from 10^5 UFC / ml to 10^4 UFC / ml). On the contrary, *M. fructicola* it is slightly more effective to control the population of *H.uvarum* and *Pichia* spp. Furthermore, *M. pulcherrima* DiSVA 269 persisted better on the grape surface than *M. fructicola*.

5. CONCLUSIONS

During the last few years one of the most relevant concerns that is related to the winemaking sector is the use of alternative methods in vineyard to replace chemical and invasive treatments. Actually, the most commonly substances used are sulfur-based compounds which, in the presence of high temperatures, have toxic effects on the vines.

The efficiency of ozonated water treatments carried out directly on the epiphytic microflora of grape berries was evaluated. Indeed, the three main microbial groups were considered: fungi, bacteria and yeasts. The results obtained from viable cells count carried out pre and post treatment samples did not show a relevant decrease of the growth and development of the spoilage population present on the berries.

Indeed, the results showed that the indigenous microbiota present on the grapes has undergone a slight modification after treatments, with a more significant reduction of *M. pulcherrima* population and with the control of *Aureobasium* spp and *Hanseniaspora* spp population.

The second part of the wineyard investigation was focused on microbial strategies with the use of *M. pulcherrima* DiSVA 269 and *M. fructicola* GAIA® as biocontrol agents.

M. pulcherrima DiSVA 269 was sprayed directly on the grapes before the harvest both in Verdicchio DOC and Montepulciano DOC vineyards. The results showed that *M. pulcherrima* DiSVA 269 persisted on the grapes for over two weeks, dominating the spoilage yeasts microbiota naturally present on the grapes and in the grape juice without a re-inoculum.

Nice results were obtained with the use both strains of *Metschnikowia* spp., in particular against the formation of molds, *A. pullulans* and *H. uvarum*. These results highlighted that the use of *Metschnikowia* spp. can be a suitable strategy to replace the use of chemical treatments and further studies should be conducted to confirm these results.

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SECOND PART

***M. PULCHERRIMA* AS BIO-PROTECTANT AT PREFERMENTATIVE AND FERMENTATIVE STAGE TO REDUCE THE USE OF SO_2**

1. **INTRODUCTION**
2. **AIM OF THE WORK**
3. **MATHERIALS AND METHODS**

3.1 Yeast Strains

3.2 Fermentation trials at laboratory scale

3.3 Fermentation trials at industrial level

3.3.1 Prefermentative stage

3.3.2 Starter inoculum and Fermentation stage

3.3.3 Biomass evolution

3.3.4 Analytical Procedures

3.3.5 Molecular characterization

3.3.6 Sensory Analysis

3.3.7 Statistical Analysis

4. RESULTS

4.1 Fermentation trials at laboratory scale: biomass evolution and main volatile compounds

4.2 Fermentation trials at industrial level

4.2.1 *M. pulcherrima* as bioprotectant agent at prefermentative stage

4.2.2 Biomass Evolution and Sugar Consumption

4.2.3 Frequency and dominance of *S. cerevisiae* starter strains

4.2.4 Main Oenological Characters of wine and Volatile Compounds of wine

4.2.5 Sensorial analysis

5. CONCLUSIONS

1. INTRODUCTION

The use of several non-*Saccharomyces* yeasts at pre fermentative stage and in sequential fermentation with *S. cerevisiae* starter strains to reduce the use of sulfur dioxide and at the same time to produce wines with distinctive sensorial properties is a strategy under investigation in winemaking. *M. pulcherrima* is a species characterized by several positive features in winemaking (Morata et al., 2019). Indeed, *M. pulcherrima* can modulate the synthesis of secondary metabolites to improve the sensorial profile of wine and to act as biocontrol agent representing a valid tool in winemaking industry. Regarding to its contribution in aroma complexity of wine, *M. pulcherrima* in mixed fermentation with *S. cerevisiae* showed a reduction of ethyl acetate production, favoring the formation of 2-phenylethyl acetate, an enhancement of acetate esters and higher alcohols (Varela et al., 2016; Zhang et al., 2018). Another important feature of this non-*Saccharomyces* yeast is the wide possess among the strains of numerous enzymatic activities such as the proteolytic activity it is important in mixed fermentation to release amino acids as nutrient for *S. cerevisiae*. In addition, the glucosidase activity promotes the release of varietal aromas from the grape (Fernández et al., 2000; Mendes Ferreira et al., 2001).

M. pulcherrima can be also used as biocontrol agent, due to the production of pulcherrimin a red pigment. This antimicrobial activity has shown effective inhibitory activity against several yeasts as *Candida*, *Brettanomyces*/*Dekkera*, *Hanseniaspora* and *Pichia* genera and fungi as *Botrytis Penicillium*, *Alternaria* and *Monilia* genera. (Csutak et al., 2013; Kántor et al., 2015; Oro et al., 2014; Saravanakumar et al, 2008).

2. THE AIM OF THE WORK

In this investigation a selected strain of *M. pulcherrima* was evaluated to set up a vinification process. After preliminary laboratory trials, this *M. pulcherrima* selected strain was inoculated at pre-fermentative stage in winemaking processes carried out at industrial level. As starter strains it was used the improved *S. cerevisiae* native strain I4, belonging to the Yeast Collection of the Department of Life and Environmental Sciences (DiSVA) of the Polytechnic University of Marche (Italy) (Agarbati et al., 2020) and compared with *S. cerevisiae* starter strain, Lalvin ICV OKAY® (Lallemand Inc., Toulouse, France). The microbial evolution of the industrial fermentations and the analytical and sensorial profile of wines were evaluated.

3. MATERIALS AND METHODS

3.1 Yeast Strains

The improved *S. cerevisiae* native strain I4, belonging to the Yeast Collection of the Department of Life and Environmental Sciences (DiSVA) of the Polytechnic University of Marche (Italy) (Agarbati et al., 2020) and

the commercial *S. cerevisiae* starter strain, Lalvin ICV OKAY® (Lallemand Inc., Toulouse, France) were used as starter strains. *M. pulcherrima* DiSVA 269 was selected on the bases of previous works on biocontrol ability (Oro et al., 2014; Oro et la., 2018). *Hanseniaspora uvarum* DiSVA 49 was used as spoilage non-*Saccharomyces* yeast and inoculated on the grape juice. All the yeast strains were maintained on YPD agar medium (yeast extract 1%, peptone 2%, dextrose 2% and agar 1.8%) at 4 ◦C for short-term storage and in YPD broth supplemented with 80% (w/v) glycerol at −80 ◦C for long-term storage.

3.2 Fermentation trials at laboratory scale

The fermentation trials were carried out using Verdicchio grape juice coming from vintage 2017. The Verdicchio grape juice had the following main analytical composition: pH 3.22; initial sugar content 212 g/L; total acidity 4.58 g/L; malic acid 2.7 g/l; nitrogen content YAN (60 mg/L) and total SO2 27 mg/l. The fermentation trials were carried out in flasks containing 200 mL of Verdicchio grape juice previously sterilized in an autoclave. The flasks were locked with a Müller valve containing sulfuric acid to allow only CO_2 to escape from the system and placed at 22 °C in thermostat under static condition in triplicate. All strains were pre-cultured in modified YPD (0.5% w/v yeast extract, 2% w/v glucose, and 0.1% w/v peptone) for 1 day at 25 °C in an orbital shaker (rotation, 150 rpm). The cells were used to inoculate the grape juice at initial concentration of 1×10^6 cells / mL for *S. Cerevisiae* DiSVA 708 and *M. pulcherrima* DiSVA 269 and 1×10^4 cells / mL for *H. uvarum* DiSVA 49. As control strains and in sequential fermentation, OKAY® was used. The fermentation kinetics were monitored by measuring the weight loss of the flasks due to the CO_2 evolution, which was followed to the end of the fermentation (i.e., constant weight for 2 consecutive days).

Biomass evolution was analyzed by viable cells counts that were made using differential and selective medium with the aim to obtain more detailed and more easily interpretable results.

WL-agar that allows the growth of all yeasts and differentiating the various genera in based on the color and morphology assumed by the colony, Lysine Agar (Oxoid, Hampshire, UK), a selective medium that does not support the growth of *S. cerevisiae*.

3.3 Fermentation trials at industrial level

3.3.1 Prefermentative stage

The bioprotectant action of the *M. pulcherrima* strain at pre-fermentative stage was carried out in three lots of Verdicchio grape juice of 600 hL each during cold chiarification and used to fill four different vats of 300 hL, 200 hL and two of 50 hL. The grapes were treated following the same winemaking procedures: soft pneumatic pressing cold clarification without SO_2 addition at 10 °C for 48 h. *M. pulcherrima* DiSVA 269 strain was

inoculated (1 × 10^6 cells/mL) in three vats of each lot while the other three were not inoculated. After 48 h the temperature was brought at 19 ± 1°C and inoculated with *S. cerevisiae* strain (1 × 10^6 cells/mL).

3.3.2 Starter inoculum and Fermentation stage

Fermentation trials at industrial level were performed using Verdicchio grape juice coming from vintage 2020. The analytical characters of the grape musts were initial sugars 216g/l, pH 3.34, total acidity 4.37 g/l, malic acid 1.7g/l, and nitrogen content 90 mg/l. The diammonium phosphate and yeast derivative (Genesis Lift® Oenofrance, Bordeaux, France) used were adjusted to 250 mg N/L as yeast assimilable nitrogen. The fermentation trials were carried out in a steel vat containing 300 hL of Verdicchio grape juice at 19 ± 1°C. The fermentations were carried out under winemaking conditions at e winery Terre Cortesi Moncaro s.r.c.l.

All the yeast strains were pre-cultured in modified YPD medium (0.5% yeast extract, 0.1% peptone and 2% glucose) for 48 h at 25 °C in an orbital shaker (150 rpm). After this period, the pre cultures were used to inoculate 30-L Bench-top bioreactor (Biostat® C; B. Braun Biotech Int., Goettingen, Germany) containing 25 L modified YPD medium for *S. cerevisiae* strains and medium containing 1% di yeast extract, 0.5% peptone and 5% invert sugar for *M. pulcherrima* strain under agitation condition (400 rpm/min) and with air flow of 1 vvm (L/L/min). The biomass production was carried out using a feed batch procedure and at the end of the process it was collected by centrifugation, washed three times with sterile distilled water and inoculated into the grape juice to obtain an initial concentration of approximately 1 × 10^6 cell/mL. The growth kinetics of the yeast strains were monitored during the fermentation at established time.

3.3.3 Biomass evolution

The growth kinetics of the yeast strains were monitored during the fermentation at established intervals using WL nutrient Agar medium (Oxoid, Hampshire, U.K.) and Lysine Agar medium (Oxoid, Hampshire, U.K.). The sugar consumption during the fermentation process was measured by Baumé (°Bé) densimeter.

3.3.4 Analytical Procedures

Total acidity, volatile acidity, pH, and ethanol content were determined according to the Official European Union Methods (2000). Enzymatic kits (Megazyme International Ireland) were used to measure the amounts of glucose and fructose (K-FRUGL) and malic acid (K-DMAL) according to the manufacturer instructions. A specific enzymatic kit (kit no. 112732; Roche Diagnostics, Germany) was used to determine the ammonium content. The free α-amino acids were evaluated following Dukes and Butzke protocol (1998). Ethyl acetate, acetaldehyde, and higher alcohols were quantified using a gas chromatograph system (GC-2014; Shimadzu, Kjoto, Japan) by direct injection. The final wines were prepared as described by Canonico et al. (2018). The solid-phase microextraction (HS-SPME) method was used to quantify the main volatile compounds as

described by Canonico et al. (2019). The compounds were desorbed by inserting the fiber Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Sigma-Aldrich, St. Louis, MO, USA) into a gas chromatograph (GC) injector.

3.3.5 Molecular characterization

Intraspecies characterization of *S. cerevisiae* isolates were carried out using primer pairs δ 12/21 as described by Legras and Karst 2003. PCR amplifications were carried out in 25 µL reaction volumes containing approximately 20 ng template, 10 mM Tris, pH 9.0, 50 mM KCl, 0.1% (v/v) Triton X-100, 0.2 mg/mL gelatin, 200 mM each dNTP, 2.5 mM MgCl2, and 1 mM each oligonucleotide primer of the δ12 and δ21 family. The amplification reactions were performed with a Biorad Thermal Cycler, using the following programme: 4 min at 95°C, followed by 35 cycles of 1 min at 95°C, 45 s at 55°C, and 2 min at 72°C, and the final extension at 72°C for 2 min. 15 µL samples of the PCR products were loaded onto 1.5% (w/v) agarose gels, and the electrophoresis was carried out at 70 V for 2 h in 0.5× TBE buffer. The DNA bands on the gel were visualized by staining with ethidium bromide, and acquisition of the images was performed under a UV lamp (UV source GelDoc 1000; BioRad). The length of the PCR products was estimated by comparing them with 100-bp marker DNA standards (GeneRuler 100-bp DNA Ladder; AB Fermentas).

3.3.6 Sensory Analysis

At the end of the fermentation, the wines obtained were transferred into filled 750 mL bottles, closed with the crown cap, and maintained at 4 °C until sensory analysis. After storage for 3 months, wines were subjected to sensory analysis based on the main sensorial descriptors. A group of ten testers using a score scale of 1 to 10, expressed their opinion regarding each wine tested. The data obtained were used to compare the wines and provide information regarding the organoleptic quality and probable consumers' acceptability of the wines obtained.

3.3.7 Statistical Analysis

Analysis of variance (ANOVA) was used to elaborate the data of analytical character of wines. The means were analyzed using the statistical software package JMP® 11. Duncan tests were used to detect the significant differences. The experimental data were significant with associated p-values < 0.05.

4.RESULTS

4.1 Fermentation trials at laboratory scale: biomass evolution and main volatile compounds

The growth kinetics of control and sequential fermentations carried out at lab scale are shown in the Figure 5.

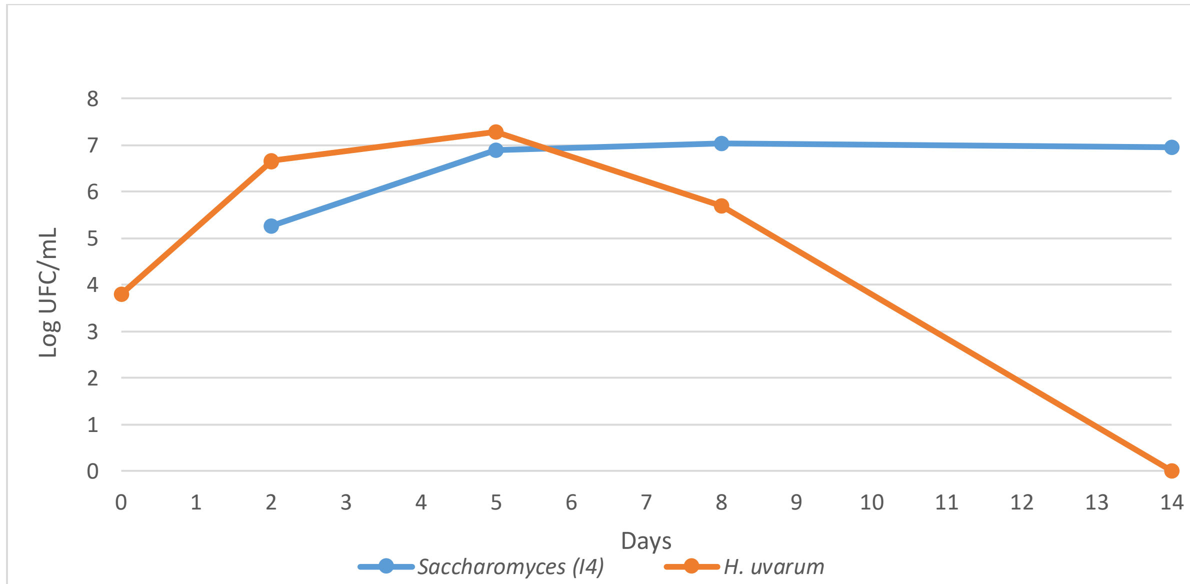

Figure 5a. Growth kinetics in control fermentation trials of *S. cerevisae* I4 ( ) and *H. uvarum* ( ) on natural grape juice.

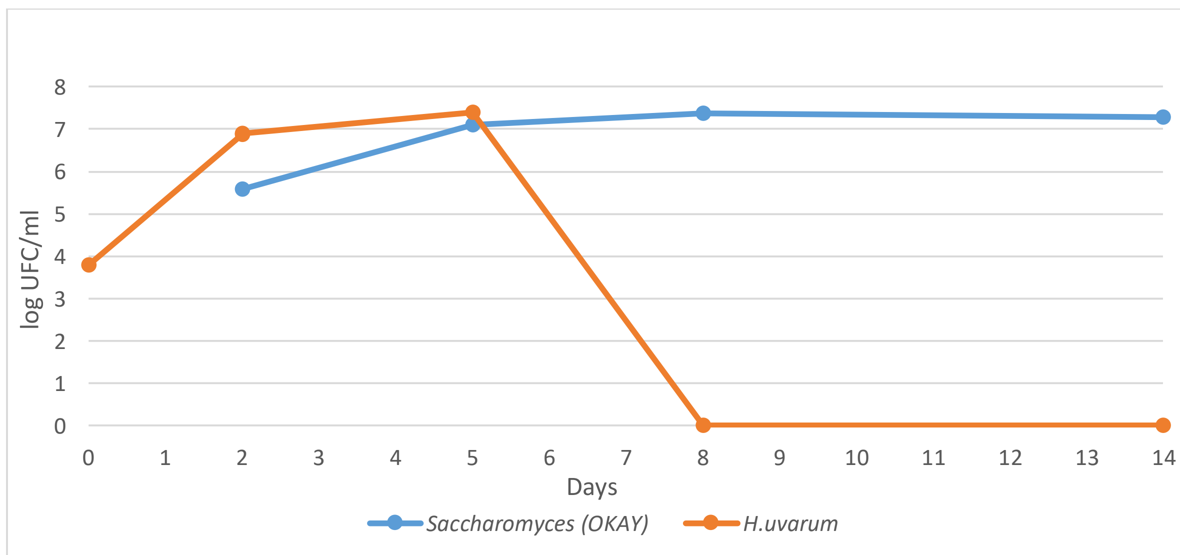

Figure 5b. Growth kinetics in control fermentation trials of *S. cerevisae* .LALVIN ICV OKAY® ( ) and *H. uvarum* yeasts ( ) on natural grape juice

S. cerevisiae pure fermentations (Figure 5a-b) showed a similar trend regarding the evolution of *S. cerevisiae* population. *H. uvarum* population showed a similar trend in both trials for the first days of fermentations achieving over 10^7 CFU /mL at 5th day of fermentation. After that, *S. cerevisiae* I4 trial (Fig. 5a) showed a slower decrease of *H. uvarum* from 5th day until the end of fermentation in comparison with *S. cerevisiae* OKAY® trial (Fig. 5b).

Regarding the sequential fermentations with *M. pulcherrima*, *H. uvarum* population during the first five days did not exceed 10^6 CFU/mL disappearing in both cases (Fig. 6 a-b) at 8th day of fermentation.

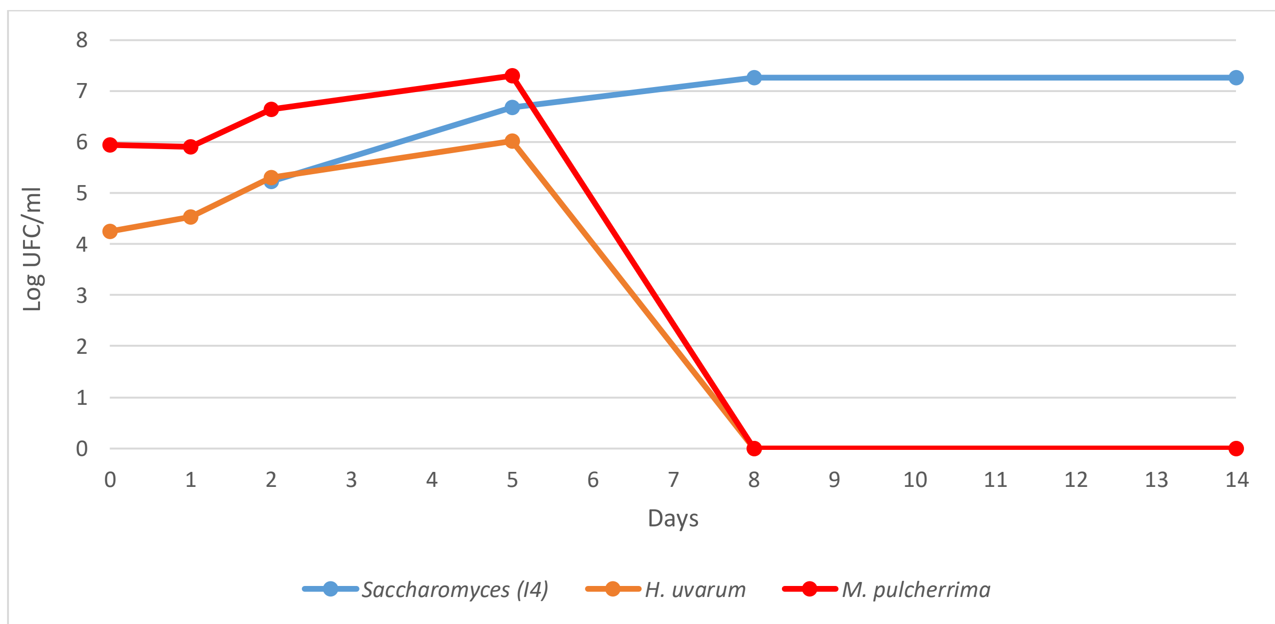

Figure 6a Growth kinetics in sequential fermentation trials of *M. pulcherrima* ( )/ *S. cerevisiae* ( )*H. uvarum* ( ) on natural grape juice.

