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Evaluation of microbiome, selection and improvement of indigenous yeast strains of typical grape varieties of Marche region

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Contents

Abstract

Vineyards and grape berry surfaces are characterized by a complex community of microorganisms, such as yeasts, bacteria and filamentous fungi, which play critical roles in grape and wine quality. Moreover, the unique composition of this microbial community can confers distinct regional and desired characteristics to the final aromatic complexity of wines. The microbiota complexity associated to grape and must can occur in fluctuations that depend on different factors, such as climatic conditions, the vineyard's geographic location, the grape's variety and integrity, viticultural practices, insect pests and vinification technology. For this reason, it could be very important to evaluate the effect of the influencing factors on grape's microbiota complexity and then on the aromatic properties of the resulting wine. *Saccharomyces cerevisiae* represents the main species selected as a starter yeast for wine fermentation, but the massive use of domesticated yeasts led to the standardization of the analytical and sensory properties of wines. Numerous studies showed high DNA polymorphism differences between *S. cerevisiae* native strains in relationship with their geographical distribution, therefore the geographical imprinting of *S. cerevisiae* strains could be used to produce wines with specific aromatic complexity that reflect the viticultural area of production. The nature offers an immense diversity of wild yeasts but often they are not able to satisfy the selective and specific exigence of an efficient fermentation process. Improved yeasts with interesting oenological properties can be obtained through genetic improvement strategies such as hybridization. Hybridization is a process that occurs spontaneously in nature when adverse environmental conditions happen. Therefore, hybrid strains obtained in laboratory are not considered genetically modified organisms (GMO),

and can be easily used in the winemaking with the purpose of obtaining innovative wines.

In the first part of the thesis there have been carried out the analyses of the grapes' microbiota composition and its evolution during spontaneous fermentation, thorough viable cell counts and culture-dependent methods. The influence of two typical varieties that characterized the winemaking areas of Marche region (Italy), and different farming systems (organic and conventional vineyard's treatments and untreated grapes) were analysed. Moreover, the effects of farming system, on fungal community of samples from Montepulciano variety, has been evaluated through high-throughput next-generation sequencing (NGS) technology, with the aim to obtain information about the whole microbial composition picture. The results obtained were then compared with those obtained from culture-dependent methods. The data obtained suggest that the yeast community colonizing the grape berry surface was influenced by both grape varieties and farming treatments, which characterized the yeast biota of spontaneous must fermentation and then could influence the quality of the resulted wines. Moreover, the use of NGS technology highlighted the superiority application of metagenomic approaches to know the whole fungal biodiversity in the samples analyzed. In the meantime, the inability of the method to distinguish live and dead microorganisms, underline the importance of cultural-dependent approaches to follow the yeasts's dynamic during a fermentation process, necessary to evaluate their potential influence in winemaking.

The second part of the research focused on the oenological properties of two *S. cerevisiae* native yeasts isolated from Verdicchio and Pecorino grapes (native varieties of the Marche region). After genotypic characterization, microvinification

trials were carried out and the most important oenological parameters were evaluated. The results highlighted the genotypic and phenotypic peculiarity of the two indigenous *S. cerevisiae* strains that could be proposed as new starter yeasts to characterise the bouquet of wines coming from Verdicchio and Pecorino grapes, valorizing the viticultural area of production.

In the third part, a further enhance of selected native strains of *S. cerevisiae* with peculiar features (no H_2S, SO_2 and acetaldheyde production), specifically required for organic wines production, was carried out using yeast hybridization approach. Four improved new *S. cerevisiae* strains were obtained and after genetic stabilization, they were subjected to molecular fingerprinting to confirm their unicity when compared with parental strains and each other. Moreover, their oenological aptitudes ware evaluated in microvinifications trials using shyntetic grape juice and compared with those obtained by unstabilized strains, parental yeasts and two commercial *S. cerevisiae* strains commoly use in winemaking.

PART I

Study of grapes mycobiome and factors influencing it

1. Introduction

The first living cells to inhabit the Earth were entities such as bacteria, viruses, yeasts and molds, algae and protozoa. These microorganisms of microscopic size, all together represented a number that far exceeded all other living cells on this planet and they played important roles, sometimes desirable and sometimes not, in the living system. Some of these microorganisms are involved in many steps of food production and therefore it is important to know their morphological, physiological, biochemical and genetical characteristics (Ray and Bhunia, 2007).

Around 1658, Athanasius Kircher was the first to see, on microscope, minute living worms in milk and meat. In the middle of seventeenth century, Theodore Schwann and Hermann Helmholtz proposed that putrefaction and fermentation were due to the action of microorganisms (Ray and Bhunia, 2007) and at the end of the same century, Luis Pasteur showed that wine was obtained by fermentative yeasts (Barnett, 1998; Beck, 2000). In the same period, Hansen and co-workers described the importance of yeasts in brewing (Barnett et al., 2001). At the end of eighteenth century, Flegel (1977) described yeasts as unicellular microorganisms able to replicate by budding or binary fission and Oberwinkler (1987) placed the yeasts in a phylogenetic framework defining them as ascomycetous or basidiomycetous fungi.

On an industrial scale, the yeasts are well-known as microorganisms widely used in bread, beer, wine and other fermented foods and beverages (Querol & Fleet, 2006). Nowadays, the yeasts are largely used even in the production of different compounds such as biofuel, fine chemicals, pharmaceuticals, proteins and recombinant proteins (Madzak et al., 2004; Porro et al., 2011; Hou et al., 2012; García-Hernández et al., 2012).

1.1 The main yeast applications

Yeasts are a heterogeneous group of eukaryotic microorganisms belonging to Ascomycetes and Basidiomycetes phyla characterized by small genomes (10–20 Mbp), simple life cycles and easily cultivable. For these reasons they have become exceptional models for different purposes, such as molecular genetics and evolutionary genomics studies and biotechnology applications (Hittinger et al., 2015). They are commonly and widely used in fermented foods and beverages (Moreira et al., 2011; Arroyo-López et al., 2012; Binetti et al., 2013), vitamin synthesis and recombinant protein production (García-Hernandéz et al., 2012; Hatoum et al., 2012) as well as in many other areas of research.

Food and fermented beverage. Different yeast species are involved in foods and beverage productions as a part of their manufacturing (used as starter culture) or like contaminants (spoilage yeasts) (Lowes et al., 2000). Industrially, these yeasts are involved in the fermentation of common foodstuffs like bread, table olives, wine, beer, kefir and koumiss (Arroyo-Lopez et al., 2012; Moreira et al., 2011). They are also involved in cheese maturation (Binetti et al., 2013) and used like multifunctional microorganisms with probiotic or antimicrobial activity (Antunes & Aguiar, 2012; Perricone et al., 2014; Oro et al., 2018).

Biofuel. During the last decades, many governments from all over the world focused their attention on the ways that could bypass the many problems caused by the use of gasoline from petroleum, such as spikes in gasoline price and climate change. In particular, the attention has been focused on the use of plant-derived as sustainable sources of biofuel (Limayem and Ricke, 2012). Corn and sugarcane have become the main source of fermentable sugars that could be used to obtain bioethanol from

Saccharomyces cerevisiae yeast (Shapouri et al. 2006). Recently, some studies have been focusing their attention on the potential use of non-edible crops to obtain biofuel, with the purpose of reducing the negative impact on food and feed markets (Wyman, 2007; Kim et al., 2014).

Enzyme. Nowadays, many enzymes of industrial importance are produced by microorganisms. *Kluyveromyces marxianus* could be used to produce inulinase enzyme to obtain fructooligosaccharides (FOS) from sucrose as a new alternative sweetener characterized by low calories and with probiotic effects (Santos and Maugeri et al., 2007). Also, *Yarrowia lipolytica* could be used to obtain citric acid from inulin. Citric acid is widely used on industrial scale as additive in food and beverage (flavoring, acidifying, conservative), in chemical cosmetics, in pharmaceutical industry and other applications (Liu et al., 2010). *Kluyveromyces lactis* is commonly used to produce β-galattosidase (lactase) employees in the production of low-lactose milk for lactose-intolerant persons (Neri et al., 2008) while *Kluyveromyces wickerhamii*, *K. marxianus* and *Stephanoascus smithiae* are involved in pectinases production. This class of enzymes are widely used in food and beverage industries for their ability to improve the clarification processes of fruit juices, wine, tea, cocoa or coffee production (da Silva et al., 2005).

Bioconversions. There are several examples on the use of yeasts as bioconversor, indeed genistein (a natural compound with healty effects on animal cells) could be obtained by bioconversion of *Fructus sophorae* by *Aspergillus niger* and Yeast (Feng et al., 2015) or *Cryptococcus curvatus* could be involved to convert carbon sources in acetyl-CoA as basic unit of fatty acid biosynthesis (Fei et al., 2011). Other

yeast species could be used in different transformations, such as chetoriductions, oxidations, hydrolysis of esters and hydrogenation of steroids.

Aromatic compounds. Many perfume industries use natural aromatic compounds biosynthesized by microorganisms and plants to meet the increasing demand of consumers for biological products. For example, *Kluyveromyces marxianus* is used as a high-producer of 2-phenylethanol (rose aroma), 2-phenylethyl acetate (fruity floral aroma) and ethyl acetate (fruity aroma) (Figure 1) (Morrisey et al., 2015) while *Ambrosiozyma monospora* is used as a producer of monoterpene. Different flavour and fragrance compounds produced by yeasts are also largely used in order to improve food and beverage's quality. Andrade and co-workers (2017) described the potential use of *Torulaspora delbrueckii* and *Candida intermedia* in cheese production as higer-producers of 3-methyl-1-butanol, octanoic acid and ethyl decanoate that are important for the taste and flavor of cheeses. Furthermore, many non-*Saccharomyces* yeast species are involved in alcoholic fermentation in order to positively contribute to the organoleptic characteristics of fermented beverage (beer, wine, cider, etc.) (Combina et al., 2005; Xufre et al. 2006; Varela et al., 2009; Ciani and Comitini, 2011; Canonico et al., 2016; Saerens and Swiegers, 2016).

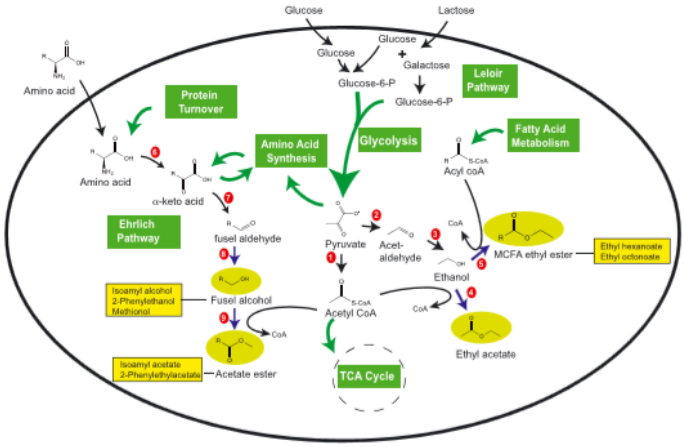

Figure 1. Metabolic pathways for synthesis of aroma and flavour molecules (Morrissey et al., 2015).

Cloning vectors. The first eukaryote cloning vector proposed was *Saccharomyces cerevisiae*. The discovery of the presence of the "2µm plasmid" in most of these yeast strains initially stimulated the proposal to use this yeast as cloning vector to obtain recombinant protein or to introduce particular characteristics or properties in a living organism (Brown, 2016). In biotechnology field, *S. cerevisiae* represent the most used yeast specie because it is not pathogenic for humans, its phenotypic and genotypic characteristics are well known, and it is easy to manipulate genetically (Krivoruchko et al., 2011; Jensen and Keasling, 2015; Krivoruchko and Nielsen, 2015). Nowadays, a lot of different high-value metabolites are produced through metabolic engineering of yeast strains such as acetyl-CoA, isoprenoids, aromatic amino acid-derived products, alkaloids, resveratrol, flavonoids, etc. This approach has become more important because it allows to bypass the problems given by the low presence of these metabolites in the natural host (usually not easily culturable in large-scale), complex procedures of extraction are required and often the chemical

synthesis of these compounds is difficult and commercially infeasible for their complex structures (Krivoruchko and Nielsen, 2015).

1.2 Yeast ecology and biodiversity in the natural environment

Yeasts are found on every continent and thanks to their different metabolic ability, they can colonize a lot of different ecological niches in atmospheric, aquatic and terrestrial environment (Hittinger et al., 2015). They belong to Fungi kingdom, together with molds, mushrooms, polypores, plant parasitic rusts and muts. Among these, important microorganisms like *Penicillium chrysogenum*, *Neurospora crassa*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* have become model organisms of numerous studies (Blackwell, 2011). The comprehension of yeast community composition in natural setting requires more studies and there must be taken into account different parameters including climatic conditions (such as temperature, pH, radiation), insect, plant, animal and human hosts, their metabolic activity and that of the other microorganisms that coexist in the same niche (Kurtzman et al., 2011; Lachance, 2013; Spencer and Spencer, 2013). The various yeast metabolic activities include their capacity to use different organic molecules as carbon source like the common fermentative sugars (e.g., fructose, glucose, rafinose, lactose, sucrose, maltose) and non-fermentable organic molecules (pentose sugars, sugar alcohols, amino sugars, organic acids, hydrocarbons and aromatic compounds) (Lahav et al., 2002). They are not able to fix nitrogen but can assimilate it from different sources and survive in a broad pH range. Yeasts were found at pH values that are difficult even for the survival of bacteria, but they can rarely grow up to

42°C. Tolerances to high osmotic pressure and salinity, antibiotics and active compounds are all descriptors that can help to understand why some yeasts can or cannot occupy a specific environment (Kurtzman et al., 2011). For these reasons, knowing all the intrinsic characteristics of yeasts (chemical, physical, and physiological) and their beneficial or competitive relationship with other organisms, can allow to describe their ability to exist and persist in a habitat and to define their fundamental niche (Hutchinson, 1959). For example, *Saccharomyces* spp. evolved the preference to use glucose through fermentative metabolism even in the presence of oxygen (Gojković et al., 2004), instead *Scheffersomyces*, *Spathaspora* and *Pichia* spp. evolved their ability to live in the guts of beetles and they are capable of fermenting xylose (Jeffries et al., 2007; Lobo et al., 2014). Most yeasts (quantitatively and qualitatively) are found in the terrestrial environment. In particular, exudates of leaves and tree trunks, flowers, fruits and soils represent a good habitat in which yeasts can found all the necessary nutrients and flourish. Nevertheless, the difficult study of the environmental habitat is due to the different conditions that occur, like different climates and chemicals compositions and the influence of the local plants and animals' activity that live inside them (Kurtzmann et al., 2011). Yeasts have been recovered not only from terrestrial environment, but also from atmospheric and aquatic ones; for example, Fuzzi and co-workers (1997) suggested that some *Rhodotorula* species can reproduce in fog. The presence of species belonging to *Rhodotorula* genus were founded even 200-400 m below the surface of the Baltic Sea (Ekendahl et al., 2003) as well as in Greenland's glaciers (Starmer et al., 2005). *Debaryomyces hansenii* represented the most common ascomycetous in marine water and *Metschnikowia* spp. was found like pathogenic to crustaceans and fish (Lachance et al., 1976; Chen et al., 2003; Moore and Strom,

2003). Earlier on 1987, Hagler and Ahearn, described an incredible yeasts density in the water: around 500cells/L for rivers, 100 for lakes, 10-50 for seawater and much more ($2.8x10^3$) for urban estuaries.

1.3 Yeast diversity in grape wine

Wine is a natural product obtained from alcoholic fermentation of grape must and the yeasts are the main microorganisms involved in this biochemical transformation. The yeasts action is influenced by certain events starting in a vineyard context, such as during agronomic practices management, grape ripening and harvesting and that goes on in the cellar during alcoholic fermentation and after bottling (Querol and Fleet, 2006). Generally, the yeast community associated to mature grape berry surfaces is very diversified and both the qualitative and quantitative changes depend on the ripeness of the grapes. In immature grape berries the yeast population ranged around $10\text{-}10^3$ CFU/ml and the predominant species were belonging to genus *Cryptococcus*, *Rhodotorula*, *Candida* and *Aureobasidium* (Fleet et al., 2002). These yeasts are also present in mature grapes, together with other predominant yeast species belonging to genus *Hanseniaspora*, *Pichia*, *Metschnikowia* and sometimes also *Saccharomyces*, *Torulaspora*, *Zygosaccharomyces*, *Kluyveromyces* and *Brettanomyces*. At the harvest time, the yeasts presence increases to $10^3\text{-}10^5$ CFU/ml (Fleet et al., 2002; Barata et al., 2008; Li et al., 2010; Milanović et al., 2013; Martins et al., 2014) until above 10^6 CFU/ml in damage grapes (Fleet et al., 2002). Regarding the main wine yeast *Saccharomyces cerevisiae*, numerous studies (Martini, 1993; Martini et al., 1996; Pretorius et al., 1999; Ciani et al., 2004) describe it as a yeast closely associated with cellars and fermentation plants. Indeed, it was usually detected on a low

concentration (less than 10-100 CFU/g) or absent in fresh grape must. During the biochemical transformation of grape juice in wine, most of non-*Saccharomyces* yeasts decline until disappearing. This happens due to the high selective conditions established by the fermentation process, such as anaerobic conditions, depletion of nutrients and the increasing levels of ethanol (Fleet, 2003; Querol and Fleet, 2006) produced by *Saccharomyces sensu stricto* group wine yeasts (Naumov et al., 2000). However, some non-*Saccharomyces* yeasts such as *Hanseniaspora*, *Candida*, *Pichia*, *Kluyveromyces* and *Metschnikowia* could survive/proliferate until mid-fermentation. Furthermore, when the wine fermentation is performed at 15–20˚C, *Hanseniaspora* and *Candida* species decrease their ethanol sensitivity and they can participate throughout the fermentation process and give their contribution to the wine's quality (Erten, 2002). In general, Kurtzman and Fell in 1998, described around 15 different yeast genera associated with grape/wine ecosystem: *Brettanomyces*/*Dekkera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*/*Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Saccharomycodes*, *Schizosaccharomyces* and *Zygosaccharomyces*.

1.4 Factors influencing yeasts community in grape wine

Yeasts are ubiquitous microorganisms that form part of the grapes' microbiota. The variety and proportion of each yeast species depend on the action of biotic and abiotic factors, such as geographic location and climatic conditions (temperature, radiation, nutrients, etc.), grape variety, viticultural practices (such as vineyard treatments, harvest technique) and the interaction with other organisms found in the same ecological niches (Pretorius et al.,1999; Fleet et al., 2002; Combina et al. 2005;

Li et al. 2010). Together, these factors exert different stress conditions on yeast life which respond differently at this stress: they can adapt or die. The basic knowledge about the relationship between yeast community composition and environment is important in order to be able to control, as much as possible, biotic and abiotic factors and consequently enhance the yeasts activity or, on the contrary, inhibit or stop them (Deak, 2006).

1.4.1 Abiotic factors influencing yeasts community

The abiotic factors include all those non-living aspects that define the characteristics of the surrounding environment and can influence, together with the biotic ones, the microbial composition of specific ecological niches.

Climatic conditions could be considered a consequence of the cumulative effect of temperature, rainfall, UV exposure, wind and sunlight. The minimum, optimum and maximum values of the yeast growth temperature cannot be expressed using absolute degrees values because the yeast sensitivity to the temperature depends on the physiological state of the cells, combined with the environmental conditions established. Generally, yeasts are mesophilic, with a grow optimum range between 20 and 30°C (Deak, 2006), with obvious exceptions. Yanagida and co-workers (1992) founded a greater frequency of *Kloeckera apiculata* in mild climates and Rementeria et al. (2003) described the same behavior, regarding *K. apiculata*, in warmer climates for yeast associated with white grape variety. The main yeast specie used in fermentation processes, *Saccharomyces cerevisiae*, can grow until 37°C but there are strains of *Kluyveromyces marxianus* able to grow and ferment even at 52°C

(Banat and Marchant 1995). Another important factor that could influence yeast community on grapes is the effect of the rainfall. Several studies have detected an increase in the total yeasts count during the years with high rainfall (Combina et al., 2005; Čadež et al., 2010). These data are not in agreement with what was founded by Comnitini and Ciani (2006). Concerning the UV exposure, there are few observations referring to its effect on yeast grow but the relative abundance of *Cryptococcus* and *Rhodotorula* species usually founded on leaves could be explained by the presence of pigment in these cells (Deak, 2006).

Grape variety and vineyard characteristics like age, size, vintage year and geographical location are among the most influencing factors of the grape yeast community (Martini et al., 1980; Rosini et al., 1982; Pretorius et al., 1999; Barata et al., 2012). Indeed, grapes are considered as a primary source of natural yeasts in wine and, as previously described, species belonging to genera *Candida*, *Hanseniaspora*, *Hansenula*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces* are known to be commonly present on the grape berries surface (Chavan et al., 2009; Francesca et al., 2010; Li et al., 2010). Concerning fermentative species of *Saccharomyces* (e.g. *S. cerevisiae*), several studies described the absence or a low amount of this yeast on healthy and undamaged grapes (Martini 1993; Pretorius 2000), while Mortimer and Polsinelli (1999) proposed that damaged berries could be the natural depositories of *S. cerevisiae*. Sabate and co-workers (2002), studying the yeast population associated to two spain red wine grapes vines, Carinyena and Garnacha, founded a higher presence of basidiomycetous in the second grape variety, while in the first one *Hanseniaspora uvarum* and *Candida zeylanoides* were described as the predominant species. Several

studies also described how the yeast population diversity varied with the vintage year (De La Torre et al., 1999; Sabate et al., 2002; Valer et al., 2007). On the contrary, some authors concluded that the grape variety did not influence microbiome composition (Poulard et al., 1981; Guerzoni and Marchetti, 1987). Furthermore, grape barriers colonization should be driven by vector dissemination and nutrient availability. Under this view it is difficult to clearly understand the precise influence of these factors on grape/grapevine microbiota.

Agronomic practices. To ensure wines that can be appreciated by consumers, winemakers must necessarily take care of all steps, from vineyard to winery, involved in winemaking. The common farming practices used in vine could be divided in two macro-groups, organic and conventional treatments. The organic farming guidelines have been defined at European level by the European Council (EC) Regulation No 834/2007 and No 889/2008 and the International Federation of Organic Agriculture Movement (IFOAM) defines organic agriculture as a "holistic production management system which promotes and enhances agro-ecosystem health, including biodiversity, biological cycles, and soil biological activity. It emphasizes the use of management practices in preference to the use of off-farm inputs, taking into account that regional conditions require locally adapted systems" (IFOAM 2005; Trioli and Hofmann 2009). Regarding the conventional treatments, they make use of common chemical plant protection products. In the last few years, several studies described the important effects of viticulture practices on wine yeast population. Cordero-Bueso and co-workers (2011), concluded that organic farming system leads to higher biodiversity about *Saccharomyces* and non-*Saccharomyces* yeasts in accordance with what was found by Viviani-Nauer et al. (1995) that

described a decrease yeast population and diversity in fermenting musts obtained from grapes treated with pesticides. In the same way, Tofalo et al. (2011) found a high non-*Saccharomyces* yeasts biodiversity in organic musts. Differently, some authors, described any effect of vineyard treatments on fermentation performance of *S. cerevisiae* (Cabras et al., 1999) or its relative abundance (Ganga and Martinez, 2004). Further, Oliva et al. (2007) described any negative effect on yeast enumeration in grapes treated with fungicides in the same day of the harvest.

1.4.2 Biotic factors influencing yeasts community

In any ecosystem populated by microorganisms, it is inevitable that direct or indirect interactions will occur between them (Boddy and Wimpenny, 1992). Yeasts can establish interactions with themselves, filamentous fungi, bacteria and higher organisms, furthermore the resulting effects of this "socialization", that can be detrimental, neutral or beneficial, stabilize the population that cohabit in a specific ecological niche (Odum, 1953; Challinor and Rose, 1954; Bull and Slater, 1982; Lachance and Starmer, 1998).

Yeast-yeast interactions. The interactions between yeasts (mutualism, commensalism, amensalism and predation) are not consistently studied but they generally regard the competition for nutrients to survive (Starmer et al. 1987; Nissen et al. 2004). The most important model of antagonistic interaction regards the capacity of yeasts to secrete proteins (killer toxins) lethal to a variety of susceptible yeasts (Young 1987; Walker et al., 1995; Marquina et al. 2002). About 9–27% of yeast species in natural communities were described as able to produce killer toxins. For example, the zygocin secreted by *Zygosaccharomyces bailii* can kill pathogenic

yeasts like *Candida albicans*, *Candida glabrata* and *Candida krusei* (Weiler and Schmitt, 2003). Often, the killer toxins facilitate the competitive interaction between yeasts during the spontaneous fermentation of grape juice, where the development of the main indigenous yeasts associated with grapes and wine, like *Hanseniaspora*, *Metschnikowia*, *Pichia, Candida* and *Saccharomyces* species, has been attributed to their level of ethanol tolerance (Fleet and Heard, 1993). Lachance and Pang (1997) described the predation phenomena in *Saccharomycopsis* species like another direct interaction between yeasts but the ecological impact of this one remains to be assessed.

Yeast-mold interaction. Different interactions occur between yeast and molds, for example, yeasts can take advantage of the molds' metabolisms leading to relies compounds, such as simple sugar or inorganic molecules, necessary for yeasts growth (Than et al., 2002), while some yeast species can be used as biocontrol agents against molds. *Pichia guilliermondii* and *Pichia anomala* can inhibit the growth of specific moulds responsible of fruit damage during postharvest storage (Björnberg and Schnürer, 1993; Suzzi et al., 1995; Druvefors et al., 2005). On the contrary, species of mycelial fungi can attack yeasts using them as a nutrient source. Fracchia and co-workers (2003) showed the possible use of yeasts exudates to stimulate hyphal growth of *Rhodotorula mucilaginosa*.

Yeast-bacteria interaction. In fermented foods and beverages there are a lot of examples of synergistic interactions between yeasts and bacteria to enhance aromatic characteristic of the final products. In the fermentation of sauerkrauts and pickles, yeasts and lactic acid bacteria live together (Buckenhüskes, 1997) as well as in red wine, the malolactic fermentation by *Oenococcus oenos* is facilitated by vitamins and

amino acids produced by yeasts (Alexandre et al., 2004). In oriental food fermentations and in dairy products (such as ripening of sausages and cheeses), several communities of yeast, molds and bacteria are involved in the productive process (Nout, 2003; Viljoen, 2001). Special interactions can be observed in biofilms development, which represent a strategy to facilitate the colonization of niches and offer protection to environmental stress. Bacteria are responsible of the most biofilm biosynthesis, but the yeasts also contribute by producing necessary molecules of adhesion (Watnick and Kolter, 2000; El-Azizi et al., 2004). Bacteriocins can be produced by lactic acid bacteria but it seems this does not affect the yeasts' life (Magnusson et al., 2003).

Yeasts-higher organisms interaction. Insects and birds play an important role in the ecological yeasts' transmission and distribution, they can transport yeasts from one side to the other of plants, flowers and fruits, including grapes and vineyards and therefore they contribute to the yeasts' community structure. Several studies reveal that the relationship between yeasts and bees, flies and birds is not random, but a coadaptation of the partners is necessary (Starmer and Fogelman, 1986; Starmer et al., 1991; Rosa et al., 2003; Lachance et al., 2003). Francesca et al. (2010, 2012) described the role of birds on the dissemination of oenological yeasts in vineyards, suggesting migratory birds as vectors of *Saccharomyces cerevisiae* cells. The latter represents the most important oenological yeast, whose origin is quite hard to retrieve (Mortimer, 2000). Depending on the vector, yeasts can be transported at different distances, indeed Goddard et al. (2010) highlighted that insects such as honey bees can disseminate *S. cerevisiae* strains at a distance of about 10 Km.

1.5 Methods of yeast community identification

Most of the yeasts are more or less ubiquitous while others seem to be restricted to specific ecological niches. Currently, Microbiology proposes different methods for yeasts identification, one of these involves the traditional techniques based on cultural dependent methods in which they are usually required specific enrichments media, many laborious and long identification tests based on biochemical, physiological and morphological yeast characteristics. Furthermore, the reproducibility of these techniques often depends on the physiological state of the studied cells. Recently, there have been developed innovative molecular biology techniques to study the dynamics of the microbial population based on non-cultural dependent methods that are faster, more accurate, with a high reproducibility and by-pass the limits related to traditional cultural dependent methods.