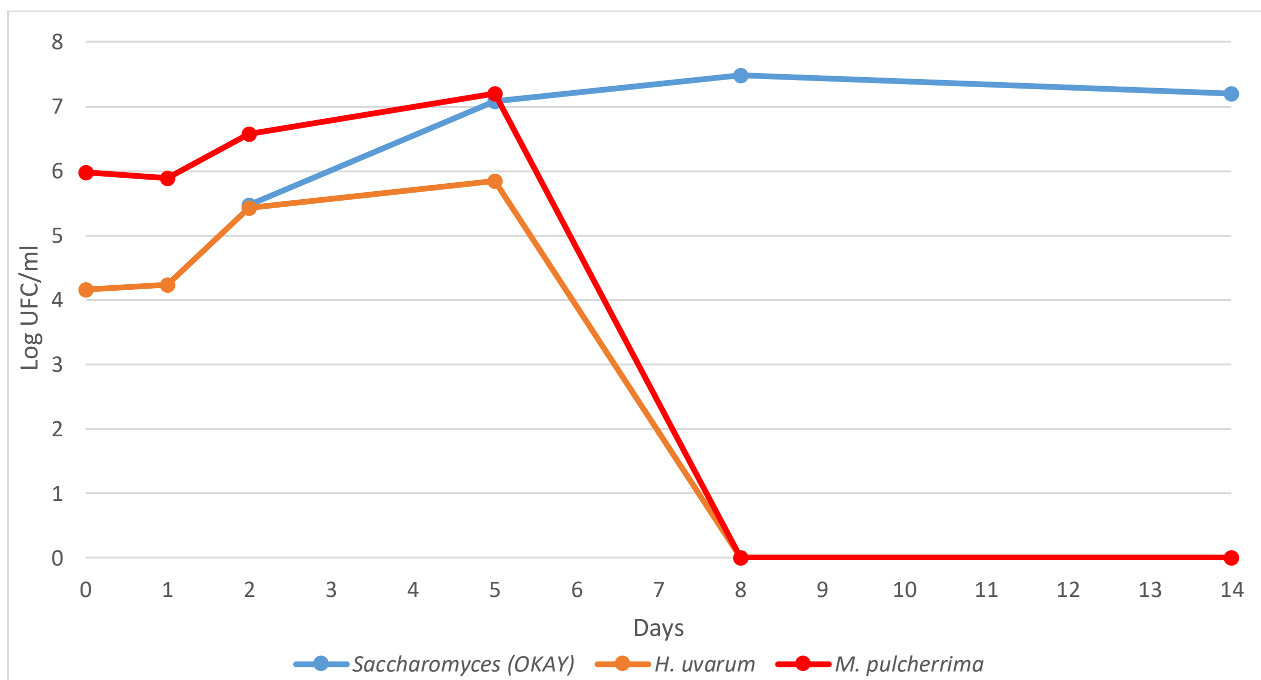

Figure 6b. Growth kinetics in sequential fermentation trials of *M. pulcherrima* ( )/ *S. cerevisiae LALVIN ICV OKAY®* ( ) and *H. uvarum* ( ) on natural grape juice.

In the figures 6 a-b are shown the mixed fermentation trials. Both *S. cerevisiae* inoculated after 48 h reach the maximum cell concentration at the 8th day remaining almost constant until the end of the fermentation process while *M. pulcherrima* population disappeared at the same time (8th day).

Regarding to the fermentation kinetics (data not shown), all fermentation tested did not show difference among them, only *S. cerevisiae* I4 pure culture exhibited a slower fermentation kinetics in comparison with the other trials, determining, in any case, the completion of fermentation at the same time.

These results indicated that *M. pulcherrima* did not affect the growth and the development of *S. cerevisiae* but, on the contrary, determined the control of the development of *H. uvarum* showing a bioactive action on *H. uvarum* population in presence of both *S. cerevisiae* strains .

The resulting wine were subject to the analysis of the main volatile compounds (Table 1).

	S. cerevisiae I4	*M. pulcherrima* /*S. cerevisiae* I4	*S. cerevisiae* OKAY®	*M. pulcherrima* /*S. cerevisiae* OKAY®
Ethyl butyrate	0.121±0.016^{b}	0.410±0.033^{a}	0.429±0.016^{a}	0.453±0.07^{a}
Isoamyl acetate	0.867±0.172^{d}	1.08±0.23^{c}	1.630±0.031^{b}	2.493±0.13^{a}
Ethyl exanoate	0.107±0.020^{b}	0.147±0.006^{a}	0.063±0.012^{c}	0.048±0.00^{c}
Hexanol	0.012±0.001^{c}	0.013±0.006^{c}	0.054±0.009^{a}	0.038±0.00^{b}
Linalol	0.079±0.044^{b}	0.117±0.055^{a}	0.043±0.007^{b}	0.062±0.01^{b}
Geraniol	0.009±0.000^{a}	0.008±0.005^{a}	0.005±0.002^{a}	0.001±0.00^{a}
β-Phenyl Ethanol	33.4±0.05^{b}	57.8±0.072^{a}	42.2±0.019^{b}	32.5±0.010^{b}

Table 1. The main volatile compounds of the fermentation trials carried out at laboratory scale. Data are means ± standard deviations. Values displaying different superscript letters (^{a,b}) within each line are significantly different according to Duncan tests ($p < 0.05$).

Regarding to the main volatile compounds produced during fermentation trials carried out at laboratory scale, the wine obtained with *S. cerevisiae* I4 pure culture show an increase of Ethyl exanoate (apple peel) and Linalol (floreal aroma) content in comparison with *S. cerevisiae* OKAY® pure culture. On the contrary *S.*

cerevisiae OKAY® pure culture led a significant increase in isoamyl acetate (banana aroma) and β-Phenyl Ethanol (rose aroma) content respect the fermentation trails *S. cerevisiae* I4 pure fermentation

M. pulcherrima in sequential fermentation with *S. cerevisiae* I4 led a significant increase in Ethyl hexanoate, linalool (floral aroma) and β-Phenyl Ethanol (rose aroma) content in comparison with other sequential fermentation with *M. pulcherrima / S. cerevisiae* OKAY®.

The sequential trial with *S. cerevisiae* OKAY® showed an increase of isoamyl acetate (banana aroma) content. Regarding to the production of hexanol and geraniol compounds, the resulting wines did not show significant differences.

In conclusion the presence of *S. cerevisiae* I4 in pure culture showed an increase in linalool and β-Phenyl Ethanol respect *S. cerevisiae* OKAY® pure culture. The sequential fermentation *M. pulcherrima/ S. cerevisiae* I4 led an increase of ethyl hexanoate, linalool and β-Phenyl ethanol content highlighting a good synergistic effect between the yeasts.

M. pulcherrima/ S. cerevisiae I4 is the best trial to control the spoilage yeasts and to produce an organic wine without organoleptic defects and with a particular aromatic bouquet.

4.2 Fermentation trials at industrial level

4.2.1 *M. pulcherrima* as bioprotectant agent at prefermentative stage

Based on the results obtained at laboratory scale, the selected strain *M. pulcherrima* (1×10^6 cells/mL) was inoculated in grape juice at prefermentative stage (for the first two days of clarification) in four different steel vats (300 hL) to test its bio control effect against wild yeasts.

In the following Figures (7 a/b – 8 a/b) are showed the results of the action of *M. pulcherrima* on wild yeasts.

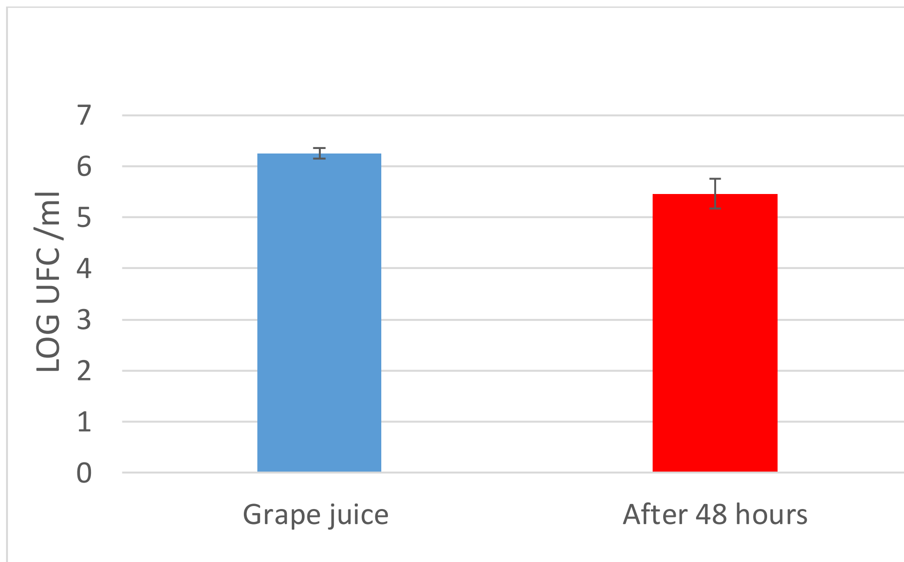

Figure 7a The bio control effect of *M. pulcherrima* on wild yeasts for LALVIN ICV OKAY® trial

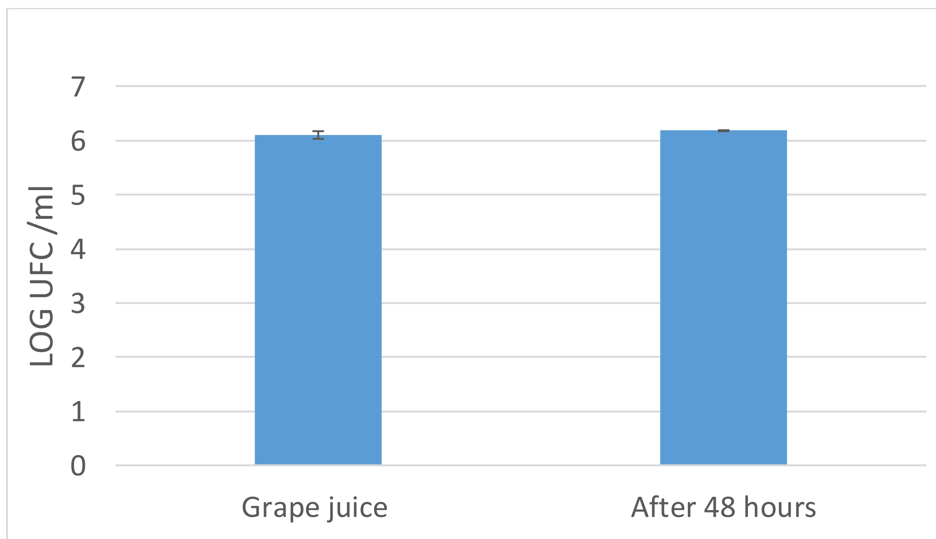

Figure 7b. The absence of bio control effect without inoculum of *M. pulcherrima* for LALVIN ICV OKAY® trial

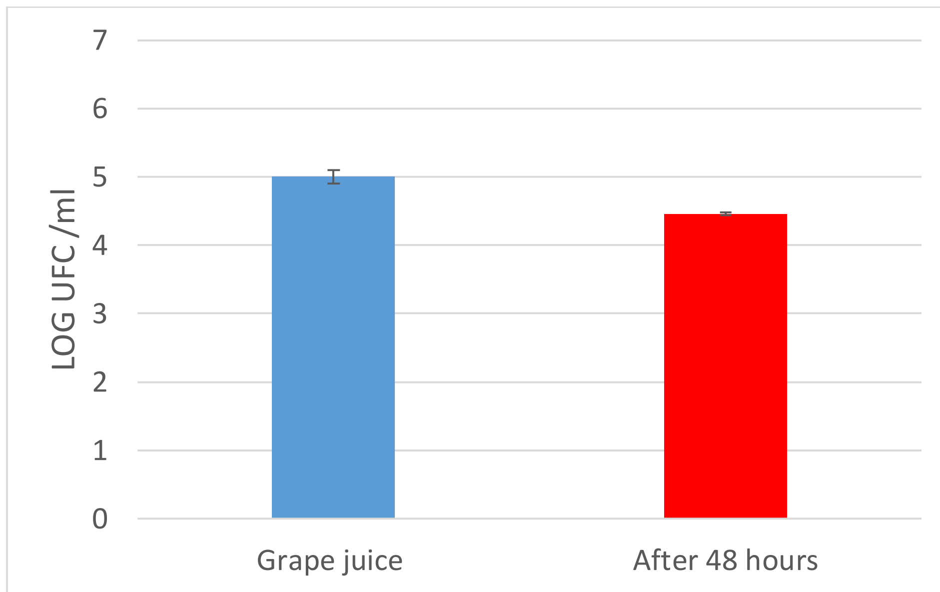

Figure 8a. The biocontrol effect of *M. pulcherrima* on wild yeasts for *S. cerevisiae* I4 trial

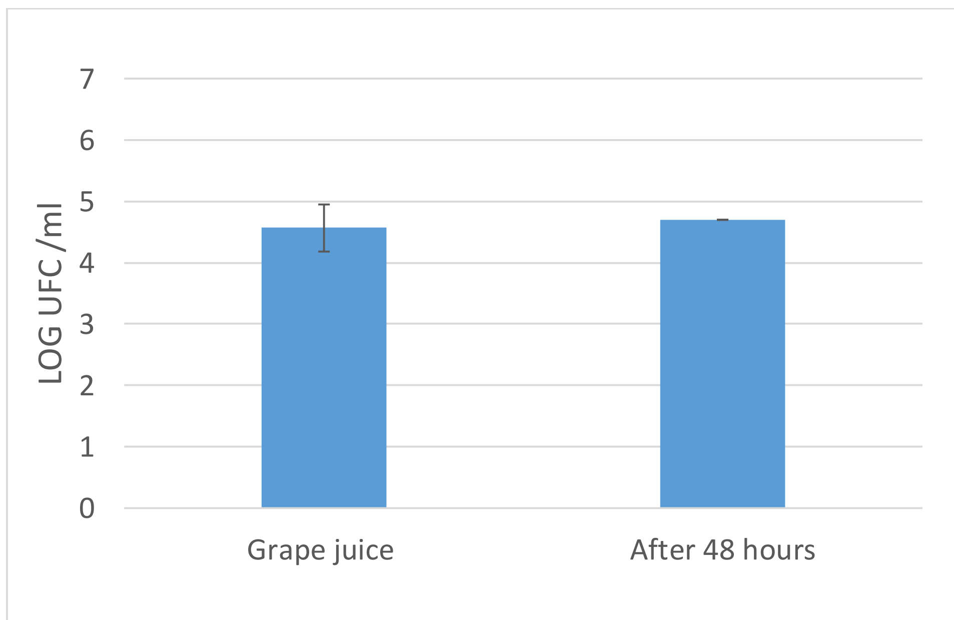

Figure 8b The absence of bio control effect without inoculum of *M. pulcherrima* for *S. cerevisiae* I4 trial

The results obtained by viable cells counts conducted on WL agar, highlighted that *M. pulcherrima* showed a significant bioactive effect reducing the wild yeasts (*H. uvarum* was the most abundant species) in both inoculated vats. In fact the graphs 7a and 8a showed a reduction of wild yeast population that was affected by the presence of *M. pulcherrima,* with an average reduction of 1 Log UFC/ml. The figures 7b and 8b showed the control theses in which *M. pulcherrima* was not inoculated. In this case, the concentration of population

of spoilage yeast was not affected remaining unchanged (c.a. 10^6 cell/mL). In all vats sulfur dioxide was not added. .

Summarizing the results obtained after two days of cold clarification of grape juice showed that the presence of *M. pulcherrima* determined a control and regulation of the development of wild yeasts particularly *H. uvarum* species exercising an effective bio-control effect.

The grape juice of the same batch, after the clarification phase, was inoculated with two *S. cerevisiae* starter strain (I4 and OKAY®). The growth kinetics during the fermentation process and the main oenological characters were evaluated.

4.2.2 Biomass Evolution and Sugar Consumption

Growth kinetics of fermentations inoculated with the starter *S. cerevisiae* I4 with and without *M. pulcherrima* strain 48 at prefermentative stage are reported in following graphs.

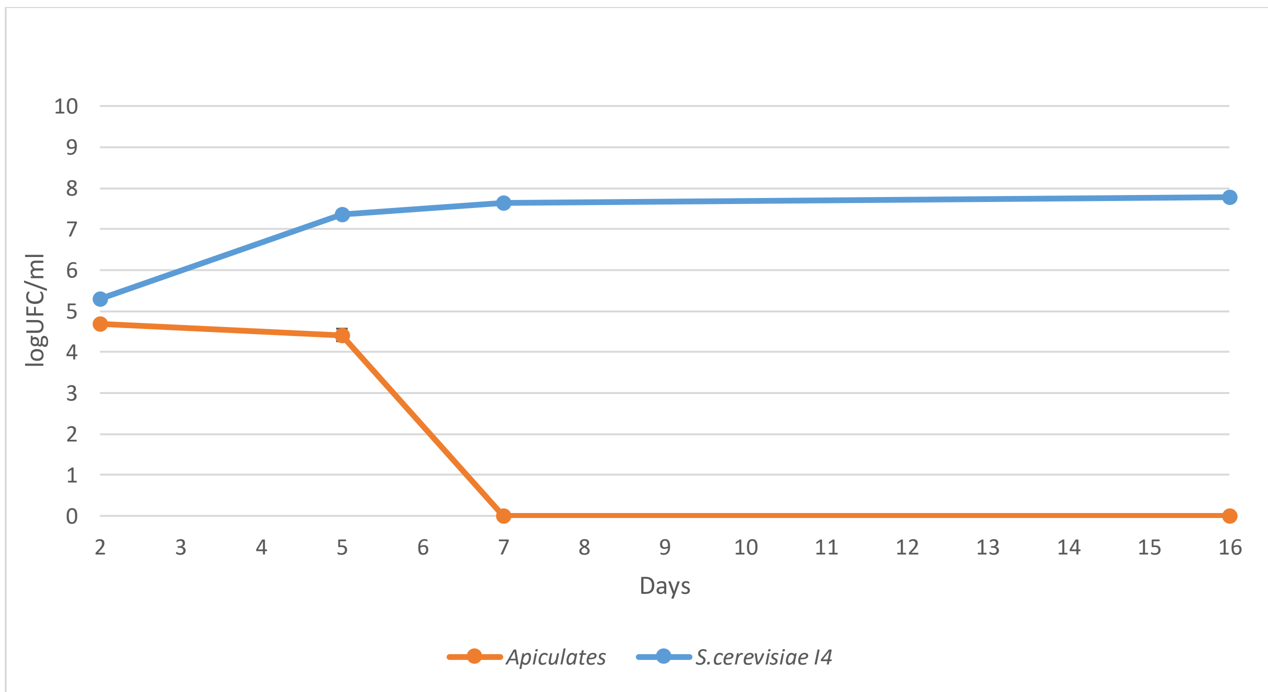

Figure 11a Growth kinetics in control fermentation trials of *S. cerevisiae* I4 ( ) and Apiculate yeasts ( ) on natural grape juice

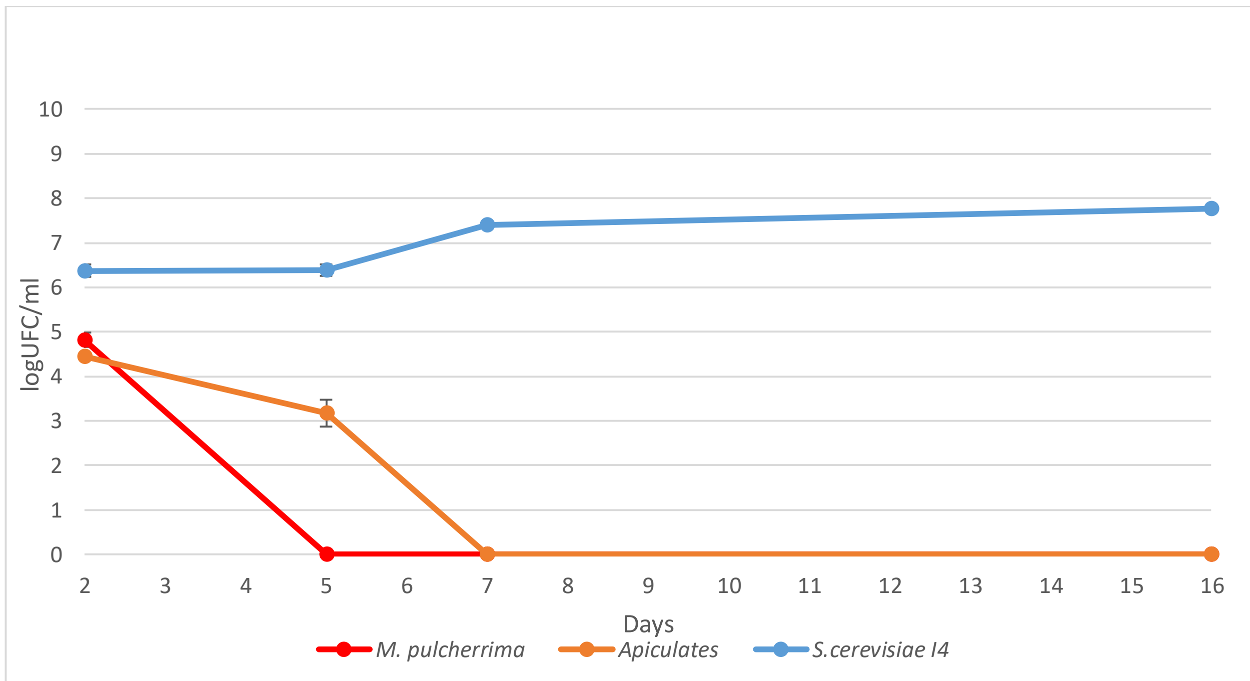

Figure 11b. Growth kinetics in sequential fermentation trials of *M. pulcherrima* ( )/ *S. cerevisiae* I4 ( ) Apiculate yeasts ( ) on natural grape juice.

The pure culture *S. cerevisiae* I4 exhibited a different trend in comparison with OKAY®. Indeed, I4 (fig. 11a) with initial inoculum level of 10^5cell/ml, exhibited the maximum cell concentration at 7th day of fermentation (10^8 cell/ml) to remain constant until the end of fermentation. The sequential fermentation *M. pulcherrima*/I4 (fig. 11b) did do not show bio-active apiculate yeasts if compared to *M. pulcherrima*/ OKAY®. However, the presence of *M. pulcherrima* improved the control of the development on apiculate if compared to I4 pure culture.

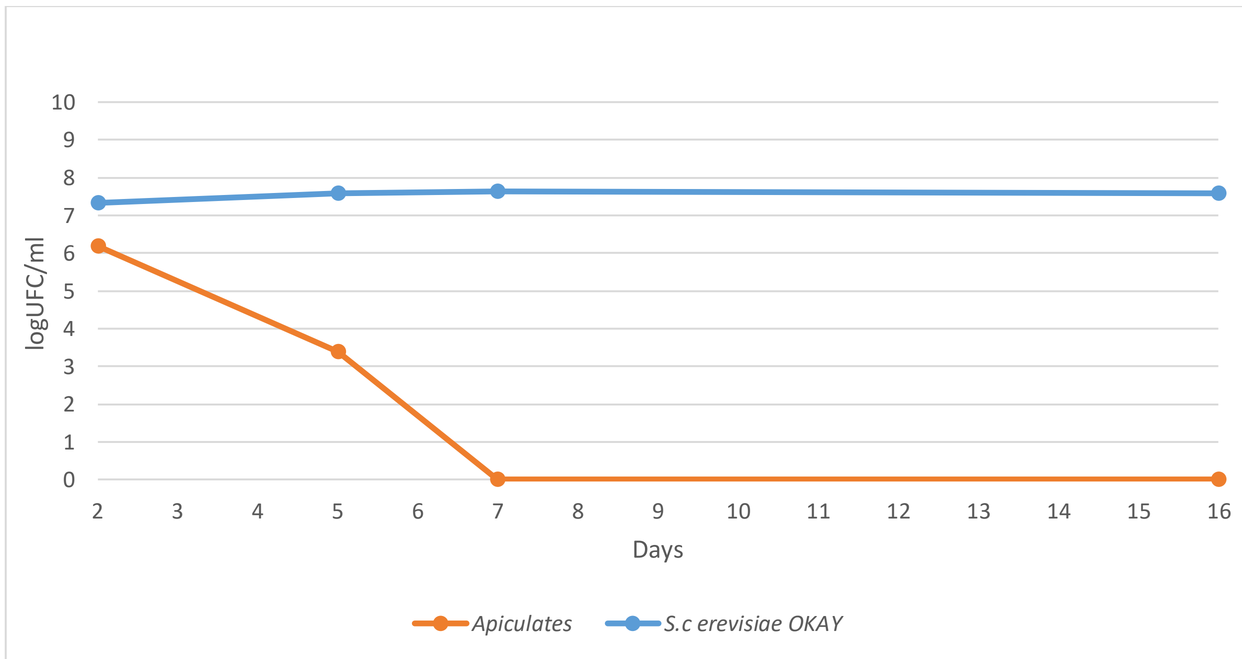

Figure 12a. Growth kinetics in control fermentation trials of *S. cerevisiae* LALVIN ICV OKAY® ( ) and Apiculate yeasts ( ) on natural grape juice.

The results of the fermentation with the inoculum of *S. cerevisiae* starter strain OKAY® exhibited a more effective control on the apiculate yeasts in comparison to I4. LALVIN ICV OKAY® (fig 14a) achieved the maximum cell concentration at 2nd day of fermentation (c.a 10^8 cell/ml) remaining constant for the entire duration of fermentation.

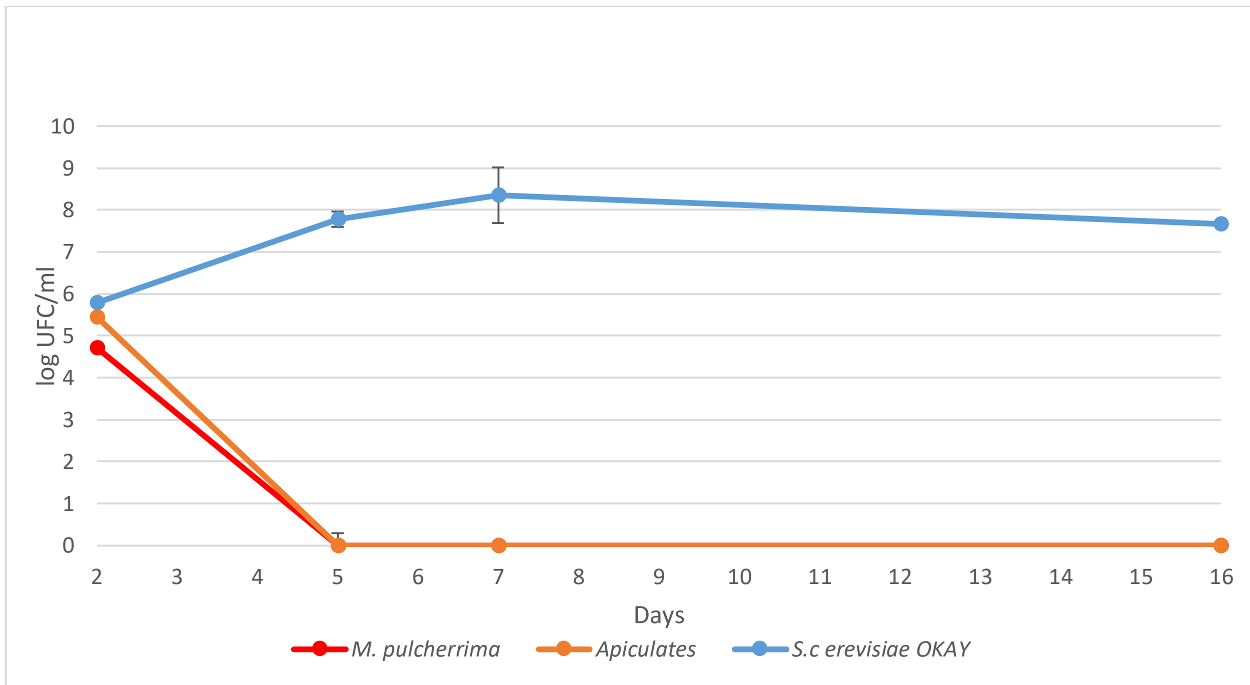

Figure 12b. Growth kinetics in sequential fermentation trials of *M. pulcherrima* ( )/ S. *cerevisiae* OKAY ( ) and Apiculate yeasts ( ) on natural grape juice.

M. pulcherrima sequential fermentation with OKAY® (fig. 12 b) highlighted a decrease of apiculate yeasts by 3rd day to disappear at 5th day of fermentation. Moreover, the biomass evolution of OKAY® did not affect by *M. pulcherrima*.

However, in both fermentation trials the apiculate yeasts disappear within the 7th day of fermentation.

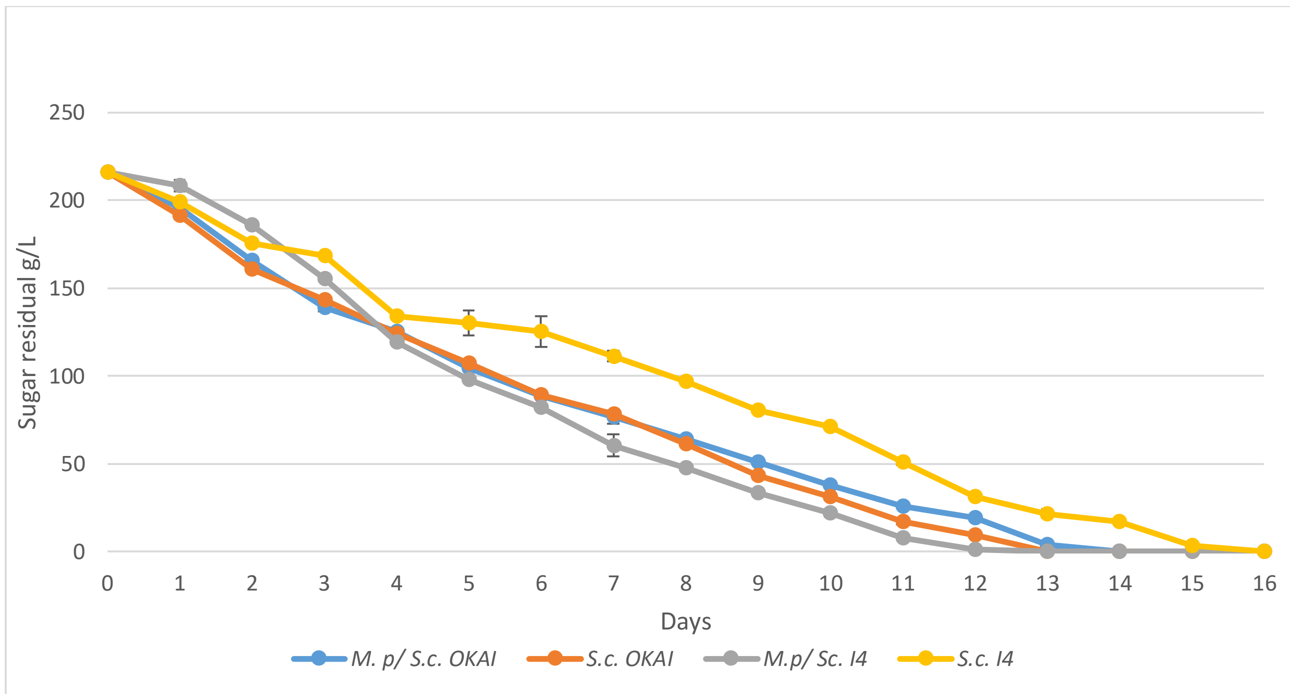

Figure 13 Sugar consumption kinetics in sequential fermentation trials *M. pulcherrima*/*S. cerevisiae* OKAY (—),*M. pulcherrima*/*S. cerevisiae* I4(—) and control *S. cerevisiae* OKAY (—), *S. cerevisiae* i4(—) on natural grape juice (NGJ) .

Regarding to sugar consumption (fig. 13), all fermentations exhibited a similar trend in fermentation kinetics with the only exception of *S. cerevisiae* I4 pure culture that exhibited a slower sugar consumption that other trials. All fermentation showed a total sugar consumption at the end of fermentation. Moreover, the results highlighted a positive interaction on fermentation kinetics of *M. pulcherrima* when used in sequential fermentation with *S. cerevisiae* I4.

4.2.3 Frequency and dominance of *S. cerevisiae* starter strains

Frequency and dominance of S. cerevisiae starter strains

The use of interdelta sequences showed that among 25 *S. cerevisiae* isolated from each thesis, the percentage of *S. cerevisiae* which was found after inoculation and at the end of fermentation was quite low for *S. cerevisiae* I4 (c.a. 50%) in both in pure and sequential fermentation Regarding to commercial starter strain the percentage of OKAY® was 70 and 90% in pure and sequential fermentation respectively .

4.2.4 Main Oenological Characters of wine and Volatile Compounds of wine

The data of the main oenological characters are reported in Table 2.

	Ethanol (%v/v)	Total Acidity (Tartatic Acid g L^{-1})	Volatile Acidity (Acetic Acid g L^{-1})	Malic Acid (g L^{-1})
M.pulcherrima/ OKAY	13.90±0.02[a]	5.07±0.04[c]	0.24±0.01[b]	1.25±0.07[b]
OKAY	14.06±0.09[a]	5.41±0.03[b]	0.25±0.01[ab]	1.55±0.07[a]
M.pulcherrima/*S. cerevisiae* I4	12.92±0.04[b]	5.12±0.014[c]	0.21±0.02[b]	1.5±0.14[a]
S. cerevisiae I4	12.93±0.06[b]	6.28±0.01[a]	0.29±0.014[a]	1.55±0.07[a]

Table 2 Main oenological characters of wines

Fermentation trials carried out with OKAY® and *M. pulcherrima* / OKAY® exhibited the highest content of ethanol in comparison with other wines. Moreover, this sequential fermentation led a significantly lower malic acid content. On the contrary, I4 led a significant increase in total and volatile acidity than the other fermentations.

The data of the main volatile compounds are reported in Table 3. Data are the means ± standard deviation. Data with different superscript letters (^{a,b,c,}) within each row are significantly different (Duncan tests; $p < 0.05$).

	M. pulcherrima/ S. cerevisiae OKAY®	*S. cerevisiae* OKAY®	*M. pulcherrima/ S. cerevisiae I4*	*S. cerevisiae* I4
Esters (mg L^{-1})				
Ethyl butyrate	0.126±0.007^{b}	0.064±0.035^{b}	0.303±0.075^{a}	0.148±0.01^{b}
Ethyl acetate	27.01 ± 0.39^{b}	42.96 ± 0.36^{a}	25.67 ± 0.83^{b}	12.28 ± 0.97^{c}
Phenyl ethyl acetate	0.038± 0.009^{c}	ND	0.10 ±0.01^{a}	0.049 ±0.02^{b}
Ethyl exanoate	0.194 ± 0.109^{a}	0.041±0.003^{a}	0.130±0.020^{a}	0.037±0.001^{a}
Ethyl octanoate	0.005± 0.001^{a}	0.006 ±0.000^{a}	0.005± 0.001^{a}	0.002 ±0.000^{a}
Isoamyl acetate	0.914±0.287^{ab}	0.517±0.171^{ab}	1.029±0.314^{a}	0.307±0.001^{b}
Alcohols (mg L^{-1})				
n- propanol	75.83±0.33^{b}	104.79±5.04^{a}	31.83±0.15^{c}	13.98±0.18^{d}
Isobutanol	11.54±0.76^{b}	13.83±0.57^{a}	13.12±0.52^{ab}	13.79±0.83^{a}
Amyl alcohol	6.41±0.90^{b}	9.76±0.09^{a}	9.87±3.41^{a}	19.50±1.35^{b}
Isoamyl alcohol	89.49±1.08^{b}	110.18±0.01^{a}	13.99±7.09^{c}	94.73±3.08^{b}
β-Phenyl Ethanol	13.12±0.33^{ab}	16.05 ± 0.20^{a}	19.04±0.27^{a}	8.08±0.23^{b}
Carbonyl Compounds (mg L^{-1})				

Acetaldehyde	1.40±0.24[c]	3.80± 0.93[c]	7.97± 1.16[b]	13.98±1.36[a]
Monoterpenes (mg L^{-1})				
Linalool	0.18±0.100[ab]	0.371±0.147[a]	0.221±0.054[ab]	0.028±0.008[b]
Geraniol	0.025±0.011[a]	0.036±0.015[a]	0.038±0.012[a]	0.014±0.008[a]
Nerol	0.074 ± 0.055[a]	0.202±0.140[a]	0.136±0.022[a]	0.028±0.008[a]
Thiols (ng L^{-1})				
3-mercaptohexan-1-ol	367.1 ± 0.0[b]	35.7 ± 0.0[d]	1215.1±0.0[a]	190.6±0.00[c]
3-mercaptoexil acetate	388.9 ± 0.0[a]	52.8± 0.0[c]	181.8±0.0[b]	17.4±0.00[d]

Table 3 Main volatile compounds of wines

Regarding to the esters content, the presence of *M. pulcherrima* with *S. cerevisiae* I4 led significant increase in ethyl butyrate, phenyl ethyl acetate and isoamyl acetate (banana flavor) content in comparison with the other trials. Ethyl acetate (fruity aroma) was significant increase in wine fermented with *S. cerevisiae* OKAY® pure culture than the other wines. No significant differences were detected for ethyl hexanoate and ethyl octanoate content. Moreover, the use of OKAY® pure culture characterized wine with a significant increase in higher alcohols, while I4 pure culture a significant increase in isobutanol content and *M. pulcherrima*/I4 highlighted a significant increase of amylic alcohol and β-phenyl ethanol.

Regarding to the monoterpenic compounds a relevant high content was detected for linalool in OKAY® fermentation trials and in sequential fermentation *M. pulcherrima/*I4 in comparison with I4 pure culture. while no significant differences were shown to produce the other terpens. The acetaldehyde was significant higher in wine fermented by I4. Regarding to the evaluation of the main volatile thiols, *M. pulcherrima* sequential inoculation led a significant increase of these compounds.,

Both sequential fermentations showed an increase of 3-mercaptohexan-1-ol and 3-mercaptoexil acetate particularly in *M. pulcherrima*/I4 trial. The increased compounds found in presence of *M. pulcherrima* in the laboratory trials are substantially confirmed by the results obtained in the winery trials particularly with the I4 starter strain confirming the positive role in the enhance of fruity characters and the complexity of wines. On the other hand, differences in the interactions with *S. cerevisiae* starter strain were also detected.

4.2.5 Sensorial analysis

The wines produced with and without *M. pulcherrima* in cold clarification underwent to sensory analysis, and the results were reported in figure 5. The testers expressed a positive judgement regarding each wine, characterized by specific aromatic notes and without defects. Wines obtained with pure *S. cerevisiae* I4 were precepted more balanced and structured and significantly characterized by citrusy, and softness note, with a low perception of bitter notes. Instead, *M. pulcherrima*/ *S. cerevisiae* I4 led a wine with stronger tropical fruit notes in comparison with the trial with only *S. cerevisiae* I4. On the other hand the wine obtained by the pure fermentation with only *S. cerevisiae* I4 showed more structure, balancing and softness respect the other wines.

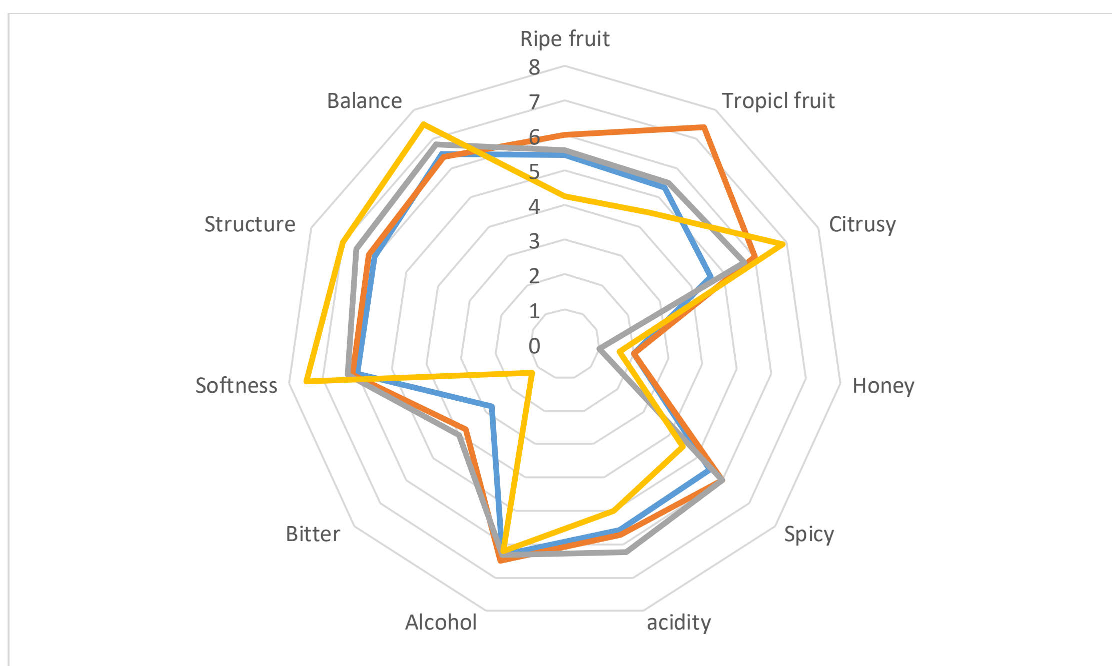

Figure 14 Sensory analysis of Verdicchio wine fermented by *S. cerevisiae* I4(——)and *S. cerevisiae* OKAY®(——) pure fermentation; *M. pucherrima* sequential fermentation with *S. cerevisiae* I4 (——) and OKAY® (——).

5 DISCUSSION AND CONCLUSIONS

After being considered alterative yeasts for decades actually the potential of non-*Saccharomyces* yeasts has been recognized. Non-*Saccharomyces* strains was used to decrease the final content of ethanol in wines (Contreras et al., 2014; Varela et al., 2016; Brou et al., 2018) or for the diversification of the product's flavor profile, increasing both the formation of fermentative aromas and the release of varietal aromas thanks to their ability to expel hydrolytic enzymes (Egli et al., 2002; Rodríguez et al., 2010; Zott et al., 2011; Belda et al., 2017). Indeed, some non-*Saccharomyces* strains are available on the market as active dry yeasts or in creamy form.

The growing demand for "natural" and certified organic wines has opened a new frontier for non-*Saccharomyces* yeasts, used as biocontrol agents to replace sulfur dioxide and to enhance the aromatic bouquet of the final product, which in recent years had been standardized using only a limited number of commercial *S. cerevisiae* strains as starter cultures.

In this regard, it was focused on the use of a selected strain of *M. pulcherrima* at pre-fermentative and fermentative stages. In particular, this yeast was inoculated in steel vats during the clarification phase instead of SO_2 and during the fermentation phase with the main objective of comparing the test carried out by pure cultures of *S. cerevisiae* and sequential inoculation of *M. pulcherrima / S. cerevisiae* for the production of organic Verdicchio wine.

The trials were conducted in lab and at industrial scale in collaboration with the Terre Cortesi Moncaro Soc. Coop Agricola wineries, that have made available the winery equipments, the grape juice and their tanks.

The results of lab trials showed that *M. pulcherrima* have a good bioactive effect on *H. uvarum*, the most representative alterative yeasts, both with OKAY® and with I4. Indeed, *M. pulcherrima* in sequential fermentation with I4 showed a relevant biocontrol activity but also the best aromatic bouquet.

Therefore, the use of *M. pulcherrima* in combination with I4 could be a suitable natural strategy to obtain a high quality organic wine by decreasing the concentration of sulfur dioxide.

These results were substantially confirmed at industrial scale. Indeed, after two days of cold clarification, the bio-control efficacy of *M. pulcherrima* on the population of apiculate yeasts was demonstrated.

In agreement with several studies, these results showed that *M. pulcherrima* exerts an antimicrobial action against *H. uvarum,* the main species of wild yeasts present in the grape juice before the fermentation, exerting a bioprotectant action also under industrial condition.

In this condition, *M. pulcherrima* led changes in microbic evolution, fermentation kinetics and in the production of flavors. Indeed, the presence of *M. pulcherrima* leads important modifications of the fermentation kinetics. This can be linked to the exhaustion of some nitrogen sources by *M. pulcherrima* during the first part of the fermentation as also suggested by the study carried out by Pauline S. et al (2020).

Regarding to the production of secondary compounds and volatile compounds, the presence of *M. pulcherrima* enhancing the production of a certain compounds as compared to the controls. Specifically, was significantly evident for the content of ethyl acetate, propanol, active amyl, 2-phenyl ethanol and linalool.