1.5.1 Culture-dependent methods

Yeasts share with molds and bacteria their natural ecological niches, therefore enrichment techniques are often required for their recovery in order to permit the development of yeasts and in the meantime suppress molds and bacteria growth. Generally, the media used for yeasts culture and isolation are made of different nutritional compounds such as sugars, as a source of energy (e.g. glucose, fructose, sucrose), proteins, digested and used as a source of nitrogen (e.g. peptone, tryptone, casitone), additional complexes, such as yeast extract, malt extract, glycerol, etc, for special growth requirements. Indeed, several growth substrates have been discovered for exigent yeasts, such as, psychrophils, resistant acids and osmophiles, through the

choice of suitable formulations and favorable incubation conditions. Lourerio and Malfeito-Ferreria (2003) identified copper sulfate as the best compound discriminating between wild yeasts (including wild *S. cerevisiae*) and fermenting yeasts in beer. In addition, they may contain one or more antibiotics against bacteria (e.g. oxytetracicline, chloramphenicol), compounds that inhibit the rapid expansion of molds (e.g. rose bengal, sodium propate, biphenyl) and/or pH indicators (e.g. green of bromocresol, bromophenol blue) (Kreger-van Rij, 2013). The cultivation of yeast is necessary to obtain pure cultures before proceeding with their identification through the analysis concerning morphological, sexual, asexual and physiological properties of the strains.

Morphological characteristics. The comparison between the various colony and cells morphologies represent the easiest method that allows to distinguish the isolates. The colonies that show the similar morphotype can be grouped together after meticulous observations of their shape, size, color and texture. The shape can be circular, irregular or filamentous; the margins can be smooth or with small or large lobes; the elevation can be flat, immersed in the agar, concentric circular, convex or with a raised area in the center; the weaving can be mucous, fluid, viscous, friable or membranous. Mucosal growth is frequently associated with the production of extracellular polysaccharides, while the membranous development results from abundant filamentous formations (Kurtzman and Fell, 2000). Most colonies are colorless, while others are colored which can be useful to help the discrimination between species. Regarding the cellular morphology, the cells may be spheroidal (e.g *S. cerevisiae*), subglobose, ellipsoidal, ovoid, obovoid, elongate, filamentous, apiculate (e.g *Hanseniaspora uvarum*), botuliform, lunate (e.g *Metschnikowia*

lunata) or triangular (*Trigonopsis variabilis*) (Kurtzman and Fell, 2000; Kreger-van Rij, 2013). As for the size, the cells and the colonies could be simply described as small, medium and large.

The morphology of the colonies, as well as the cellular one, is highly dependent on the growth substrates. Thus, groups of isolates showing similar morphology should be traced to colonies of the same development stage and from the same culture medium (Kreger-van Rij, 2013).

Sexual and asexual characteristics. Yeasts could be characterized by sexual and asexual reproduction and studying these characteristics could facilitate the yeasts identification process. Regarding asexual reproduction, this can appear by budding, fission or by a mix of both actions. During the budding, on the surface of the mother cell it appears a small evagination (bud or blastospore) that increases in size becoming a new cell that separates itself from the parental cell. Budding can occur involving all layers of the mother cell (holoblastic budding) or from a rupture in the mother cell wall where an innermost layer evaginates forming a new cell (eteroblastic budding). In the first budding type, no further buds can originate from the same cell surface site, contrary to eteroblastic type. Holoblastic budding has been described as a characteristic of *Saccharomycetales* (Von Arx, 1981) and eteroblastic ones as characteristics of basidiomycetous yeasts. In terms of cell surface formation location, budding can be monopolar, bipolar or multilateral (Figure 2).

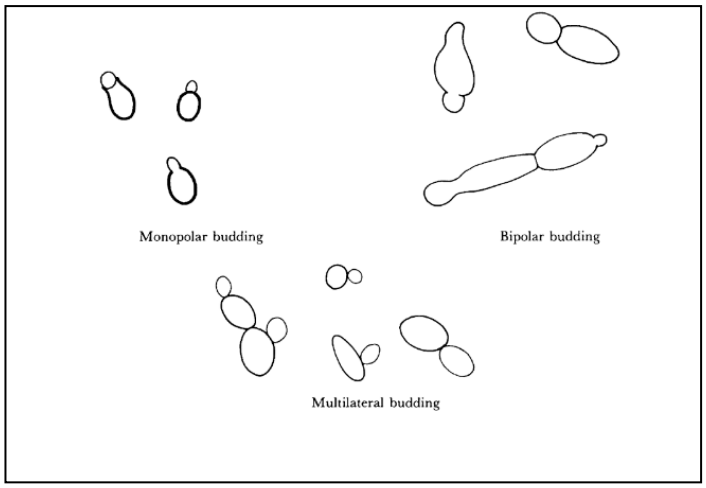

Figure 2. Monopolar, bipolar or multilateral yeast budding (Kreger-van Rij, 2013)

The typical *Schizosaccharomyces* genus vegetative reproduction is by fission, with the parental cell dividing itself in two new cells (arthrospores) after the formation of a transverse septum long axis of the cell (Figure 3a). Scars or annellations can remain on the surface of the young cells (Streiblová, 1971). Another relatively rare vegetative reproduction mode (typical of *Sterigmatomyces* genus) is through the formation of one or more tubular protuberances on mother cell surface, which originates at the ends new cells that separate themelves after maturation (Figure 3b) (Kreger-van Rij, 2013).

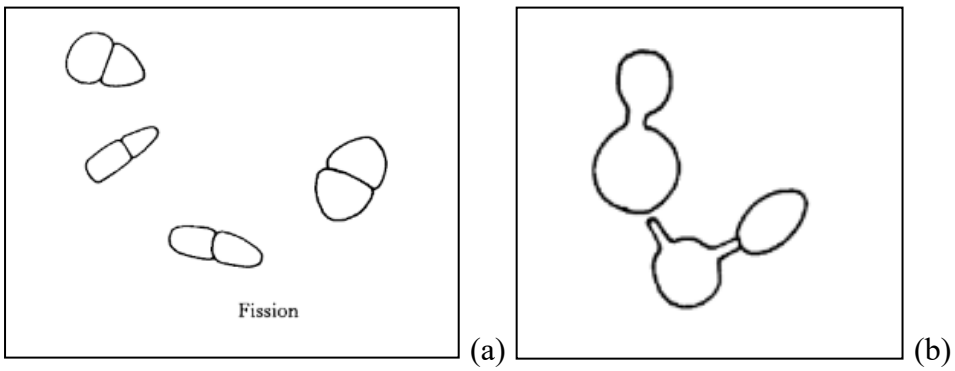

Figure 3. a) Yeast fission reproduction; b) vegetative yeast reproduction by tubular protuberances formation (Kreger-van Rij, 2013)

Concerning sexual reproduction, diploid or polyploid yeasts can obtain haploid spores by meiotic division. The ascus is the site where the spores, generally four, are formed in the ascomycetous yeasts while in the basidiomycetous yeasts, the spores' formation, numerically less than four, is delimited to the basidium. Yeasts that are characterized by sexual reproduction are called perfect yeasts, on the contrary, imperfect yeasts lack sexual stages. Haploid spores can be of two sexual types (mating types) called MATa and MATα and are able to mate with another haploid cell of opposite sex generating a stable diploid cell a/α (Michaelis and Herskowitz, 1988; Herskowitz, 1988). A yeast strain can be defined as heterotallic when the resulting spores have a definite sexual type and the diploid progeny is formed only after the coupling of the two spores with the opposite MAT, or homotallic when the resulting spores have both mating types and therefore are called self-diploidizing (Romano, 2005; Kreger-van Rij, 2013). Sexual agglutination could be associated with heterothallism (e.g. *Saccharomyces kluyveri*) and within the same species, different strains can be homothallic or eterothallic (such as *Pichia membranifaciens* and *Pichia spartinae*) (Wickerham, 1958).

Physiological characteristics. Yeast species and strains identification could be facilitated through the study of their physiological and biochemical properties. Usually, carbon and nitrogen utilization, fermentation properties, enzymatic activities, specific growth factor requirements, growth temperature and antibiotics susceptibility are investigated in order to better differentiate, characterize and describe yeasts. The result obtained is not unmistakable, indeed it strongly depend on the techniques employed and the yeast culture physiological state analyzed. Therefore, in order to conduct these trials, it is important for the yeasts to be in an

optimum nutritional state (Kurtzman and Fell, 2000; Kreger-van Rij, 2013). Yeasts belonging to genera *Saccharomyces*, *Zygosaccharomyces*, *Torulaspora*, *Kluyveromyces* are fermenting microorganisms able to use vigorously glucose, while yeasts belonging to genera *Hansenula* include strong fermentative and non-fermentative yeasts. *Lipomyces* and *Steringmatomyces* genera include exclusively non-fermentative yeasts. All the species belonging to *Hansenula*, *Citeromyces*, *Wickerhamiella*, *Pachysolen* genera are able to use nitrate, whilst *Pichia*, *Kluyveromyces*, *Debaryomyces* and *Saccharomyces* genera are not able to use them. Species that have the capacity of using nitrate are also capable of using nitrite, but this is not true on the contrary, in fact *Debaryomyces hansenii* and *Pichia pinus* can use nitrite but no nitrate. Regarding specific growth factor requirements, *Hanseniaspora* spp. and *Kloeckera* spp. need m-inositol and pantothenic acid, while *Metschnokowia* spp. and *Dekkera/Brettanomyces* spp. require biotin and thiamine. Most yeast species' optimal growth temperature is between 20 and 28°C. However, there are exceptions related to the natural habitat of the yeasts, as it happens for the species living in the polar sites, which grow well from 15 to 4°C, while some *Hansenula* and *Kluyveromyces* species grow well from 45 to 48°C (Kreger-van Rij, 2013).

Molecular analyses. Traditional microbial methods for yeasts identification are often subject to a personal interpretation of the results by the researcher, therefore these approaches, alone, are not adequate to obtain yeasts identification to the species level (Samelis et al., 1994; Coppola et al., 2000). In the last 20 years, the attention of many researchers was focused on the development of molecular methods for the classification of isolated strains. Genomic traits, such as ITS1-5.8S-ITS2, are a

common use for wine yeast differentiation to species level (Combina et al., 2005; Nisiotou and Nychas, 2007; Zott et al., 2008). The study of the polymorphisms in length of the restriction fragments (RFLP) of the rDNA-ITS regions represents an officially accepted method in the yeast taxonomy. This method involves PCR amplification of the ITS regions and analysis of the electrophoretic profiles obtained after digestion with restriction endonucleases. These regions show a low intraspecific polymorphism and a high interspecific variability, for this reason the RFLP of the 5.8S-ITS region is an excellent tool for yeast identification (Guillamon et al., 1998). This technique has been used for the classification of *Saccharomyces* species, *Kluyveromyces*, *Hanseniaspora* and *Candida*, as well as for the identification of vine yeasts (Esteve-Zarzoso et al., 1999; Caggia et al., 2001; Esteve-Zarzoso et al., 2001; Sabate et al., 2002; Heras-Vazquez et al., 2003; de Llanos Frutos et al., 2004; Arroyo-Lòpez et al., 2006).

1.5.2 Culture-independent methods

The traditional cultural-dependent methods applied in yeast classification have the disadvantage that they only reveal the cultivable microbes that generally represent less than 1% of the natural environmental microbes (Amann et al., 1995) Additionally, some stressful growth conditions can induce the yeasts in a peculiar metabolic condition where they appear viable-but-not-cultivable (VBNC), making their detection impossible (Millet and Lonvaud-Funel, 2000; Divol and Lonvaud-Funel, 2005). This way it is impossible to have a complete picture of ecological diversity in natural environments. For this reason, it has become crucial to have availability of molecular cultural-independent tools that allow to study the whole

microbial community (Giraffa and Neviani, 2001). Furthermore, these methods are characterized by greater speed, reproducibility and accuracy (Bokulich and Mills, 2012).

Fluorescence *in situ* hybridization (FISH). This technique allows to directly analyze microbial communities within their native environment. The method involves the use of fluorescence-labeled probes targeting specific DNA regions, usually rDNA. It is possible to analyze (detection and enumeration) different species at the same time using different specie-specific probes, each labeled with a specific fluorophore. The cells can be fixed *in situ* and the fluorescence can be observed by fluorescence microscopy or by flow cytometry if the cells are suspended in a fluid (Bottari et al., 2006).

Quantitative PCR (QPCR). New progresses in bioinformatics and molecular knowledge allowed the development of this technique applied in microbial ecology studies. This method uses the PCR technique principle, but all the process is monitored in real-time using fluorescent molecules. QPCR has been proposed for identification and quantification of microbial populations in food and beverage (Postollec et al., 2011). For example, some researchers used real-time PCR to detect lactic acid bacteria strains able to synthesize biogenic amine (Lucas et al., 2008; Arena et al., 2011) or exopolysaccharide (Ibarburu et al., 2010) in wine, otherwise it can be used to study the metabolic pathway involved in sulfite production in *S. cerevisiae*. Sulfites represents one of the major off-flavor produced in wine fermentations by yeasts (Mendes-Ferreira et al., 2010).

Denaturing gradient gel electrophoresis (DGGE). The technique separates the DNA fragments, obtained after specific PCR, exploiting a chemical gradient through

a polyacrylamide gel under electric current. The PCR amplicons are of the same size but of different sequences. The most commonly used target for the amplification is rDNA, which contains both highly conserved regions and variable regions within the species. After the electrophoretic run, the bands can be observed under UV light and, in order to determine the most dominant microorganisms present in a sample, it is necessary to extract each fragment, reamplifie and sequence it (Ercolini et al., 2004). Ampe and co-workers (1999) applied DGGE technologies to study bacterial communities in food, such as Cocolin and co-workers (2000) that used it in fungi identification during wine fermentation. Despite the advantageous technology, there are some disadvantages that make DGGE unsuitable for large-scale studies of microbial ecology because it is time-consuming and costly and does not provide reliable relative abundant information (Bokulich et al., 2012).

Terminal restriction fragment length polymorphism (TRFLP). Samples containing mixed microbial DNA must be amplified using universal primers labeled with fluorophore and the amplicons must be digested using specific restriction enzymes. The fragments are separated by capillary electrophoresis and the results are compared with the electrophoretic profiles present in database to obtain information about microbial population composition. Fluorescence intensity can be utilized to obtain pseudo-quantitative information (Marsh, 2005; Schutte et al., 2008). TRFLP was first developed to study bacterial communities and only subsequently it was used for studying food systems. Some researchers used this method to study microbial composition in food and fermented beverage such as cheese, yogurt, beer and wine (Rademaker et al., 2005; Rademaker et al., 2006; Sanchez et al., 2006; Marcobal et al., 2008; Bokulich et al., 2012a; Bokulich et al., 2012b; Bokulich et al., 2012c).

Despite the fact that TRFLP shows more advantages than DGGE, such as a low-tech, high-throughput method, rapidity and efficiency, it has been little used by microbiologists for microbial ecology studies.

Next-generation sequencing (NGS). This technology allows obtaining and analyzing sequences of DNA belonging to heterogeneous microbial community from different environmental sites. Before obtaining the sequences, it is necessary to amplify specie-specific DNA, usually 16S rRNA and ITS regions for prokaryotic and fungi respectively, using universal PCR primers. Pyrosequencing (Margulies et al., 2005) was the first commercially available NGS system, which can generate sequences of about 600 bp with an amount of coverage of about 10^6 reads per run. Some studies described the use of pyrosequencing to study bacteria or fungi communities in fermented foods (Kim et al., 2011; Koyanagi et al., 2011; Li et al., 2011; Jung et al., 2012; Kiyohara et al., 2012; Nam et al., 2012). Subsequently, Illumina sequencing platforms (Figure 4) represented an evolution of NGS technology (Bennet, 2004), which produce sequences of about 150 bp with an amount of coverage of about 10^9 reads per run and a greater multiplex capacity with lower cost per sample compared with pyrosequencing (Kuczynski et al., 2012). These Illumina advantages favored its application in microbial ecology studies, such as the observation of environmental microbial populations and their evolution (in diversity counts and/or functional activity) in relationship with climatic conditions or treatments.

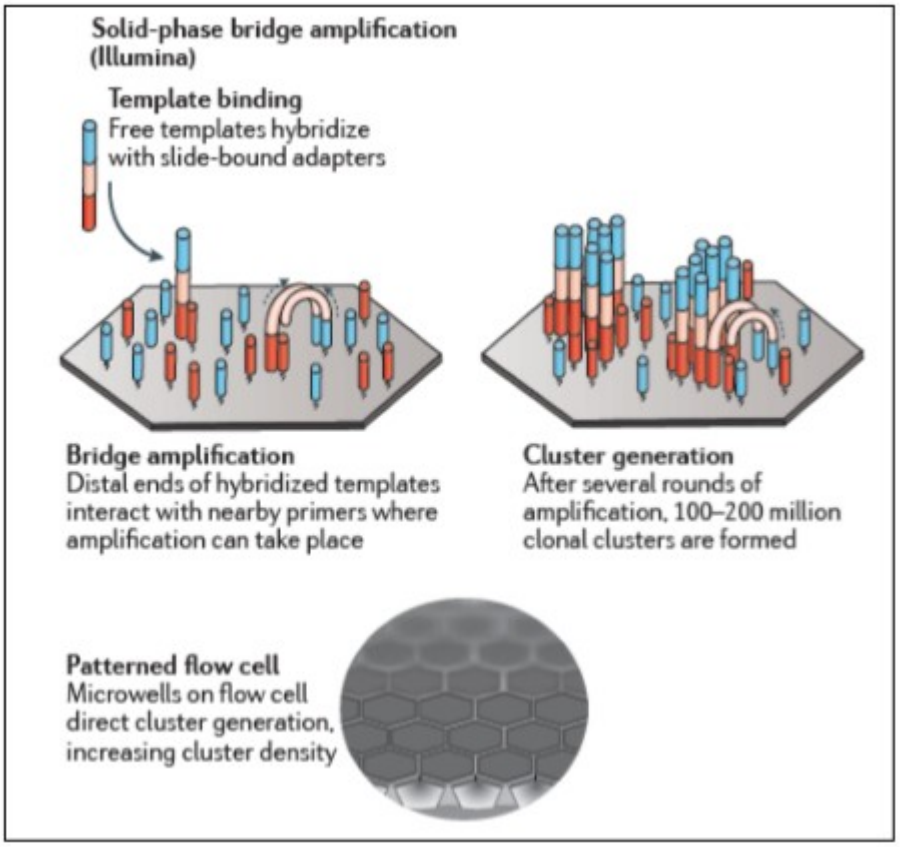

Figure 4. Illumina sequencing process, demonstrating bridge amplification and cluster generation (Goodwin et al., 2016).

2. Aim of the work

Nowadays, many wineries produce wine by spontaneous fermentations conducted by indigenous yeasts present on the grape surface and in the cellar. These yeasts are responsible not only for alcoholic fermentation, but they can also contribute positively or negatively to the aromatic properties of the resulting wine (Setati et al., 2012). For this reason, to know the species composition of these communities becomes of significant importance to control the fermentation process and to predict the final quality of the wines.

The aim of the study was to analyze the effect of agronomic practices on yeast population associated with grape berries at the harvest time and evaluate the dynamic evolution of the yeasts' community during a spontaneous fermentation process. The analyzed grapes, coming from two different vineyards, Verdicchio and Montepulciano, typical varieties of Marche region (central Italy), are subject of two different agronomic managements: Organic and Conventional. The first one includes the use of sulfur and copper as regulated by the European Council (EC) with the Regulation No 834/2007 and No 889/2008 and the International Federation of Organic Agriculture Movement (IFOAM); the second one encloses the use of the common agrochemical products. The data were compared with that obtained by non-treated grapes.

The first aim was to analyze the quantitative and qualitative evaluation of yeast population present on Verdicchio and Montepulciano grapes at the harvest time and during the evolution of the spontaneous fermentations. The analyses were conducted using cultural-dependent methods evaluating the combination effect of grape variety

and vineyard treatments on grape microbiome composition and yeast species dynamic during spontaneous grape juice fermentation.

Another purpose was to extend the evaluation of yeast community using cultural-independent methods to bypass the limit related to the use of cultural-dependent methods for yeast detection and to obtain information about the whole microbial composition picture of grapes at the harvest time and their evolution during a spontaneous fermentation (culturable and non-culturable species). In this context, samples from Montepulciano variety were treated by Next Generation Sequencing (NGS) technology using Illumina sequencing platform and amplifying specie-specific ITS regions. Subsequently, NGS results were then compared with cultural-dependent methods.

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Article: **“The impact of fungicide treatments on yeast biota of Verdicchio and Montepulciano grape varieties”**

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The impact of fungicide treatments on yeast biota of Verdicchio and Montepulciano grape varieties.

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Running title: Variety and fungicide effects on grape yeast

Abstract

Yeast species that colonize the surface of grape berries at harvest time play an important role during the winemaking process. In this study, the use of culturable microbial techniques permitted a quantitative and qualitative inventory of the different yeast species present on the grape berry surfaces of Montepulciano and Verdicchio varieties when treated with conventional and organic fungicides.

The results show that the most widespread yeast species at harvest time were *Aureobasidium pullulans* and *Hanseniaspora uvarum*, which are considered normal resident species and independent of the grape varieties and treatments applied. Specific differences when comparing the grape varieties were observed in species and were detected at a lower frequency; *Pichia* spp. were prevalent in Verdicchio, whereas *Lachancea thermotolerans* and *Zygoascus meyerae* were found in Montepulciano.

In both vineyards, the farming treatments improved the competitiveness of *A. pullulans*, which was probably due to its reduced susceptibility to treatments that improved the competition toward other fungi. In contrast, the fermenting yeast *H. uvarum* was negatively affected by fungicide treatments and showed a reduced presence if compared with untreated grapes.

Organic treatments directly impacted the occurrence of *Issachenkia terricola* in Montepulciano grapes and *Debaryomyces hansenii* and *Pichia membranifaciens* in Verdicchio. Conversely, a negative effect of organic treatments was found toward *Metschnikowia pulcherrima* and *Starmerella bacillaris*.

Overall, the data suggest that the yeast community colonizing the grape berry surface was influenced by both grape variety and farming treatments, which characterized the yeast biota of spontaneous must fermentation.

Keywords: yeast diversity, organic vineyard, conventional vineyard, grape berry, spontaneous fermentation

Introduction

Grapes represent a complex ecological niche where filamentous fungi, yeasts and bacteria cohabitate. The microbiome includes species at a concentration that mainly depends on the grape ripening stage and the availability of nutrients. However, the microbial communities of grapes may be affected by many other variables, such as pedoclimatic factors, viticultural practices, diseases and pests that could modify grape integrity [1]. In general, the yeast populations of mature grapes are comprised by 10^3 and 10^5 cells/g, but higher values (approximately one log) have also been found on damaged berries where the availability of sugar and nutrients is higher [2].

Among biotic factors, microbial vectors, such as bees and wasps, can actively transfer yeasts to the grape surfaces [3-5] where it can established synergistic or antagonistic behaviours between various genera and species of bacteria, yeasts and molds that cohabit together. The microbiome composition and complexity also depend on the interactions between individuals, and the resulting consortium is generally stable over time. Relative to abiotic factors, the climatic and microclimatic

conditions, including the effect of temperature, UV exposure, rainfall, sunlight and winds, can influence microbial populations. However, the results are often unclear because it is not easy to apply the scientific method to the function of natural events. For instance, rainy vintages lead to higher use of phytochemicals and show higher fungal proliferation and higher berry damage in conjunction with lower UV irradiation [6].

Concerning the total yeast counts, Combina et al. [7] found that rainy years increased yeast presence. This climatic condition probably increases the berry volume and permits the release of juice in joint areas, such as the part between the pedicel and the berry, and higher exosmosis leads to nutrients on the grape surface. With careful and sound berry sampling, Čadež et al. [8] found that colder harvests with higher rainfall lead to increased yeast counts. In contrast, Comitini and Ciani [9] found 10-fold less total counts in years with high rainfall. In addition, the geographic location, grape variety and vineyard age and size can influence the composition and occurrence of microflora that are present on the surface of grape berries.

Another important aspect is related to vineyard treatments. Viviani-Nauer et al. [10] found that pesticides decreased the yeast population and diversity in fermenting musts, whereas Cabras et al. [11] reported the absence of an effect on the fermentation activity of *Saccharomyces cerevisiae* by different fungicides and a stimulation of fermentation by *Kloeckera apiculata* was observed. Ganga and Martínez [12] detected less diversity of non-*Saccharomyce*s species in association with the systemic use of chemical fungicides against *Botrytis cinerea.*

Čadež et al. [8], with careful berry selection, showed that after the safety interval, fungicides against *B. cinerea* had a minor impact on the composition of grape berry microbiota and untreated grapes were less contaminated. Recent works

concerning the differences between organic and conventional farming systems concluded that organic farming leads to higher biodiversity both in *S. cerevisiae* and in non-*Saccharomyces* yeasts [13-16].

In this study, the yeast culturable biota of the grape surface of two Italian varieties was monitored at harvest time and during the spontaneous fermentations of grape samples when conducted under sterile conditions using conventional culture methods. The influence on the yeast community of conventional and organic treatments was also evaluated by comparing the samples with untreated grapes.

Materials and methods

Viticultural habitats and grape sampling

The grapes used in this study were obtained from two vineyards of two typical grape varieties of the Marche region, in the center of Italy: Verdicchio and Montepulciano. In particular, the Verdicchio vineyard is located in the Montecarotto locality (43°31'41''N, 13°03'59''E; 334 m altitude) within the *Denominazione di Origine Controllata* (D.O.C.), and the main climatic condition in September (sampling period) was 18.7 °C for air temperature, had 50.4% humidity and included 9 rainy days. Montepulciano vineyard is located in the Sirolo locality (43°31'20N, 13°36'53''E; 97 m altitude), and the main climatic condition in October (sampling period) was 14.9 °C for air temperature, had 82% humidity and included 15 rainy days. Both vineyards have employed three different management systems: organic,

conventional and with no treatment. To exclude any cross-contamination between different treatments, the minimum distance between each block of rows was approximately one kilometer from each other for all of the grapes; and within the same vineyard, the grapes are exposed to the same slope, sun, and shade and have similar soil characteristics. The harvest was carried out, in both varieties, at full ripeness (15 September for Verdicchio; 10 October for Montepulciano).

In the organic treatment (both varieties: Montepulciano and Verdicchio), a Bordeaux mixture (20 g/L of copper (II) sulfate + 13 g/L of calcium hydroxide with pH 6.6) and sulfur (Microthiol disperss, UPL EUROPE Ltd., Warrington WA3 6YN, Great Britain) were used. For both vineyards, 15 consecutive treatments were performed from April 20th, 2016, to August 17th, 2016.

In the conventional Verdicchio treatment, viticulture commonly included chemical compounds with fungicide activity and were employed as follows: copper-oxychloride (Coprantol WG, Sygenta Italia Spa, Casalmorano, Cremona, Italy) (1), sulfur (Tiovit jet, Sygenta Italia Spa, Casalmorano, Cremona, Italy) (1), cyclohexanol + 1,2- propanediol + abamectin + 2,6-di-terbutylp-cresol (Vertimec 1.9 ec, Sygenta Italia Spa, Casalmorano, Cremona, Italy) (1), iprovalicarb + technical copper oxychloride (Melody compact WG, Bayer Crop Science, Monheim am Rein, Germany) (1), sulfur (Selenium free) + terpene alcohols + sodium salt of an aromatic polymer (Heliosulfure S, Biogard, Cesena, Italy) (12), a Bordeaux mixture (11), coppery sulfur (1), and phosphorus pentoxide + potassium oxide (Landamine PK, BMS Micro-Nutrients N.V., Bornem, Belgium) (1). Twelve consecutive treatments were performed from April 18th, 2016, to August 12th, 2016.

In the conventional Montepulciano treatment, viticulture commonly included chemical compounds with fungicide activity and were employed as follows:

spiroxamina (Prospher300 CS, Bayer Crop Science, Monheim am Rein, Germany), copper-oxychloride (Coprantol, Sygenta Italia Spa, Casalmorano, Cremona, Italy), sulfur (Tiovit jet, Sygenta Italia Spa, Casalmorano, Cremona, Italy), fosetyl-Al+copper sulfate (R6 Erresei Bordeaux WG, Bayer Crop Science, Monheim am Rein, Germany), Metalaxyl-M14+ copper-oxychloride (RidomilGold, Sygenta Italia Spa, Casalmorano, Cremona, Italy), quinoxyfen+myclobutanil+coformulants (Arius System Plus, Dow AgroSciences, Indianapolis, Indiana, USA), copper sulfate and sulfur. Nine consecutive treatments were performed from March 10th, 2016, to July 17th, 2016.