The conclusion from this study highlighted that *M. pulcherrima* has an antimicrobial effect against *H. uvarum* making it a potential SO_2 reduction or replacement agent to control the development of wild yeasts. Another relevant aspect in the use of *M. pulcherrima* is the highly specific behavior in relation to the starter *S. cerevisiae* regarding to the production of secondary and volatile compounds indicating that the positive interactions between *M. pulcherrima* DiSVA 269 and *S. cerevisiae* I4.

These differences arose from metabolic interactions between the two species, which can be attributed to the regulations in *S. cerevisiae* linked to the environmental changes induced by the growth of *M. pulcherrima* (nutrient consumption, metabolite production ...) (Comitini F., et al. 2021; M Sadoudi M., et al 2017) . However, the metabolic and molecular basis governing these interactions are unclear and will require new studies to fully understand what are the necessary characteristics that make two microorganisms excellent fermentation partners.

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THIRD PART

Non-*SACCHAROMYCES* YEASTS IN SEQUENTIAL FERMENTATION WITH NATIVE *S. CEREVISIAE*: BIOCONTROL OF INDIGENOUS MICROBIOTA AND IMPACT ON AROMATIC PROFILE OF HIGH QUALITY ORGANIC WINE

1. **INTRODUCTION**
2. **AIM OF THE WORK**
3. **MATHERIALS AND METHODS**
4. **RESULTS**
   - **4.1 Evaluation of bioprotectant activity of *T. delbrueckii* DiSVA 130 and sugar consumption**
     - **4.1.1 Biomass Evolution and Sugar Consumption**
     - **4.1.2 Main Oenological Characters of wine and Volatile Compounds of wines**
     - **4.1.3 Sensory analysis**
   - **4.2 Set up mixed fermentation using *T. delbrueckii* and *M. pulcherrima* strains in combination with improved native *S. cerevisiae* strains**
     - **4.2.1 Biomass Evolution and Sugar Consumption**
     - **4.2.2 Main Oenological Characters of wine and Volatile Compounds of wines**
     - **4.2.3 Sensory analysis**
5. **DISCUSSIONS AND CONLUSION**

1. INTRODUCTION

Nowadays, the use of selected cultures is a suitable strategy to improve organoleptic profiles and specific aroma compounds for the production of fine wines (Comitini F. et al. 2017). Non-*Saccharomyces* yeasts initially were considered as a source of microbiological problems or insignificant appearances during fermentation, in modern oenology their role was reconsidered.

In this regard the non- *Saccharomyces* yeast must have specific oenological features that make it easy to apply as a selected commercial yeast and it must be able to characterize of final wines.

On the other hand, many researcher focused their work to select indigenous yeasts in order to identify the best strain of specific grape variety, production area and winemaking technique (Gayevskiy V. et al. 2011). The use of selected native cultures comes from the excessive and massive use of *S. cerevisiae* which stabilize the fermentation microbiologically but on the other hand standardize the sensory and olfactory connotations of the product (Di Gianvito et al. 2022).

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In this context, in recent years, there is a growing diffusion of sequential or mixed fermentations are based on the use of *S. cerevisiae* as a starter strain and non-*Saccharomyces* yeast, with the aim to improve the organoleptic qualities of wine and the complexity of aromatic notes.(Ciani & Comitini 2019). Beyond the enhancement of aromatic bouquet many researchers focused their work on the use of non-*Saccharomyces* yeasts to implement the action of biocontrol against spoilage yeast of the grape juice to reduce of the added sulfur dioxide or exploiting their metabolic activities to reduce the ethanol content of the finale wine-(Varela et al., 2017; Mateo J.J. et al. 2016; Branco P. et al. 2021)

2. THE AIM OF THE WORK

In the previous invstigation it was found an highly specific behavior of the selected non-Saccharomyces yeast M. pulcherrima in relation to the starter *S. cerevisiae* regarding to the production of secondary and volatile compounds. In this investigation it was evaluated several non-*Saccharomyces* wine yeasts in mixed fermentation with improved native strains of *S. cerevisiae* isolated from Verdicchio DOC wine . A set of trials was focused on the evaluation of selected *T. delbrueckii* strain DISVA 130 as bio protectant agent in presence or in absence of SO_2in sequential fermentation with I4 improved native strains. Subsequently a combination of a mixed fermentation of several yeast species: non *Saccharomyces* strains *Metschnikowia fruticola* commercial (GAIA.), *M. pulcherrima* DiSVA 269 non-commercial, *T. delbrueckii* DISVA 130 non-commercial, *T. delbrueckii* commercial (ALPHA.) and *S. cerevisiae* OKAY ® (commercial), and improved native B4 and I4 strains . The final goal is to set up a vinification process reducing the use of SO_2 and improve the analytical composition and aromatic bouquet through the combination of selected yeasts in sequential fermentation.

3. MATHERIALS AND METHODS

3.1 Yeasts strains and Biomass production

The improved *S. cerevisiae* native strain I4 and B4 belonging to the Yeast Collection of the Department of Life and Environmental Sciences (DiSVA) of the Polytechnic University of Marche (Italy) (Agarbati et al., 2020) was used in sequential fermentations and as control.

M. pulcherrima DiSVA 269 was selected on the bases of previous works on biocontrol ability (Oro et al., 2014; Oro et la., 2018). *T. delbrueckii* DISVA 130 was selected for its capacity to characterize the final wine. These two native non-*Saccharomyces* yeasts are compared with the other two non-*Saccharomyces* commercial yeasts *T. delbrueckii* ALPHA® and *M. pulcherrima* GAIA®.

The effects of non-*Saccharomyces* yeasts were evaluating in sequential fermentation with *S. cerevisiae* commercial starter strain LALVIN ICV OKAY® and native *S. cerevisiae* B4 and I4. The yeasts strains were maintained on YPD agar medium (yeast extract 1%, peptone 2%, dextrose 2% and agar 1.8%) at 4 ◦C for short-term storage and in YPD broth supplemented with 80% (w/v) glycerol at −80 ◦C for long-term storage.

M. pulcherrima DiSVA 269 was pre-cultured using TYPE A medium (1% yeast extract, 0.5% peptone, 5% inverted sugar all *w/v*). *T. delbrueckii* DiSVA 130 and native *S. cerevisiae* B4 and I4

were pre-cultured in modified YPD (0.5% w/v yeast extract, 2% w/v glucose, and 0.1% w/v peptone) for 1 day at 25 °C in an orbital shaker (rotation, 150 rpm).

The biomass at the end of the process was collected by centrifugation, washed three times with sterile distilled water and inoculated into the grape juice to obtain an initial concentration of approximately 1×10^6 cell/mL for each yeasts. The growth kinetics of the yeast strains were monitored during the fermentation at established time.

The other commercial strain was rehydrated with bidistilled water as reported on the protocol and then used to carried out the fermentation trials.

3.2 Starter inoculum and Fermentation stage

Fermentation trials were performed using Verdicchio grape juice coming from vintage 2021. The analytical characters of the Verdicchio grape juice had the following main analytical composition: pH 3.22; initial sugar content 230 g/L; total acidity 4.48 g/L; malic acid 2.3 g/l; nitrogen content YAN (60 mg/L) and total SO_2 14 mg/l. The diammonium phosphate and yeast derivative (Genesis Lift® Oenofrance, Bordeaux, France) used were adjusted to 250 mg N/L as yeast assimilable nitrogen.

The first set of the fermentation trials was carried out in duplicate in a steel vat containing 40 L of Verdicchio grape juice in presence and in the absence of SO_2 at 22 ± 1°C.

The fermentation kinetics were monitored by measuring the sugar consumption. The trials were carried out with and without 30 mg/l of SO_2.

The second set of fermentation trials were carried out without SO_2 added, in duplicate, in glass demijohn containing 5 L of bio grape juice at 19 ± 1°C. The fermentations were carried out under winemaking conditions at the winery Terre Cortesi Moncaro s.r.c.l.

3.3 Biomass evolution

The growth kinetics of the yeast strains were monitored with viable cells counts during the fermentation at established intervals using WL nutrient Agar medium (Oxoid, Hampshire, U.K.) and Lysine Agar medium (Oxoid, Hampshire, U.K.). The sugar consumption during the fermentation process was measured by Baumé (°Bé) densimeter.

3.4 Analytical Procedures

Total acidity, volatile acidity, pH, and ethanol content were determined according to the Official European Union Methods (2000). Enzymatic kits (Megazyme International Ireland) were used to measure the amounts of glucose and fructose (K-FRUGL) and malic acid (K-DMAL) according to

the manufacturer instructions. A specific enzymatic kit (kit no. 112732; Roche Diagnostics, Germany) was used to determine the ammonium content. The free α-amino acids were evaluated following Dukes and Butzke protocol (1998). Ethyl acetate, acetaldehyde, and higher alcohols were quantified using a gas chromatograph system (GC-2014; Shimadzu, Kjoto, Japan) by direct injection. The final wines were prepared as described by Canonico et al. (2018). The solid-phase microextraction (HS-SPME) method was used to quantify the main volatile compounds as described by Canonico et al. (2019). The compounds were desorbed by inserting the fiber Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Sigma-Aldrich, St. Louis, MO, USA) into a gas chromatograph (GC) injector.

3.5 Sensory Analysis

At the end of the fermentation, the wines obtained were transferred into filled 750 mL bottles, closed with the crown cap, and maintained at 4 °C until sensory analysis. After storage for 3 months, wines were subjected to sensory analysis based on the principal sensorial features. A group of ten testers using a score scale of 1 to 10, expressed their opinion regarding each wine tested. The data obtained were used to compare the wines and provide information regarding the organoleptic quality and probable consumers' acceptability of the wines obtained.

3.6 Statistical Analysis

Analysis of variance (ANOVA) was used to elaborate the data of analytical character of wines. The means were analyzed using the statistical software package JMP® 11. Duncan tests were used to detect the significant differences. The experimental data were significant with associated p-values < 0.05.

4. RESULTS

4.1 Evaluation of bioprotectant activity of *T. delbrueckii* DiSVA 130

4.1.1 Biomass evolution and sugar consumption

Growth kinetics of control fermentations inoculated with *S. cerevisiae* I4 with and without SO_2 added, were reported in following graphs.

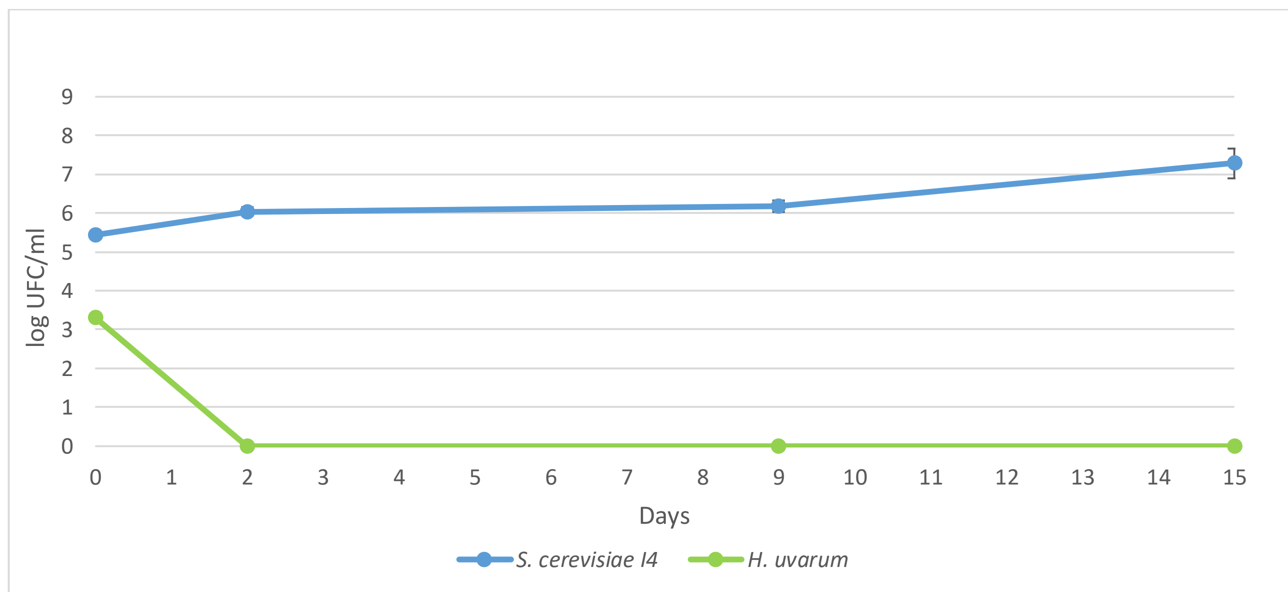

Figure 15a Growth kinetics of *S. cerevisiae* I4 (—●—) and spoilage *H.uvarum* (—●—) on natural grape juice with SO_2

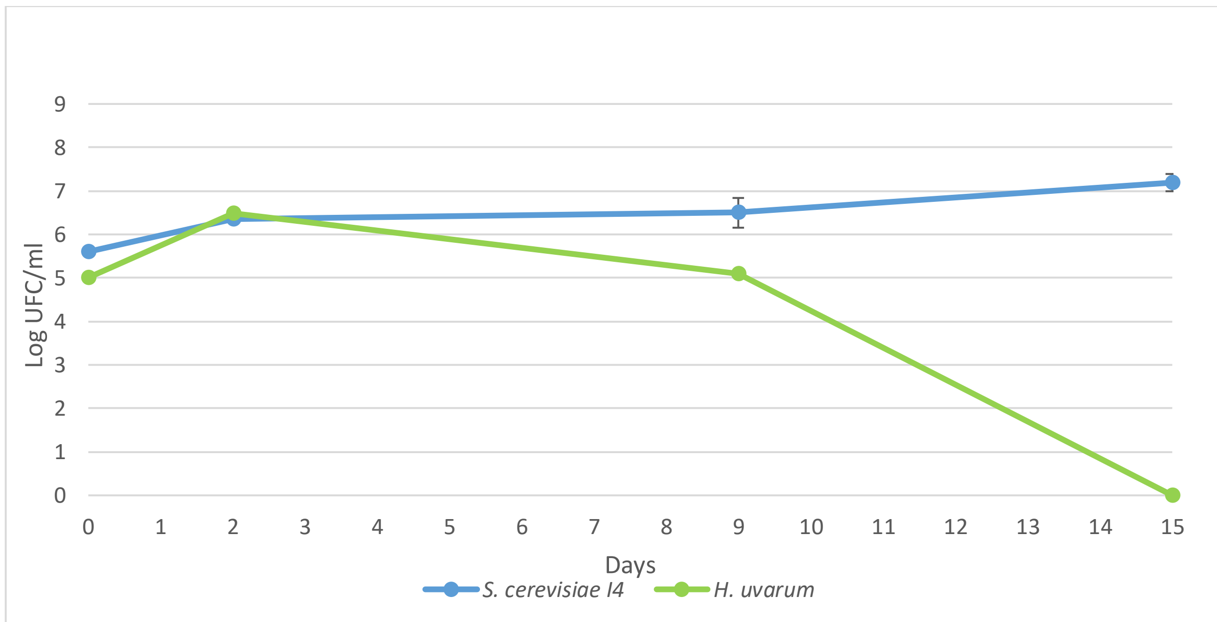

Figure 15b Growth kinetics of *S. cerevisiae* I4 (—●—) and spoilage *H.uvarum* (—●—) on natural grape juice without SO_2

The results showed that *S. cerevisiae* I4 starter strain with and without SO_2 exhibited a similar trend during the fermentation with an initial inoculum of 10^6cell/ml, have reached the maximum cell concentration at 15th day of fermentation (10^7 cell/ml) to remain constant until the end of fermentation.

H. uvarum showed a different trend. As expected, with the addition of SO_2 apiculate population decreased faster from 10^3 cell / ml to 0 cell / ml after two days .

Without SO_2 *H. uvarum* (fig. 15b) persisted during whole fermentation; at the beginning the concentration was 10^5 cell / ml, increasing to 10^7 cell / ml to slowly decrease until the end of fermentationindicating that the addition of SO_2 strongly affected *H. uvarum* population in pure fermentation carried out by *S. cerevisiae* I4.

Growth kinetics of control fermentations inoculated with *S. cerevisiae* LALVIN ICV OKAY® with and without SO_2 added are reported in following graphs

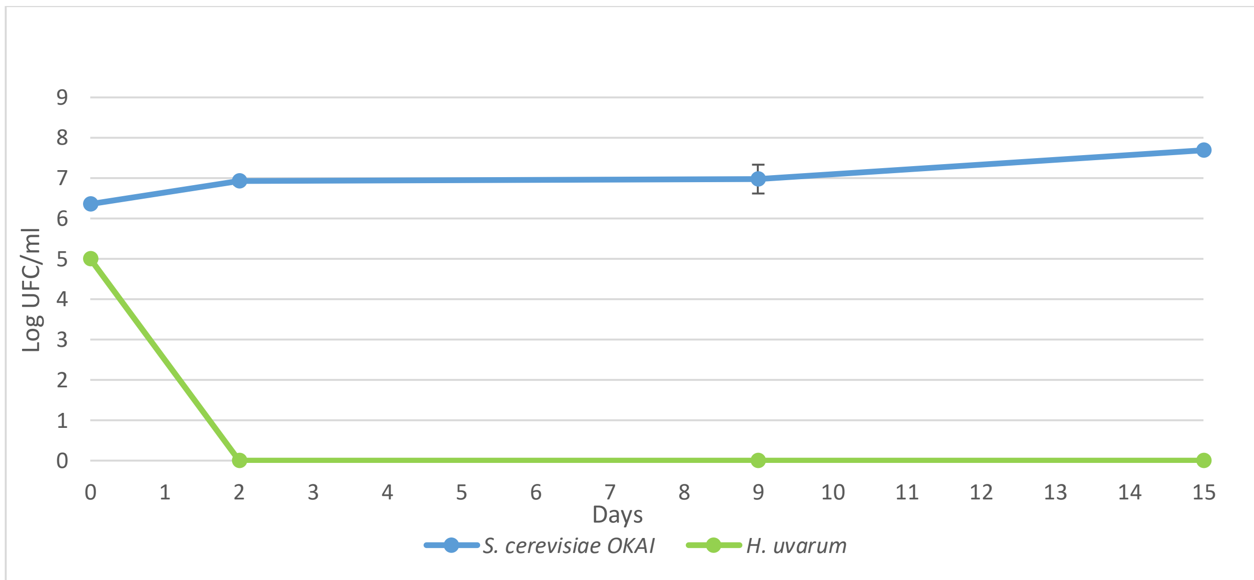

Figure 16a Growth kinetics of *S. cerevisiae* OKAY® (—●—) and spoilage *H.uvarum* (—●—) on natural grape juice with SO_2

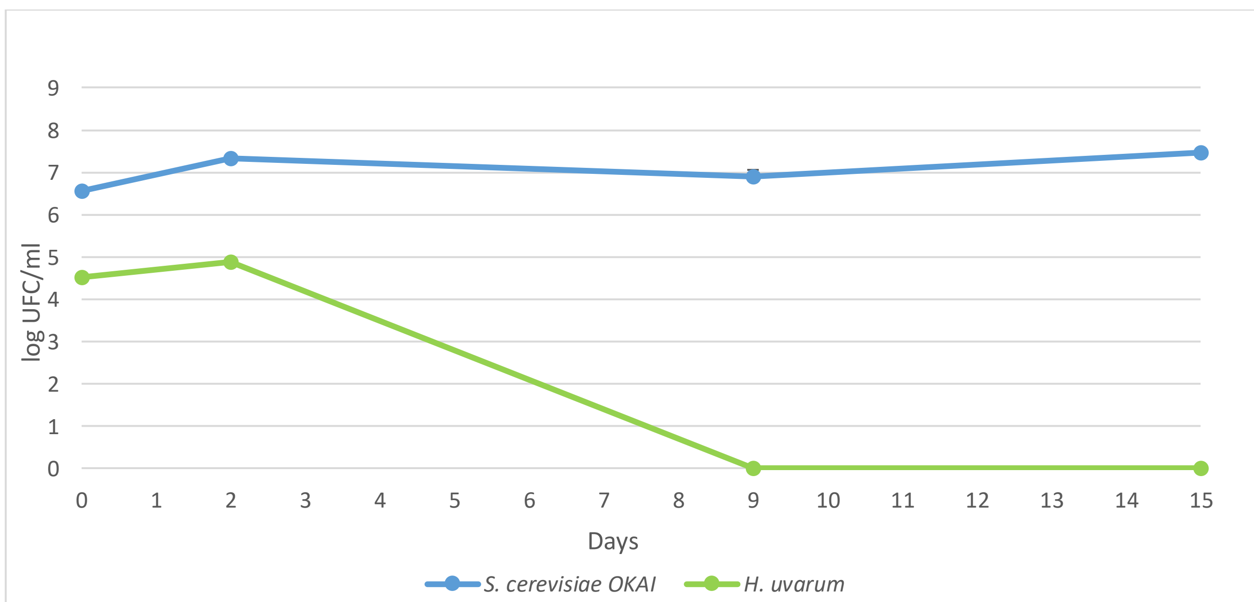

Figure 16b Growth kinetics of *S. cerevisiae* OKAY® (—●—) and spoilage *H.uvarum* (—●—) on natural grape juice without SO_2

The results showed that *S. cerevisiae* commercial starter strain LALVIN ICV OKAY® in presence and in the absence of SO_2 exhibited a similar trend during the fermentation. *S. cerevisiae* OKAY® with initial inoculum level of 10^6cell/ml, exhibited the maximum cell concentration at 15th day of fermentation (10^8 cell/ml) to remain constant until the end of fermentation.

The addition of SO_2 determined the disappearance of *H. uvarum* population at second day . On the contrary, in absence of SO_2 *H.uvarum* population decreased slower during the fermentation until the 9th day . Moreover, *H. uvarum* is affected by the presence of OKAY® since in the trial without SO_2 added the population decreases much faster in comparison with *S. cerevisiae* I4 trial control.

Growth kinetics of fermentations inoculated with *T. delbrueckii* DISVA 130 in combination with *S. cerevisiae* I4, in presence and absence of SO_2 added, are reported in Figures 16a and 16b.

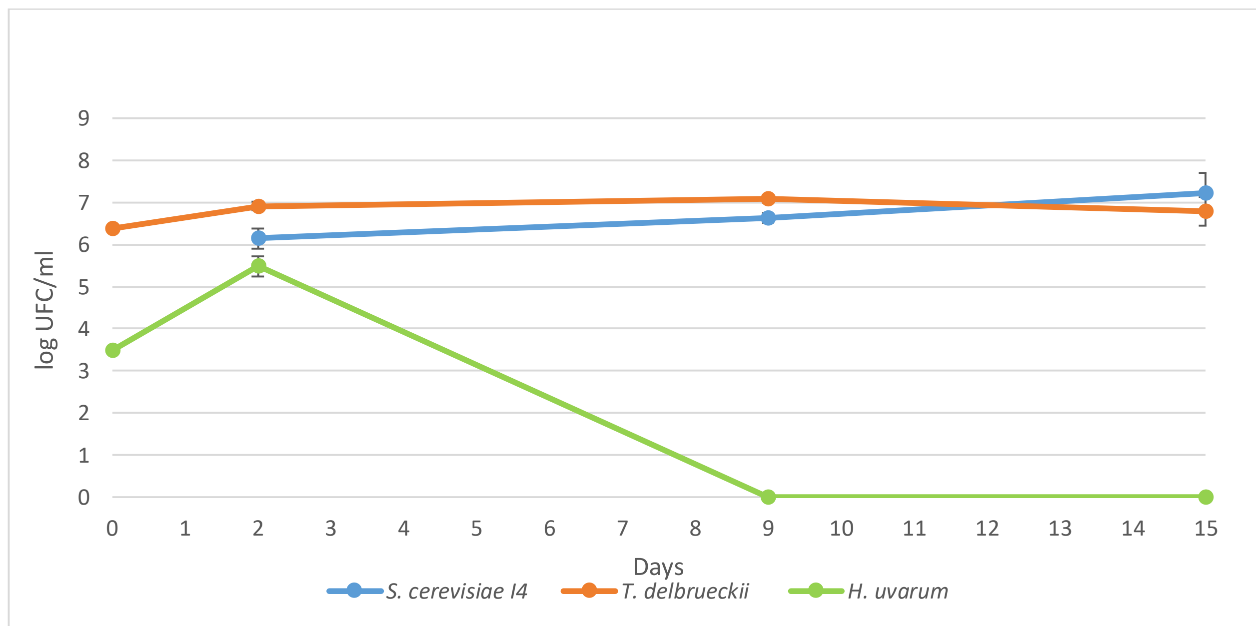

Figure 17a Growth kinetics in sequential fermentation trials of *T. delbrueckii* DISVA 130 (—)/ S. *cerevisiae* I4(—) and spoilage *H.uvarum* (—) on natural grape juice with SO_2

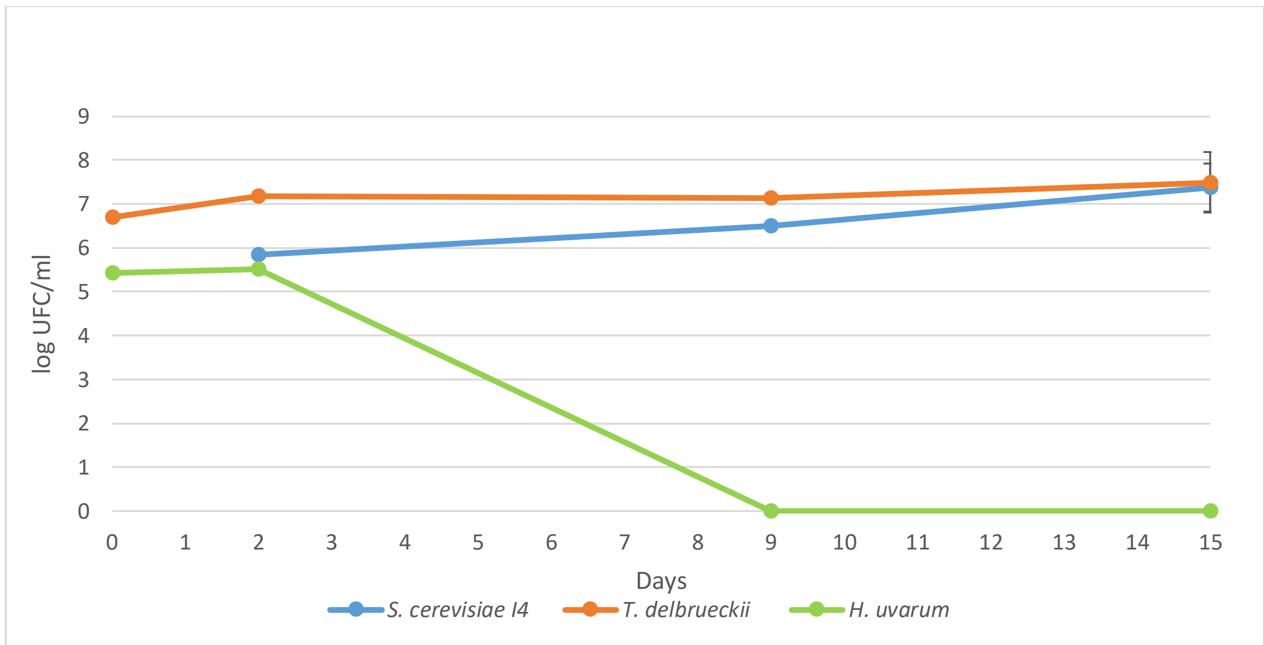

Figure 17b Growth kinetics in sequential fermentation trials of *T. delbrueckii* DISVA 130 (—●—)/ *S. cerevisiae* I4 (—●—) and spoilage *H.uvarum* (—●—) on natural grape juice without SO_2

Both sequential fermentation *T. delbrueckii* DISVA 130 / *S. cerevisiae* I4 exhibited a similar trend.

Indeed, *T. delbrueckii* DISVA 130 with initial inoculum level of 10^6cell/ml, show a constant trend until the end of fermentation achieved the maximum cell concentration at 2nd day of fermentation (10^7 cell/ml) to remain constant until the end of fermentation., .

S. cerevisiae I4 with initial inoculum level of 10^6cell/ml, exhibited the maximum cell concentration at 15th day of fermentation (10^7 cell/ml) to remain constant until the end of fermentation.

H. uvarum population decreased from the second day of fermentation from (10^5 cell / ml) to 0 cell / ml by the 9th day of fermentation.

In absence of SO_2 *H. uvarum* population exhibited the same trend of the trial with SO_2 added. The presence of *T. delbrueckii* DISVA 130 effectively reduced the population of *H. uvarum* highlighting the bioprotectant ability.

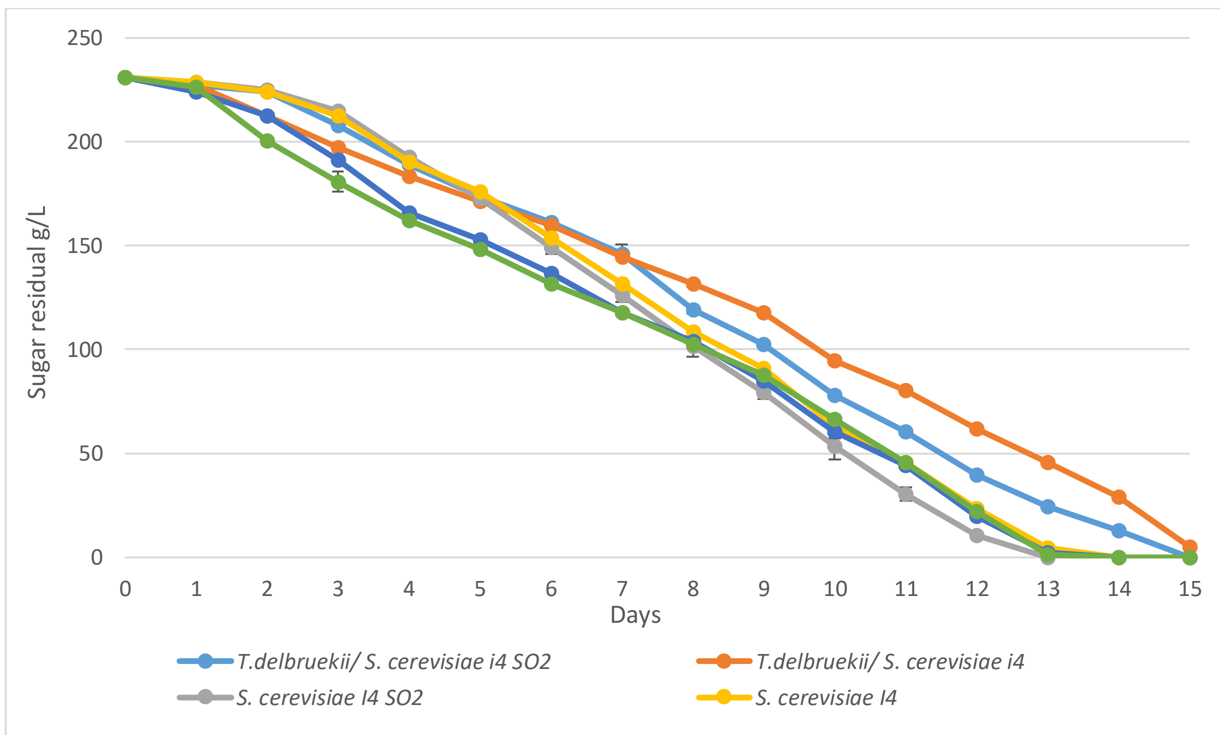

Figure 18 Sugar consumption kinetics in sequential fermentation trials *T. delbrueckii* DiSVA 130 /*S. cerevisiae* I4 (SO_2) ( ),*T. delbrueckii* DiSVA 130 /*S. cerevisiae* I4 (without SO_2) ( ) , *S. cerevisiae* I4 (SO_2) ( ),*S. cerevisiae* I4 (without SO_2) ( ), *S. cerevisiae* OKAY® (SO_2) ( ) and *S. cerevisiae* OKAY® (without SO_2) ( ) on natural grape juice (NGJ) .

Regarding to sugar consumption (fig. 17) all fermentations showed a total sugar consumption at the end of fermentation. all fermentations exhibited a similar trend in fermentation kinetics with the only exception of sequential fermentation *T. delbrueckii* DiSVA 130 /*S. cerevisiae* I4 (without SO_2) that consumed sugars more slowly. Moreover, the results highlighted a positive interaction on fermentation kinetics of *T. delbrueckii* DiSVA 130 when used in sequential fermentation with *S. cerevisiae* I4.

4.1.2 Main Oenological Characters of wine and Volatile Compounds of wines

The data of the main oenological characters of the wines obtained were reported in Table 1.

	Ethanol (%v/v)	Total Acidity (Tartatic Acid g L^{-1})	Volatile Acidity (Acetic Acid g L^{-1})	Malic Acid (g L^{-1})
S. cerevisiae OKAY® SO_2	14.69±0.01[a]	5.685±0.02[c]	0.3±0.00[a]	0.9±0.00[b]
S. cerevisiae OKAY®	14.88± 0.02[a]	5.27±0,01[c]	0.31±0.014[a]	0.7±0.00[b]
S. cerevisiae I4 SO_2	14.05±0.12[b]	6.29±0.05[a]	0.225±0.007[a]	1.25±0.07[a]
S. cerevisiae I4	14.35±0.1[b]	5.77±0.00[ab]	0.305±0.007[a]	1± 0.00[ab]
T. delbrueckii DiSVA 130 / *S. cerevisiae* I4 SO_2	13.9±0.02[c]	6.14±0.14[a]	0.25±0.00[a]	1.35±0.07[b]
T. delbrueckii DiSVA 130 / *S. cerevisiae* I4	13.86±0.09[c]	5.355±0.03[c]	0.29±0.00[a]	0.45±0.07[b]

Table 4 Main Oenological Characters of wines

Fermentation trials carried out with OKAY®, in presence and in the absence of SO_2 exhibited the highest content of ethanol in comparison with other wines. Moreover, this fermentation led a significantly lower malic acid content together with the sequential fermentation *T. delbrueckii* DiSVA 130 / *S. cerevisiae* I4 in absence of SO_2. On the contrary, the trials with *S. cerevisiae* I4 led a significant increase in total and volatile acidity than the other fermentations .

	T. delbrueckii DiSVA 130 / I4 SO_2 mg/L	*T. delbrueckii* DiSVA 130 / I4 mg/L	I4 SO_2 mg/L	I4 mg/L	OKAY® SO_2 mg/L	OKAY® mg/L
ESTERS						
Ethyl butyrate	1.04± 0.50[ab]	0.44 ± 0,08[c]	0.65±0.22[bc]	0.6±0.035[bc]	1.21±0.02[ab]	1.49± 0.37[a]
Ethyl acetate	14.18±1.06[b]	19.75±0.7[b]	10.439±0.68[b]	38.201±0.86[a]	19.71±2.17[b]	36.35±1.61[a]
Ethyl exanoate	1.44± 0.70[ab]	1.60±0.46[ab]	2.53±0.97[a]	1.91±0.10[ab]	1.06±0.23[b]	1.60±0.92[ab]
Isoamyl acetate	1.79±0.66[a]	1,98± 2.28[a]	1.35±0.46[a]	1.09±0.02[a]	0.94 ±0,04[a]	0.85±0.435[a]
ALCOHOLS						
n-propanol	27.904±0.713[b]	35.734±2,103[b]	39.655± 0.260[b]	37.032±2.636[b]	86.630±0.94[a]	94.148±1.51[a]
Isobutanol	32.634± 0.04[a]	25.211± 0.85[a]	10.957± 2.02[b]	19.211±0.516[b]	17.559± 0.184[b]	12.561±0.632[b]
Amyl alcohol	20.74±1.50[a]	25.690±0.92[a]	19.211±0.51[c]	14.909± 0.08[b]	12.601±2.27[bc]	12.245±1.51[c]

Isoamilic alcohol	171.56± 2.71[a]	192.248±1.68[a]	137.156±0.99 [c]	125.505 ± 0.13[b]	132.53±2.18[bc]	145.105±1.,57 [c]
β-Phenyl ethanol	15.62±0.53[b]	25.41±0.649[a]	18.89±0.027[ac]	15.82±0.211[c]	13.,93± 0.091[bc]	10.05±0.5[bc]
CARBONYL COMPOUNDS						
acetaldehyde	4.792± 0.50[abc]	14.188±0.350[bc]	27.904±0.314 [a]	32.634±1.85[ab]	20.745±2.83[abc]	17.560±0.145[c]
MONOTERPENS						
Linalol	0.07±0.014[a]	0.12±0.01[a]	0.15±0.118[a]	0.18±0.13[a]	0.19±0.07[a]	0.12±0.076[a]
Geraniol	0.011±0.008[a]	0.01±0.004[a]	0.015±0.002[a]	0.013±0.003 a	0.008±0.00[a]	0.001±0.058[a]
Nerol	0.008±0.005[a]	0.008±0.002[a]	0.00±0.00	0009±0001[a]	0.006±0.003[a]	0.004±0.004[a]

Table 5. Main volatile compounds produced

Regarding to the esters content (fruits aroma), the presence of *T. delbrueckii* DiSVA 130 with *S. cerevisiae* I4 without SO_2 led an increase in phenyl ethyl acetate and isoamyl acetate (banana flavor)

content in comparison with the other trials in presence of SO_2. Ethyl acetate was significant increase in all trials without the SO_2. No significant differences were detected for ethyl hexanoate. Moreover, the use of OKAY® pure culture in presence and in the absence of SO_2led an increase of n-propanol in comparison with the other wine while the sequential fermentation *T. delbrueckii* DiSVA 130 / I4 led a significant increase in isobutanol content and *T. delbrueckii* DiSVA 130 /I4 without SO_2 highlighted a significant increase of amylic alcohol and β-phenyl ethanol. Considering monoterpenic compounds (terpenes that contribute to the classic floral aroma), *T. delbrueckii* DiSVA 130 /I4 in presence of SO_2 produce wine with a low content of linalool content but the results regarding geraniol and nerol did not showed significant differences. The acetaldehyde was significant higher in wine fermented by I4 in presence and in the absence of SO_2.The results showed that the presence or the absence of SO_2 at the start of fermentation characterized the composition of the final wines.

4.1.3 Sensorial analysis

The wines produced by pure and sequential fermentations underwent to sensory analysis, and the results are reported in figure 19. The testers expressed a positive judgment regarding each wine, characterized by specific aromatic notes and without defects. In particular the wines obtained were characterized significantly to have a similar structure, softness, alcohol content and smell; the toasted and spicy notes were absent, on the contrary of the hints of honey, citrus fruits and fruity notes present above all in the thesis *T. delbrueckii* DiSVA 130 with *S. cerevisiae* I4 in presence and absence of SO_2 and *S. cerevisiae* LALVIN ICV OKAY®.

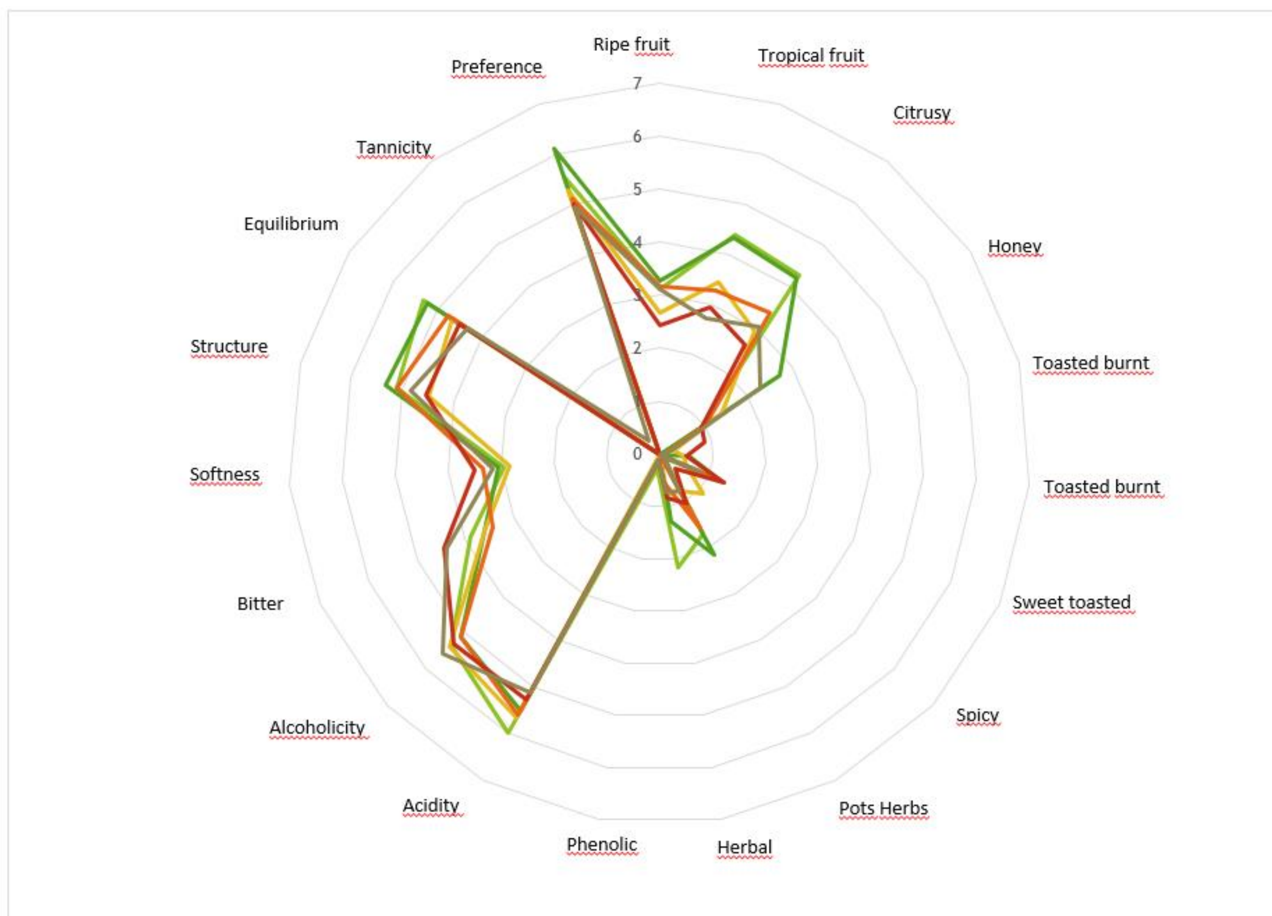

Figure 19. Sensory analysis of Verdicchio wine fermented by *S. cerevisiae* OKAY® with SO_2(━━);*S. cerevisiae* OKAY® in absence of SO_2 (━━); *S. cerevisiae* I4 with SO_2 (━━); *S. cerevisiae* I4 in absence of SO_2 (━━); *T. delbrueckii* DiSVA 130 / *S. cerevisiae* I4 with SO_2 (━━); *T. delbrueckii* DiSVA 130 / *S. cerevisiae* I4 in absence of SO_2 (━━);

In conclusion the sequential fermentation *T. delbrueckii* DiSVA 130/ *S. cerevisiae* I4 in absence of SO_2 obtained the highest score.