During the harvest period, several grape samplings were performed using sterile plastic bags. Each sample consisted of an undamaged ripe grape bunch (approximately 1 kg per bunch). In total, 50 samples were collected: ten samples of organic and conventional Montepulciano grapes; five samples of non-treated Montepulciano grapes; thirteen samples of organic Verdicchio grapes; ten samples of conventional Verdicchio grapes and two samples of no-treated Verdicchio grapes. All of the samples were immediately transported to the laboratory on ice for processing.

Spontaneous fermentations

The grapes were placed into sterile bags and were hand-crushed and shaken at 120 rpm for 30 minutes on an MAXQ 4450 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). One milliliter of each fresh must was collected and used for yeast isolation and enumeration. The remaining grape juice with skins was

transferred into 250 mL sterile Erlenmeyer flasks, closed with Pasteur bungs (to allow CO_2 to escape from the system) and set up for spontaneous fermentation at 25 °C under static conditions. After 7 and 15 days from the start to the spontaneous fermentation, the samples were collected to evaluate the yeast population by viable cell counts.

Yeast enumeration and isolation

Samples from fresh musts and during fermentation were collected and used for monitoring the yeast populations at the beginning and after 7 and 15 days of fermentation. For total yeast enumeration, serial decimal dilutions in sterile water were prepared and then spread on Wallerstein (WL) nutrient agar (Merck KGaA, Germany) supplemented with 0.005% chloramphenicol (Thermo Fisher GmbH, Germany) and 0.02% biphenyl (Sigma-Aldrich, <Saint Louis, Missouri, USA) to suppress the bacteria and reduce the growth of molds, respectively. The plates were incubated at 25 °C for four days. After macro- and micro-morphological analysis and in proportion to their frequencies, the yeast isolation was conducted on plates that contained between 100 and 300 colonies. Approximately 10% of the colonies per plate were isolated on YPD agar (1% Yeast Extract, 2% Peptone, 2% D-glucose, and 2% Agar) from each sample and at each time of sampling [17,18]. The total isolates were 1,240. The yeast strains were preserved in 40% (v/v) glycerol at -80 °C.

DNA extraction and yeast identification

The 1,240 isolated strains, that showed identical macro- and micro-morphological characteristics, were grouped and representative isolates were used for genomic DNA analysis. DNA was extracted according to the method described by Stringini et al. [19]. Using primer set ITS1 (5'-TCCGTAGGTGAACCTCGCG-3') and ITS4 (5'-TCCTCCGCTTTATTGATATGC-3') [20], the ITS1-5.8S rRNA-ITS2 region was amplified by PCR. The PCR was performed as described by Esteve-Zarzoso and co-workers [21]. The PCR products were separated in 1.5% (w/v) agarose gel (stained with ethidium bromide) using 0.5x TBE buffer by horizontal electrophoresis (Bio-Rad, Hercules, USA). The identities of the representative yeasts were obtained by sequencing. The BLAST program [22] and the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) were used to compare the sequences provided with those already in the data library. The inclusion of obtained sequences into the NCBI GenBank data library has been completed under the accession numbers from MK351988 to MK352096.

Statistical analysis

The relative abundance of species was obtained by calculating the corresponding portion of each species with respect to the total yeast detected in the samples and based on the colony counts. The analysis of variance was conducted using the JMP 11 from SAS program. Furthermore, the results obtained from the

analysis of microbial diversity on the grape surface of different vineyards employing different agronomic practices and yeast dynamics during spontaneous fermentation were examined with Unscrambler 7.5 software (CAMO ASA, Oslo, Norway) to obtain the Principal component analysis (PCA).

Results

Effect of grape variety on the yeast community at harvest time

Microbial community associated with the grape surface of the Verdicchio and Montepulciano varieties was evaluated. From the general framework (Fig 1), it was observed that both grape varieties presented an abundance of yeasts, such as *Aureobasidium pullulans* and *Cryptococcus* spp., with oxidative metabolism. Together, these represent 60% and 40% of the total yeasts present on grape berries of Verdicchio and Montepulciano, respectively. In particular, out of the 60% associated with Verdicchio grapes, 50% belong to *A. pullulans* and 10% to *Cryprococcus* spp. The 40% of yeasts with oxidative metabolism associated with Montepulciano were 30% *A. pullulans*, 7% *Cryptococcus* spp. and 3% minor representative species. In terms of relative abundance and among the fermenting yeasts, *Hanseniaspora uvarum* (22% Verdicchio grapes and 43% Montepulciano grapes) and *Starmerella bacillaris* (13% Verdicchio grapes and 7% Montepulciano grapes) were the most

abundant and constantly present species found in both varieties. The Montepulciano variety was characterized by a consistent presence of *Issatchenkia terricola* (7%). Both grape varieties showed the presence of weak fermenting yeasts such as *Metschnikowia pulcherrima* and *Debaryomyces hansenii* (1.0-1.5%) and *Candida californica* (<1%). At low relative abundance (1.0-2.0%), some yeast species were found in one or the other grape varieties. *Pichia sporocuriosa*, *Pichia fermentans* and *Pichia membranifaciens* were found only in the Verdicchio grape variety, whereas species such as *Lachancea thermotolerans*, *Zygoascus meyerae* and *Rhodotorula* spp. were only found in grapes from the Montepulciano variety. In summary, no substantial differences were found among the two grape varieties regarding the main yeast species (oxidative and low fermenting species) that colonized the grape berry surface. A statistical significant difference was found for the relative abundance of *H. uvarum* more present on Montepulciano grape variety. This difference, in addition to the variety effect, could also due to other intrinsic variables of their cultivation management such as the different harvest time of grapes (Table 1 supplemental material). On the other hand, differences were detected in species found at a low frequency (often fermenting species) that were only isolated in one or another grape variety.

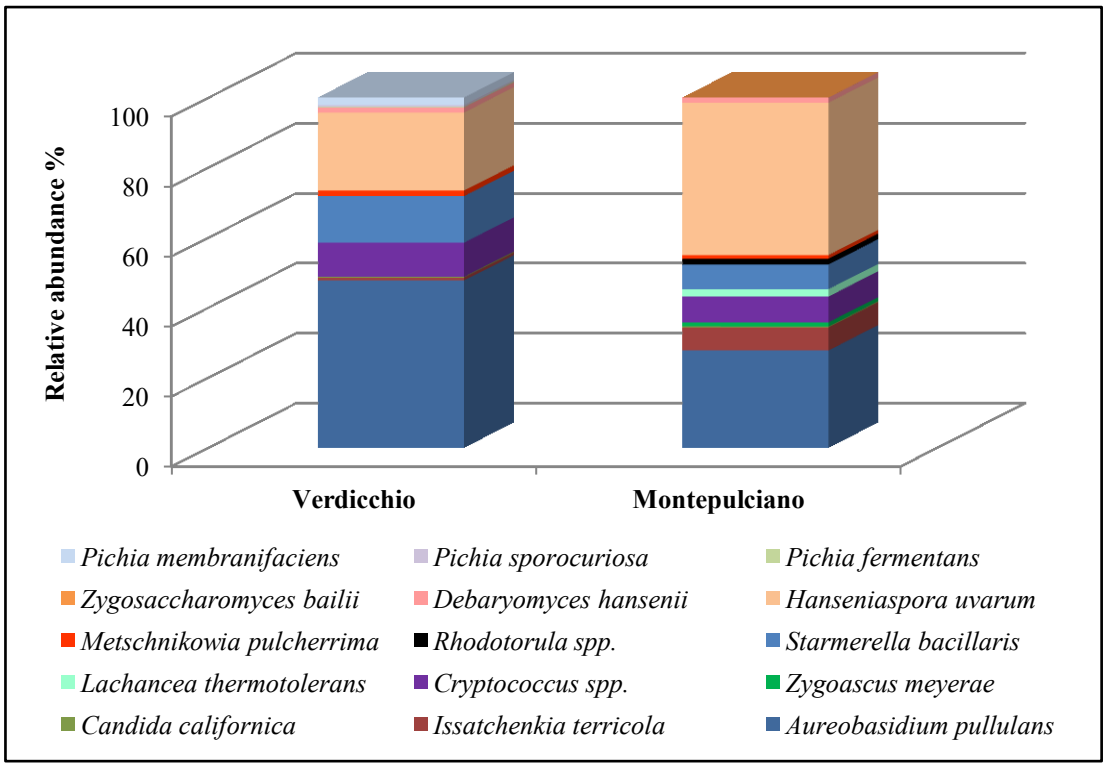


Fig 1. Mean values (%) of the initial yeast community in Verdicchio and Montepulciano grapes.

The influence of fungicide treatments on the yeast community at harvest time

The yeast community that colonizes grape surfaces was analyzed for the influence of the fungicide treatments. Both Verdicchio and Montepulciano varieties have been subjected to an organic and a conventional treatment. The results showed that in Verdicchio samples (Fig 2), the yeast-like *A. pullulans* was favorite by fungicide treatments in comparison to untreated samples (t = 0.05) probably due to the lower competition of the yeasts affected by treatments. Indeed, in both organic

and conventional samples, this yeast was the most abundant species (44% and 60%, respectively) while in untreated grapes it was only 5% of the whole yeast population. The same behavior was observed in Montepulciano samples (22%, 45% and 10% in organic, conventional and untreated samples, respectively) (Fig 2). Conventional treatments seem to exert more selective pressure on the yeast community and favor the colonization of *A. pullulans* (around half of the yeast community). Similar to *A. pullulans*, the occurrence of *Cryptococcus* spp. seemed to be positively influenced by the treatments (and this was absent in the untreated grapes). Different from *A. pullulans*, the organic treatments positively affected the colonization of *Cryptococcus* spp. in comparison with conventional treatments in both Verdicchio and Montepulciano varieties even if only in Verdicchio variety a significant difference was found (Table 2 supplemental materials) (16% and 3% in organic and conventional Verdicchio grapes, respectively, and 7% and 1% in organic and conventional Montepulciano grapes, respectively).

H. uvarum was the second most abundant species in the treated grapes of both varieties. This apiculate yeast did not seem to be influenced by the type of treatment (26% and 20% in organic and conventional Verdicchio grapes and 36% and 40% in organic and conventional Montepulciano grapes, respectively). In untreated grapes, *H. uvarum* was the most abundant species (47% and 75% in Verdicchio and Montepulciano varieties, respectively) and showed significant differences in comparison with both treated grapes (organic and conventional) but only in Montepulciano variety (Table 3 supplemental materials).

S. bacillaris species showed a wide variability among the treatments/varieties. In the Verdicchio variety, *S. bacillaris* decreased in the treated grapes (9% in organic samples and 16% in conventional samples) compared to the untreated ones

(29%) (Fig 2). In Montepulciano, *S. bacillaris* was more present in organic grapes (17%), absent in conventional samples and poorly present in untreated samples (4%).

Organic treatments favorably affected *I. terricola* and showed an abundance of 14% in organic grapes and only 5% in untreated grapes, and *I. terricola* was almost absent in conventional samples of the Montepulciano variety while it was present at a very low relative abundance (<1%) in the Verdicchio variety. A positive effect of organic treatments was also exerted on some low abundance species only present in the Verdicchio variety, such as *P. membranifaciens* (4%) and *P. sporocuriosa* (1%), that were only found in the grapes treated with copper and sulfur. Differently, a negative effect of organic treatments was shown toward *M. pulcherrima*. Indeed, this yeast species was significantly present in untreated (13 and 3% in Verdicchio and Montepulciano grapes, respectively) and poorly present or absent in treated grapes (Table 2 and 3 supplemental material). *D. hansenii* was detected only in treated samples, while *P. fermentans* and *C. californica* were generally found in untreated grapes (2.5% and 3%, respectively). *Rhodotorula* spp., a ubiquitous yeast, was present only after the treatments (1.67% and 2.57% in Montepulciano organic and conventional samples, respectively). Fermenting yeasts detected only in the Montepulciano grape variety showed a different response toward fungicide treatments. *L. thermotolerans* was only present in conventional treatment samples (5.5%), whereas *Z. meyerae* and *Zygosaccharomyces bailii* were mainly detected in untreated samples and almost absent in conventional grapes.

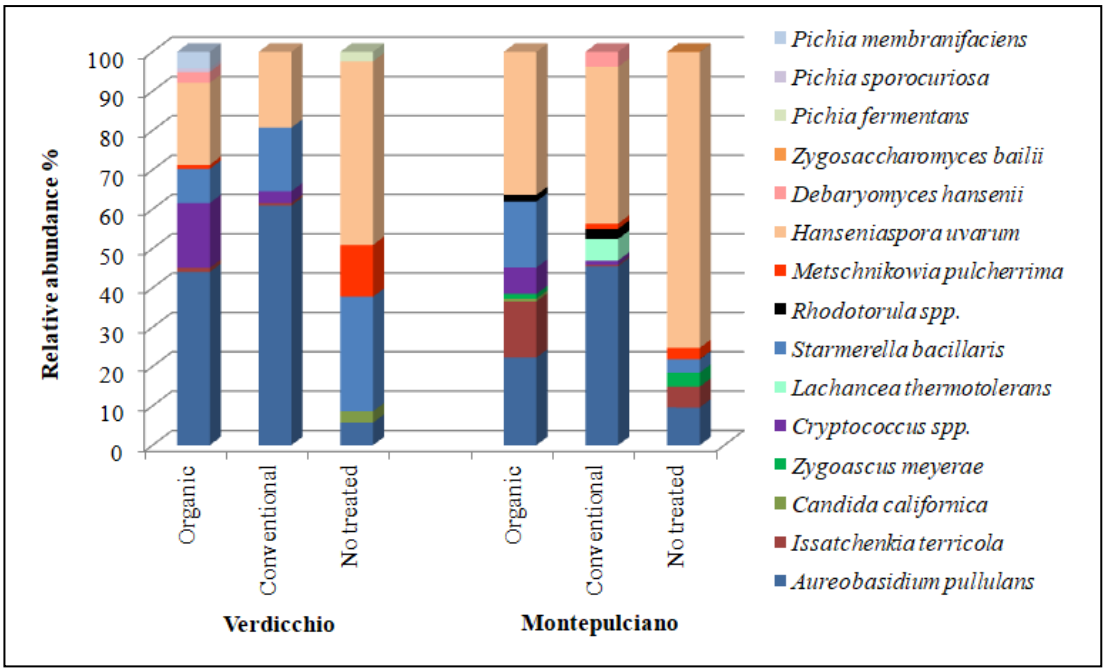


Fig 2. The average percentages of yeast species detected in organic, conventional and non-treated samples of Verdicchio and Montepulciano grapes at harvest time.

Yeasts dynamics during spontaneous fermentation

Middle fermentation

The microbial community evolution during spontaneous fermentation was monitored through viable counts after 7 and 15 days from the start of fermentation. After 7 days (approximately middle fermentation) the yeast population increased by approximately two log (from 8.0×10^5 CFU/ml to 6.0×10^7 CFU/ml) in Verdicchio samples and only one log in Montepulciano samples (from 1.7×10^6 CFU/ml to 1.5×10^7 CFU/ml) (Table 4 and 5 supplemental materials). As expected, the environmental conditions determined a selection in favor of fermenting yeasts.

Indeed, the oxidative yeasts *A. pullulans* and *Cryptococcus* spp. disappeared from all of the samples. *H. uvarum*, which was already well represented at the beginning of fermentation (22%), became the dominant species in all of the Verdicchio trials (Fig 3) and the only occasionally fermenting yeast species (*I. terricola*, *S. bacillaris*, *C. californica*, *M. pulcherrima*, *P. fermentans*, *Torulaspora delbreuckii*, and *Candida diversa*) were found at low relative abundance (all together at approximately 4%). This picture is nearly confirmed in Montepulciano treated samples where other yeast species participated in the fermentation process. In untreated samples, *H. uvarum* was present at only 4% of the total yeast population, but other fermenting species *S. bacillaris* (37%), *Z. bailii* (34%) and *C. californica* (26%) appeared. *S. bacillaris* and *C. californica* were present in all Montepulciano samples even if they were more abundant in untreated samples. Montepulciano samples can be differently recognized by a relevant presence of: i) *I. terricola* in organic samples, ii) *L. thermotolerans* in conventional samples, and iii) *Z. bailii* in untreated samples (Fig 3).

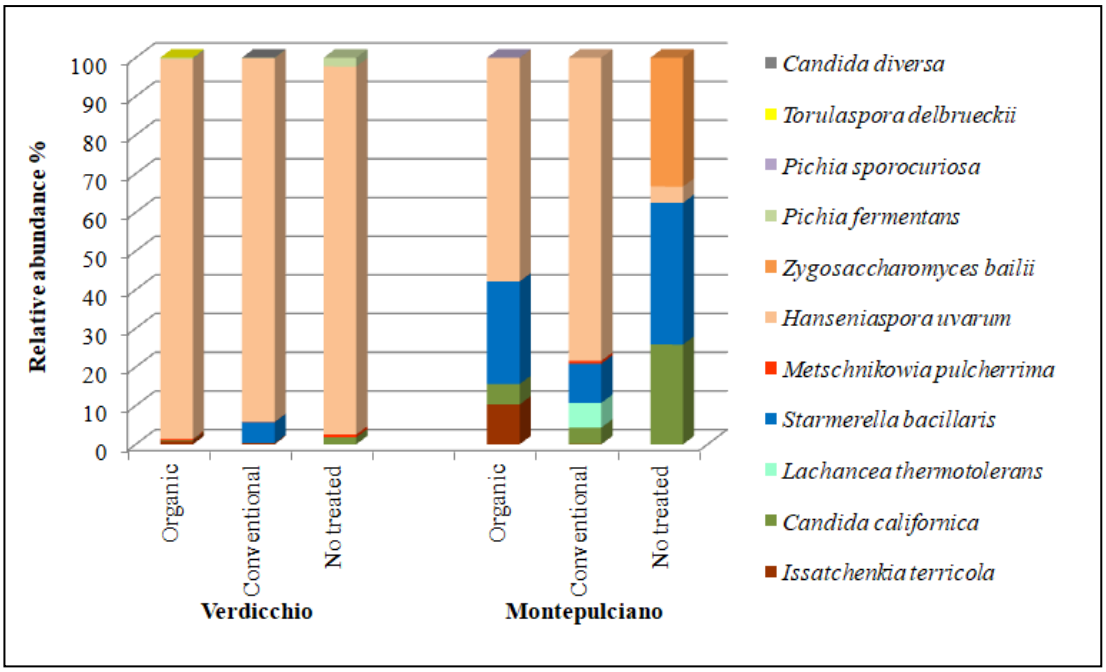


Fig 3. Average percentages of yeast species detected in organic, conventional and non-treated samples of Verdicchio and Montepulciano samples after 7 days of fermentation.

End of fermentation

The results of the microbiological analysis conducted after 15 days of spontaneous fermentation are shown in Fig 4. Due to the reduced size of each grape juice sample, the presence and participation of the fermentation process of the strongest fermenting yeast, *S. cerevisiae* and *T. delbrueckii*, were very limited (<1% and 3%, respectively, and only in Verdicchio conventional samples). *H. uvarum* remained the dominant species in organic and conventional Verdicchio samples (50% and 67.5%, respectively), while it showed a significant reduced presence in Montepulciano treated samples (7% in organic and 17% in conventional samples). In

organic samples, *C. californica* (33% and 21% in Verdicchio and Montepulciano samples, respectively) and *S. bacillaris* (10% and 44% in Verdicchio and Montepulciano samples, respectively) were the other dominant species. The other yeast species were *P. fermentans* (6%) in Verdicchio samples and *Z. bailii* (13%), *D. hansenii* (12%) and *I. terricola* (3%) in Montepulciano samples. In conventional samples other than *H. uvarum*, the species were *W. anomalus* (13%), *S. bacillaris* (10%) and *I. terricola* (5%) in Verdicchio varieties and *P. sporocuriosa* (32%), *C. californica* (12%), *L. thermotolerans* (11%), *D. hansenii* (11%), *Z. bailii* (11%) and *S. bacillaris* (5%) in Montepulciano samples. In untreated samples, *H. uvarum* was not found in either variety where the species was detected: *Pichia kudiavzevii* (52%) and *S. bacillaris* (48%) in the Verdicchio variety and *Z. bailii* (56%) and *C. californica* (44%) in the Montepulciano variety.

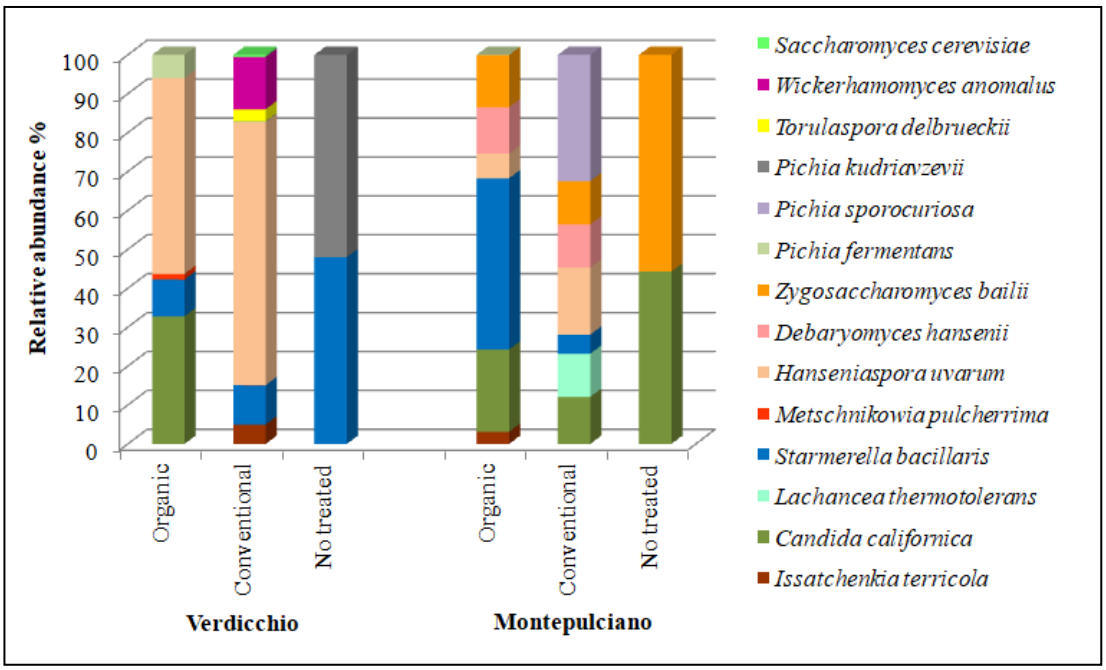


Fig 4. Average percentages of yeast species detected in organic, conventional and non-treated samples of Verdicchio and Montepulciano samples after 15 days of fermentation.

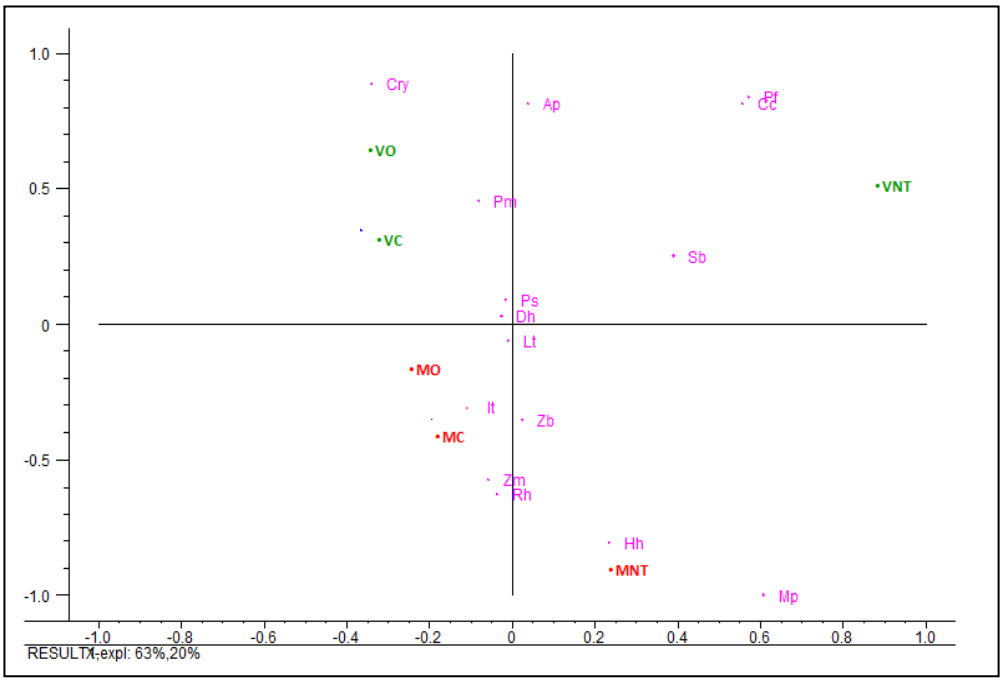
Principal component analysis (PCA) of the yeast community

The PCA of the overall yeast microbiome of the grape berry surface at the harvest period and during a spontaneous fermentation revealed a yeast population diversity among the samples of grapes subjected to organic and conventional fungicide practices and untreated samples coming from two different varieties/vineyards (Fig 5). The biplots were obtained by evaluating the relative yeast species abundance, the grape varieties and the fungicide treatments.

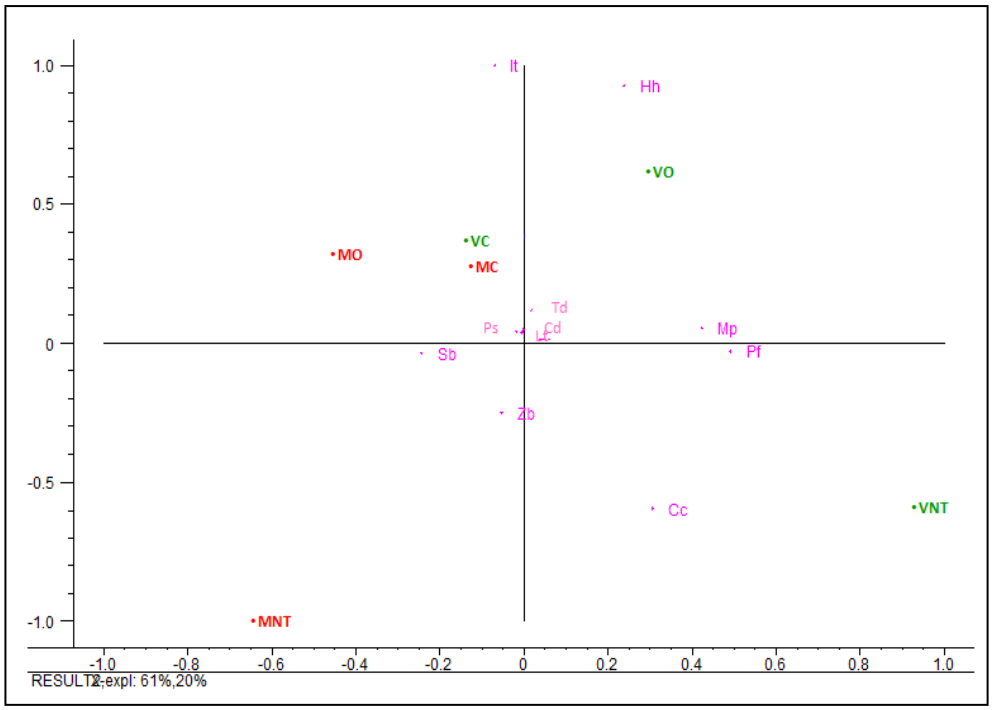
At the harvest time, PC1 (63%) showed a differentiation between untreated (right quadrants) and treated samples independently by the type of treatment while PC2 (20%) distinguished between Verdicchio (upper quadrants) and Montepulciano (lower quadrants) samples (Fig 5a). These data suggest an evident impact of fungicide treatments on yeast biota associated with the grape berry surface. Moreover, the grape varieties showed a different yeasts colonization, although different harvest times (with consequent different climatic conditions) and agronomic management could contribute to this yeast differentiation. In this regard, *M. pulcherrima* and *H. uvarum* in Montepulciano and *P. fermentans* and *C. californica* in Verdicchio varieties were the main characterizing species of untreated samples, whereas oxidative yeasts species mainly differentiated the treated samples. The spatial distribution of PCA confirmed that the species that linked the two grape varieties were: *Z. meyerae*, *I. terricola*, *Rhodotorula* spp., and *Z. bailii* for Montepulciano and *P. membranifaciens*, *P. sporocuriosa* and *P. fermentans* for Verdicchio.