4.2 Set up mixed fermentation using *T. delbrueckii* and *M. pulcherrima* strains in combination with improved native *S. cerevisiae* strains

4.2.1 Biomass Evolution and Sugar Consumption

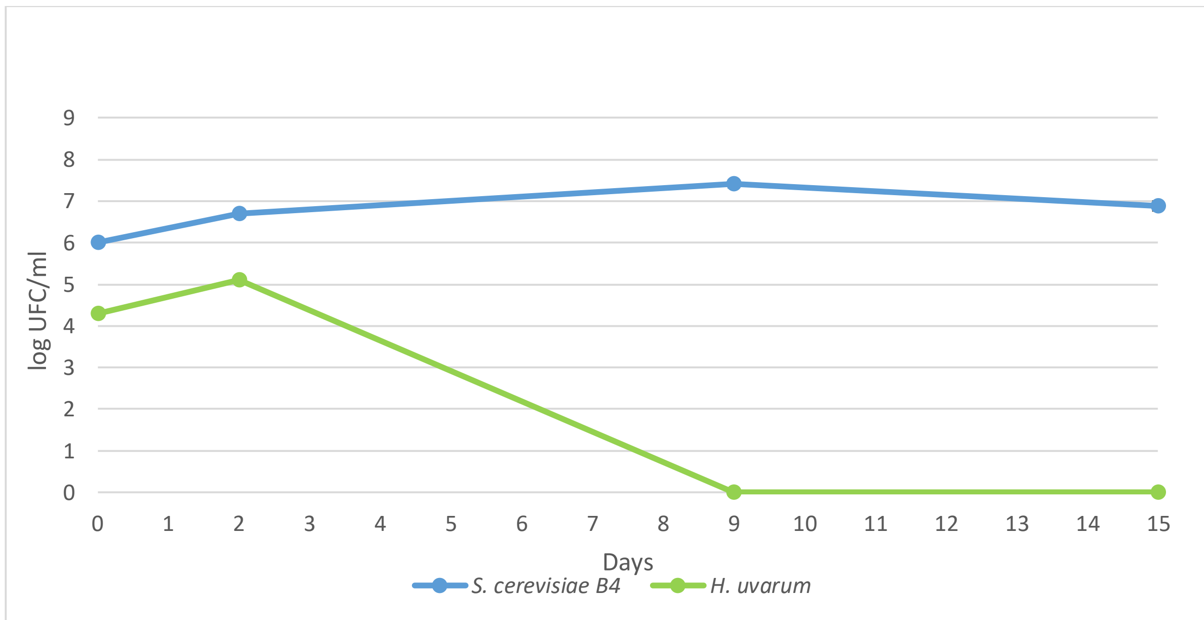

Figure 20 Growth kinetics of *S. cerevisiae* B4 ( )*H. uvarum* ( )

The control trials with the inoculum of *S. cerevisiae* B4 showed that with initial inoculum level of 10^6cell/ml increase the concentration constantly, achieved the maximum cell concentration at 9th day of fermentation (10^7 cell/ml) to remain constant until the end of fermentation.

The concentration of *H. uvarum* population showed a slowly enhancement reaching the maximum cell concentration at 2nd day of fermentation (10^5 cell/ml).

Growth kinetics of sequential fermentations *M. pulcherrima* DiSVA 269 / *S. cerevisiae* B4, is reported in following graphs.

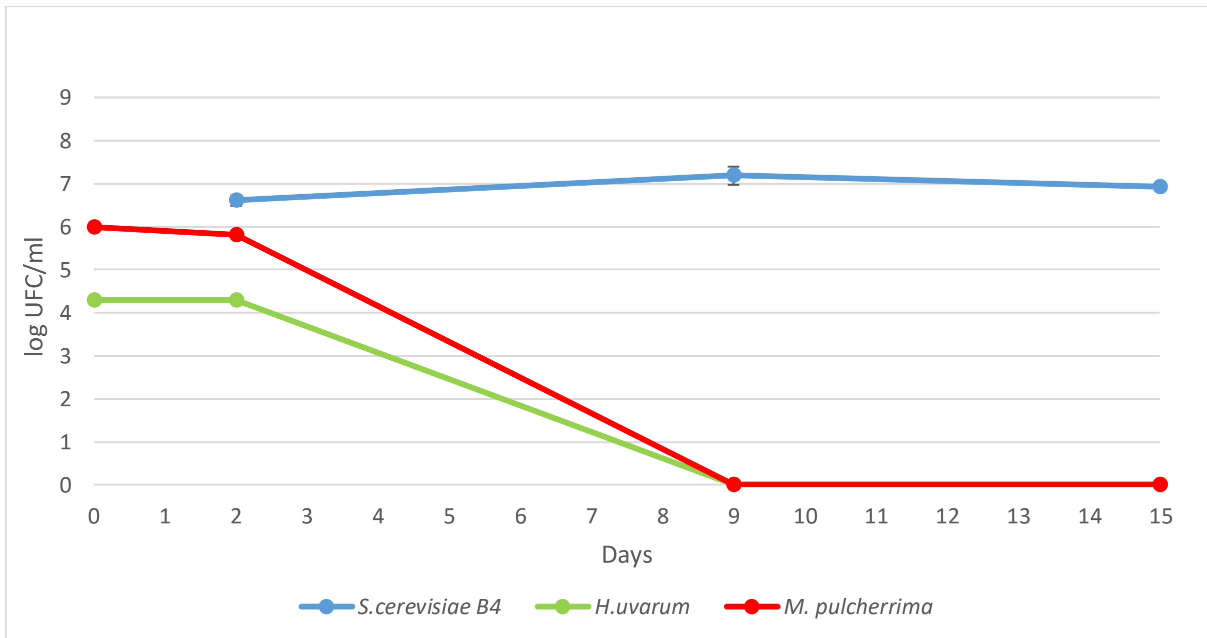

Figure 21 Growth kinetics of *S. cerevisiae* B4 (), *H. uvarum* ()*M. pulcherrima* DiSVA 269 () on natural grape juice

The graph showed that *M. pulcherrima* DiSVA 269 with initial inoculum level of 10^6cell/ml, slowly decreases by the 2nd day of fermentation until the 9th day of fermentation (0 cell / ml).

S. cerevisiae B4 with initial inoculum level of 10^6 cell/ml, exhibited the maximum cell concentration at 9th day of fermentation (10^7 cell/ml) to remain constant until the end of fermentation. *S. cerevisiae* B4 do not affect by the presence of *M. pulcherrima* DiSVA 269.

H. uvarum population decrease from the second day of fermentation from 10^4 cell / ml unitil the by the 9th day of fermentation.

The results showed that *M. pulcherrima* DiSVA 269 determined a better control on *H. uvarum* population indicating that *H. uvarum* population was affected by the bioactive effect of *M. pulcherrima* DiSVA 269.

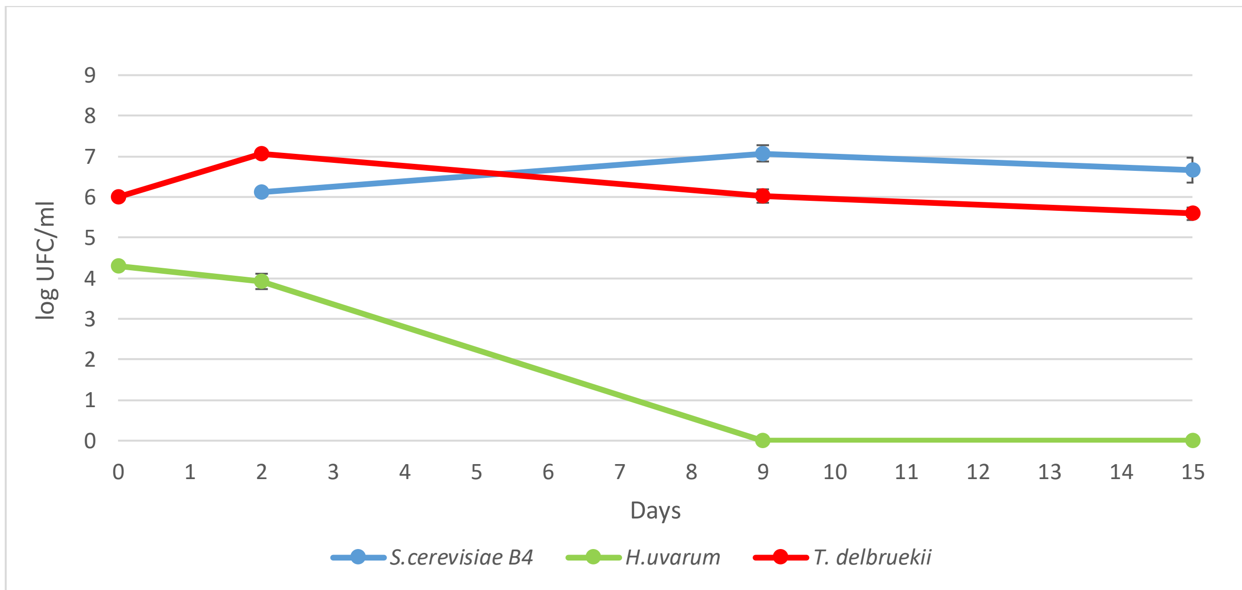

Figure 22 Growth kinetics of *S. cerevisiae* B4 (), *H. uvarum* ()*T. delbrueckii* DiSVA 130 () on natural grape juice

The result of this trial showed that *T. delbrueckii* DiSVA 130 with initial inoculum level of 10^6cell/ml, showed a constant presence until the end of fermentation achieved the maximum cell concentration at 2nd day of fermentation (10^7 cell/ml) that slowly decreased until the end of fermentation.

S. cerevisiae B4 with initial inoculum level of 10^6cell/ml, achieved the maximum cell concentration at 9th day of fermentation (10^7 cell/ml) to remain constant until the end of fermentation. *H. uvarum* population decrease from the beginning of fermentation from (10^4 cell / ml) to 0 cell / ml by the 9th day of fermentation.

Theese results showed that *H. uvarum* population was affected by the presence of *T. delbrueckii* DiSVA 130 particularly in the first two days of fermentation and persisting until the end of fermentation.

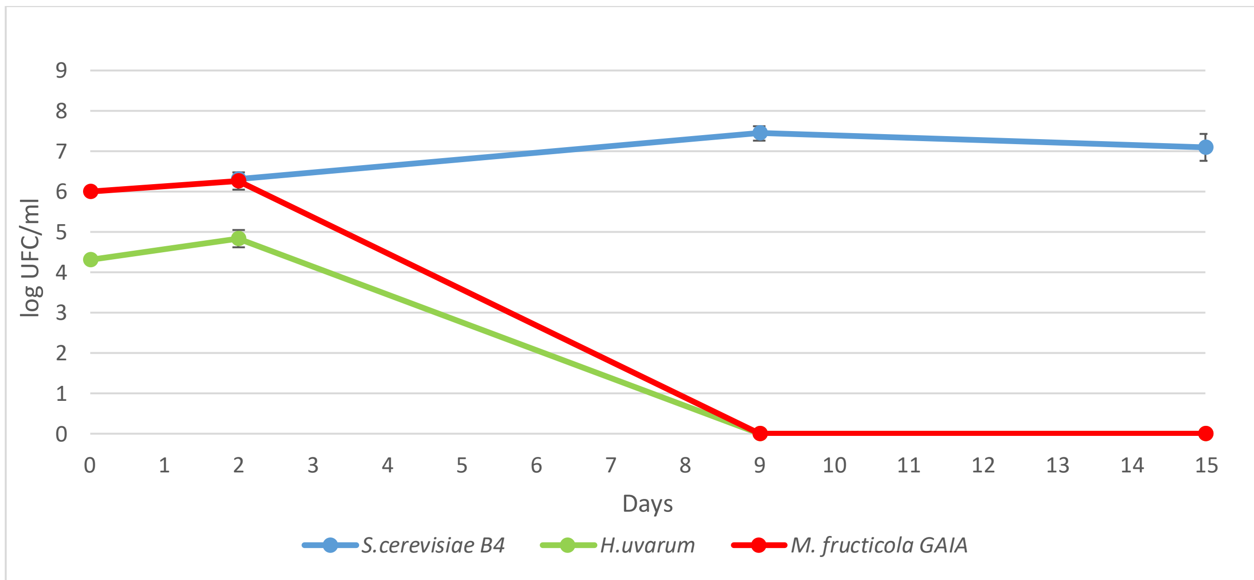

Figure 23 Growth kinetics of *S. cerevisiae* B4 (—●—),*H. uvarum* (—●—) *M. fructicola* GAIA® (—●—) on natural grape juice

In this trial *M. fructicola* GAIA® with initial inoculum level of 10^6cell/ml, persisted during two days of fermentation and then decreased until the 9th day of fermentation (0 cell / ml).

S. cerevisiae B4 with initial inoculum level of 10^6cell/ml, exhibited the maximum cell concentration at 9th day of fermentation (more than 10^7 cell/ml) to remain constant until the end of fermentation (10^7 cell/ml). *S. cerevisiae* B4 was not affected by the presence of *M. fructicola* GAIA® .

The concentration of *H. uvarum* showed an increase of 1 Log order during the first two days of fermentation (10^5 cell/ml) and then decreased until 0 cell / ml at the 9th day of fermentation.

M. fructicola GAIA® showed less effectively control on *H. uvarum* population in comparison with *M. pulcherrima* DiSVA 269.

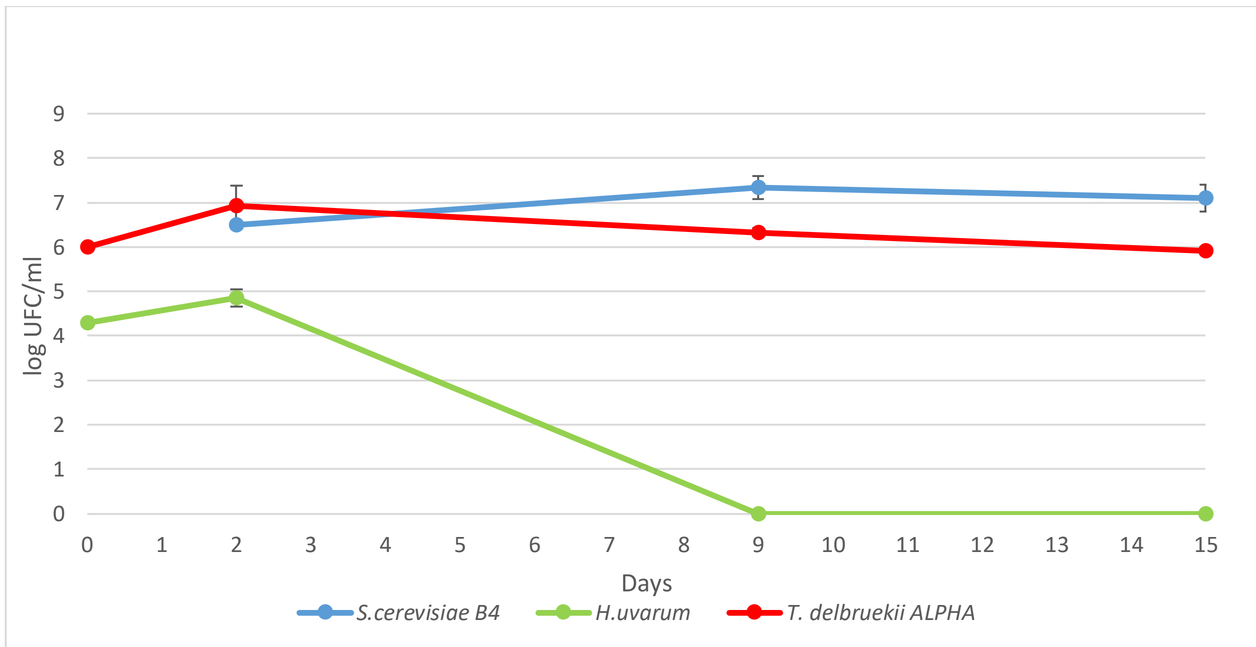

Figure 24 Growth kinetics of *S. cerevisiae* B4 ( ), *H. uvarum* ( )*T. delbruekii* ALPHA®( ) on natural grape juice

T. delbrueckii ALPHA® with initial inoculum level of 10^6cell/ml, showed an increase of one Log order reaching the maximum cell concentration at 2nd day of fermentation maintaining a constant trend until the end of fermentation .

S. cerevisiae B4 with initial inoculum level of 10^6cell/ml, achieved the maximum cell concentration at 9th day of fermentation (10^7 cell/ml) to remain constant until the end of fermentation. *S. cerevisiae* B4 was not affected by the presence of the other two yeasts.

The concentration of *H. uvarum* population showed a similar behavior of mixed fermentation with *M. fructicola* Gaia® .

In conclusion, the results showed that the sequential fermentation trial with *M. pulcherrima* DiSVA 269 showed a bio-control effect more effective on *H. uvarum* population in comparison with the two *Metschnikowia* spp. commercial strains.

On the contrary, both *T. delbrueckii* spp. showed a similar constant trend for the whole duration of fermentation. *S. cerevisiae* B4 showed a similar trend in all trials highlighting that is not affected by the presence of the non-*Saccharomyces* yeasts.

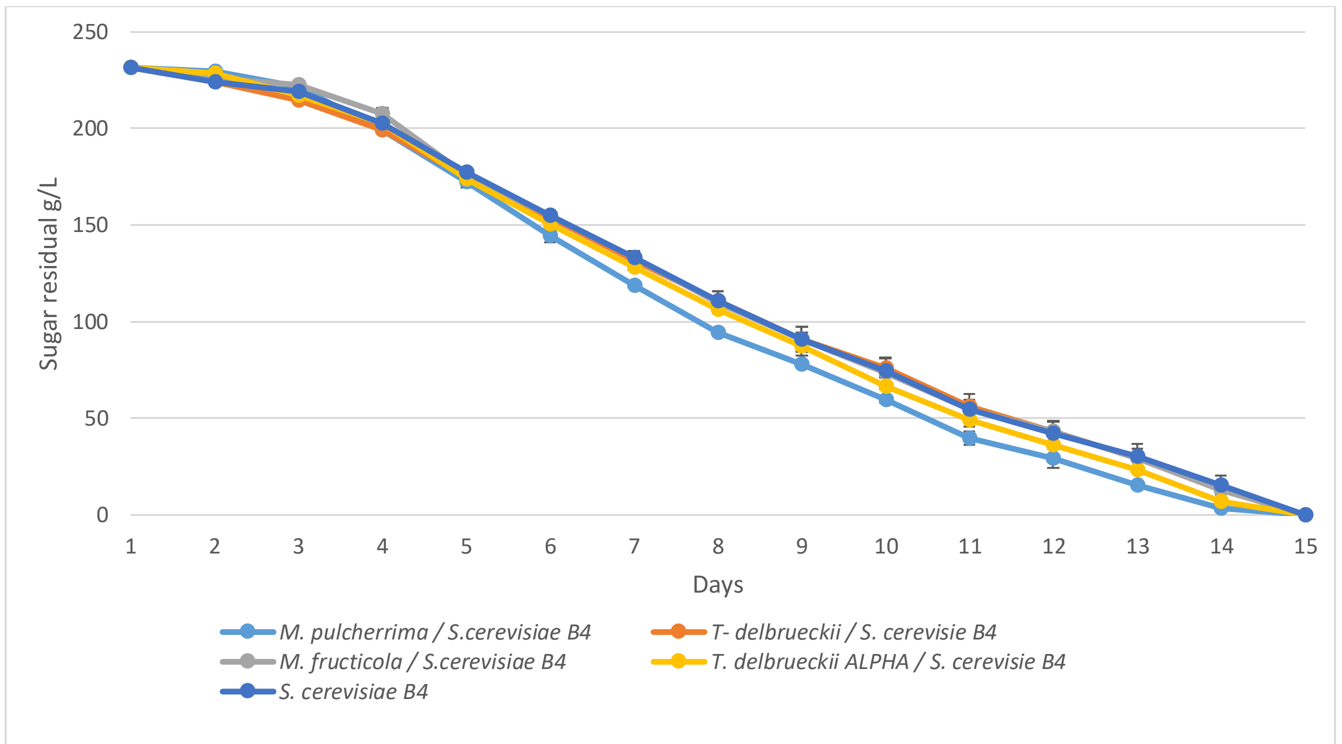

Figure 25 Sugar consumption kinetics in sequential fermentation trials *M. pulcherrima* DiSVA 269 /*S. cerevisiae* B4 ( ),*T. delbrueckii* DiSVA 130 /*S. cerevisiae* B4( ) , *M. fructicola* GAIA® /*S. cerevisiae* B4( ),*T. delbrueckii* ALPHA®/*S. cerevisiae* B4 ( ), *S. cerevisiae* B4 ( )

Regarding to sugar consumption (fig. 25), all fermentations exhibited a similar trend in fermentation kinetics. All fermentation showed a total sugar consumption at the end of fermentation.

Moreover, the results highlighted a positive interaction on fermentation kinetics of non-*Saccharomyces* yeasts when used in sequential fermentation with *S. cerevisiae* B4.

4.2.1 Main Oenological Characters and Volatile Compounds of wines

	Ethanol (%v/v)	Total Acidity (Tartatic Acid g L^{-1})	Volatile Acidity (Acetic Acid g L^{-1})	Malic Acid (g L^{-1})
S. cerevisiae B4	13.43±0.00[b]	5.52±0.02[a]	0.25±0.063[a]	1.2±0.00[a]
M. pulcherrima DiSVA 269 / *S. cerevisiae* B4	13.51± 0.21[b]	5.56±0.04[a]	0.25±0.01[a]	1.15±0.07[a]
M. fructicola GAIA® / *S. cerevisiae* B4	13.53±0.14[b]	5.61±0.09[a]	0.23±0.02[a]	1.15±0.07[a]
T. delbrueckii DiSVA 130 / *S. cerevisiae* B4	13.77± 0.10[a]	5.55±0.14[a]	0.23±0.007[a]	1.2±0.14[a]
T. delbrueckii ALPHA® / *S. cerevisiae B4*	13.71±0.02[a]	5.52±0.06[a]	0.25±0.028[a]	1.1±0.00[a]

Table 6 Main Oenological Characters of wines

Sequential fermentation trials carried out with *T. delbrueckii* DiSVA 130 and *T. delbrueckii* ALPHA® exhibited the highest content of ethanol in comparison with other wines. Moreover, this sequential fermentation led a significantly lower malic acid content.

S. cerevisiae B4 control trial exhibited the lowest ethanol content while the sequential fermentation *M. fructicola* GAIA® / *S. cerevisiae* B4 exhibited the highest value of total acidity.