At 7 days of fermentation (Fig 5b), a general reduction and simplification of the yeast community was observed. However, the untreated samples remained separated from the treated samples (down and upper quadrants, respectively). PC1 (61% of variance explained) distinguished Verdicchio samples from those of Montepulciano even if conventional samples were more closely related than organic ones.

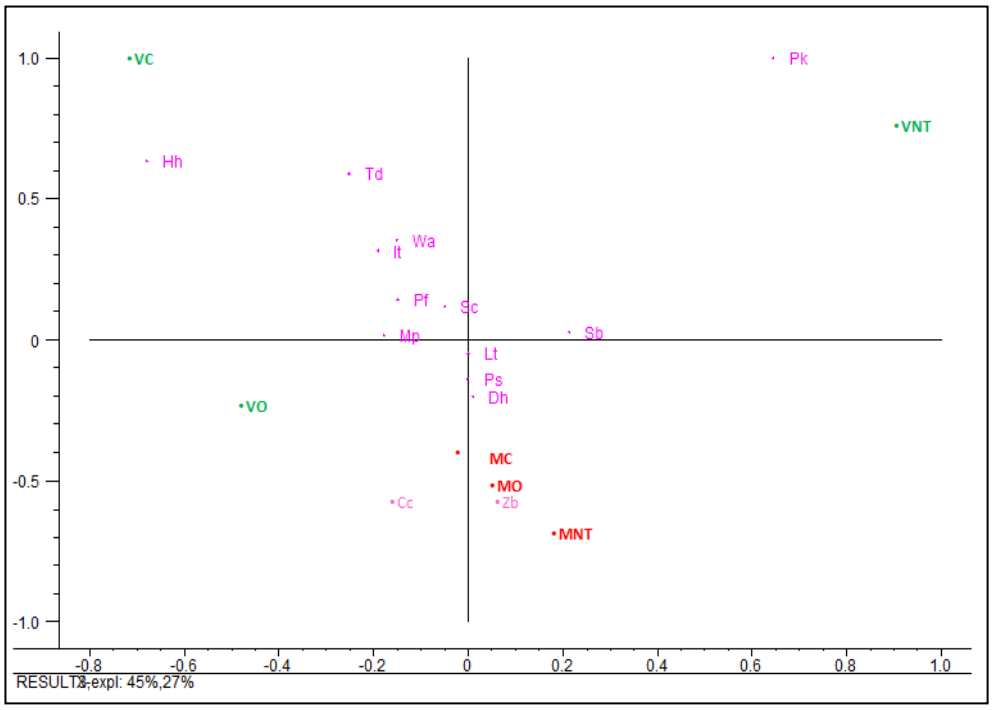
At the end of fermentation (Fig 5c), all of the Montepulciano samples were grouped in the right/lower quadrant and mainly characterized by *C. californica* and *Z. bailii*. Furthermore, *P. sporocuriosa* characterized Montepulciano conventional samples and *S. bacillaris* characterized Montepulciano organic samples. In contrast, all of the Verdicchio samples were differently distributed in the graphic space and indicated remarkable differences for the relevant presence of *P. kudriavzevii* (untreated samples), *H. uvarum* and *T. delbrueckii* (conventional samples), and *C. californica* organic samples).



(a)



(b)



(c)

Fig 5. Principal component analysis related to the yeast community of samples coming from Verdicchio (V) and Montepulciano (M) vineyards subjected to organic (O) and conventional (C) fungicide and non-treated (NT) treatments. (a) The yeast community on the grape surface detected at harvest time; (b) the yeast community of

samples at 7 days of spontaneous fermentation; and (c) the yeast community of the samples at 15 days of spontaneous fermentation. *A. pullulans* (Ap); *I. terricola* (It); *C. californica* (Cc); *Z. meyerae* (Zm); *Cryptococcus* spp. (Cry); *L. termotolerans* (Lt); *S. bacillaris* (Sb); *Rhodotorula* spp. (Rh); *M. pulcherrima* (Mp); *H. uvarum* (Hu); *D. hansenii* (Dh); *Z. bailii* (Zb); *P. fermentans* (Pf); *P. sporocuriosa* (Ps); *P. membranifaciens* (Pm); *T. delbrueckii* (Td); *C. diversa* (Cd); *P. kudriavzevii* (Pk); *W. anomanuls* (Wa); *S. cerevisiae* (Sc)

Discussion

In recent years, the investigation of the geographic distribution of the microbial community of wine grapes revealed a geographic delineation of the yeast communities conditioned by several factors such as cultivar, vintage, climate and agricultural practices [23-26].

The influence of farming practices used in the vineyard and on the yeast biota associated with the grape berry surface was recently investigated [18, 27-29]. In the present study, the impact of organic and conventional treatments on the occurrence of yeast species in two Italian varieties was evaluated. The total yeasts recovered at harvest time were from 10^5 to 10^6 CFU/ml and in accordance with the yeast presence in grapes described in previous studies [7,30].

A. pullulans was the first and most abundant species found in both treated Verdicchio samples and in conventional Montepulciano grapes and in agreement with results obtained from Setati et al. [29]. In organic Montepulciano samples, *A. pullulans* represented the second most abundant species found, and this disagrees the

results obtained by Renouf et al. [30] that did not find *A. pullulans* on the grape surface. These yeasts seem to be favorably affected by farming treatments, which is probably due to their improved competitiveness towards other fungi in the presence of fungicides and their capacity to detoxify $CuSO_4$ as reported by Schmid et al. [31]. Their results support the key role of the yeast-like fungus *A. pullulans* in explaining the functional differences between organic and conventional agricultural systems. Indeed, it has long been known that *Aureobasidium* can utilize inorganic sulfur and is able to absorb, and in this way detoxify, copper [32-33]. In untreated grapes, *A. pullulans* represents only a minor component of the whole yeast population.

H. uvarum was the most abundant fermenting species in both varieties, although the fungicide treatments significantly reduced their presence. These results, which confirmed previous results, are in accordance with the current literature reviewed by Pretorius [34].

A negative effect of both conventional and organic treatments was detected toward *M. pulcherrima* since a significant decrease in treated samples was found. The same results were described by Milanović and co-workers [18] but only in organic samples. This finding highlighted that *M. pulcherrima,* antagonistic and antimicrobial yeast [35-38], is negatively influenced by fungicide treatments and particularly organic ones. Considering that this yeast species revealed a positive contribution to the analytical and aromatic composition and complexity of wine [39-41], fungicide treatments may reduce this yeast's positive contribution to fermentation and wine composition.

The monitoring of fermentation conducted at the laboratory scale elucidated the relationship between yeast occurrence on the grape surface and the potential influence during applicative wine management.

According to Bagheri et al. [42], our results clearly showed a decline at the start of fermentation of the oxidative strains; this decline was probably due to the anaerobic conditions created by the fermentation process. As expected, in the middle of fermentation, *H. uvarum* became the most representative species in all of the samples [18]. At this stage, *P. fermentans* and *C. californica* in Verdicchio and *Z. meyerae* and *Z. bailii* in Montepulciano seems to characterize the yeast biota of the two varieties. Regarding the fermenting yeast *L. thermotolerans*, the results obtained seem to highlight the favorable effect of conventional treatments on this species that was only found in Montepulciano. Cordero-Bueso et al. [13] described *L. thermotolerans* as the predominant non-*Saccharomyces* species found in both organic and conventional samples without relevant differences between the treatments. In our study, this yeast was present on the grape berry surface at harvest time and survived until the end of spontaneous fermentation. Its initial concentration probably plays a significant role in establishing yeast-yeast interaction that allows itself to compete and survive during fermentation [42]. In contrast to *L. thermotolerans*, *Z. bailii* was found in all of the samples of the Montepulciano varieties; this outcome indicated that this species was not affected by organic or conventional treatments.

In Verdicchio samples, the fermenting yeasts, *W. anomalus* and *T. delbrueckii*, seems to characterize the conventional samples. The presence of *T. delbrueckii* agrees with the results of Cordero-Bueso et al. [13] that detected this species in Barbera musts coming from conventional grapes. *T. delbrueckii* was found at a low frequency only at the end of fermentation as reported by Pinto et al. [43], whereas Bagheri et al. [42] found *T. delbrueckii* only in samples coming from an integrated vineyard. However, *W. anomalus* represented the second most abundant

species probably due to its capacity to persist at the typical end-fermentation conditions [44-45]. Indeed, it established its ability to tolerate up to a 12% ethanol concentration and to produce a killer toxin to compete against other yeasts [46-47].

The strong fermenting yeast *S. cerevisiae* was poorly detected and emerged at the end of fermentation and only in Verdicchio conventional samples. These data confirm that the best fermenting yeasts is very poorly present on the grape berry surface at harvest time [17, 48]. On the other hand, fermenting yeasts present at low frequencies at harvest time, took over the spontaneous fermentation differently depending on the fungicide treatments. This is the case of *C. californica* in organic samples of Verdicchio variety and *S. bacillaris* in organic samples of Montepulciano variety (both species absent or present at very low frequencies in the conventional samples). Differences in the dominant yeast species at the end of fermentation in the grape variety were also found (high presence of Z. *bailii i*n Montepulciano and *H.uvarum* in Verdicchio) and could be due to the overall differences between the varieties (characteristic of grapes, time of harvest and agronomic management).

Overall, the data suggest that the yeast community colonizing the grape berry surface was influenced by agricultural treatments. *A. pullulans* and *H. uvarum* were the dominant yeast species at harvest time even if their relative frequencies were strongly influenced by fungicide treatments. Fermenting yeast species differently colonized the grape surface and characterized microfermentations trials of Verdicchio and Montepulciano varieties. These fermenting yeast population changes from varieties are conditioned by pesticide treatments and could be expected to have some impact on the fermentation process and wine composition and their evaluation should receive further attention.

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Supplemental materials

Table 1. Analysis of variance (ANOVA) of Verdicchio and Montepulciano samples at harvest time. The significant differences were determined using t-Test, and the data were considered significant if the associated P values was <0.05. Data with different letters (A, B) within each row are significantly different.

Yeast species	Grape varieties	
	Verdicchio	**Montepulciano**
A. pullulans	A	A
I. terricola	A	A
C. californica	A	A
Cryptococcus spp.	A	A
S. bacillaris	A	A
M. pulcherrima	A	A
H. uvarum	B	A
D. hansenii	A	A
P. fermentans	nd*	nd
P. sporocuriosa	Nd	nd
P. membranifaciens	Nd	nd
Rhodotorula spp.	Nd	nd
Z. bailii	Nd	nd
Z. meyerae	Nd	nd
L. thermotolerans	Nd	nd

*nd= t-Test not detected. In particular, *P. fermentans*, *P. sporocuriosa* and *P. membranifaciens* species were detected only in Verdicchio samples, while *Rhodotorula* spp., *Z. bailii*, *Z. meyerae* and *L. thermotolerans* species were detected only in Montepulciano samples.

Table 2. Analysis of variance (ANOVA) of Verdicchio samples at harvest time and after 7 and 15 days of spontaneous fermentation. Letters O, C and NT indicated organic, conventional and untreated farming management, respectively. For each yeast species detected and for each sampling time, the different letters (A, B) indicated significant differences between the samples ($p < 0.05$) using t-Test.

Yeast species	Harvest			7 days			15 days		
	O	**C**	**NT**	**O**	**C**	**NT**	**O**	**C**	**NT**
A. pullulans	AB	A	B	nd*	nd	nd	nd	nd	nd
I. terricola	A	A	A	A	A	A	B	A	AB
C. californica	B	B	A	B	B	A	A	B	AB
Cryptococcus spp.	A	B	AB	Nd	nd	nd	nd	nd	nd
S. bacillaris	A	A	A	A	A	A	A	A	A
M. pulcherrima	B	B	A	AB	B	A	A	A	A
H. uvarum	A	A	A	A	A	A	A	A	A
D. hansenii	A	A	A	Nd	nd	nd	nd	nd	nd
P. fermentans	B	B	A	B	B	A	A	A	A
P. sporocuriosa	A	A	A	Nd	nd	nd	nd	nd	nd
P. membranifaciens	A	A	A	Nd	nd	nd	nd	nd	nd
T. delbrueckii	nd	nd	nd	A	A	A	A	A	A
C. diversa	nd	nd	nd	A	A	A	nd	nd	nd
P. kudriavzewii	nd	nd	nd	Nd	nd	nd	B	B	A
W. anomalus	nd	nd	nd	Nd	nd	nd	A	A	A
S. cerevisiae	nd	nd	nd	Nd	nd	nd	A	A	A

*nd= not detected

Table 3. Analysis of variance (ANOVA) of Montepulciano samples at harvest time and after 7 and 15 days of spontaneous fermentation. Letters O, C and NT indicated organic, conventional and untreated farming management, respectively. For each yeast species detected and for each sampling time, the different letters (A, B) indicated significant differences between the samples ($p < 0.05$) using t-Test.

Yeast species	Harvest			7 days			15 days		
	O	**C**	**NT**	**O**	**C**	**NT**	**O**	**C**	**NT**
A. pullulans	AB	A	B	nd*	nd	nd	nd	nd	nd
I. terricola	A	A	A	A	B	AB	A	A	A
C. californica	A	A	A	A	A	A	A	A	A
Cryptococcus spp.	A	A	A	nd	nd	nd	nd	nd	nd
S. bacillaris	A	B	AB	A	A	A	A	B	B
M. pulcherrima	B	AB	A	A	A	A	nd	nd	nd
H. uvarum	B	B	A	A	A	B	A	A	A
D. hansenii	A	A	A	nd	nd	nd	A	A	A
Rhodotorula spp.	A	A	A	nd	nd	nd	nd	nd	nd
Z. bailii	A	A	A	A	A	A	A	A	A
Z. meyerae	A	A	A	nd	nd	nd	nd	nd	nd
L. thermotolerans	A	A	A	A	A	A	A	A	A
P. fermentans	nd	nd	nd	nd	nd	nd	A	A	A
P. sporocuriosa	nd	nd	nd	A	A	A	B	A	AB

*nd= not detected

Table 4. Viable cell count (CFU/ml) of Verdicchio samples (V) subjected to organic (VO), conventional (VC) and untreated (VNT) farming managements, at harvest time, after 7 and 15 days of spontaneous fermentation.

Samples	Sampling time	Yeast species (CFU/ml)															
		A. pullulans	*I. terricola*	*C. californica*	*Cryptococcus spp.*	*S. bacillaris*	*M. pulcherrima*	*H. uvarum*	*D. hansenii*	*P. fermentans*	*P. sporocuriosa*	*P. membranifaciens*	*T. delbrueckii*	*C. diversa*	*P. kudriavzewii*	*W. anomalus*	*S. cerevisiae*
VO1	Harvest	1,10E+04			4,00E+03	1,00E+03	1,10E+04										
	7 days							3,70E+07		1,00E+05							
	15 days						1,51E+05	2,67E+07									
VO2	Harvest	2,20E+04						7,35E+04				8,00E+03					
	7 days		2,00E+04	8,00E+05			7,05E+05	5,78E+07		2,20E+05							
	15 days							9,30E+05		3,07E+06							
VO3	Harvest	8,00E+03			5,00E+03												
	7 days						1,00E+05	8,84E+07									
	15 days							2,91E+07									
VO4	Harvest	2,30E+04			4,00E+03		1,00E+03										
	7 days						1,00E+04	5,17E+07									
	15 days						1,45E+06	2,96E+07									
VO5	Harvest				1,40E+04			4,15E+04				7,00E+03					
	7 days		2,22E+06					7,87E+07		8,55E+05							
	15 days					4,90E+04											
VO6	Harvest	9,00E+03	1,00E+03		5,00E+03	1,00E+04											
	7 days						1,00E+04	7,83E+07									
	15 days			1,31E+06													
VO7	Harvest	8,00E+03			6,00E+03	2,00E+03						5,00E+03					
	7 days			8,00E+04				2,75E+07		7,50E+05							
	15 days			6,35E+06		6,00E+04		2,10E+05									
VO8	Harvest	1,00E+03			1,00E+03												
	7 days		5,00E+05				2,00E+04	5,79E+07		2,00E+04							
	15 days			1,98E+06				1,07E+07									
VO9	Harvest	4,65E+04						6,50E+04									
	7 days		1,10E+05				2,05E+05	8,42E+07		1,00E+04			2,00E+04				
	15 days			8,22E+06				3,65E+07									
VO10	Harvest	3,10E+04			6,00E+03	1,40E+04		4,00E+03									
	7 days		2,95E+06					5,81E+07		5,00E+05			1,00E+04				
	15 days			2,89E+06		2,40E+05	2,00E+04										
VO11	Harvest	6,10E+04			3,00E+03	4,00E+03		4,00E+03	2,00E+05								
	7 days		1,31E+06				7,85E+05	5,65E+07									
	15 days			9,40E+06			8,35E+04	5,26E+07		1,50E+05							
VO12	Harvest	1,20E+05				1,00E+05		1,41E+05				1,00E+03					
	7 days		1,00E+04					4,44E+07									
	15 days			2,25E+04			7,25E+04	4,60E+05									
VO13	Harvest	1,00E+04	8,00E+03			7,00E+03		3,60E+04			1,00E+04	1,00E+04					
	7 days		2,85E+05	1,00E+05		1,00E+05		2,19E+07									
	15 days			2,42E+05		3,95E+04											

	Harvest	7,80E+04			7,00E+03									
VC1	7 days		3,00E+04					3,21E+07	3,00E+05					
	15 days		5,05E+06					2,95E+07	3,20E+05					1,66E+06
	Harvest	2,70E+04												
VC2	7 days						5,50E+04	9,51E+07						
	15 days							1,15E+07			2,21E+06		8,41E+06	
	Harvest	5,35E+04	1,35E+04					1,47E+05						
VC3	7 days		2,16E+06				9,00E+04	7,67E+07						
	15 days		2,32E+06					1,66E+07			3,76E+06			
	Harvest	2,70E+04						3,10E+04						
VC4	7 days							3,74E+07						
	15 days							1,55E+07				1,31E+05		
	Harvest	3,70E+04						3,00E+04						
VC5	7 days							4,53E+07						
	15 days		3,16E+06					2,11E+07			5,00E+04			
	Harvest	5,00E+03			1,00E+03									
VC6	7 days							6,07E+07	1,30E+05			1,00E+04		
	15 days							8,76E+06			1,90E+05		1,87E+07	
	Harvest	3,00E+03												
VC7	7 days		1,00E+05					5,58E+07						
	15 days		8,00E+05					9,36E+06						
	Harvest	2,15E+04				3,26E+05		4,50E+04						
VC8	7 days					1,14E+07		9,60E+06						
	15 days					1,00E+04		1,00E+03						
	Harvest	2,05E+04			5,50E+04	8,51E+05		1,58E+05						
VC9	7 days							4,32E+07	1,00E+05					
	15 days													
	Harvest	1,24E+05												
VC10	7 days						1,70E+05	7,37E+07	2,00E+04					
	15 days						1,00E+04	2,53E+07	2,40E+05		1,00E+04		4,79E+06	
	Harvest	3,55E+04		1,55E+04		6,70E+04	2,20E+04	2,45E+05	1,45E+04					
VNT1	7 days			3,03E+06			7,00E+04	9,04E+07	4,38E+06					
	15 days					1,12E+06						4,65E+04		
	Harvest	1,10E+05		8,00E+04		1,77E+06	8,90E+05	1,37E+06	5,00E+04					
VNT2	7 days			3,05E+05			8,65E+05	6,13E+07	5,00E+04					
	15 days											1,00E+03		

Table 5. Viable cell count (CFU/ml) Montepulciano samples (M) subjected to organic (MO), conventional (MC) and untreated (MNT) farming managements, at harvest time and after 7 and 15 days of spontaneous fermentation.

Samples	Sampling time	Yeast species (CFU/ml)													
		A. pullulans	*I. terricola*	*C. californica*	*Cryptococcus spp.*	*S. bacillaris*	*M. pulcherrima*	*H. uvarum*	*D. hansenii*	*P. fermentans*	*P. sporocuriosa*	*Z. bailii*	*L. thermotolerans*	*Rhodotorula spp.*	*Z. meyerae*
MO1	Harvest	5,00E+03												1,00E+03	
	7 days						5,00E+05	1,39E+08							
	15 days														
MO2	Harvest	1,00E+03	1,00E+04												
	7 days		4,20E+05					5,51E+07							
	15 days		1,59E+07			4,96E+07									
MO3	Harvest	1,00E+04				6,40E+05		2,47E+05							
	7 days					6,20E+05									
	15 days											7,60E+04			
MO4	Harvest	6,55E+04			1,00E+03	1,00E+03		1,55E+04							
	7 days		1,00E+06	1,00E+06		6,50E+06		1,00E+05							
	15 days			1,27E+05		3,00E+04		1,00E+04							
MO5	Harvest	1,00E+03			1,73E+05			6,70E+04							2,20E+04
	7 days		7,50E+05			4,50E+06		1,41E+07							
	15 days		3,00E+05			8,90E+06			2,00E+05						
MO6	Harvest	7,00E+04	2,43E+06			4,40E+05		1,75E+06							9,00E+04
	7 days		2,00E+06	1,80E+06		1,11E+07		1,87E+07							
	15 days								3,34E+05						
MO7	Harvest	4,30E+04				1,00E+04		3,55E+04							
	7 days			4,25E+05				5,99E+07			3,15E+05				
	15 days		2,00E+05	1,13E+07				1,22E+07		8,00E+04					
MO8	Harvest					2,80E+05		6,10E+05							3,00E+04
	7 days			1,14E+07		5,20E+06		1,58E+07							
	15 days					1,23E+07			6,20E+05						
MO9	Harvest		8,50E+03			3,20E+05		1,16E+06							7,00E+03
	7 days		1,21E+07			3,20E+06		9,50E+06							
	15 days			4,00E+05		2,00E+05									
MO10	Harvest			7,00E+04		3,00E+05		9,30E+05							
	7 days		1,23E+07			1,70E+06		2,45E+07							
	15 days					1,90E+05						5,00E+04			
MC1	Harvest	8,05E+04												1,40E+04	
	7 days		1,70E+05					7,42E+07							
	15 days														
MC2	Harvest	2,75E+04			2,00E+03		2,00E+03	3,25E+04						2,00E+03	
	7 days		2,00E+04				4,88E+06	1,30E+08							
	15 days										2,00E+03				
MC3	Harvest	7,40E+04			2,00E+03		3,00E+03	1,15E+04							
	7 days		1,00E+04				1,44E+06	6,92E+07							
	15 days										5,00E+03				

	Harvest	3,65E+04						3,00E+03									2,00E+03		
MC4	7 days		1,00E+04				1,41E+06	1,28E+08											
	15 days			3,44E+07		4,10E+06		3,51E+07											
	Harvest	9,55E+04	2,00E+03		2,00E+03			3,00E+03						1,25E+05					
MC5	7 days						3,00E+04	2,91E+07						5,18E+07					
	15 days													8,14E+07					
	Harvest						2,96E+05	4,22E+06											1,00E+04
MC6	7 days			1,00E+04		4,10E+05													
	15 days												1,83E+05						
	Harvest	5,40E+04			4,00E+03			3,70E+04									3,00E+03		
MC7	7 days						3,40E+05	6,61E+07											
	15 days							1,00E+05			1,19E+06								
	Harvest	1,60E+04						1,80E+04											
MC8	7 days						2,00E+04	1,03E+08											
	15 days							8,60E+07											
	Harvest		2,00E+03		4,00E+03		1,40E+04	2,65E+06											2,00E+03
MC9	7 days			1,38E+07		2,80E+06		2,09E+07											
	15 days			1,22E+07		7,50E+06													
	Harvest	5,25E+04						1,50E+05	1,29E+05										
MC10	7 days							6,88E+07											
	15 days					3,70E+05			7,20E+07										
	Harvest	1,20E+05	4,00E+03				2,00E+03	1,01E+06											
MNT1	7 days																		
	15 days																		
	Harvest	2,10E+04					8,00E+03	1,52E+05											
MNT2	7 days																		
	15 days																		
	Harvest					2,00E+04	4,00E+04	7,20E+05											1,70E+05
MNT3	7 days			1,72E+06		2,20E+05		2,80E+05											
	15 days			6,60E+05									8,50E+04						
	Harvest		7,50E+05			2,80E+05	1,40E+05	1,70E+06					9,50E+03						
MNT4	7 days												6,00E+04						
	15 days												2,90E+05						
	Harvest	1,70E+05				3,30E+04	5,00E+03	4,50E+05									1,00E+03		
MNT5	7 days					6,00E+04													
	15 days																		

Article: “The influence of agronomic treatments on grape mycobiota evaluated by NGS and culture-dependent methods”

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The influence of agronomic treatments on grape mycobiota evaluated by NGS and culture-dependent methods

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Running title: Agronomic treatments and grape mycobiota

Abstract

In the present study it was evaluated the impact of different agronomic treatments (organic, conventional) and not-treated samples on yeast community of grape berry of Montepulciano variety. The yeasts dynamic during the spontaneous fermentation using culture-dependent and -independent methods was evaluated. Results showed a reduction of yeast biodiversity by conventional treatments determining a negative influence on fermenting yeasts in favor of oxidative yeasts such as *Aerobasidium pullulans*. *Starmerella bacillaris* was significantly more present in organic samples (detected by NGS method) while *Hanseniaspopra uvarum* was significant lower present in not-treated samples (detected by culture dependent method). The fermenting yeast species developed during the spontaneous fermentation, often undetectable at harvest time, were differently present in function of the agronomic treatments used.

Culture dependent and independent methods showed the same dominant yeast species with the exception of *H. uvarum* and *Zygosaccharomyces bailii* during the spontaneous fermentation. Differently, regarding the lower abundant species, the NGS method was able to detect a great biodiversity in comparison with culture-dependent method. The overall results indicated that the farming system may play an important role on the fermentation behavior and consequently on the final composition of resulting wines.

Importance

Yeast community composition of grape berries plays an important role in winemaking process determining also a characterization of geographic viticultural area. Vineyard farming system affect the mycobiota and consequently the impact on fermenting yeasts during wine fermentation. The evaluation of mycobiota of grape berries by NGS technology substantially confirm the results of culture-dependent methods, highlighting a wide biodiversity of species present at low frequency. Culture-dependent and independent methods gave a different picture of yeasts community during fermentation process.

Keywords: mycobiota of grape, organic treatment, conventional treatment, NGS, culture-dependent method.

Introduction

The fruit surface, and specifically grape berries, is a complex and specific ecologic niche colonized by different microorganisms such as filamentous fungi, yeasts and bacteria with different physiological characteristics (Barata et al., 2012; Abdelfattah et al. 2016;Madden et al., 2018;). Several environmental factors such as geographical region, climatic condition (temperature, humidity, UV radiation, etc.), availability of nutrients and farming treatments could influence the composition of microbiota (Pretorius, 2000; Comitini and Ciani, 2008; Chavan et al., 2009; Bokulich

et al., 2014; Taylor et al. 2014; Zhang et al., 2019). The possible interactions among the factors could also affect biodiversity and stability of microbiota, grapevine health and as final consequence, the quality of wines (Swiegers and Pretorius, 2005; Barata et al., 2012). Furthermore, also bees and wasps can play an important role on the occurrence of microorganisms , influencing the transfer from one side and the other, including grape surfaces (Francesca et al., 2012; Stefanini et al., 2012). The fungi species often found on grapes are saprophytic moulds such as *Cladosporium* spp., *Penicillium* spp., *Aspergiullus* spp., they have not the ability to grow in wine and then they are irrelevant to winemaking. Instead there are other microorganisms, such as yeasts, acetic acid bacteria and lactic acid bacteria that are part of the so called wine microbial consortium (WMC) because they are able to survive or grow on grape juice and wine (Barata et al., 2012) and could influence its final quality. Concerning yeasts, they could be grouped in: species easily controllable or technologically irrelevant (such as *Basidiomycetous* and *Ascomycetous* species), oxidative or weakly fermenting species present at pre-fermentation stages and/or at the beginning of fermentation (such as *Hanseniaspora* spp., *Candida* spp., *Pichia* spp., *Metschnikowia* spp.), strong fermenting yeasts liable for wine fermentation (belonging to *Saccharomyces* spp.) (Arroyo-López et al., 2010; Barata et al., 2012), and spoilage yeasts (such as *Dekkera bruxellensis, Zygosaccharomyces bailii*) responsible for wine alterations (Loureiro and Querol,1999; Malfeito-Ferrera, 2011). Several studies reported that one of the most important factor that influence microbial community composition associated with grape berries are the vineyard agronomic practices(Regueiro et al., 1993; Viviani-Nauer et al., 1995; Ganga and Martinez, 2004; Valero et al., 2007; Comitini and Ciani, 2008; Cordero-Bueso et al., 2011; Tello et al., 2012; Milanovic et al., 2013; Escribano-Viana et al., 2018).

Indeed, Cordero-Bueso and co-workers (2011) showed a greater biodiversity of yeast species when the vineyard was treated with organic practices instead conventional ones. On the contrary, Comitini and Ciani (2008) found a drastic reduction in the yeast diversity when organic fungicides were applied. More recently, Escribano-Viana et al., (2018) found that the bio-fungicide did not show significant impact on the wine microbiota whereas the chemical fungicide caused a reduction of microbial community richness and diversity.

Regarding to *S. cerevisiae* presence, Ganga and Martinez (2004) showed any effect on the enumeration of this fermenting yeast after fungicide application. Tello et al. (2012) described beneficial effect of organic farming system on *S. cerevisiae* strains biodiversity while Milanović and co-workers (2013) found greater strain biodiversity in conventional samples than organic ones.

To investigate on the microbial composition of grape barriers and to monitor their evolution during must fermentation is relevant to understand the relationship among the different microorganisms that cohabit during winemaking process (Bokulich et al., 2014; Piao et al., 2015). The use of culture-dependent techniques, allow to detect only cultivable microorganisms associated with grape berries and wine. However, there are many viable, but non-cultivable wine microorganisms, that could not be analyzed under conventional technique, therefore, this method of study, leads an incomplete knowledge about the composition and dynamics of the microbial community involved in winemaking (Oliver, 2005; Cocolin et al., 2013; Piao et al., 2015). Recent advances in sequencing technologies based on culture-independent techniques allow to capture a large proportion of microbes using high-throughput next generation sequencing obtaining a more complete microbial ecology picture

even if the methodology and the interpretation of data should be set up (Bokulich et al., 2012; De Filippis et al., 2013; Wang et al., 2015; Abdelfattah et al. 2016).

In this study we investigated on yeast community of grape berry surface of Montepulciano variety, subjected to different agronomic treatments using both culture-dependent and -independent approaches. The yeasts dynamics composition during the spontaneous fermentation was also evaluated.

Materials and methods

Vineyard treatments and grape sampling

The grapes used in this study were obtained from Montepulciano vine, an autochthonous vineyard of the center of Italy. In particular, these vines are situated in Sirolo locality (43°31'20N, 13°36'53''E; 97 m altitude), in Marche region and during the sampling time (October 2016) the main climatic conditions were 14.9 °C for air temperature, 82% of humidity and there have been 15 rainy days. The vineyard included three blocks of rows and each block has employed different agronomic practices like as organic, conventional and with no treatment. The distance between the blocks was about one kilometer to exclude cross-contaminations between the treatments.

The organic treatment was performed in 15 consecutive applications from April 20th to August 17th and included a Bordeaux mixture (20 g/L of copper (II) sulfate + 13 g/L of calcium hydroxide with pH 6.6) and sulfur (Microthiol disperss, UPL EUROPE Ltd., Warrington WA3 6YN, Great Britain).

The conventional treatment was performed in 9 consecutive applications from March 10th to July 17th and included chemical compounds such as spiroxamina (Prospher300 CS, Bayer Crop Science, Monheim am Rein, Germany), copper-oxychloride (Coprantol, Sygenta Italia Spa, Casalmorano, Cremona, Italy), sulfur (Tiovit jet, Sygenta Italia Spa, Casalmorano, Cremona, Italy), fosetyl-Al+copper sulfate (R6 Erresei Bordeaux WG, Bayer Crop Science, Monheim am Rein, Germany), Metalaxyl-M14+ copper-oxychloride (RidomilGold, Sygenta Italia Spa, Casalmorano, Cremona, Italy), quinoxyfen+myclobutanil+coformulants (Arius System Plus, Dow AgroSciences, Indianapolis, Indiana, USA), copper sulfate and sulfur.

The grape samplings consisted in the collection in sterile plastic bag of about 1 kg of undamaged ripe grape bunch for each sample and immediately transported to the laboratory on ice for processing. In particular were collected seven organic (MO), seven conventional (MC) and three not treated (MNT) samples.

Grape juice spontaneous fermentations

The grapes, as soon as arrived in the laboratory, were hand-crushed and shaken at 120 rpm for 30 minutes on MAXQ 4450 shaker (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Part of each grape juice was used for yeast counts and total microbial DNA extraction while the remaining fresh must (skin of grape included) was used for set up a spontaneous fermentation. The spontaneous fermentations were carried out in 250 mL sterile Erlenmeyer flasks closed with Pasteur bungs to allow CO_2 to escape and placed at 25 °C under static conditions. Monitoring of the microbial population composition at the beginning and their

evolution at 7th and 15th day from the start to the fermentation has been done through viable counts and high-throughput next generation sequencing (NGS).

Viable counts and yeast isolations

The total yeast enumeration was carried out by taking 1 mL of fresh musts and samples at 7th and 15th day of fermentation, serial decimal dilutions in sterile water were prepared and spread on Wallerstein (WL) nutrient agar (Merck KGaA, Germany) supplemented with 0.02% biphenyl (Sigma-Aldrich, Saint Louis, Missouri, USA) and 0.005% chloramphenicol (Thermo Fisher GmbH, Germany) to prevent molds and bacteria growth respectively. The plates were incubated at 25 °C for five days and those that contained between 30 and 300 colonies were analyzed for cell counts, macro- and micro-morphological characteristics and used for yeast isolation. The yeast isolation was carried out on YPD agar (1% Yeast Extract, 2% Peptone, 2% D-glucose, and 2% Agar) collecting approximately 10% of the colonies per plate (Stringini et al., 2008; Milanović et al., 2013). These yeasts were maintained in 40% (v/v) glycerol at -80 °C.

Yeasts identification

The 700 isolated strains were grouped based on the same macro- and micro-morphological features and representative isolates were used for genomic DNA analysis according to the method described by Stringini et al. (2008). The internal transcribed spacer ITS1-5.8S rRNA-ITS2 region was amplified by PCR using the primer set ITS1 (5'-TCCGTAGGTGAACCTCGCG-3') and ITS4 (5'-TCCTCCGCTTTATTGATATGC-3') (White et al., 1990) as described by Esteve-

Zarzoso and co-workers (1999). Horizontal electrophoresis (Bio-Rad, Hercules, USA) has been used to analyze the PCR products using 1.5% (w/v) agarose gel with ethidium bromide, in 0.5x TBE buffer. The representative yeast species were identified by sequencing and through use BLAST program (Altschul et al., 1997), the sequences provided were compared with those already present in the data library GenBank (http://www.ncbi.nlm.nih.gov/BLAST).

Total DNA extraction and Next Generation Sequencing (NGS) analysis

To obtain the total microbial DNA that represent well mixed microbial consortia of samples, 1 mL of each fresh juice and each sample at 7^{th} and 15^{th} day of spontaneous fermentation was taken. The total DNA extraction was carried out following the protocol of the Soil Kit DNA Extraction (Qiagen, Hilde, Germany) and the extracts were stored at -20 °C until further analysis.

The presence of fungal genome was confirmed using primer set NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) to amplify the region 26S rDNA D1/D2 as described by Kurtzman and Robnett (1998).

Next Generation Sequencing (NGS) analyzes were performed using primers BITS (5'-GAGATCCRTTGYTRAAAGTT-3') and B58S3 (5'-ACCTGCGGARGGATCA-3') (Bokulich and Mills, 2013) to amplify fungal internal transcribed spacer (ITS) region. Library preparation of the samples was carried out using Illumina paired-end kit, cluster generation, and 350-bp paired-end sequencing on an Illumina Miseq.

NGS data processing

The fastq files obtained from sequencing were processed using a custom script based on the QIIME software suite (Caporaso et al., 2010). In detail, paired-end reads pairs were assembled to reconstruct the complete BITS/ B58S3 amplicons. Forward reads of unmerged pairs were also included in the analysis. Quality control retained sequences with mean sequence quality score > 15 while sequences with mismatched primers were omitted. In order to calculate fungal taxonomy, ITS rRNA Operational Taxonomic Units (OTUs) were defined at ≥ 99 % sequence homology using uclust (Edgar, 2010) and OTUs with less than 10 sequences were filtered. All reads were classified to the lowest possible taxonomic rank using QIIME (Caporaso et al., 2010) and a reference dataset from the UNITE database (Kõljalg et al 2013).

The microbial richness of the samples (alpha-diversity) were calculated with Shannon indexes calculated for 10 sub-samplings of sequenced read pools and represented by rarefaction curves. The alpha-diversity could also be represented by box-and-whisker plot. In detail, the bottom and top of the box were the first and the third quartiles, and the band inside the box was the median. Moreover, the ends of the whiskers represented the minimum and maximum of all the data of the sample. Similarities between samples (beta-diversity) were calculated by weighted uniFrac (Lozupone and Knight, 2005). The range of similarities is calculated between the values 0 and 1. PCoA (principal component analysis) presentations of beta-diversity were performed using QIIME (Caporaso et al., 2010). In the PCoA each dot represented a sample that is distributed in tridimensional space according to its own bacterial composition.

Statistical analysis

Comparisons between different groups were tested by ANOVA (Analysis of Variance) calculated through SPSS software (www.ibm.com/software/it/analytics/spss/). Moreover, we also calculated the post hoc analysis LSD (least significant difference) for multiple comparison.

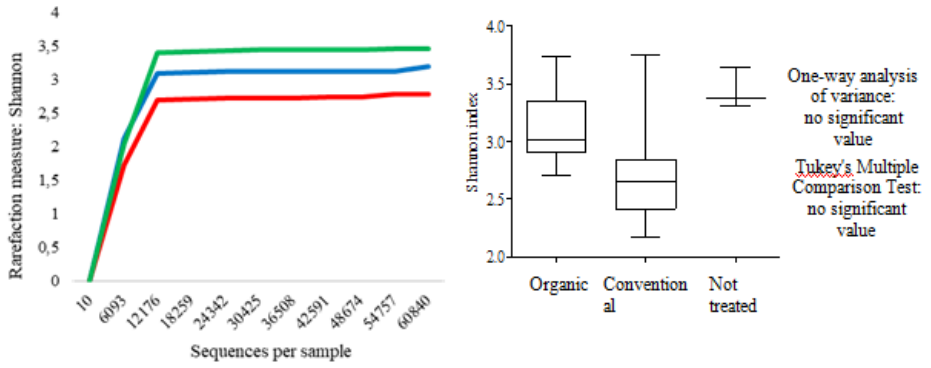
Results

Effects of agronomic treatments on fungal community at harvest time

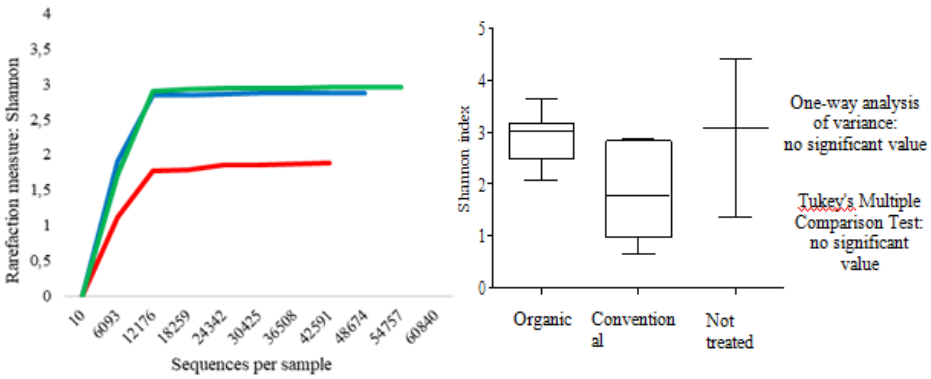
Culture-independent analysis (NGS)

The fungal population associated with grape surface of Montepulciano variety was evaluated by culture-independent method using Next Generation Sequencing (NGS). Samples MNT, MO and MC were compared.

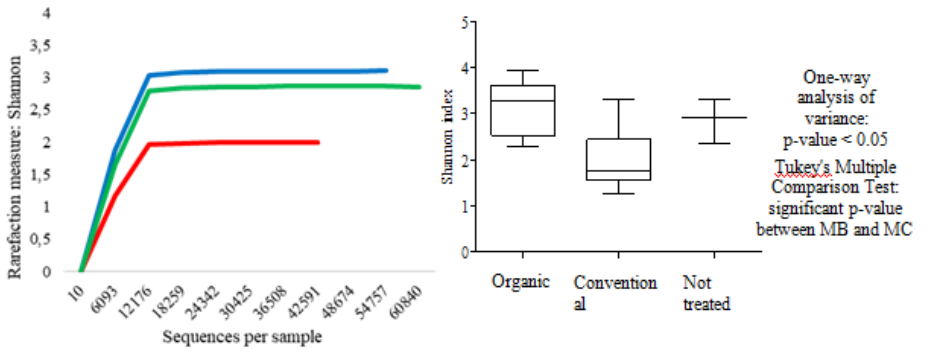
Rarefaction curves of fungal population characterizing MO, MC and MNT samples were calculated through Shannon index, as showed in Figure 1. In all three samples time (harvest, 7^{th} and 15^{th} day of spontaneous fermentation) the plateauing of the three curves related to the diversity indices indicated that the main part of the fungal diversity has been detected .. In detail, the MNT grapes displayed the highest biodiversity at the harvest time followed by MO and MC ones (Figure 1a). At 7^{th} day of spontaneous fermentation the biodiversity of MO and MNT grapes was similar and it was higher than conventional grapes (Figure 1b). At 15^{th} day of fermentation, the MO samples showed the highest biodiversity followed by MNT and MC samples (Figure 1c). Significant differences were found only at 15^{th} day (between MB and MC) for the higher homogeneity of the samples in comparison with the others.



(a)



(b)



(c)

Figure 1. Rarefaction curves (on the left) and boxes-and-whisker plots (on the right) generated for mean values of fungal ITS sequences obtained from organic ( ), conventional ( ) and not treated ( ) grapes. The results were obtained using the Shannon index. (a), (b), (c) represented rarefaction curves and box-and-whisker plots referred to harvest time and at 7th and 15th day of spontaneous fermentation, respectively.

At harvest time the high-throughput sequencing technology allowed to clearly identify 164 species (yeasts and filamentous fungi). Other fungi were classified only at higher taxonomical level. Unknown fungi were also detected (Figure 2). Mean values of relative abundance revealed that the population was mainly represented by the oxidative yeast-like *Aerobasidium pullulans* followed by the fermentative *Hanseniaspora uvarum* species. The relative abundance of the two species was similar in the MO samples (26.09% of *A. pullulans*, 19.10% of *H. uvarum*), while in MC samples predominated *A. pullulans* (45.12 %) over *H. uvarum* (20.81%). *A. pullulans* represented more than 50% of the total fungal population of MNT grapes, while only 9.30% of *H. uvarum* was detected. Conventional treatments affected the presence of *A. pullulans* since significant enhancement of the relative abundance was found in MC samples while *H. uvarum* did not seem influenced by treatments (Table 1 supplemental materials). *Starmerella bacillaris* fermentative yeast was positively influenced by organic treatments (9.96%, 0.53% and 2.99% in MO, MC and MNT respectively), while *Lachancea thermotolerans* was found only in MC samples (3.35%). *Zygoascus meyerae* was found in MO and MNT samples (0.23% and 0.17% respectively) and it was not detected in MC samples. *Rhodotorula nothofagi* and *Metschnikowia pulcherrima* were found in MNT samples (1.64% and 0.87 %, respectively), while *Pichia terricola* was detected in MO (1.34%) and MC (1.89%) samples. Filamentous fungi such as *Botrytis caroliniana*, *Alternaria* genus, *Cladosporium ramotenellum* and *Cladosporium delicatulum* showed a relevant presence in all samples. Analyzing the mean values of relative abundance, these species exhibited the same trend: they appeared more abundant in MO samples, followed by MC and MNT ones (*B. caroliniana*: 7.87%, 4.89% and 3.10%; *Alternaria* genus: 4.41%, 3.38% and 2.86%; *C. ramotenellum*: 6.93%, 2.46% and

1.55%; *C. delicatulum*: 9.43%, 6.78% and 5.78% in MO, MC and MNT samples respectively). Only *C. ramotenellum* showed a significant increase in relative abundance in MO samples (Table 1 supplemental materials).

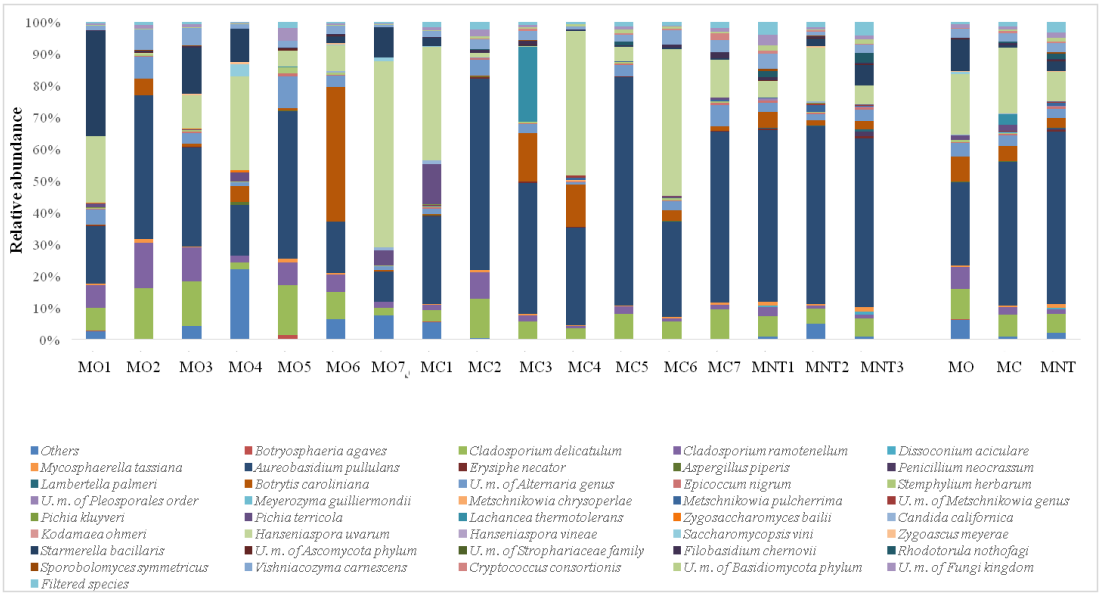


Figure 2. Relative abundance of grape's fungal community detected by NGS at harvest time in organic (MO), conventional (MC) and not treated (MNT) samples. The number associated to the samples represent the replicates for each treatment. To the right of the graph, mean values of each treatment were represented (MO, MC and MNT). Only the taxa > 0.5% were showed.

Culture-dependent analysis

The results of culture-dependent method were showed in Figure 3. Culture-dependent method allowed to detect only 12 yeast species and other 2 identified at genus level. As showed by NGS analysis, *A. pullulans* and *H. uvarum* were confirmed to be the yeasts mainly detected also in culture dependent approach.

Likewise to NGS analysis, MC samples showed higher relative abundance of *A. pullulans* than that showed by MB and MNT samples but not statistically significant (Figure 3 and Table 2 supplemental materials). *H. uvarum* was more abundant species isolated from all samples without significant differences among the MO, MC and MNT samples. As NGS analysis, *S. bacillaris* was mainly found in MO samples (20.94%) and *L. thermotolerans* was found only in MC samples (7.84%). Differently to NGS, *P. terricola* was found in MO, MC and MNT samples. *Z. meyerae* was not detected in MC samples (0.01% of relative abundance with NGS) while it was found in MO and MNT as showed by NGS analysis. By culture-dependent method, *Rhodotorula* genus was found only in MC samples (1.13%) while *M. pulcherrima* characterized MNT samples showing the same trend described by NGS.

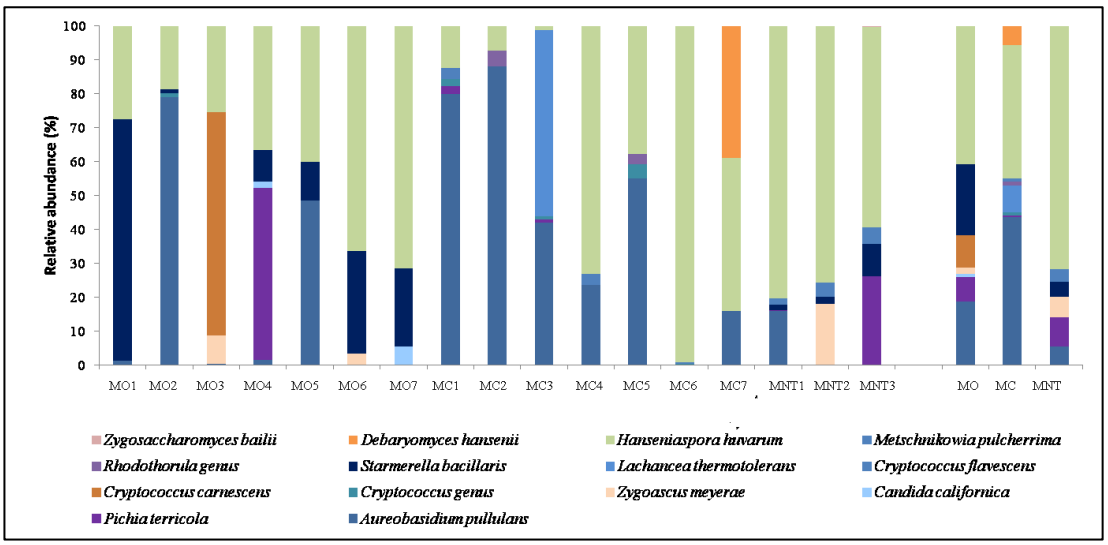


Figure 3. Relative abundance of grape's yeast community detected by culture-dependent method at harvest time in organic (MO), conventional (MC) and not treated (MNT) samples. The number associated to the samples represent the replicates for each treatment. To the right of the graph, mean values of each treatment were represented (MO, MC and MNT).

Effects of agronomic treatments on fungal community at 7th day of spontaneous fermentation

Culture-independent analysis (NGS)

The population dynamic at 7th day of spontaneous fermentation evaluated by NGS revealed 71 fungal species (Figure 4). Other fungi were classified at higher taxonomical levels. At this stage of fermentation as expected, *H. uvarum* represented the most abundant specie in MO, MC and MNT samples (40.30%, 63.61% and 41.71% respectively) while the oxidative yeast-like *A. pullulans* decreased in all samples. The same trend was observed for molds, which were found < 1% of relative abundance. *S. bacillaris* confirmed the significant higher presence in MO samples in comparison with the other treatments, as observed at the harvest time. In the same way, also *L. thermotolerans* was found only in MC samples (9.96%). *M. pulcherrima* was only detected in MC samples (1.26%) while *P. terricola* become appreciable in MNT samples (13.85%), undetected at the harvest time. Others fermentative species, unrevealed at the harvest time, become detectable at this stage of fermentation. In particular, *Candida californica* was found in MO MC and MNT samples without differences. Also *Pichia kluyveri* become detectable in MNT samples (1.08%).

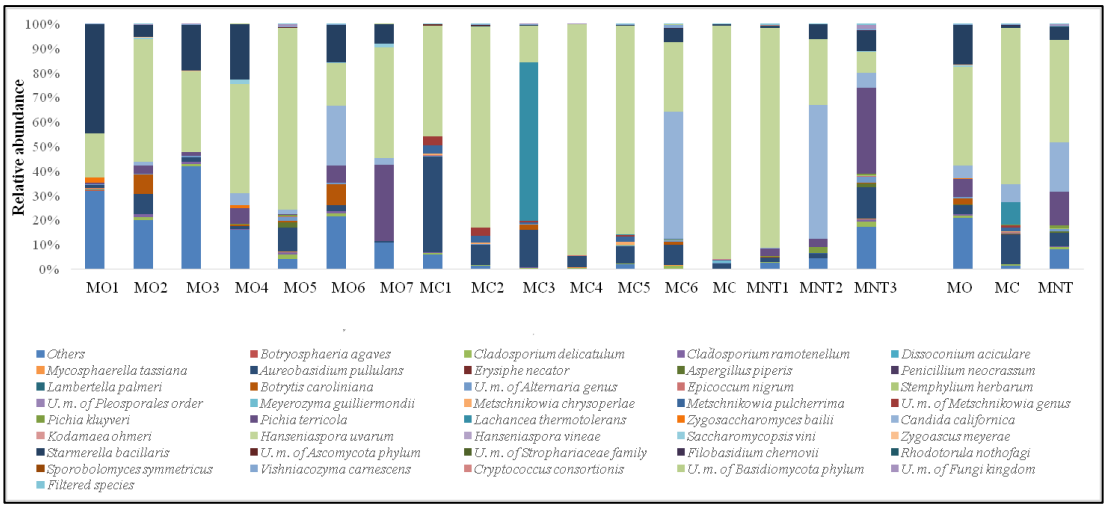


Figure 4. Relative abundance of fungal community detected by NGS at 7th day of spontaneous fermentation in organic (MO), conventional (MC) and not treated (MNT) samples. The number associated to the samples represent the replicates for each treatment. To the right of the graph, mean values of each treatment were represented (MO, MC and MNT). Only the taxa > 0.5% were showed.

Culture-dependent analysis

The results obtained by culture-dependent method after 7th days are showed in Figure 5. As observed through NGS, *H. uvarum* was the most abundant specie in MO and MC samples (48.69% and 76.86% respectively) while it was lower represented in MNT samples (4.21%) where the species mainly detected were *S. bacillaris*, *C. californica* and *Zygosaccharomyces bailii* (undetected with NGS) (36.64%, 25.81% and 33.33%, respectively). *S. bacillaris* and *C. californica* were also found in MO and MC samples with a relative abundance comparable to the NGS results. The results of NGS were also confirmed for *L. thermotolerans* and *M.*

pulcherrima in MC samples by culture-dependent method. Differently, using the culture-dependent method *P. terricola* was only detected in MO samples (7.63%).

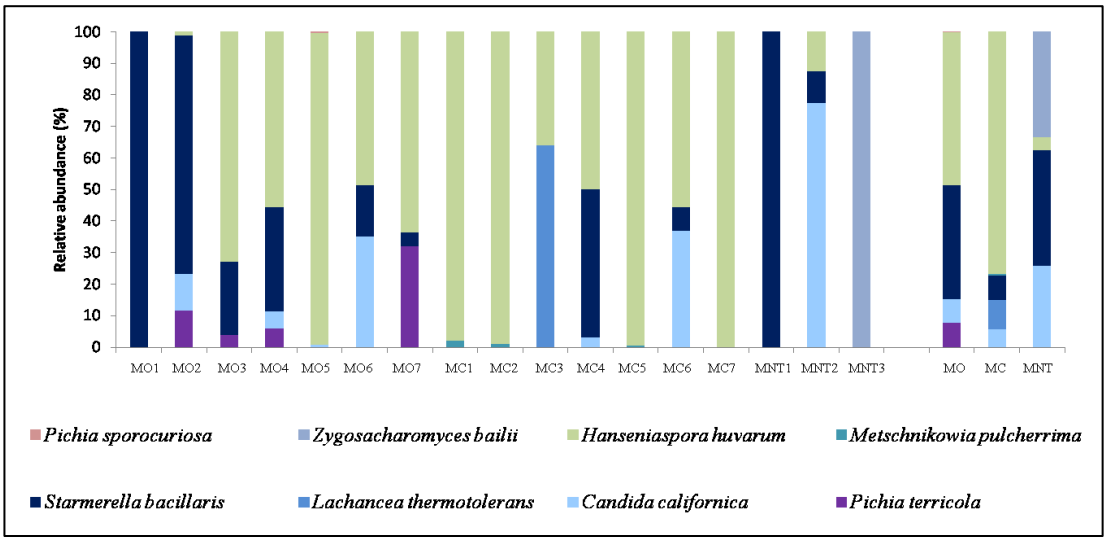


Figure 5. Relative abundance of yeast species detected by culture-dependent method at 7th day of spontaneous fermentation in organic (MO), conventional (MC) and not treated (MNT) samples. The number associated to the samples represent the replicates for each treatment. To the right of the graph, mean values of each treatment were represented (MO, MC and MNT).