	S. cerevisiae B4 mg/L	*M. pulcherrima* DiSVA 269 / *S. cerevisiae B4* mg/L	*M. fructicola* GAIA® / *S. cerevisiae* B4 mg/L	*T. delbrueckii* DiSVA 130 / *S. cerevisiae B4* mg/L	*T. delbrueckii* ALPHA® / *S. cerevisiae B4* mg/L	*M. pulcherrima* DiSVA 269 / *S. cerevisiae B4* mg/L
ESTERS						
Ethyil butyrate	0.40±0.10[b]	0.29± 0.35[b]	1.88±0.60[a]	0.31 ± 0.01[b]	0.52±0.19[b]	0.29± 0.35[b]
Ethyl acetate	26.42±4.29[a]	29.32±1.32[a]	25.03±2.44[a]	59.88±2.14[a]	33.67±6.71[a]	29.32±1.32[a]
Ethyl exanoate	0.03±0.05[a]	2.76±0.33[b]	0.01±0.02[a]	2.90±0.41[b]	3.39±0.35[b]	2.76±0.33[b]
Isoamyl acetate	0.09±0.01[a]	3.37±0.71[a]	1.22±0.40[a]	0.95±0.08[a]	1.03±0.04[a]	3.37±0.71[a]
ALCOHOLS						
n-propanol	37.01±3.09[b]	33.09±7.83[ab]	21.91±1.08[a]	39.25±1.40[b]	38.73±0.79[b]	33.09±7.83[ab]
Isobutanol	15.38±1.72[a]	11.78±3.84[a]	16.77±1.75[a]	26.67±5.20[a]	11.92±13.30[a]	11.78±3.84[a]
Amyl alcohol	12.99±0.26[a]	11.17±0.16[a]	19.46±0.81[a]	39.762±8.28[a]	14.33±3.77[a]	11.17±0.16[a]
Isoamilic alcohol	123.34±8.01[a]	119.61±25.34[a]	147.57±3.44[a]	67.11±58.45[a]	126.30±2.71[a]	119.61±25.34[a]

β-Phenyl ethanol	0.8±0.01[a]	7.7±0.00[b]	1.7±0.02[a]	7.4±0.16[b]	9.1±0.02[b]	7.7±0.00[b]
CARBONYL COMPOUNDS						
acetaldehyde	19.23±0.50[ab]	9.18±1.40[c]	23.64±0.17[a]	14.25±6.27[bc]	14.23±2.87[bc]	9.18±1.40[c]
MONOTERPENS						
Linalol	003±000[bc]	001±000[bc]	0±0[c]	0.20±0.07[ab]	0.22±0.14[a]	0.01±0.00[bc]

Table 7 Main volatile compounds

Regarding to the esters production (fruits aroma), the *M. fructicola* GAIA® / *S. cerevisiae* B4 trial produced Ethyl butyrate was the highest producer . On the contrary, the presence of *M. pulcherrima* DiSVA 269 in combination with *S. cerevisiae* B4 led an increase in Phenyl Ethyl acetate and Isoamyl acetate (banana flavor) content in comparison with the other trials. Ethyl acetate was significant increase in all trials above all for the sequential fermentation *T. delbrueckii* DiSVA 130 / *S. cerevisiae* B4. Regarding to the Ethyl hexanoate content only the sequential fermentation *M. fructicola* GAIA® / *S. cerevisiae* B4 and the control *S. cerevisiae* B4 have produced a lower content than the other trials.

No significant differences were detected for the n-propanol, only the trials with *M. fructicola* GAIA® produced a lower content than the other trials while the sequential fermentation *T. delbrueckii* 130 / *S. cerevisiae* B4 led a significant increase in isobutanol content and amylic alcohol. Moreover, the presence of *T. delbrueckii* spp. determined a significant increase of β-phenyl ethanol (rose aroma).

Considering monoterpenic compounds (terpenes that contribute to the classic floral aroma), *T. delbrueckii* spp. determined a greater content of linalool in comparison with the other wines.

The acetaldehyde was significant higher in wine fermented by *M. fructicola GAIA® / S. cerevisiae B4* and the control *S. cerevisiae B4*.

4.2.3 Sensory analysis

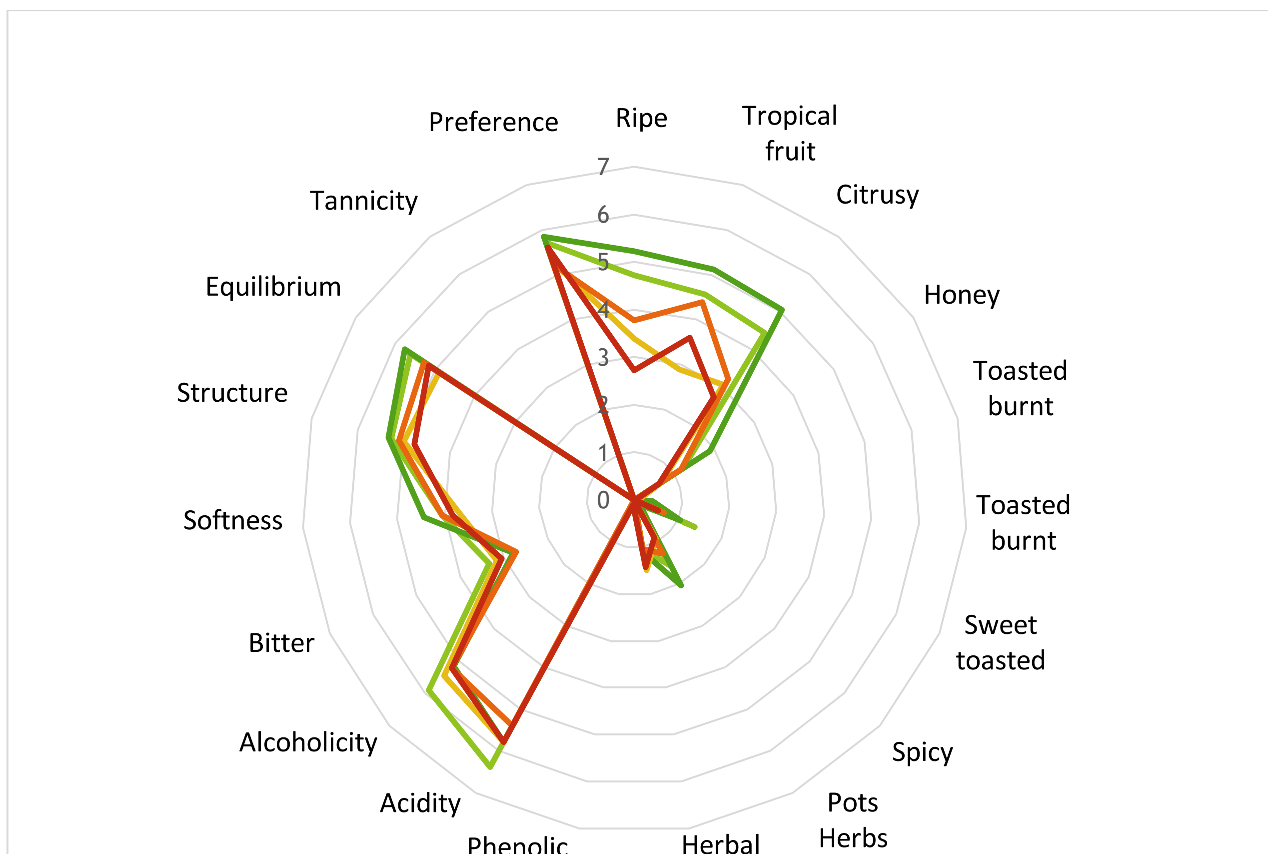

Figure 26 Sensory analysis of Verdicchio wine fermented by *S. cerevisiae* B4( ); *T. delbrueckii* DiSVA 130 / *S. cerevisiae* B4 ( );*T. delbrueckii* ALPHA® / *S. cerevisiae* B4 ( ); *M. pucherrima* DiSVA 269 / *S. cerevisiae* B4 ( ) and *M. pucherrima* GAIA® / *S. cerevisiae* B4 ( ).

Also in this case the wines produced by pure and sequential fermentations underwent to sensory analysis, and the results are reported in figure 26. The testers expressed a positive judgment regarding each wine, characterized by specific aromatic notes and without defects. In particular, the wines obtained were characterized significantly to have a similar structure, softness, alcohol content and smell; in particular the sequential fermentation *M. pulcherrima* DiSVA 269 / *S. cerevisiae* B4 expressed the highest alcohol and total acidity content Moreover the toasted and spicy notes were absent, while fruity notes were more detected in the trial *T. delbrueckii* DiSVA 130 / *S. cerevisiae* B4. In conclusion the sequential fermentation *T. delbrueckii* DiSVA 130 / *S. cerevisiae* B4 took the highest score.

From both tasting panels it emerges that the presence of *T. delbrueckii* DiSVA 130 in combination with both native *S. cerevisiae* I4 and B4 enhance the aromatic bouquet and organoleptic characteristics of final wines

5. DISCUSSION AND CONCLUSIONS

During the recent years the use of non-*Saccharomyces* yeasts in winemaking process allowed to obtain countless benefits, from structural and aromatic point of view of final wines highlighting the positive role of some non-*Saccharomyces* yeasts.

This study was focused on the use of two selected strains *M. pulcherrima* DiSVA 269 an *T. delbrueckii* DiSVA 130. In particular it was evaluated its role in the bio protection action and their influence of analytical composition and aromatic bouquet of wines. These non *Saccharomyces* yeasts were tested together with improved native *S. cerevisiae* strains and compared with commercial strains of the same genera. The final objective was the production of wines with low sulphites content and improved aromatic and sensorial profile (Ciani M. & Comitini F., 2019).

Regarding to bio control effect *M. pulcherrima* DiSVA 269 exhibited a strong and broad-spectrum effect against indigenous yeasts as *H. uvarum,*. Likewise, the results highlighted that *T. delbrueckii* DiSVA 130 spp. also exhibited a bio-control effect against wild yeasts.

The first fermentation trials of this study highlighted the ability of *T. delbrueckii* DiSVA 130 to counteract the apiculate yeasts. This result is more evident in the sequential trial with *S. cerevisiae* I4 trial respect OKAY® without added SO_2. Therefore, in wines produced with only *S. cerevisiae* I4 without added SO_2 it would be useful to exploit the bio-control capacity of *T. delbureckii* DiSVA 130.

In the second set of fermentation trials , the bio control activities of *T. delbureckii* DiSVA 130 e *M. pulcherrima* DiSVA 269 was confirmed using the improved native strain B4 without SO_2 added.

These results confirmed the bio-active effect of non-*Saccharomyces* yeasts combined with *S. cerevisiae* yeast thanks to their synergistic activity as shown data obtained from other studies (Comitini & Ciani 2010; Oro L. et al. 2016).

Moreover, the second part of this study highlighted that the presence of *M. pulcherrima* DiSVA 269 reduce the ethanol content of the final wines in comparison with the other sequential fermentation. In this case several studies highlighted the ability of this strain to reduce the ethanol content in wines with values below 15% as reported in many works (Sadoudi M. 2017; Canonico L. et al. 2019; Hranilovic A. 2020).

In conclusion, the results of this work confirming the important role of biotechnologies for the control and improvement of fermentation process, showed relevant specific yeast -yeast interaction among non-*Saccharomyces* and *S. cerevisiae* strains in; fermentation performance, analytical composition as well as aromatic and sensorial profile of wine. Thus, allow us to open up the complex bio-protection mechanism and bio-control effect on microbial populations to reduce the use of sulfur dioxide, how to reduce or manage the production of ethanol and how to enhance the aromatic bouquet.

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CHAPTER 4

Starmerella bombicola and *Saccharomyces cerevisiae* in Wine Sequential Fermentation in Aeration Condition: Evaluation of Ethanol Reduction and Analytical Profile

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ABSTRACT

In the last few decades, the increase of ethanol in wine, due to global climate change and consumers' choice is one of the main concerns in winemaking. One of the most promising approaches in reducing the ethanol content in wine is the use of non-*Saccharomyces* yeasts in cofermentation or sequential fermentation with *Saccharomyces cerevisiae*. In this work, we evaluate the use of *Starmerella bombicola* and *S. cerevisiae* in sequential fermentation under aeration condition with the aim of reducing the ethanol content with valuable analytical profile. After a preliminary screening in synthetic grape juice, bench-top fermentation trials were conducted in natural grape juice by evaluating the aeration condition (20 mL/L/min during the first 72 h) on ethanol reduction and on the analytical profile of wines. The results showed that *S. bombicola/S. cerevisiae* sequential fermentation under aeration condition determined an ethanol reduction of 1.46% (v/v) compared with *S. cerevisiae* pure fermentation. Aeration condition did not negatively affect the analytical profile of sequential fermentation *S. bombicola/S. cerevisiae* particularly an overproduction of volatile acidity

ethyl acetate. On the other hand, these conditions strongly improved the production of Glycerol and Succinic Acid that positively affect the structure and body of wine.

Keywords: ethanol reduction; Starmerella bombicola; oxygen; wine; analytical profile

1. INTRODUCTION

Ethanol is the main product in wine produced by yeast during alcoholic fermentation. During the last two decades, in many different geographical areas, the average alcohol level has risen about 2% (v/v)

[1]. Generally, the alcohol level in wine is between 12 and 14% (v/v) with some exception the different varieties of wines [2]. The climatic changes recorded in recent years have resulted in grapes with high sugar concentrations, which is reflected in wines with increased ethanol content. Wines with high ethanol content are associated with health issues, economic and quality aspects [3–12]. Indeed, high alcohol levels in wine compromise the organoleptic properties of the product increasing the hotness and viscosity, and decreasing sweetness, intensity, and aromatic flavors [13–20]. The combination of these aspects (organoleptic, economic and health issues) in wine with high ethanol content has led to the development of technological to produce wines with a reduced alcohol level without affecting flavour profile and sensorial characteristics [21]. For these reasons, many strategies in reduce ethanol content in wine during the winemaking process have been proposed, such as viticultural, pre-fermentation, fermentation and post fermentation practices [1,8,22,23]. A suitable strategy for reducing the alcohol level of wine is the use of non-*Saccharomyces* yeasts able to use different pathways for sugar convert (respiration, alcoholic fermentation, and glycerol-pyruvic metabolism) [24–26]. Biotechnological processes under different fermentation conditions with non-*Saccharomyces* in co-culture or sequential fermentation with *S. cerevisiae* starter strain were proposed [22,27–32]. In sequential inoculation, the non-*Saccharomyces* yeast strain is inoculated in the grape juice in the first stage of fermentation (48–72 h). This procedure allows the non-*Saccharomyces* strain to take advantage favouring its metabolic activity. In particular, the non-*Saccharomyces* yeasts could affect the wines by producing a low ethanol yield, low volatile acidity and/or enhancement of specific analytical and aromatic compounds [33–36]. Different researches showed that the physiological features of *Metschnikowia pulcherrima Lachancea thermotolerans, Torulaspora delbrueckii, Starmerella* and *Zygosaccharomyces* spp. strains are suitable for lower ethanol content in wine in the presence of oxygen. According with the results obtained by controlled aeration fermentations the ethanol reduction was for *M. pulcherrima* 1.6% (v/v), *T. delbrueckii* 0.9% (v/v), *Z. bailii* 1.0% (v/v), and *S. bacillaris* 0.7% (v/v) compared with *S. cerevisiae* wine [31,37]. In recent previous work, *Starmerella bombicola* (formerly *Candida stellata*) was evaluated for ethanol reduction in wine in a static condition and in a immobilized form with promising results [32]. However, immobilized cells are a modality of inoculum, quite complex and difficult to apply under an industrial vinification condition. Previously, a strain of *S. bombicola* was proposed to enhance the glycerol content of wine in immobilized form to overcome its low fermentation rate [38]. Indeed, the general enological traits of *S. bombicola* strains showed low fermentation rate and low fermentation power (4–5% vol. of ethanol) together with some interesting positive features as high glycerol and succinic acid production. In the present work, with the aim to reduce the ethanol content in wine, *S.*

bombicola/ S. cerevisiae sequential fermentations were evaluated under partial aeration condition. The analytical composition and aromatic profile of the final wines were also evaluated.

2. MATERIALS AND METHODS

2.1. Yeast Strains

The non-*Saccharomyces* yeast strain used in this study was *S. bombicola* DiSVA 66 (formerly named *Candida stellata* DBVPG 3827; Industrial Yeast Collection of the University of Perugia) and evaluated in a previous work in immobilized form [32]. *S. cerevisiae* commercial strain Lalvin EC1118 (Lallemand Inc., Toulouse, France) was used in pure (control) and sequential fermentation trials. These strains were maintained on YPD agar medium (1% yeast extract, 2% peptone, 2% D-glucose, and 1.8% agar) at 25 _C for 48 h, and stored at 4 _C.

2.2. Preliminary Screening on Synthetic Grape Juice (SGJ)

Modified YPD medium (0.5% yeast extract, 0.1% peptone, 2% dextrose, all w/v) was used to obtain biomass for fermentation trials. *S. bombicola* cells were incubated at 25 _C for 72 h in a rotary shaker (150 rpm). This biomass was harvested by centrifugation, washed three times with sterile distilled water. To optimize the cell concentration of S. bombicola, screening was conducted on SGJ, and prepared according to the protocol reported by Ciani and Ferraro [38]. The trials were carried out in 100 mL flask containing 70 mL SGJ under static and agitation condition (200 rpm rotary shaker) at 22 _ 1 _C in triplicate. The inoculum of S. bombicola was 1 x 10^8 cells/mL and 5 x 10^7 cells/mL followed three days, by S. cerevisiae (1 x 10^6 cells/mL). Ethanol content, volatile acidity and glycerol content were analyzed. The fermentation trial, which showed the best reduction in alcohol content was selected to set up fermentation in Natural Grape Juice (NGJ).

2.3. Natural Grape Juice (NGJ) Fermentation Trials

Natural grape juice (NGJ) (Verdicchio white grape variety), used for trials, showed the following characteristics: pH, 3.32; total acidity, 5.17 g/L; free SO2, 9 mg/L; total SO2, 18 mg/L; malic acid, 3.1 g/L; initial sugar content, 218 g/L; yeast assimilable nitrogen (YAN) 121 mg N/L. The 2-L Bench-top bioreactor (Biostat® B; B. Braun Biotech Int., Goettingen, Germany) containing 1.5 L of natural grape juice under gentle agitation (60 rpm/min) was used for fermentation trials. The temperature was 22 °C with an inoculation level of 5 x 10^7 cells/mL of *S. bombicola* obtained using the procedure described above. Aerobic condition was maintained using 20 mL/L/min of air flow during the initial 72 h, while in semi-anaerobic condition no aeration was applied. In sequential fermentations, *S. cerevisiae* was inoculated after 72 h (1 x 10^6 cells/mL). Pure fermentations of *S. cerevisiae* (inoculum

1 x 10^6 cells/mL) were used as controls under gentle agitation (60 rpm/min, semi-anaerobic condition). A specific enzymatic kit (Megazyme International Wicklow Ireland) was used to evaluate the sugar consumption during the fermentation to monitor fermentation kinetics. Biomass evolution was evaluated by viable cell count (CFU/mL) on Lysine Agar selective medium and WL nutrient agar (Oxoid, Hampshire, UK) [39]. Wild non-*Saccharomyces* yeasts (WNS) were easily distinguished by S. bombicola through a macro- and microscopic characterization of colony on WL nutrient agar. The fermentations were carried out in triplicate.

2.4. Analytical Procedures

Total acidity, volatile acidity, pH and ethanol content were determined according to the Official European Union Methods [40]. The final samples, prepared following the procedure of Canonico et al. [41], were directly injected into a gas chromatography system (GC-2014; Shimadzu, Kjoto, Japan) to quantify acetaldehyde, ethyl acetate, n-propanol, isobutanol, amyl and isoamyl alcohols. Solid-phase microextraction (HS-SPME) method with the fiber. Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Sigma-Aldrich, St. Louis, MO, USA) was used to determine the main volatile compounds desorbed by inserting the fiber into gas chromatograph GC (GC-2014; Shimadzu, Kjoto, Japan) The compounds were identified and quantified using external calibration curves [42]. Glucose and fructose (K-FRUGL), glycerol (K-GCROL) and succinic acid (K-SUCC) were analyzed using specific enzyme kits (Megazyme International,Wicklow Ireland).

2.5. Statistical Analysis

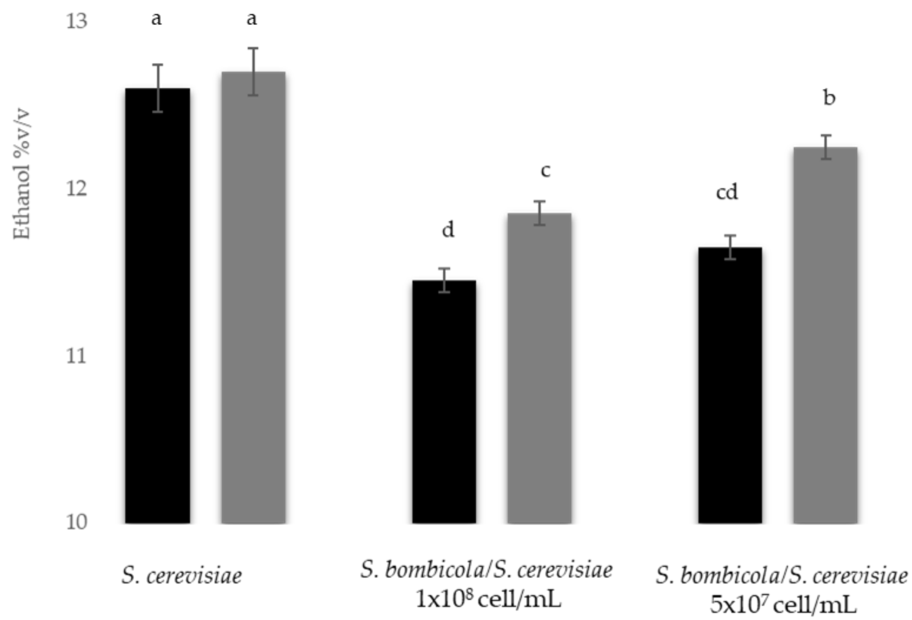
Experimental data for the main analytical characters of wine have been subjected to analysis of variance (ANOVA) using the statistical software package JMP® 11. Duncan test was used to determine the significant differences (p-values were <0.05).

3. RESULTS

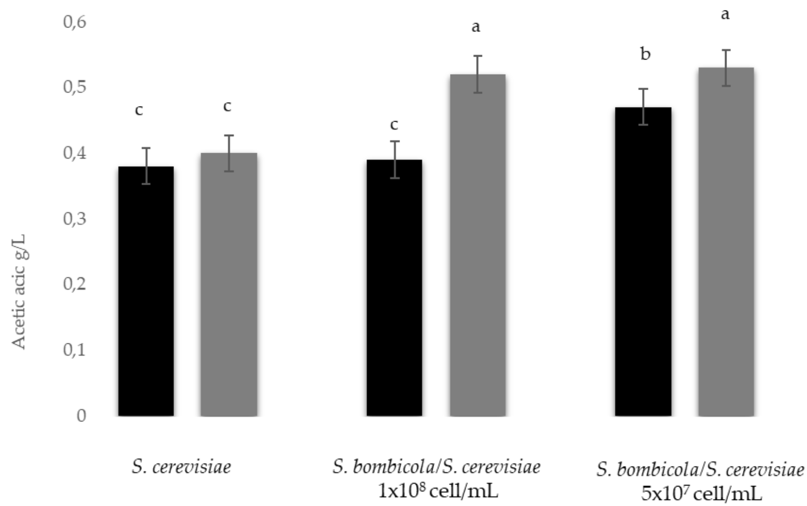
3.1. Preliminary Screening on Synthetic Grape Juice

The results of the ethanol content of screening trials, carried out under static and agitation conditions, was reported in Figure 1A. *S. bombicola/S. cerevisiae* sequential fermentation in agitation condition significant enhanced the ethanol reduction if compared with static one and *S. cerevisiae* pure culture both in static and agitation condition. In particular, *S. bombicola/S. cerevisiae* sequential fermentation

10^8 cells/mL and 5 x 10^7 cells/mL achieved an ethanol reduction of 1.25% (v/v), and 1.05% (v/v), respectively in comparison with *S. cerevisiae* pure culture (in both conditions). Moreover, the ethanol content in the trials with inoculum level 5 x 10^7 cells/mL in agitation condition was comparable with that obtained with 10^8 cells/mL.



(A)



(B)

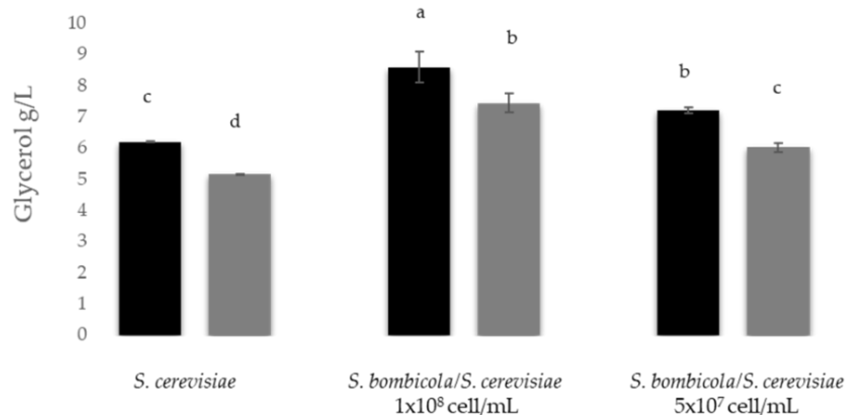


Figure 1. Ethanol content (a), volatile acidity (b) and glycerol content (c) of preliminary screening in sequential fermentation in static and agitation condition in *Synthetic Grape Juice* . ■ Agitation condition; ■ static condition.

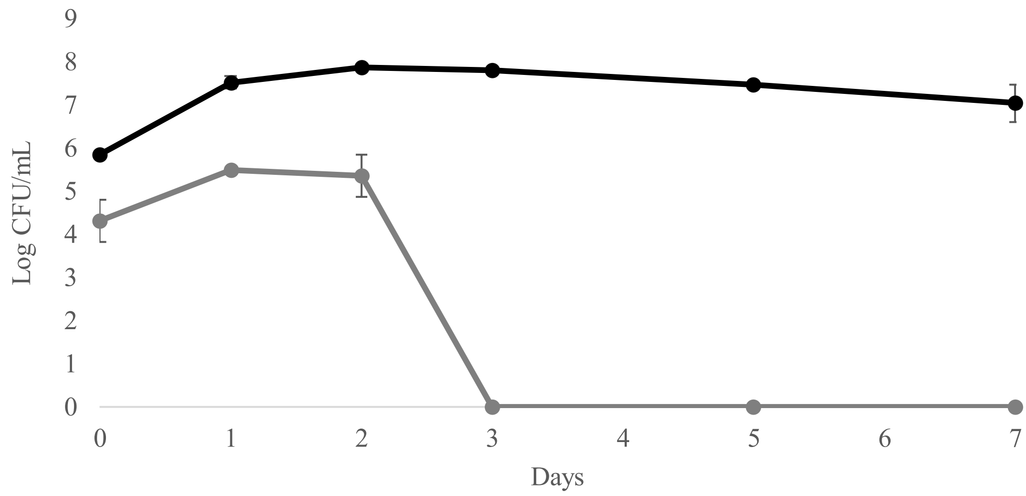
In relation to the volatile acidity (Figure 1B), the fermentation trials at different inoculum level of *S. bombicola* showed in general similar values exhibited by *S. cerevisiae*. A significant increase was detected only with *S. bombicola/S. cerevisiae* sequential fermentation in static condition using different inoculation levels (0.53 g/L acetic acid). The aeration conditions determined a general enhancement of glycerol production in all fermentation trials. In particular, a significant increase was showed in *S. bombicola/S. cerevisiae* 1 x 10^8 cells/mL sequential fermentations (8.58 g/L) compared with *S. cerevisiae* pure culture with the exception of *S. bombicola/S. cerevisiae* 5 x 10^7 cells/mL in static condition (Figure 1C). Considering the similar results obtained and the most practice application in vinery condition of the lower inoculum level, *S. bombicola* at inoculum level 5 x 10^7 cell/mL was identified for the further bench-top fermentation trials in NGJ. Using the following fermentation conditions: Semi-anaerobic (gently agitation 60 rpm) and aeration flow of 20 mL/L/min during the first 72 h.

3.2. Bench-Top Fermentation Trials

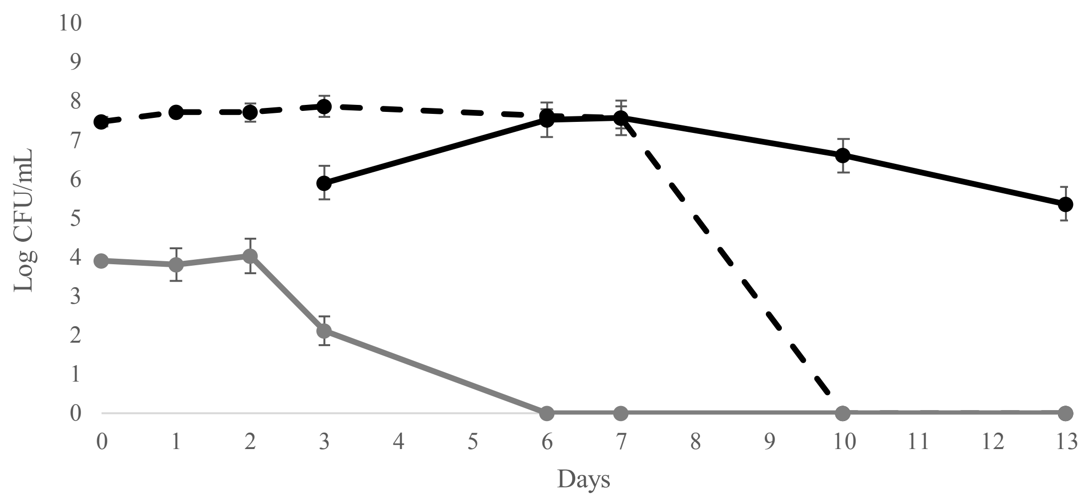
3.2.1. Biomass Evolution and Sugar Consumption in Natural Grape Juice (NGJ)

The growth kinetics are reported in Figure 2. The pure *S. cerevisiae* fermentation (Figure 2A) achieved the maximum cell concentration (c.a. 10^8 cells/mL) in 2 days maintaining this cell concentration until the end of the fermentation. When S. cerevisiae reached the maximum cell

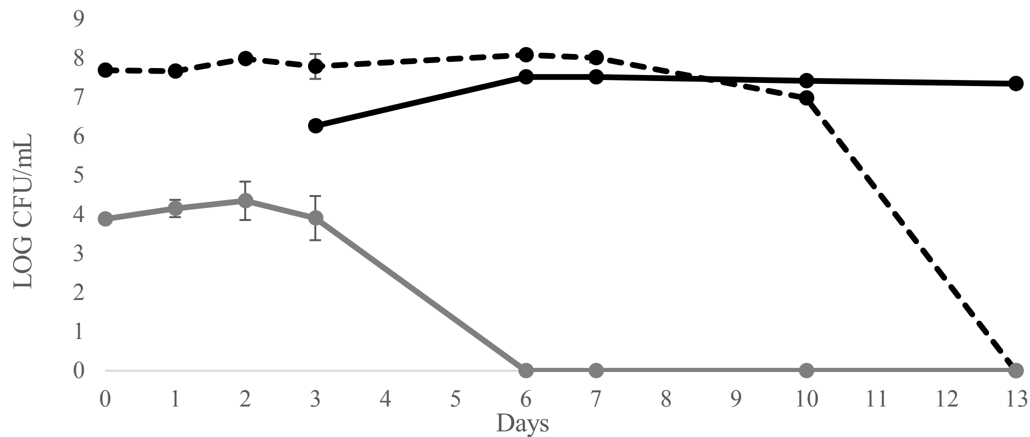
concentration, the wild non-Saccharomyces yeasts (WNS) present in the natural grape juice, decreased until disappeared.



(A)



(B)



(C)

Figure 2. Growth kinetics in sequential fermentation trials *S. bombicola*/*S. cerevisiae* and control *S. cerevisiae* on natural grape juice (NGJ). ( ) *S. cerevisiae;* ( ) Wild non-*Saccharomyces;* ( ) *S. bombicola*. (a) control-pure fermentation with *S. cerevisiae* inoculum; (b) semi-aerobic condition (no aeration); (c) with aeration (20 mL/L min of air flux during the first 72 h).

A similar trend in biomass evolution of *S. cerevisiae* and WNS was shown in semi-anaerobic conditions (Figure 2b). Regarding to *S. bombicola* population differences between semi-anaerobic and air flow addition were shown. The sequential fermentations carried out with air flow (20 mL/L/min of air flux during the first 72 h) (Figure 2 c) showed that achieved high level (> 10^7 cfu/mL) until 10th day while in semi-aerobic condition high biomass concentration where maintained only until 7th day (Figure 2b). The same biomass evolution that in semi-aerobic condition in WNS was shown. Moreover, *S. bombicola* fermentation trials with air flow showed a higher persistence of viable cells in comparison with sequential fermentation in semi-anaerobic condition. Regarding to the evolution of WNS, *S. cerevisiae* pure culture showed a strong control of WNS that disappeared at 3th day of fermentation, while WNS with *S. bombicola*/*S. cerevisiae* sequential fermentation disappeared at 6th day of fermentation (in both conditions: with and without air flux).

The duration of fermentation process was approximately 13 days for both the sequential fermentations while *S. cerevisiae* pure culture, as expected, completed the fermentation in 7 days.

The sugar consumption (Figure 3) confirmed the fermentation trend: the sequential fermentations showed a comparable trend *S. cerevisiae* pure culture exhibited a faster fermentation kinetics with a half of the sugar consumed after 24h of fermentation.

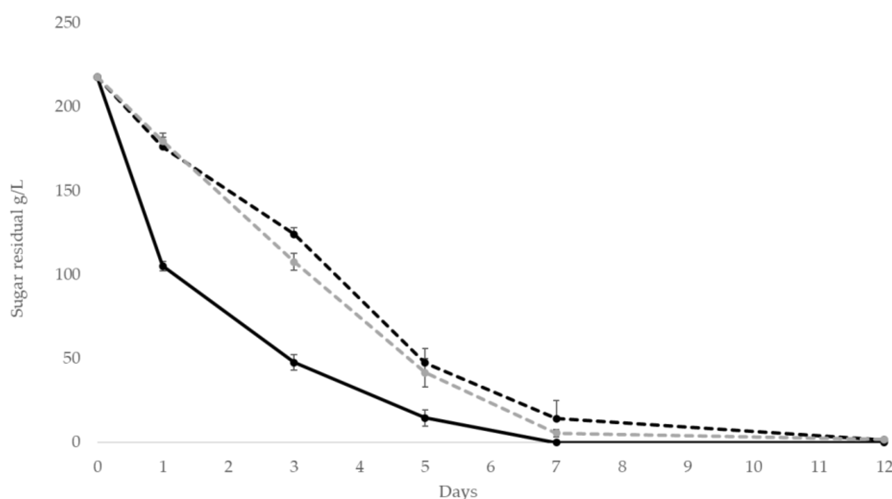


Figure 3. Sugar consumption kinetics in sequential fermentation trials *S. bombicola*/*S. cerevisiae* and control *S. cerevisiae* on natural grape juice (NGJ) in static and aeration condition. (—●—)*S. cerevisiae;* (- -●- -) *S. bombicola/ S. cerevisiae* static and (- -●- -) *S. bombicola/ S. cerevisiae* 20 mL/L/min oxygen

3.2.2. Main fermentation parameters in Natural Grape Juice (NGJ)

The main fermentation parameters determined at the end of fermentation are reported in Table 1.

Fermentation Trials	Sugar Consumed (g/L)	Ethanol (% *v/v*)	Ethanol Yield (g/g) %	Glycerol (g/L)	Volatile Acidity (as Acetic Acid g/L)	Succinic Acid (g/L)
S. cerevisiae pure culture	218 ± 0.00 [a]	12.12 ± 0.11 [a]	44.03 ± 0.46 [a]	3.08 ± 0.27 [c]	0.35 ± 0.01 [a]	0.25 ± 0.21 [b]
S. bombicola/S. cerevisiae static condition	216.44 ± 0.47 [a,b]	11.91 ± 0.11 [b]	43.45 ± 1.13 [a]	7.30 ± 0.12 [b]	0.29 ± 0.02 [b]	0.61 ± 0.14 [b]
S. bombicola/S. cerevisiae 20 mL/L/min oxygen	215.03 ± 0.99 [b]	10.66 ± 0.08 [c]	38.99 ± 0.73 [b]	10.50 ± 0.12 [a]	0.29 ± 0.00 [b]	2.69 ± 0.10 [a]

Table 1. Main parameters of NGJ fermentation trials

The initial sugar concentration was 218 g/L. Data are means ± standard deviations from three independent experiments. Data with different superscript letters ([a,b,c]) within each Column are different according to Duncan tests(0.05%).

In relation to the ethanol content, in comparison with *S. cerevisiae* pure culture, *S. bombicola/ S.cerevisiae* air flow exhibited an ethanol reduction of 1.46% (v/v). Whereas, *S. bombicola/ S. cerevisiae* static condition led an ethanol reduction of 0.21% (v/v). This trend was reflected by the ethanol yield that was significant significantly lower in *S. bombicola/S. cerevisiae* with air flow in comparison with other fermentation trials. While, volatile acidity amounts were comparable among the fermentations, sequential fermentation led a general increase in final glycerol content. In particular, *S. bombicola* sequential fermentation air flow supplied showed a significant increase in this compound (more than 3-fold of *S. cerevisiae*). However, the results also exhibited an increase in

glycerol content in static sequential fermentations, indicating the effect of *S. bombicola* in glycerol production. Aeration condition also determined a significant increase in succinic acid.

3.2.3. The Main Volatile Compounds in Natural Grape Juice

The data of the main volatile compounds are reported in Table 2.

mg/L	Fermentation Trials		
ESTERS	*S. cerevisiae* Pure Culture	*S. bombicola/S. cerevisiae* 20 mL/L/min	*S. bombicola/S. cerevisiae* semi anaerobic condition
Ethyl butyrate	0.41 ± 0.02 [a,b]	1.08 ± 0.35 [a]	0.40 ± 0.39 [b]
Ethyl acetate	30.58 ± 1.27 [a]	26.17 ± 2.51 [b]	21.58 ± 1.04 [c]
Ethyl hexanoate	0.06 ± 0.004 [a]	0.04 ± 0.011 [a]	0.03 ± 0.019 [a]
Isoamyl acetate	2.017 ± 0.05 [a,b]	0.91 ± 0.34 [b]	2.71 ± 1.18 [a]
ALCOHOLS			
n-propanol	34.00 ± 2.04 [b]	69.63 ± 0.06 [a]	33.74 ± 0.31 [b]
Isobutanol	14.33 ± 0.16 [c]	35.34 ± 1.21 [a]	19.43 ± 2.04 [b]
Amyl alcohol	4.89 ± 1.77 [a]	3.82 ± 0.28 [a]	1.30 ± 0.24 [b]
Isoamyl alcohol	64.50 ± 2.63 [a]	45.47 ± 1.36 [b]	29.31 ± 0.42 [c]
β-Phenyl ethanol	30.1 ± 0.018 [a,b]	24.8 ± 0.28 [b]	35.8 ± 0.07 [a]
CARBONYL COMPOUNDS			
Acetaldehyde	10.59 ± 0.19 [b]	30.12 ± 2.22 [a]	9.26 ± 0.53 [b]
MONOTERPENS			
Linalool	0.08 ± 0.01 [a]	0.05 ± 0.001 [a]	0.12 ± 0.05 [a]
Geraniol	0.09 ± 0.018 [a,b]	0.007 ± 0.0004 [b]	0.10 ± 0.05 [a]

Table 2. The main volatile compounds of S. cerevisiae pure culture and sequential fermentations with or without air flow addition. Data are means _ standard deviations from three independent experiments. Data with different superscript letters (a,b,c) within each Column are different according to Duncan tests (0.05%).

In relation to the higher alcohols, the sequential fermentation trials with air flow led a significant increase in n-Propanol and Isobutanol in comparison with the other fermentation trials, while Amylic alcohol was comparable with *S. cerevisiae* pure culture. On the contrary, the wine obtained with *S. bombicola/S. cerevisiae* sequential fermentation in aerobic condition, showed a lower amount of β-Phenyl ethanol (rose aroma) than the other wines.

The behaviour of sequential fermentations was variable in the group of esters compounds. Indeed, it was not possible to define a general trend. Indeed, sequential fermentation with 20 mL/L/min of air flow exhibited a significant increase in Ethyl butyrate content than other trials and a comparable amount of Ethyl hexanoate with other fermentations. *S. bombicola/S. cerevisiae* sequential

fermentation in static condition led an increase in isoamyl acetate (banana aroma) content, and a significant decrease of this aroma compound in aerobic condition if compared with *S. cerevisiae* control trial. *S. bombicola/S. cerevisiae* sequential fermentation with air flow affected the acetaldehyde content in comparison with other fermentation trials without negative influence on the aromatic profile of wines. In relation to the main mono-terpens (Linalool and Geraniol), the resulting wines did not show a significant difference.

4.DISCUSSION

In recent years, one of the most relevant concerns related to winemaking sector, is the progressive increase of ethanol content. Among microbiological strategies proposed to decrease alcohol level in wine the use of non-*Saccharomyces* yeasts in co-fermentation or sequential fermentation with *S. cerevisiae* starter strains under aerobic and anaerobic conditions were proposed [24,28,29,31,32,43,44,45,46,47,48]. Several studies reported that the use of air flow during the early stage of fermentation affect yeast physiology and metabolism favouring the fermentation performance of yeasts [49-53]. In particular, in *S. cerevisiae* respiration is repressed by high concentrations of sugars even in the presence of oxygen, whereas in general non-*Saccharomyces* wine yeasts are able to aerobically respire sugar, modulating the production of ethanol, glycerol and other by-products [28,47,54,55,56].

In this work, the effect of aeration on ethanol content, population dynamics and analytical profile of wines using free cells of *S. bombicola* /*S. cerevisiae* sequential inoculation were evaluated. *S. bombicola* strain used in this work, was investigated in a previous work in immobilized form and in anaerobic condition [32], determining an ethanol reduction of 1.6 % (*v/v*) using 10% (*w/w*) of beads corresponding to an inoculation level of 10^8 cells /mL. Here, a comparable result was obtained (1.46% *v/v*) but with a lower inoculum of free cells (5×10^7 cells/mL) and in presence of initial concentration of 10^4 cells/mL of WNS. Free cell inoculation is an easily procedure to apply at industrial level in winemaking sector in comparison to the use of immobilized cells that in the other side allows high inoculum level.

The ethanol reduction achieved in the present work could be, at least in part, explained by the relevant increase in glycerol as previously reported [38]. A similar result was obtained with *C. zemplinina* (synonym *Starmerella bacillaris*, a closely related species with similar oenological features of *S. bombicola*), that was widely investigated to produce wines with less ethanol levels and higher glycerol content [26].

On the other hand, these results confirmed that the oxygen addition decreased the ethanol production of *S. bombicola* cells highlighting an increase of growth and sugar utilization kinetics. However, different metabolic behaviour of various non-*Saccharomyces* species was exhibited with oxygen supplied, highlighting that it is not possible to delineate a general trend within non-*Saccharomyces* yeasts. [57].

S. bombicola in sequential fermentation confirmed the highest production of glycerol and succinic acid as previously reported [38]. Moreover, the results showed a positive role of oxygen on cell growth and development of *S. bombicola.* On the other hand, this significant enhancement of by-products together with respiration activity do not completely justify the ethanol reduction obtained and other fermentation products that were not evaluated in this investigation need to be explored.

One of the most negative features in mixed or sequential fermentation non-*Saccharomyces* /*S. cerevisiae* yeasts in aeration condition is the increase of acetic acid, compound responsible of sour and bitter taste [28,48,52,58]. In this study, *S. bombicola* in sequential fermentation both in anaerobic and aerobic condition limiting the air flow in the first 72 h (before the inoculum of *S. cerevisiae*) showed an acetic acid content very closed to that exhibited by *S.* cerevisiae indicating a positive interaction between the two yeast strains.

Conversely, ethyl butyrate and higher alcohols increased with oxygen supplementation. This trend could be related to the oxygen supplementation. Indeed, Valero et al. [59] and Shekhawat et al. [52] showed an increase in the concentration of esters and higher alcohols in aeration condition. The supplementation of oxygen revealed a correlation between alcohols content, the growth of non-*Saccharomyces* yeasts, and oxygen levels. However, it is not possible to define a general effect of oxygen on the volatile profile of the wine. Indeed, different factors such as yeast strains, fermentation conditions and grape variety concurrently may affect the aroma composition of wines [31,60,61].

In conclusion, the results obtained highlighted the ability of *S. bombicola* strain DiSVA 66, in sequential fermentation and under partial aeration conditions, to make wines with reduced alcohol content maintaining, at the same time, an effective analytical profile. Obviously, it is necessary to set up the modalities of its use in function of the physiological and fermentation characteristics of the non-*Saccharomyces* specie/strain.

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