Effects of agronomic treatments on fungal community at 15th day of spontaneous fermentation

Culture-independent analysis (NGS)

NGS results of the fungal community composition at 15th day of spontaneous fermentation were showed in Figure 6. The fungal community found was very similar to that found at 7th day of fermentation. Indeed, at this stage of fermentation

74 species were identified, and *H. uvarum* confirmed to be the most abundant species detected in all samples, from 29% to 50% of relative abundance. At lower relative abundance were present *S. bacillaris* (significant higher in MO samples; Table 1 Supplemental materials) and *C. californica* in all samples. *L. thermotolerans* and *M. pulcherrima* confirmed their presence only in MC samples, *P. kluyveri* was confirmed to be present in MNT samples, and *P. terricola* was found in MO and MNT samples (10.17% and 5.01%, respectively). An emerging fermenting species, not detected before, was *Meyerozyma guillermondii* that characterize MO and MNT samples (8.99% and 21.47%, respectively) but absent in MC samples. Poorly found (≤ 0.5%) high-fermentative yeasts at this stage of fermentation. In particular, *Torulaspora delbrueckii* in MO and MNT samples and *Z. bailii* in MO and MC samples. *Z. meyerae* remained detectable only in MO samples (0.72%).

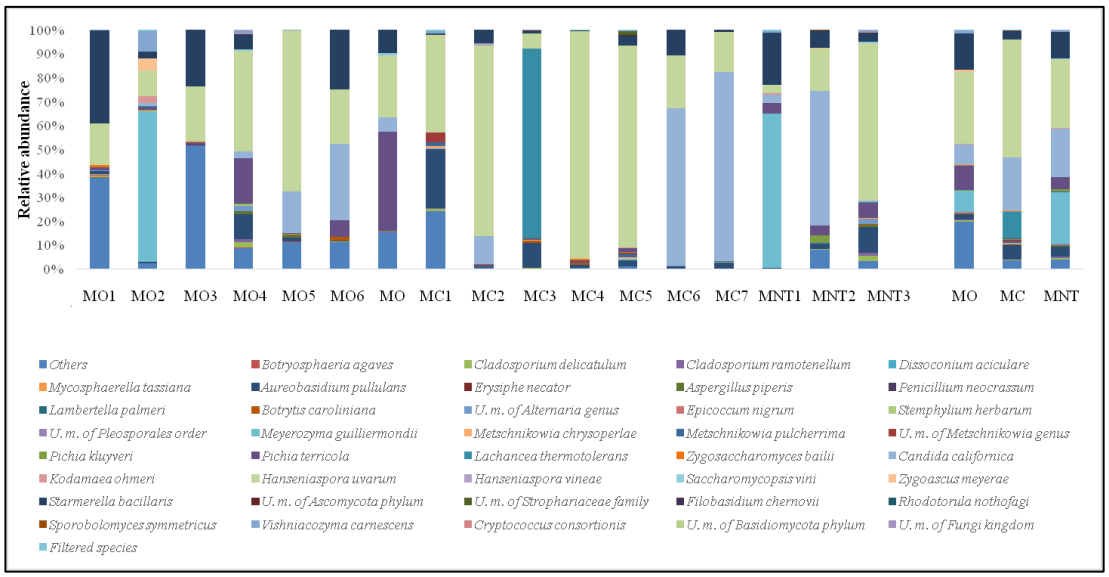


Figure 6. Relative abundance of fungal community detected by NGS at 15th day of spontaneous fermentation in organic (MO), conventional (MC) and not treated (MNT) samples. The number associated to the samples represent the replicates for

each treatment. To the right of the graph, mean values of each treatment were represented (MO, MC and MNT). Only the taxa > 0.5% were showed.

Culture-dependent analysis

The relative abundances of yeasts by culture-dependent method were showed in Figure 7. The comparison between NGS and conventional methods showed, at this stage of fermentation, some differences in relative abundance since some low fermenting yeasts could be present at viable but not cultivable condition. Indeed, differently from NGS results, using culture-dependent method, *H. uvarum* was found at lower relative abundance in all samples (8.18%, 15.06% and not detected in MO, MC and MNT samples respectively). The MNT samples were dominated by two highly fermenting species: *Z. bailii* (55.71%) (just detected by NGS; 0.02%) and *C. californica* (44.30%). The relative abundance of *C. californica* in MO and MC samples was comparable to NGS results. MO samples were dominated by *S. bacillaris* (41.00%) and *Debaryomyces hansenii*, (15.27%) (not detected by NGS). In MC samples the presence of *L. thermotolerans* was confirmed using both culture-independent and -dependent methods, while the presence of the fermentative yeast *D. hansenii*, not detected by NGS, was arisen.

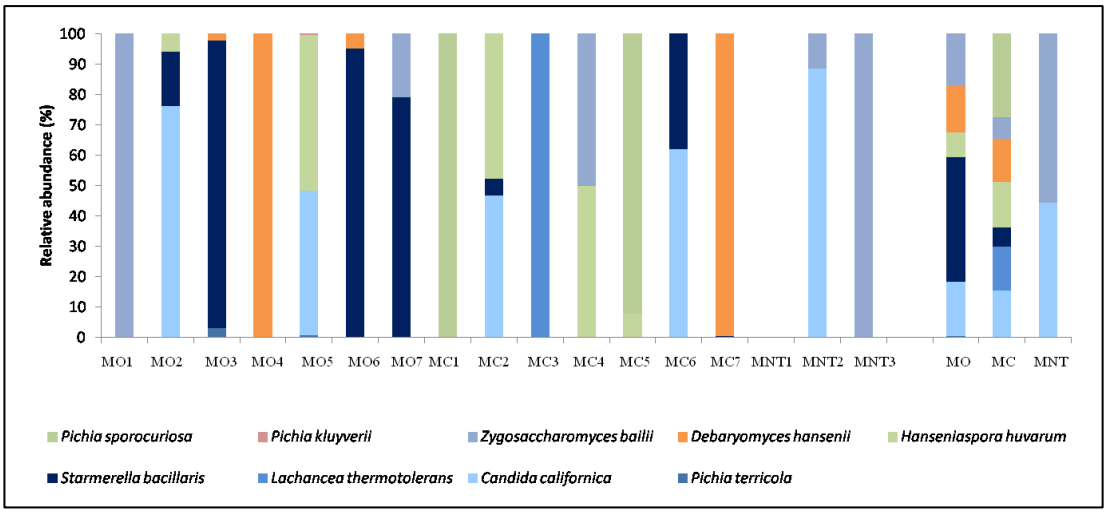


Figure 7. Relative abundance of yeast species detected by culture-dependent method at 15th day of spontaneous fermentation in organic (MO), conventional (MC) and not treated (MNT) samples. The number associated to the samples represent the replicates for each treatment. To the right of the graph, mean values of each treatment were represented (MO, MC and MNT).

Principal Component Analysis

The fungal community was also subjected to Principal Component Analysis (PCoA) and the distribution of the samples in the three-dimensional plot graphic, at harvest time, at 7th and 15th day of fermentation, was showed in Figure 8. At harvest time (Figure 8a) the total variance explained was 63.87% (PC 1 35.82%, PC 2 17.68%, PC 3 10.37%). The graphic distribution of the samples highlighted a clear distinction between MO and MC samples, while the MNT samples were closely related to MO ones.

At 7^{th} day of spontaneous fermentation Figure 8b showed a clear distinction in fungal community composition among the three treatments. At 15^{th} day of spontaneous fermentation MC samples grouped separately, while MO and MNT samples showed some overlaps (Figure 8c).

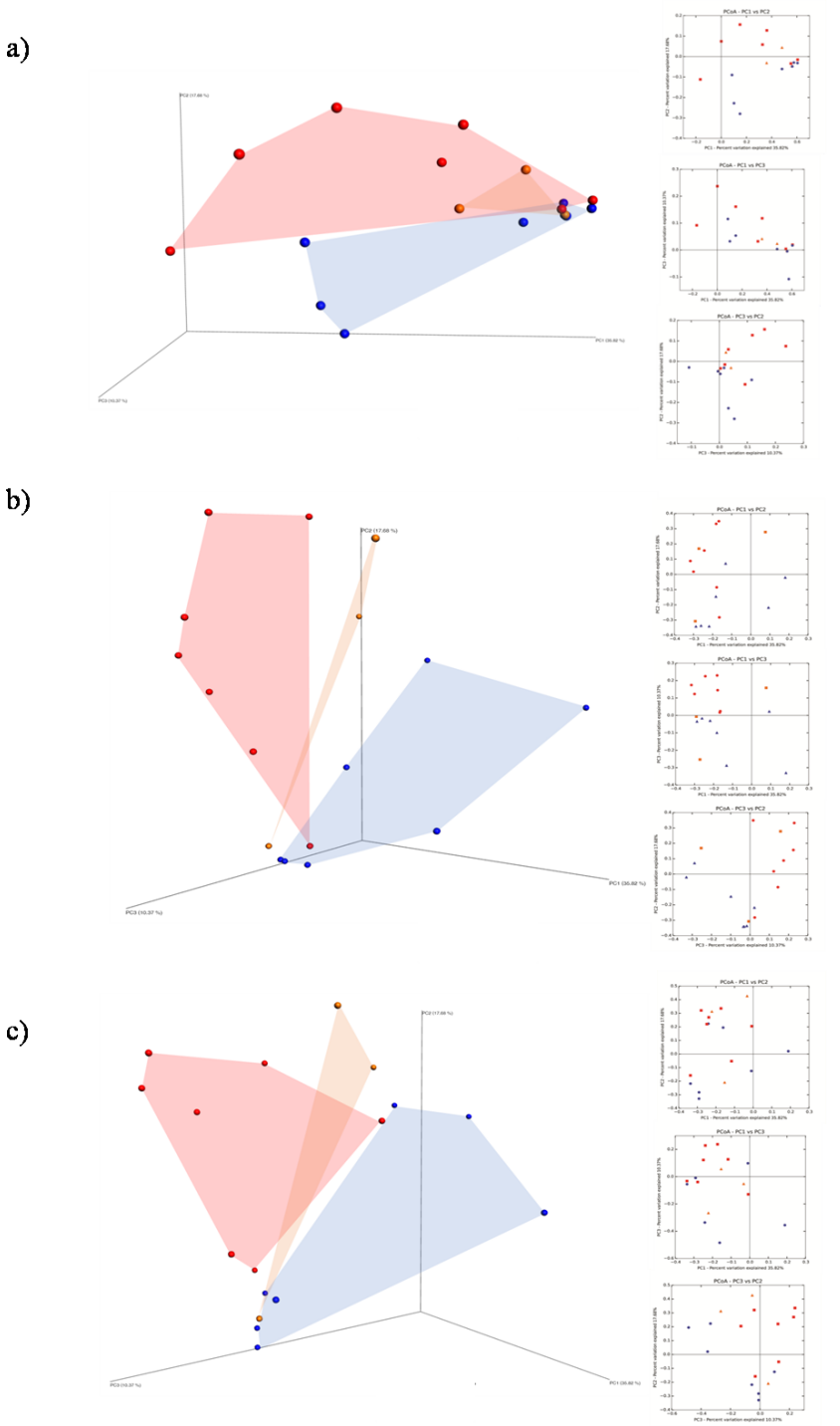


Figure 8. Principal Coordinate Analysis (PCoA) based on the fungal ITS sequences identified in organic (MO) , conventional (MC) and not treated (MNT) samples at the harvest time (a), at 7th day (b) and at 15th day (c) of spontaneous fermentation. Percentages shown along the axes represent the proportion of dissimilarities captured by the axes.

Discussion

The knowledge of complex dynamic microbial ecosystem associated to grape berries surface, represented by yeasts, filamentous fungi and bacteria (Barata et al., 2012) is crucial to better understand the involvement during winemaking process, with consequently repercussions on wine quality (Fleet, 2003; Verginer et al., 2010; Nisiotou et al., 2011; Bokulich et al., 2014). The study of fungal diversity during winemaking process using culture-dependent methods can led risks such as an incomplete microbial detection and identification, linked to different microbial kinetics or VBNC state of such species or for low abundant strains (Rantsiou et al., 2005). Recently, the development of NGS technology allowed to obtain a more exhaustive information about microbial communities associated to grape berries, fresh must and winery (Bokulich et al., 2014; Pinto et al., 2014; Valera et al., 2015; Wang et al., 2015).

In the present study, the impact of organic and conventional treatments on the occurrence of fungal community in Montepulciano variety was evaluated by NGS technology and culture-dependent methods, comparing overall results obtained. At the harvest time, 164 fungal species were identified by NGS, while only 14 yeast species were identified by culture-dependent methods. However, both methodologies detected *A. pullulans* and *H. uvarum* as the most abundant species found in all samples at the harvest time. The occurrence of *A.pullulans* seems to be influenced by treatments: indeed it was the most abundant species detected in conventional samples. These data confirmed the results of previous studies (Comitini and Ciani 2008; Čadež et al. 2010; Setati et al., 2012) that found *A. pullulans* as the most abundant species on grape surface at the harvest time. Although this yeast is

considered irrelevant in fermentation process for its inability to ferment sugars, it represents a common resident of grape berry (Barata et al., 2012). Regarding *H. uvarum* no relevant differences at harvest time among the treatments was found using both methodologies.

The presence of *S. bacillaris* characterized MO samples, while *L. thermotolerans* was only found in MC samples. These data are in agreement with those of Ghosh et al. (2015) that described *S. bacillaris* as the dominant yeast species in biodynamic Cabernet Sauvignon fresh must and Cordero-Bueso and co-workers (2011) that described *L. thermotolerans* as predominant non-*Saccharomyces* yeast found in organic and conventional samples. *M. pulcherrima* was only found in MC and MNT samples using both technologies, confirming the results obtained by Milanović et al. (2013) that showed the negative effect of the organic treatments on this species. The fungal dynamic at7th day of spontaneous fermentation showed, as expected, a reduction of species and the predominance of *H. uvarum* using both methodologies (Pretorius, 2000; Beltran et al., 2002; David et al., 2014; Wang et al., 2015). However, an overestimation of this yeast using NGS methodology at 15th day of fermentation was found since culture-dependent method relieved absence or slightly presence in all samples. This result could be due to the detection by NGS method of died and/or viable but non cultivable cells showing at this time a warp picture of fermentative yeast population. Indeed, at this time, using culture-dependent method, a predominance of *S. bacillaris*, *C. californica* and *Z. bailii* in MNT samples was found. The fermenting yeasts *C. californica* and *P. kluyveri*, seemed to be negatively influenced by treatments. Other fermenting yeasts (*M. guillermondii*, *T. delbrueckii* and *Z. meyerae*) were detected in MO and/or in MNT samples only by NGS analysis. *D. hansenii* (MO and MC samples) and *Z. bailii*

(MNT samples) were detected only by culture-dependent method. It is necessary underline that the failure in some species identification by NGS, could be due to a significant portion of relative abundance described as unidentified yeasts or fungi. In this regard, the choice of the target used during microbial metabarcoding and the availability of an exhaustive reference database for the target chosen become very important. To date, only few fungal databases are available, therefore a more exhaustive database information of bioinformatics package could be necessary to improve the sensitivity of the method (Kioroglou et al., 2018; Stefanini and Cavalieri, 2018). In the condition tested, *S. cerevisiae* was very poorly detected and only using NGS method (about 0.003% at 7^{th} and 15^{th} day of spontaneous fermentation), in agreement with previous works (Rosini et al., 1982; Martini et al 1996; Fleet et al., 2002).

In conclusion, the two methods used in the present study revealed the same species concerning the dominant yeast species present in the samples, with some exceptions (*H. uvarum* at 7^{th} and 15^{th} day of fermentation; *Z. bailii* at 15^{th} day of fermentation). Regarding the lower abundant species the NGS method was able to identify a great biodiversity in comparison with culture-dependent method.

The influence of agronomic treatments on grape yeast community composition highlighted the lost of yeast biodiversity with conventional treatments. Chemical compounds seem to negatively affect the fermenting yeasts in favor of oxidative yeasts such as *A. pullulans*. Moreover, *S. bacillaris* was more present in Mo samples (detected by NGS method) while *H. uvarum* was significant lower in MNT samples (culture dependent method). In this regard, the fermenting yeast species, often undetected at harvest time, become detectable during the fermentation process and resulted different in function of the agronomic treatments applied. For

these reasons, the different farming system may play an important role on the fermentation behavior and consequently on the final composition of resulting wines.

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Supplemental materials

Table 1. Analysis of variance (ANOVA) of filamentous fungi detected by NGS in Montepulciano samples at harvest time and at 7th and 15th day of spontaneous fermentation. The significant differences were determined using t-Test (the associated P values considered was <0.05). The presence of different letters (A, B) within each row highlight the data significantly different. Only the fungal species > 0.5% of relative abundance were represented.

Cultural-independent method (NGS)	**Harvest time**			**at 7th day**			**at 15th day**		
fungal species	**MO**	**MC**	**MNT**	**MO**	**MC**	**MNT**	**MO**	**MC**	**MNT**
Botryosphaeria agaves	A	A	A	A	B	AB	A	B	AB
Cladosporium delicatulum	A	A	A	A	A	A	A	A	A
Cladosporium ramotenellum	A	B	B	A	B	AB	A	A	A
Dissoconium aciculare	nd	nd	*	nd	nd	nd	nd	nd	nd
Mycosphaerella tassiana	A	A	A	A	A	A	A	A	A
Aureobasidium pullulans	B	A	A	A	A	A	A	A	A
Erysiphe necator	B	AB	A	nd	nd	nd	nd	nd	nd
Aspergillus piperis	*	nd	nd	A	nd	A	A	nd	A
Penicillium neocrassum	nd	nd	*	nd	nd	nd	nd	nd	nd
Lambertella palmeri	nd	nd	*	nd	nd	nd	nd	nd	nd
Botrytis caroliniana	A	A	A	A	A	A	A	A	A
U. m. of Alternaria genus	A	A	A	A	A	A	A	A	A
Epicoccum nigrum	A	A	A	A	A	A	A	A	A
Stemphylium herbarum	A	A	A	A	A	A	A	A	A
U. m. of Pleosporales order	B	B	A	nd	nd	nd	nd	nd	nd
Meyerozyma guilliermondii	nd	nd	nd	nd	nd	nd	A	nd	A
Metschnikowia chrysoperlae	nd	nd	nd	nd	*	nd	nd	*	nd

Metschnikowia pulcherrima	nd	nd	*	nd	*	nd	nd	*	nd
U. m. of Metschnikowia genus	nd	*	nd	nd	*	nd	nd	*	nd
Pichia kluyveri	nd	nd	nd	nd	nd	*	nd	nd	*
Pichia terricola	A	A	nd	A	A	A	A	nd	A
Lachancea thermotolerans	nd	*	nd	nd	*	nd	nd	*	nd
Zygosaccharomyces bailii	A	nd	A	*	nd	nd	A	A	nd
Candida californica	nd	nd	nd	A	A	A	A	A	A
Kodamaea ohmeri	nd	nd	nd	nd	nd	nd	A	nd	A
Hanseniaspora uvarum	A	A	A	A	A	A	A	A	A
Hanseniaspora vineae	nd	nd	nd	nd	nd	nd	nd	*	nd
Saccharomycopsis vini	*	nd	nd	*	nd	nd	A	nd	A
Zygoascus meyerae	A	nd	A	A	nd	A	*	nd	nd
Starmerella bacillaris	A	B	AB	A	B	AB	A	B	AB
U. m. of Ascomycota phylum	A	A	A	nd	nd	nd	nd	nd	nd
U. m. of Strophariaceae family	nd	nd	nd	nd	nd	nd	nd	*	nd
Filobasidium chernovii	A	A	A	A	A	A	A	A	A
Rhodotorula nothofagi	nd	nd	*	nd	nd	nd	nd	*	nd
Sporobolomyces symmetricus	B	B	A	A	nd	A	A	nd	A
Vishniacozyma carnescens	A	A	A	A	A	A	A	A	A
Cryptococcus consortionis	B	A	AB	nd	nd	nd	nd	nd	nd
U. m. of Basidiomycota phylum	C	B	A	nd	nd	nd	A	A	A
U. m. of Fungi kingdom	A	A	A	A	A	A	A	A	A

nd = not detected

* = fungi detected in only one treatment

Table 2. Analysis of variance (ANOVA) of yeast species detected by cultural-dependent method in Montepulciano samples, at harvest time and at 7th and 15th day of spontaneous fermentation. The significant differences were determined using t-Test (the associated P values considered was <0.05). The presence of different letters (A, B) within each row highlight the data significantly different. Only the yeast species > 0.5% of relative abundance were represented.

Cultural-dependent method	**Harvest time**			**at 7th day**			**at 15th day**		
Yeast species	**MO**	**MC**	**MNT**	**MO**	**MC**	**MNT**	**MO**	**MC**	**MNT**
Aureobasidium pullulans	A	A	A	nd	nd	nd	nd	nd	nd
Pichia terricola	A	A	A	*	nd	nd	*	nd	nd
Candida californica	*	nd	nd	A	A	A	A	A	A
Zygoascus meyerae	A	nd	A	nd	nd	nd	nd	nd	nd
Cryptococcus genus	A	A	nd	nd	nd	nd	nd	nd	nd
Cryptococcus carnescens	*	nd	nd	nd	nd	nd	nd	nd	nd
Cryptococcus flavescens	nd	nd	nd	nd	nd	nd	nd	nd	nd
Lachancea thermotolerans	nd	*	nd	nd	*	nd	nd	*	nd
Starmerella bacillaris	A	nd	A	A	A	A	A	A	A
Rhodothorula genus	nd	A	nd	nd	nd	nd	nd	nd	nd
Metschnikowia pulcherrima	nd	B	A	nd	*	nd	nd	nd	nd
Hanseniaspora uvarum	A	A	A	AB	A	B	A	A	nd
Debaryomyces hansenii	nd	*	nd	nd	nd	nd	A	A	nd
Zygosaccharomyces bailii	nd	nd	*	nd	nd	*	A	A	A
Pichia sporocuriosa	nd	nd	nd	*	nd	nd	nd	*	nd
Pichia kluyveri	nd	nd	nd	nd	nd	nd	*(0.05%)	nd	nd

nd = not detected

* = fungi detected in only one treatment

PART II

Oenological characterisation of selected indigenous *Saccharomyces cerevisiae* strains coming from Marche region

1. Introduction

1.1 *Saccharomyces cerevisiae* as a starter yeast for the alcoholic fermentation of wine

Saccharomyces cerevisiae is the main specie selected as a starter yeast for the alcoholic fermentation of wine. Generally, this yeast is present on grapes surfaces at very low concentration (10-100 CFU/g) and detectable only after enrichment cultures (Rosini et al., 1982; Martini et al., 1996; Fleet et al., 2002; Mannazzu et al., 2002). On the contrary, it appears to be strictly associated with cellar environment (Rosini et al., 1988; Vaughan-Martini and Martini, 1995; Ciani et al., 1997, Ciani et al., 2004). Disputes concerning the natural habitat of *S. cerevisiae* have led to speculate on the use of this yeast in wine production (Martini et al., 1996, Mortimer and Polsinelli, 1999). In vinification, the ones that drive the whole alcoholic fermentation process are the pure cultures of *S. cerevisiae* selected strains. They are added to freshly pressed grape must ensuring a quickly depletation of the alcoholic fermentation and prevent slowdown fermentations and off-flavours production (Lambrechts and Pretorius, 2000; Swiegers et al., 2005; Tofalo et al., 2014). This procedure, adopted by most winemakers, guarantees defect-free products and ensures the same wine organoleptic characteristics every vintage. Nowadays, there are hundreds of commercial yeast cultures that can be used as starter. They must possess three fundamental properties:

- high fermentation power described as the maximum ethanol production in sugars excess conditions;

• high fermentation purity described as the ratio between volatile acidity production and ethanol formation - ratio that must tend to zero value;

• high fermentation rate evaluated during the first phases of fermentation.

The development of new technologies and exigent consumer requests have led to a continuous evolution of the selection criteria of starter strains for oenology. Indeed, the role of the yeast culture used in winemaking becomes fundamental not only for driving the fermentation process but also for other aspects involved in important oenological aspects such as:

• resistance to drying, keeping a high vitality;

• genetic stability;

• good resistance to sulfur dioxide;

• killer factor;

• type of development (e.g. pulverulent, flocculent, in aggregates);

• foaming power;

• ability to conduct fermentation at different temperatures;

• film-forming power;

• production of secondary fermentation compounds (e.g. acetaldehyde, glycerol, acetic acid, higher alcohols, sulfur compounds, aromatic compounds);

• autologous power;

• interaction with other yeast and malo-lactic bacteria.

The combination of these characteristics ensures the successful completion of the chemical-physical fermentation process, as well as the transformation of molecules present in the must and the formation of new metabolites, which can enrich the wine

at level sensory, increasing its organoleptic complexity and quality (Giudici and Zambonelli, 1992; Vincenzini et al., 2005; Tofalo et al., 2014).

1.2 The main by-products of fermentation that influence the flavor of wine

The organoleptic characteristics of the wine (appearance, aroma and flavor) come from a series of processes that occur during winemaking, from vineyard (varietal aromas) to the cellar (fermentation and post-fermentation aromas) (Lambrechts and Pretorius 2000; Swiegers and Pretorius 2005). In addition to the choice of grape variety, whose composition is significantly important for the final product (González-Barreiro et al., 2015), the choice of the microorganism to be used, as well as the fermentation conditions, are fundamental aspects for producing wines with specific aromatic bouquets (Lambrechts and Pretorius 2000; Swiegers et al., 2005; Swiegers et al., 2006). Indeed, during the alcoholic fermentation, *S. cerevisiae* releases aromatic metabolites, ex novo or from inactive precursors present in grape juice, which can negatively or positively influence the quality and sensorial properties of the wines. (Lema et al., 1996; Lambrechts and Pretorius, 2000; Swiegers and Pretorius, 2007). More than 1000 aromatic compounds have been identified, of which about 400 are produced by yeast metabolism (Nykanen, 1986). Ethanol and carbon dioxide, together with glycerol, organic acids, sulfur compounds, acetaldehyde, higher alcohols and volatile compounds are responsible for the "fermentation bouquets" (Rapp and Versini, 1991; Callejon et al., 2010), which, if present in excess, can contribute negatively to the wine quality.

Glycerol is responsible for the "body of wine" contributing to "fullness" and "sweetness" characteristics. *Saccharomyces* yeasts can produce from 2 to 10 g/L of glycerol, depending on the species and the belonging strain.

Acetaldehyde is the most important carbonyl compound that is formed during fermentation. It is an acetate, acetoin and ethanol precursor and plays a key role in isobutanol and active amyl alcohol biosynthesis. The acetaldehyde content in wine can vary from 10 to over 300 mg/L and the evaluation of its amount is used as an indicator of the degree oxidation. The acetaldehyde content is about 80 mg/L and 30 mg/L in white and red wines respectively. High concentration of acetaldehyde (100-125 mg/L) give pungent and irritating odor, while low concentration gives pleasant herbaceous and fruity aroma to the wine. The amount of acetaldehyde in wines depends on many factors: the medium composition, the substances used in must clarification process, the anaerobiosis conditions, the fermentation temperature, the sulfur dioxide and the yeast species employed (Romano, 2005).

Sulfur compounds include mainly hydrogen sulphide (H_2S) and sulfur dioxide (SO_2), coming from the reduction of sulfates present in the grape must. *S. cerevisiae* yeast produces hydrogen sulfide in different amounts (from few of 1mg/L to 4-5 mg/L) in relationship to the yeast strain used and the must composition. H_2S can participate in the production of disulfides and mercaptans, which lead to negative effects (such as rotten egg and burnt rubber) on the wine aroma (Zambonelli, 2003). Also, the production of SO_2 by yeasts is variable within the same specie and during the starter yeast selection, the preference should go to the strains that produce low levels of sulfur dioxide (10-20 mg/L maximum) to avoid off-flavour production (Vincenzini et

al., 2005) and negative effects on human health as described by FAO/OMS Committee Codex.

Higher alcohols are mainly represented by n-propanol, isobutanol, active amyl alcohol (2-methyl-1-butanol), isoamyl alcohol (3-methyl-1-butanol) and 2-phenylethanol. They are produced from the amino acids present in the must (threonine, valine, isoleucine, leucine, phenylalanine) and from glucose metabolism (Boulton et al., 1999, Eden et al 2001, Dickinson et al., 2003). In wines, the content of higher alcohols can vary from 100 to 500 mg/L: small quantities contribute positively, while too high quantities contribute negatively to the final wine quality (Amerine et al., 1980). The production of higher alcohols by yeasts is influenced by several factors: medium composition, oxygen availability, nitrogenous source, fermentation temperature and initial concentration of sugar. All yeasts are able to produce higher alcohols, but the quality and quantity of these compounds are yeast species dependent. *S. cerevisiae* and *Saccharomycodes ludwigii* are examples of high-producers, while *Hanseniaspora uvarum* and *Candida stellata* are examples of low producers of higher alcohols. The n-propanol and the isobutanol increase the volatility of other aromatic substances enhancing them (Romano, 2005). Isoamyl alcohol may produce a burnt odor if present in excess, while 2-phenylethanol liberates a characteristic floral rose fragrance (Swiegers et al., 2005; Vincenzini et al., 2005).

Volatile compounds synthesized by yeasts include volatile acids (cheese and sweat aroma), esters acetates and ethyl esters (fruity and floral aromas), aldehydes (aromas of butter, fruit and hazelnuts) (Stashenko et al. 1992, Lambrechts and Pretorius 2000, Delfini et al 2001, Russell, 2003). Also, in volatile compounds production, the must

composition, the fermentation conditions and the yeast strain used are factors that influence their content in the wine. The main ester is ethyl acetate which gives a typical aroma of vinegar and fruit. Concentrations ranging from 50 to 80 mg/L are favorable while more quantities are negative. The amount of ethyl acetate in wine is yeast specie-dependent (Rojas et al., 2001). Non-*Saccharomyces* yeasts are high producers of ethyl acetate (30-120 mg/L) compared to *S. cerevisiae* (Romano, 2005). Other volatile compounds are also important, such as ethyl butyrate (blackberry and blueberry aroma), ethyl hexanoate (pineapple aroma), ethyl octanoate (toasted fragrance, bread aroma), isoamyl acetate (banana flavour) and phenylethyl acetate (rose and honey notes) (Lee et al., 2012). Terpenes, such as linalool, geraniol and nerol (floral aroma) are involved in wine aromatic complexity (especially in young white wines) and their perception threshold levels are very low. Among norisoprenoids, β-damascenone is the most important, both for white and red wines and gives aromatic notes of exotic flowers (such as orchid, bougainvillaea, passionflower), cooked apples and tea (Guth, 1997).

1.3 *Terroir* concept in wine production

1.3.1 Definition of *Terroir*

The clear definition of *terroir* (French word derived from Latin '*terratorium*') often results difficult and ambiguous but substantially is related to three meanings (Rey et al., 1998):

- territoriality associated to the country region;
- a small ground area with specific qualities or agricultural properties;

- specific geographic area in which there are native people with their personality, spirit and lifestyle.

The *terroir* concept is expressed by a complex set of facets such as origin, persistence, specificity and personality, each described by different factors that define its uniqueness (Figure 1) (Vaudour, 2002).

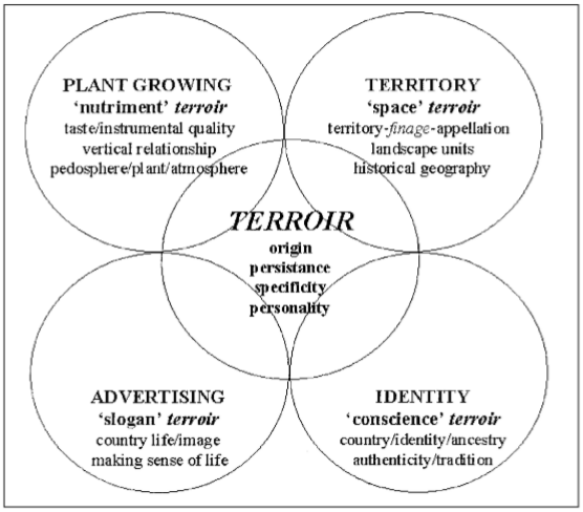

Figure 1. Terroir concept defined by different factors (Vaudour, 2002)

The "nutriment" factor is referred to the natural properties (technological and agronomical) and potentialities of a farmed environment, responsible of specific productions. In particular, regarding vine and wine farming, different wines should be characterized by distinctive taste and quality related to their geographical distribution and properties of terrains production (Vaudour, 2002). When the socio-economic context is combined with the concept just described, can be introduced the "space" *terroir* aspect (Dion, 1990; Unwin, 1991). The "conscience" *terroir* is referred to a specific geographical place with sociological, ideological, cultural, mythical, mystical aspects that describe the identity and the memory of the site. The dissemination, through advertising slogans, of typical, original and quality products

(Carbonneau, 1995, Bonnamour, 1999) combined with rural, ecological and community values (Letablier and Nicolas, 1994) of the production place, represent what is defined "slogan" *terroir*. A perfect combination of these factors represents the very heart of the *terroir* notion.

1.3.2 Grapes and wines associated with the *terroir* perspective

Grapes, as well as wine, are made up by numerous and different chemical compounds that characterize them. Long since, some studies highlighted many differences regarding chemical compounds composition (such as volatile compounds, phenols, free amino acids, trace elements) in relationship with the geographical distribution of the vineyard and winery (Mulet et al., 1992; Forcén et al., 1993; McDonald et al., 1998; Peña et al., 1999; Liu et al., 2008). These differences have been described not only between different countries but also within the same country, between distinct regions or areas (Mulet et al., 1992; Alvarez et al., 1997; Li and Hardy, 1999; Thiel et al., 2004; Martin et al., 2012; Geana et al., 2013).

Regarding the sensory analysis of wine coming from defined geographical location, when it was conducted thorough a panel test by competent judges the results showed differences in both space and time. This could be due to the different research conditions (such as duration of the research in time, number and size of samples, range of aromatic descriptors) and an approximate description of location of production. For these reasons, more precise descriptions of the area of production is necessary for a real and meticulous description about the geographical differentiation of wine aroma complexity and quality (Vaudour, 2002).

1.3.3 Microbial *terroir* in winemaking

The *terroir* concept as above described, must be revised including also a "microbial aspect" (Bokulich et al., 2014; Taylor et al., 2014). In the past, the role of the commensal microbial flora (bacteria and fungi species) that coexists with the grapevine plants has been ignored but they could be involved in key roles in winemaking, such as to influence flavour and productivity grapes and the organoleptic characteristics of wine (Gilbert et al., 2014). Compant et al. (2011) described the important role of bacteria and fungi associated with grapevine plants: they can contribute to health and plant productivity or used as biological control for grapevine pathogens. Bokulich and co-workers (2014) described the microbial community of four of the major wine growing regions in California and their results highlighted that different winegrowing regions maintain different microbial communities with some influences from the grape variety and the year of production considering the inter annual climate variations. Two years before, Setati and co-workers (2012) showed higher yeast heterogeneity in grape samples collected within and between vineyards located in the same geographical area but employed on different agronomic systems.

However, to better know the vine microbiome and its role in wine production, is necessary a greater characterization of the vine-associated microbiota and its potential regulatory functions.

Regarding the main wine yeast *S. cerevisiae*, numerous studies based on molecular approach, such as the study of DNA polymorphism, showed that indigenous *S. cerevisiae* strains are characterized by a high genetic diversity (Blanco et al., 2006; Agnolucci et al., 2007; Romano et al., 2008; Mercado et al., 2011) in relationship

with their geographical distribution. Knight and Goddard (2015), described *S. cerevisiae* populations with specific regional genotype without difference within the region suggesting that specific native strains could be associated with the *terroir* concept and the typical aroma profile in wines. In this regard, each wine yeast strain can influence the wine flavour by different aroma compounds production (Torrens et al., 2008) due to the metabolisation of flavour active compounds present in different grape varieties (Álvarez-Pérez et al., 2012). In this context, the biogeography signature of *S. cerevisiae* populations could be utilised by wine producers as valorization of their wines linked to the territory of production (Romano et al., 2003; Aa et al., 2006; Camarasa et al., 2011; Pretorius et al., 2012; Tofalo et al., 2013). This led an increasing demand of indigenous *S. cerevisiae* strains by winemakers, that could be used as starter yeasts to ensure the correct develop of the alcoholic fermentation and to give the representative imprinting of the viticultural area of production (Callejon et al., 2010; Orlić et al., 2010). Therefore it has become very important to analyze the *S. cerevisiae* strains diversity like a strategy to select local wine yeasts, potentially better adapted to each oenological area (Guillamón et al., 1996; Torija et al., 2001; Capece et al., 2016) with the purpose to obtain wines with chemical and sensory properties that could reflect their geographic origin (Valero et al., 2005; Villanova and Sieiro,2006; Callejon et al.,2010; Tofalo et al., 2014; Capozzi et al., 2015).

2. Aim of the work

In the last few years, the attention of winemakers has been focused to obtain wines with “typical” sensory properties related to the specific area of production. The use of indigenous *S. cerevisiae* yeast as a starter strain, isolated from the environment and with oenological characteristics related to the territory of vineyard, seems to be an interesting approach to obtain wines with geographically recognizable aroma complexity. This aspect is in agree with the consumers’ demand, which requires high quality, originality and territoriality products combined with natural wine obtained by vineyards and winemaking practices environmentally friendly.

The aim of this study was to assess the oenological properties of two indigenous yeasts isolated from grapes of Verdicchio and Pecorino varieties and identified as *S. cerevisiae*. These two varieties, together with Passerina, represent native grape cultivars of the Marche region (Italy), cultivated in winemaking area of Ascoli Piceno.

In the first step a genotyping analysis was performed to confirm the unique profile of the two unknown yeasts, about interdelta sequences, when compared with those obtained from the commercial strains. Subsequently, microvinification trials were carried out using organic Verdicchio must, evaluating their fermentation power, expressed like the maximum ethanol production in a sugars excess condition, and evaluating their fermentative performance at 22°C and 16°C. Fermentation kinetics were evaluated during alcoholic fermentation, as well as the main analytical characteristics and volatile compounds of the final wines. The results were compared with those obtained by commercial strains used as controls and subjected at the same fermentation parameters.

Overall, the results obtained highlight a genotypic and phenotypic specificity of the two indigenous *S. cerevisiae* yeasts that could be proposed as a new starter cultures to characterize the bouquet complexity of wine belonging to the Piceno DOC area.

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Article

Fitness of Selected Indigenous *Saccharomyces cerevisiae* Strains for White Piceno DOC Wines Production

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Abstract: Verdicchio, Passerina and Pecorino are native grape cultivars of the Marche region, cultivated in winemaking area of Ascoli Piceno, in central Italy. In particular, Passerina and Pecorino varieties have been abandoned and forgotten for a long time and only in recent years are rediscovered and appreciated. Here, two indigenous yeasts, isolated from grapes of Verdicchio and Pecorino varieties and identified as *Saccharomyces cerevisiae*, were evaluated and compared with commercial starters cultures widely used for the vinification of Piceno DOC (Denominazione Origine Controllata) area at industrial scale. A polyphasic approach, including yeast genotyping, phenotypic traits evaluation and fermentative performance in natural grape juice, was applied to evaluate the fitness of strains. Using interdelta primers, the two selected cultures showed a unique profile while the results of microvinifications showed that both indigenous strains exhibited good enological parameters and fermentative aptitude comparable with that shown by commercial strains used as controls. The profile of volatile compounds of wines of indigenous strains was characterized by a significant high production of isoamyl acetate and ethyl esters at 22 °C and phenyl ethyl acetate at 16 °C. Overall results indicate that the two indigenous selected yeasts showed a genetic and phenotypic specificity and they could be profitably used to characterize the Piceno DOC area wines.

Keywords: *Saccharomyces cerevisiae*; indigenous yeast; wine; starters

1. Introduction

The territory of Piceno is one of the areas with the greatest wine vocation within Marche region, in the center of Italy. The Piceno territory was initially known for its red wines, the first to obtain national awards, progressively combined by native white wines, rediscovered by local producers and winemakers who reassessed their potential. In particular, the Offida DOCG (Denominazione Origine Controllata Garantita) wines refer to Pecorino and Passerina varieties that originate wines characterized by a straw yellow color with greenish and yellowish reflections, respectively, a good level of acidity, smell of floral notes (white flowers), fruity pineapple, hints of anise and sage, taste are fresh and mineral sapid. Fermentation of these white wines are generally carried out at low temperatures (from 16 °C to 22 °C). In the last years, as reported by "Consorziovinipiceni" [1], Passerina wines have undergone the greatest increase in the sale of bottles in Italy in 2015. In particular, the official data

showed that Passerina and Pecorino increased by 34.2% and 19.9%, respectively.

Quality perception is a combination of both sensory and chemical approaches that influence wine experts and consumers. The aroma of wine is a combination of volatile compounds originating from grapes (varietal aromas), secondary products formed during the wine fermentation (fermentative aromas) and aging (post-fermentative aromas) [2]. The main volatile compounds are produced by yeast during alcoholic fermentation, and significantly impact the flavor and overall quality of wines [3].

Molina et al. [4] evaluated the influence of fermentation temperature on the production of yeast-derived aroma compounds together with the expression of genes involved, during the fermentation of a defined must at 15 and 28 °C. They found that the production of volatile aroma compounds varied according to yeast growth stage. In particular, higher concentrations of compounds related to fresh and fruity aromas were found at low temperature, while higher concentrations of flowery related aroma compounds were found at 28 °C. In addition, Torijia et al. [5] demonstrated that low temperatures restricted yeast growth and lengthened the fermentations. In particular, low temperature alcoholic fermentations are becoming more frequent due to the wish to produce wines with more pronounced aromatic profiles. The yeast influence on wine aroma is not only species-dependent but relevant variations are also showed at strain level [6,7]. In this regard, each wine yeast strain produces different aroma compounds, higher alcohols, acetate esters, ethyl esters and aldehydes that characterize the final wine bouquet [4]. Indeed, different strains of *S. cerevisiae* can metabolize flavor active compounds that characterized different grape varieties to produce volatile compounds [8]. For this reason, the choice of wine yeast is crucial for the development of the desired wine style. On the other hand, after the wide diffusion of the use of selected *S. cerevisiae* starter cultures, many studies were conducted with the aim to select, from various habitats, yeast strains with physiological characteristics functional to be used as commercial starter. In recent years, there is increasing interest among winemakers to select local strains with the aim to select starter cultures potentially well adapted to specific grape must reflecting the biodiversity of a given region, which support the notion that specific indigenous yeast strains can be associated with a "*terroir*" [9–13]. The use of indigenous yeast strains that possess suitable oenological characteristics and also closely related to the territory of vineyard, is desirable for their better adaptation to the environmental conditions and may contribute to the maintenance of the "typical" sensory properties of the wines of each specific region. On this basis, the aim of this work was to characterize two selected indigenous yeast strains isolated from vineyards of Piceno DOC area and to evaluate the fermentative fitness in comparison with the commercial starter strains widely used for the production of Piceno DOC wines.

2. Materials and Methods

2.1. Yeast Strains

Ten different commercial *S. cerevisiae* starter strains were used to compare the genomic differences at strain level of two indigenous *S. cerevisiae* (Pe1 and G4). The commercial dry starter used were: Lalvin EC1118, Lalvin ICV OKAY, NEM, Enoferm BDX, Uvaferm CM, BC, CEG, and VRB YSEO (Lallemand Inc., Toulouse, France); VIN13 (Anchor Wine Yeast, Cape Town, South Africa); and Zymaflore F15 (Laffort, Bordeaux Cedex, France). All these dry yeasts were rehydrated following the manufacture instructions and the isolated pure cultures were used for genetic and physiological evaluation essays. For the fermentation trials, three starter cultures, VIN13, OKAY and EC1118, were used as control strains.

The selected indigenous *S. cerevisiae* (Pe1 and G4) came from Pecorino and Verdicchio grapes in the Offida DOC area. These yeasts were previously identified by D1 and D2 26S-DNA sequence [14].

All of the yeast strains were maintained at 4 °C for short-term storage on yeast extract–peptone–dextrose (YPD) agar medium (Oxoid, Basingstoke, UK) and for long-term storage in YPD broth supplemented with 80% (*w*/*v*) glycerol at −80 °C.

2.2. Molecular Identification and Characterization of the S. cerevisiae Isolates

Pure yeast cultures of all commercial *S. cerevisiae* and two indigenous selected culture were pre-grown on YPD agar at 25 °C for 3–4 days. The cells were then transferred to screwcap tubes with 5 µL of water molecular biology grade and the DNA was extracted at 95 °C for 10 min in Biorad Thermal Cycler [15]. PCR amplifications were carried out as described by Legras and Karst [16] using primer delta 2 (5′-GTGGATTTTTTATTCCAAC-3) and primer delta 12 (5′-TCAACAATGGAATCCCAAC-3′).

The amplification reactions were performed with a Biorad Thermal Cycler, using the following programme: 4 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 46 °C, and 90 s at 72 °C, and the final extension at 72 °C for 10 min. Amplification products were separated by electrophoresis on 1.5% (*w*/*v*) agarose gels submitted to 100 V for 1 h in 0.5×TBE buffer.

2.3. Fermentation Power Assay (Maximum Ethanol Production)

To determine the fermentation power of OKAY, VIN13, EC1118, Pe1 and G4, grape juice coming from vintage 2016 was adjusted to 30% sugar content with sucrose (Carlo Erba, reagents S.r.l., Comaredo, Milano). The trials were carried out on 70 mL of pasteurized Verdicchio grape juice at 25 °C under static condition in triplicate. The pasteurization process was carried out at 100 °C for 10 min. All strains were pre-cultured in modified YPD (0.5% *w*/*v* yeast extract, 2% *w*/*v* glucose, 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 approximately 1×10^6 cells/mL for each yeast.

2.4. Microfermentation Trials

To evaluate the fermentative aptitudes of the indigenous *S. cerevisiae* strains, two sets of fermentation trials were carried out at different fermentation temperatures (22 °C and 16 °C) on Verdicchio grape juice coming from vintage 2015. The Verdicchio grape juice had the following main analytical composition: pH 3.26; initial sugar content 217 g/L; total acidity 4.53 g/L; and nitrogen content YAN (111 mg/L). The fermentation trials were carried out in flasks containing 350 mL of Verdicchio grape juice. 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 or 16 °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 approximately 1×10^6 cells/mL for each yeast. As control strains, VIN13, OKAY and EC1118 were 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).

2.5. Analytical Procedures

To determine the glycerol and sugar concentrations, specific enzyme kits (Megazyme International Ireland, Wicklow, Ireland) were used. Ethanol content, volatile acidity and total SO_2 were measured using the current analytical methods according to the Official European Union Methods [17]. Ammonium was determined using an enzymatic method (kit no. 112732; Roche Diagnostics, Germany), while the free α-amino acids were quantized following the procedures described by Dukes and Butzke [18], using the *o*-phthaldialdehyde/*N*-acetyl-l-cysteine spectrophotometric assay.

Yeast assimilable nitrogen was calculated as the sum of the concentrations of the free α-amino acids and the ammonium.

Fermentation power (FP) indicates the maximum amount of ethanol (as % *v*/*v*) in the presence of an excess of sugar. Fermentation vigor (FV) was evaluated as the amount of carbon dioxide produced after 3 days of fermentation [19].

Acetaldehyde, ethyl acetate, n-propanol, isobutanol, amyl and isoamyl alcohols and acetoin, were quantified by direct injection into a gas–liquid chromatography system. Each sample was prepared and analyzed as reported by Canonico et al. [20].

The volatile compounds were determined by solid-phase microextraction (HS-SPME) method. Each sample (5 mL) was placed in vial containing 1 g NaCl closed with a septum-type cap. HS-SPME was carried out under magnetic stirring for 10 min at 25 °C. After this period, an amount of 3-octanol as the internal standard (1.6 mg/L) was added and the solution was heated to 40 °C and extracted with a fiber Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) for 30 min by insertion into the vial headspace. The compounds were desorbed by inserting the fiber into Shimadzu gas chromatograph GC injector for 5 min. A glass capillary column was used: 0.25 µm Supelcowax 10 (length, 60 m; internal diameter, 0.32 mm). The fiber was insert in split-splitless modes, as reported by Canonico et al. [20].

2.6 *Statistical Analysis*

The experimental data related to wines were elaborated by one-way ANOVA. The means were analyzed using the STATISTICA 7 (Statsoft, Tulsa, OK, USA). The significant differences were determined using Duncan tests, and the data were considered significant if the associated p values were <0.05. Principal component analysis (PCA) was used to discriminate among the means of volatile compounds. The PCA was carried out using the Unscrambler 7.5 software (CAMO ASA, Oslo, Norway). The mean data were normalized to neutralize any influence of hidden factors.

3. Results

3.1. Biotyping of the Selected Cultures

he results of biotyping carried out using interdelta primers are reported in Figure1. The comparison with ten of the most diffused *S. cerevisiae* commercial strains widely used during Piceno DOCG fermentations indicated that the indigenous strains Pe1 and G4 showed unique profiles and can be easily distinguished from the other strains.

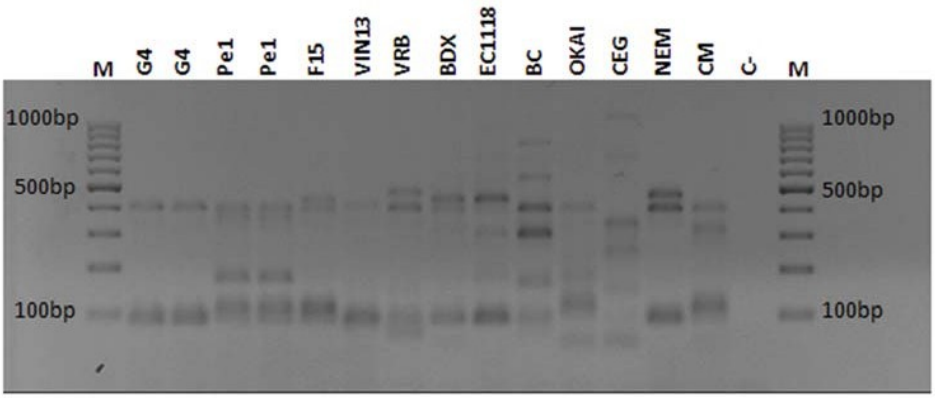

Figure 1. Molecular characterization of commercial and indigenous *S. cerevisiae*. PCR inter-δ primers were used to amplify the corresponding genes of strains. The representative gel shows the profiles of these selected strains; indigenous strains are reported in duplicate. Lane M: Gene ruler 100 bp (Fermentans), as indicated on the left and right of gel. Lane C-: indicated as negative control.

3.2. Fermentation Power (FP) and Fermentation Vigour(FV) Test Assay

Results of microfermentation trials carried out to determine FP (fermentation power) and FV (fermentation vigor) are shown in Table1. Under the condition tested, VIN13 and EC1118 showed the best performance achieving a FP higher that 14.0% *v*/*v* of ethanol. They were followed by Pe1 with FV of 13.8% *v*/*v*, OKAY (13.0% *v*/*v*) and G4 (12.2% *v*/*v*) with variable residual sugars. Regarding to the evaluation of FV, the best performance was exhibited by Pe1 (4.5 g evolved by the apparatus in the first three days of fermentation) followed by VIN13 (4.0 g) and the other strains that showed lower FR. Moreover, Figure2shows the lower fermentation kinetics of G4 strain in comparison with the other strains tested, while Pe1 exhibited an intermediate behavior between the *S. cerevisiae* commercial strains.

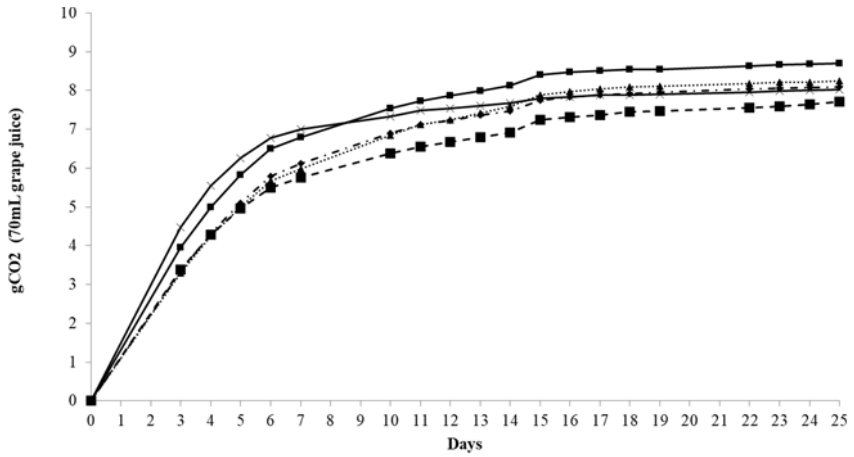

Figure 2. Fermentation power (FP) of wines: OKAY ( ); VIN13 ( ); EC1118); Pe1 ( ); and G4 ( ).

Table 1. Fermentation power and fermentation vigor in high sugar Verdicchio grape juice (fermentation power assay).

Strains	Ethanol (% *v*/*v*)	Sugar residual (g/L)	*Fermentation rate (gCO_2/3days)
OKAY	13.05±0.27^{c}	24.04±2.75^{bc}	3.34±0.26^{b}
VIN13	14.34±0.10^{a}	7.36±2.92^{d}	3.95±0.34^{ab}
EC1118	14.23±0.07^{a}	21.09±0.80^{c}	3.30±0.16^{a}
Pe1	13.78±0.10^{b}	27.38±2.34^{b}	4.47±0.01^{a}
G4	12.15±0.06^{d}	35.90±0.21^{a}	3.38±0.40^{b}

* Evaluated at Day 3 of fermentation (g of CO_2 evolved by 350 mL of substrate). Data are means standard deviations. Statistical analysis did not include the base wine. Data with different superscript letters (^{a,b,c,d}) within each column are significantly different (Duncan test; $p < 0.05$).

3.3. Microfermentation Trials Carried Out at 16 °C and 22 °C

The results of fermentation kinetics of microfermentations carried out at 16 °C and 22 °C are reported in Figure3. The data of the fermentation trials carried out at 22 °C showed comparable fermentation kinetics among all *S. cerevisiae* strains tested, highlighting no differences between the indigenous and commercial strains. Only Pe1, after 16 days of fermentation, exhibited higher fermentation kinetics in comparison with the other strains.

The fermentations carried out at 16 °C showed slower fermentation kinetics in comparison with the same trails carried out at 22 °C without relevant differences among the strains tested (31 days instead 22 days at 22 °C). However, OKAY showed slower fermentation kinetics in comparison with the other strains. Therefore, in both fermentation conditions, the two indigenous strains exhibited a kinetics comparable to that showed by *S. cerevisiae* commercial starter strains.

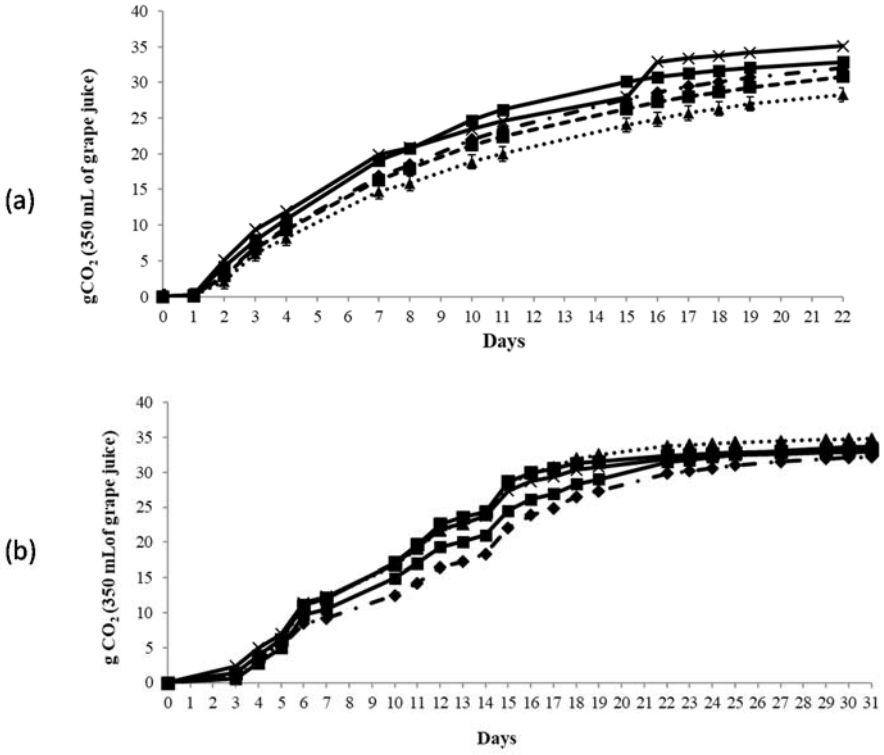

Figure 3. Fermentation kinetics of wines at two different temperature: (**a**) 22 °C; and (**b**) 16 °C OKAY ( ); VIN13 ( ); EC1118 ( ); Pe1 ( ); and G4 ( ).

3.4. Main Analytical Characteristics

To evaluate the main analytical characteristics, two sets of microfermentation trials at 16 °C and 22 °C were carried out in Verdicchio natural grape juice. Three commercial strains, OKAY, EC1118 and VIN13, widely used in Piceno DOCG wines fermentation, were chosen as controls. Results shown in Table2 indicated that the main analytical compounds did not generally show relevant differences among the strains. Only G4 strain showed a slight increase of volatile acidity (0.64 g/L at 22 °C) while both indigenous yeasts exhibited a significant increase in glycerol production at 16 °C. A decrease of total SO_2 production was seen for all strains at 22 °C.

3.5. Main Volatile Compounds

The evaluation of the main volatile compounds produced by *S. cerevisiae* strains revealed significant differences among the yeast cultures also strongly influenced by the fermentation temperature (Table3).

The commercial strain OKAY at 22 °C showed significant higher production of esters such as ethyl butyrate, isoamyl acetate, phenyl ethyl acetate (supplementary material) and higher alcohols as *n*-propanol and β-phenyl ethanol (Table3). At 22 °C, Pe1 and G4 fermentation trials were characterized by high amounts of isoamyl acetate and in general of ethyl esters (ethyl hexanoate and ethyl octanoate). The other strains showed intermediate production of esters. EC1118 strain was characterized by a high production of acetaldehyde, isobutanol, amyl and isoamyl alcohols at both temperatures of fermentation (22 °C and 16 °C). Moreover, at 16 °C, EC1118 showed relevant and generalized enhancement of esters. In microfermentations carried out at 16 °C, some differences in esters and higher alcohols were found. With the exception of OKAY, the strains showed a generalized increase of esters at lower temperature. However, in Pe1 and G4 indigenous strains, there was a reduction of isoamyl acetate and a significant enhancement of phenyl ethyl acetate. Of particular relevance was the high production of acetaldehyde at both temperatures tested by EC1118 that could be negatively affect the analytical profile of white wines.

Table 2. The main analytical characteristics of each *S. cerevisiae* strains on Verdicchio grape juice.

	22 °C						16 °C					
Strains	**Ethanol (% *v/v*)**	**Volatile Acidity (Acetic Acid g/L)**	**Glycerol (g/L)**	**SO_2 (mg/L)**	**Residual Sugar (g/L)**	**Fermentation Rate (g CO_2/3 Days)**	**Ethanol (% *v/v*)**	**Volatile Acidity (Acetic Acid g/L)**	**Glycerol (g/L)**	**SO_2 (mg/L)**	**Residual Sugar (g/L)**	**Fermentation Rate (g CO_2/3 Days)**
OKAY	12.26 ± 0.00 ^{a}	0.38 ± 0.03 ^{b}	4.77 ± 0.39 ^{a}	5.76 ± 1.35 ^{ab}	0.04 ± 0.00 ^{a}	6.97 ± 1.36 ^{b}	11.77 ± 0.07 ^{b,c}	0.53 ± 0.04 ^{a}	4.56 ± 0.08 ^{e}	14.12 ± 1.30 ^{b}	0.03 ± 0.02 ^{a}	1.28 ± 0.04 ^{b,c}
VIN13	12.00 ± 0.06 ^{a}	0.38 ± 0.03 ^{b}	3.81 ± 0.05 ^{b}	7.67 ± 0.91 ^{a}	0.01 ± 0.00 ^{c}	7.97 ± 0.46 ^{a,b}	12.45 ± 0.21 ^{a}	0.41 ± 0.04 ^{b}	5.47 ± 0.13 ^{c}	14.88 ± 0.22 ^{b}	0.01 ± 0.00 ^{a}	1.39 ± 0.34 ^{b}
EC1118	11.56 ± 0.27 ^{b}	0.44 ± 0.03 ^{b}	4.46 ± 0.13 ^{a}	0.16 ± 0.22 ^{c}	0.01 ± 0.01 ^{b,c}	6.04 ± 0.88 ^{b}	11.33 ± 0.23 ^{c}	0.38 ± 0.01 ^{b}	4.99 ± 0.04 ^{d}	16.39 ± 0.09 ^{a,b}	0.04 ± 0.03 ^{a}	0.71 ± 0.19 ^{b,c}
Pe1	11.96 ± 0.05 ^{a}	0.40 ± 0.05 ^{b}	4.57 ± 0.10 ^{a}	3.36 ± 0.67 ^{b}	0.04 ± 0.01 ^{a,b}	9.41 ± 0.40 ^{a}	11.50 ± 0.21 ^{b,c}	0.44 ± 0.04 ^{b}	6.42 ± 0.12 ^{a}	16.48 ± 2.50 ^{a,b}	0.02 ± 0.01 ^{a}	2.37 ± 0.46 ^{a}
G4	11.96 ± 0.00 ^{a}	0.64 ± 0.02 ^{a}	4.93 ± 0.07 ^{a}	9.44 ± 2.94 ^{a}	0.02 ± 0.02 ^{a,b,c}	6.75 ± 0.48 ^{b}	11.92 ± 0.11 ^{b}	0.54 ± 0.01 ^{a}	5.82 ± 0.02 ^{b}	18.56 ± 0.45 ^{a}	0.01 ± 0.00 ^{a}	0.57 ± 0.19 ^{c}

Data are means ± standard deviations. Data with different superscript letters (^{a,b,c,d,e}) within each column are significantly different (Duncan test; $p < 0.05$).

Table 3. The main volatile compounds of each *S. cerevisaie* strains on Verdicchio grape juice

	22°C					16°C				
Esters	**OKAY**	**VIN13**	**EC1118**	**Pe1**	**G4**	**OKAY**	**VIN13**	**EC1118**	**Pe1**	**G4**
Ethyl acetate	23.46±0.51^{b}	20.51±0.10^{c}	33.25±0.01^{a}	14.47±0.33^{e}	16.74±0.15^{d}	25.53±0.15^{b}	25.62±0.46^{b}	31.03±0.37^{a}	15.92±0.12^{c}	15.95±0.53^{c}
Alchols										
n-propanol	40.54±0.20^{a}	18.41±0.16^{c}	19.62±0.06^{b}	13.76±0.04^{e}	15.705±0.20^{d}	41.70±0.24^{a}	24.58±0.01^{b}	21.34±0.31^{c}	16.00±0.49^{e}	19.95±0.56^{d}
Isobutanol	5.166±0.17^{d}	7.049±0.08^{c}	13.681±0.02^{a}	6.777±0.20^{c}	9.548±0.30^{b}	10.20±0.50^{b}	7.41±0.37^{c}	11.88±0.30^{a}	8.44±0.46^{c}	11.60±0.58^{a}
Amilyc alchol	13.35±0.05^{d}	14.70±0.31^{c}	18.82±0.53^{a}	11.45±0.02^{e}	16.71±0.04^{b}	10.67±0.40^{c}	12.34±0.30^{b}	10.77±0.07^{c}	12.43±0.51^{b}	14.14±0.62^{a}
Isoamilyc alchol	94.43±0.47^{c}	96.04±0.40^{b}	105.49±0.30^{a}	80.38±0.48^{e}	82.54±0.25^{d}	74.55±0.37^{d}	80.82±0.74^{c}	112.43±2.87^{a}	61.91±0.59^{e}	88.64±0.36^{b}
β-Phenyl ethanol	34.95±7.02^{a}	11.32±0.32^{b}	13.03±0.36^{b}	11.04±1.12^{b}	12.13±0.77^{b}	11.25±0.11^{c}	14.3±0.09^{b}	16.7±0.05^{a}	10.82±0.01^{c}	13.2±0.03^{b}
Carbonil compounds										
Acetaldehyde	10.57±0.14^{e}	20.38±0.48^{d}	91.33±0.29^{a}	53.77±0.12^{b}	32.355±0.24^{c}	13.07±0.44^{d}	19.56±0.40^{c}	134.901±0.80^{a}	30.86±2.67^{b}	9.90±0.13^{d}
Acetoin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Data are means ± standard deviations. Data with different superscript letters (^{a,b,c,d,e}) within each row are significantly different (Duncan test; $p < 0.05$). ND = not detected.

The data on the volatile compounds were subjected to Principal Component Analysis (PCA) (Figure4). The distribution of strains in the biplot graphic highlighted two main effects. PC2 grouped the strains in function of the fermentation temperature separating 16 °C trials upper quadrants from the 22 °C trials (lower quadrants). Only Pe1 was placed close to the center. On the other hand, PC1 differentiated the strains in function of the volatile compounds assayed. At 22 °C, indigenous strains were separated from the other strains mainly for isoamyl acetate production. At 16 °C, all strains were separated for ethyl hexanoate with the exception of Pe1 strain.

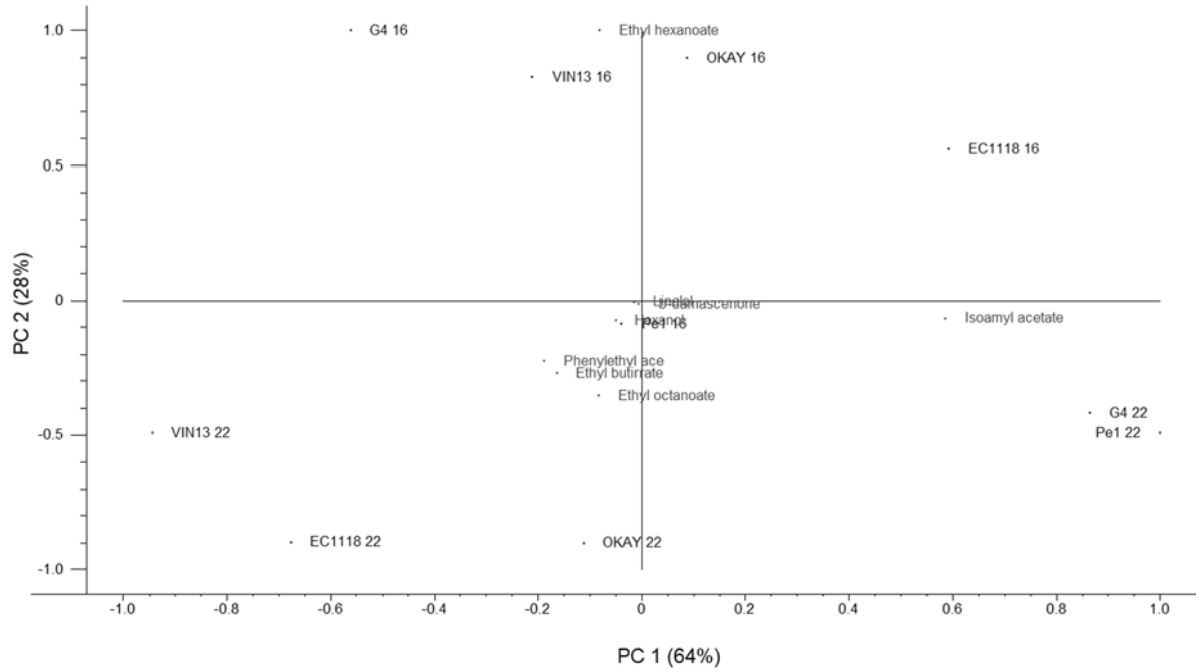

Figure 4. Principal component analysis based on the data for the volatile compounds in the wines produced by different *S. cerevisiae* strains. The numbers 16 and 22 associated to each strain refer to the fermentation temperature (16 °C and 22 °C).

4. Discussion

In recent years, geographic distribution of microbial community of wine grapes has been investigated revealing regional microbial signatures and the geographic delineation of yeast communities with wines [21,22]. This distribution is conditioned by several factors such as cultivar, vintage and climate and could be influenced by agricultural practices [15,23,24]. In addition, preliminary evidence on the association of wine microbiome and phenotype at regional level suggests a microbial contribution to regional characteristic defined as "*terroir*" [25,26]. Indeed, there is preliminary evidence that yeast biota related to specific vineyard strongly influence the final wine composition [15,21,27–29]. Actually, the concept of the "indigenous" yeast is related to the preservation of microorganism naturally present in grapes and winery.

In this context, we evaluated the enological aptitude of two *S. cerevisiae* isolated from the regional winemaking areas Piceno DOC. The molecular characterization using the polymorphism of inter-δ regions showed specific patterns of selected *S. cerevisiae* strains isolated from Offida DOCG area in comparison with the most common *S. cerevisiae* commercial strains tested.

The same approach was used by Schuller et al. [30] to investigate the intraspecific genetic diversity of *S. cerevisiae* associated with the vineyard environment. To assess the oenological aptitude of two *S. cerevisiae* native Pe1 and G4 strains, microfermentations on natural grape juice were carried out. The influence of indigenous *S. cerevisiae* strains on the analytical and volatile compositions of wine

was investigated for Montepulciano d'Abruzzo and Malvar winemaking areas. In both investigations, distinctive analytical profiles of wines were shown [31,32].

In this work, the two native strains Pe1 and G4 did not differ significantly from the compared commercial strains for the main analytical characteristics. On the other hand, they showed a specific volatile profile that could be useful to further characterize Piceno DOC wines, emphasizing their aromatic composition. In this regard, we found that the fermentation temperature plays an important role on flavor of final wine. Indeed, an increase of some volatile compounds, such as isoamyl acetate, ethyl hexanoate and ethyl octanoate, in microfermentations carried out at 22 °C was exhibited by two indigenous strains. Similarly, a reduction of total SO_2 production was found at 22 °C in comparison with 16 °C fermentation trials without significant differences between the two indigenous and commercial starter strains.

In conclusion, the present work emphasizes the potential use of selected indigenous *S. cerevisiae* for the fermentation of Piceno DOC wines. In particular, these selected strains (Pe1 and G4) could be used as a suitable strategy to influence the aromatic complexity of Piceno DOC wines.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2311-5637/4/2/37/s1 .

Author Contributions: A.A., L.C., M.C. and F.C. contributed equally to this manuscript. All authors participated in the design and discussion of the research. A.A. carried out the experimental part of the work. A.A., L.C., M.C. and F.C. carried out the analysis of the data and wrote the manuscript. All the authors have read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Supplementary material: The main volatile compounds of each *S. cerevisiae* strains on Verdicchio grape juice

Volatile compounds (mg L^{-1})	22 °C					16 °C				
Esters	OKAY	VIN13	EC1118	Pe1	G4	OKAY	VIN13	EC1118	Pe1	G4
Ethyl butyrate	0.904±0.07^{a}	0.166±0.02^{b}	0.186±0.00^{b}	0.074±0.00^{c}	0.144±0.01^{bc}	0.629±0.02^{a}	0.225±0.05^{b}	0.302±0.04^{b}	0.614±0.05^{a}	0.265±0.05^{b}
Phenylethylacetate	0.906±0.08^{a}	0.251±0.01^{b}	0.228±0.00^{b}	0.323±0.05^{b}	0.294±0.01^{b}	0.203±0.04^{c}	0.623±0.04^{b}	0.929±0.01^{a}	0.843±0.05^{a}	0.717±0.05^{b}
Ethyl hexanoate	0.096±0.04^{ab}	0.103±0.02^{ab}	0.054±0.01^{b}	0.120±0.01^{a}	0.154±0.02^{a}	1.13±0.01^{b}	0.938±0.01^{c}	1.68±0.04^{a}	0.566±0.01^{d}	0.973±0.00^{c}
Ethyl octanoate	0.151±0.02^{ab}	0.098±0.00^{c}	0.172±0.01^{a}	0.126±0.01^{bc}	0.110±0.01^{b}	0.001±0.00^{c}	0.065±0.00^{a}	0.006±0.00^{c}	0.019±0.00^{b}	0.009±0.00^{c}
Isoamylacetate	1.624±0.10^{a}	0.156±0.02^{b}	0.243±0.03^{b}	1.712±0.01^{a}	1.563±0.19^{a}	1.67±0.27^{b}	1.159±0.06^{c}	4.34±0.25^{a}	1.66±0.06^{b}	0.800±0.07^{c}
Alchols										
Hexanol	0.074±0.01^{a}	0.051±0.00^{b}	0.072±0.00^{a}	0.045±0.00b^{c}	0.038±0.01^{c}	0.075±0.00^{ab}	0.085±0.01^{ab}	0.089±0.01^{a}	0.070±0.01^{b}	0.075±0.00^{c}
Terpens										
Linalol	0.058±0.02^{a}	0.024±0.00^{b}	0.004±0.00^{b}	0.014±0.01^{b}	0.021±0.01^{b}	0.034±0.00^{b}	0.038±0.00^{b}	0.023±0.00^{c}	0.034±0.00^{b}	0.041±0.00^{a}
B-damascenone	0.021±0.01^{a}	0.006±0.00^{b}	0.007±0.00^{b}	0.009±0.00^{ab}	0.011±0.01^{ab}	0.002±0.00^{c}	0.014±0.00^{b}	0.004±0.00^{c}	0.025±0.00^{a}	0.019±0.00^{ab}

Data are data means ± standard deviations. Data with different superscript letters ($^{a, b, c, d}$) within each row are significantly different (Duncan test; P <0.05)

PART III

Selective hybridization of *Saccharomyces cerevisiae* oenological strains and its characterization in winemaking

1. Introduction

1.1 Life cycle of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, the yeast commonly used in enology, is characterized by a complex life cycle (Landry et al., 2006) which includes two types of reproduction: asexual or sexual. In asexual reproduction, or cell proliferation, the yeast reproduces by budding, giving rise to an ellipsoidal daughter cell, identical to the mother cell (Barton, 1950; Agar and Douglas, 1955; Nurse, 1985) (Figure 1). In the presence of optimal nutritional conditions, yeast cells double in number every 100 minutes.

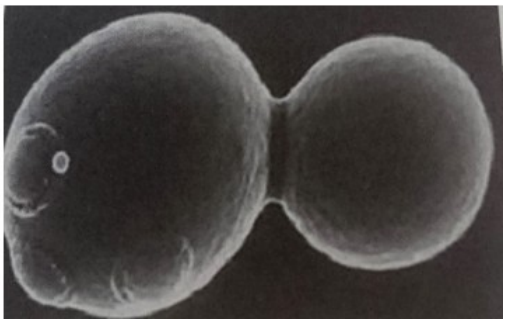

Figure 1. Cell proliferation of *S. cerevisiae* yeast.

In cell proliferation, the cell cycle is characterized by four phases: G1 phase, in which the cell monitors its dimensions, the external environment and synthesizes RNA and proteins; S phase, in which the replication of the chromosomes takes place; G2 phase, in which the cell controls the replication of all the chromosomes and prepares itself for mitosis; M phase, which includes mitosis and cytokinesis. During mitosis step the chromosomes of the cell are divided between the two daughter cells, while during cytokinesis, the cytoplasm division occurs (Michaelis & Herskowitz,

1988). In particular conditions, such as the lack of nutrients available, the cell cycle arrests in G1 phase with consequence stop of cell proliferation. Cell proliferation can restart with the restoration of favorable environmental conditions. Cell proliferation can stop even in presence of neighboring cells with different mating type and then they undergo to fusion (Herskowitz, 1988). Strains of *S. cerevisiae* can be stably haploid, with 16 chromosomes, diploids, with 32 chromosomes or polyploids with *n*-copies of chromosomes.

Regarding sexual reproduction, the cells can originate haploid spores by meiosis process, that led to a reduction of the chromosomal equipment in a single copy (haploid equipment). Moreover, through the so-called crossing-over process, the cells occur in a genetic recombination of the chromosomes, one of the main events involved in the evolution of eukaryotic organisms. The spores can be of two sexual types (mating types): MATa and MATα. The mating type of haploid cells are defined by the allele, a or α, present in the mating-type locus *MAT*, located in the chromosome III (Strathern et al. 1982). MATα cells produce a peptide of 13 aminoacids, the sexual factor α, while MATa cells produce a peptide of 12 aminoacids, the sexual factor a. These peptides represent coupling factors that facilitate cell fusion. In particular, haploid cells of opposite sex can combine and give rise to diploid cells a/α (Herskowitz, 1988) (Figure 2). In optimal nutritional conditions, the a/α cells can continue to develop and divide, maintaining their diploid state.

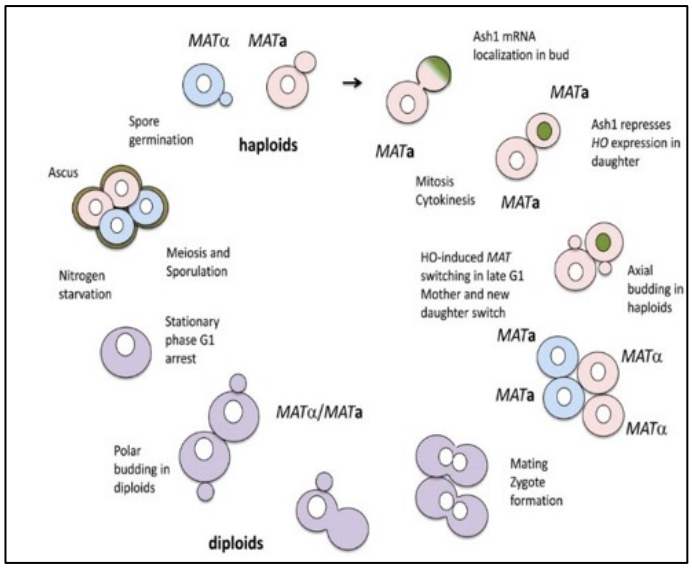

Figure 2. Sexual reproduction of *S. cerevisiae*

1.1.1 Homothallic and heterothallic yeasts

Under conditions of nutritional deficiencies, the diploid cell a/α, through the meiosis process, can produce a bag-like called asco with four spores inside, two MATa and two MATα. The mature spores can emerge from the asco, germinate and begin a haploid life cycle. The stability of mating type specificity is influenced by the *HO* gene that encode for an enzyme with endonuclease activity (Mortimer and Hawthorne 1969; Strathern et al. 1982). In heterothallic strains the mating type specificity is stable and they are characterized by non-functioning version of this gene (*ho*), on the contrary, in homothallic strains cells of one mating type switch frequently to the other mating type due to the active function of the gene *HO* (Rabin 1970; Oshima and Takano 1971; Hicks and Herskowitz, 1976). For this reason, homothallic yeasts are able to self-diploidizing. The endonuclease anzyme drive the mating-type switch, catalyzing a double-strand break at the MAT locus, this leads to

a programmed gene rearrangement event that restore the mating information but with opposite meaning. This is due to a shift on the MAT locus and subsequent activation of silent loci, HML and HMR, located at the opposite ends of chromosome III and bringing the same information of MAT locus (Figure 3).

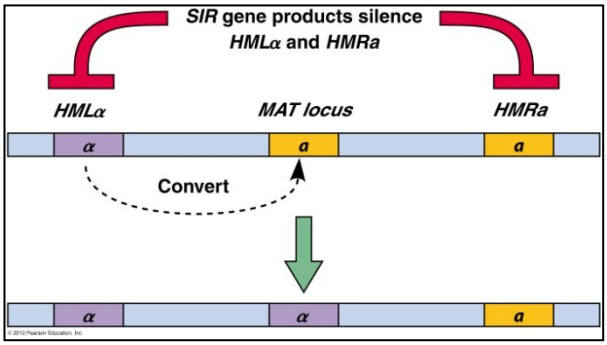

Figure 3. MAT locus and mating-type switch of *S. cerevisiae*

In particular, HML locus brings "α" information while HMR brings "a" information (Houston & Broach, 2006; Tsabar and Haber, 2013).

1.2 Genetic improvement of *S. cerevisiae* yeasts by hybridization

Nowadays, the preservation of typical wines, linked to the combination of indigenous yeasts/territory (*Terroir* concept) as valorization and identification of a specific geographical area, becomes very important for both winemakers and consumers (Steensels et al., 2014). The spontaneous fermentations driven by native yeasts, although interesting for their high aromatic complexity potential, are risky for the difficulty in process control, with the possibility of slowing down the fermentation

process and off-flavor production that leads to obtaining low quality wines and economic losses (Pretorius, 2000; Bauer et al., 2004). For these reasons, since the 1960s, numerous strains of *S. cerevisiae* have been selected to be used as fermentation starters and bypass the drawbacks related to the spontaneous fermentations. The choice of the yeast strain to be used in winemaking is made by winemakers, considering its oenological characteristics in order to give desired traits to the final product. Despite the immense diversity of natural yeasts, the extremely selective and specific conditions of industrial fermentations require a combination of phenotypic traits that may not be commonly found in nature. While the physiological behavior of wild yeasts is dedicated exclusively to survival and reproduction, most industrial fermentations require the maximization of processes and characteristics that may not be beneficial in natural environments. Hence the need to implement vine yeasts selection and isolation programs and subsequent evaluation of their oenological properties. This could represent an interesting pool of biological material from which to start designing new starter yeasts through genetic improvement strategies (Giudici et al, 2006; De Vero et al., 2013). Various techniques have been developed, which may involve specific genes or the entire genome, to artificially increase the existing diversity among yeasts and then generate variants that are improved for their applications in the industrial field, compared to selected native strains (Barre et al. al., 1993; Giudici et al., 2005). Probably, the simplest way to generate artificial diversity in yeasts is the targeted crossover, such as sexual hybridization, with the aim to create new strains with a combination of characteristics of the selected parental strains. With this approach, the characters can be theoretically improved even beyond the phenotypic boundaries of the parental strains, a phenomenon called hybrid vigor (Lippman and Zamir, 2007). This

phenomenon occurs occasionally only for some characters and generally the incidence is low (Marullo et al., 2006; Timberlake et al., 2011; Zörgö et al., 2012). The crossing of different yeasts to produce hybrid yeasts involves three phases: the induction of spore formation (sporulation), spores' isolation and the hybridization that depends on the fusion of spores or haploid cells produced for budding from the spores (Figure 4). When two spores merge, a diploid zygote is produced and produces diploid cells by budding. The demonstration that yeast hybridization is possible has opened the "door" to the production of improved yeast strains to be used in industrial processes, for example in bread making, in brewing, in the production of alcohol and wine (Fowell, 1955).

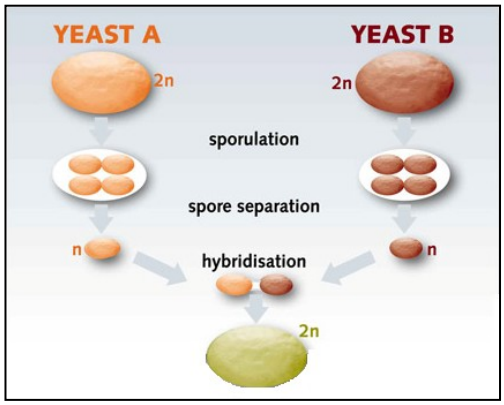

Figure 4. Yeast hybridization

1.2.1 Hybridization techniques

Direct mating. It consists in the crossing of two carefully selected parental strains based on their interesting phenotype. In the case of yeast strains, there are three distinct approaches: cell-cell, spore-cell and spore-spore. The applicability of these approaches depends on the life cycle of parental strains. If both parental strains are

heterotallic, it is possible to perform a pre-screening of the aploid spores (Figure 5) of both parental and choose those with the best characteristics for hybridization (Blasco et al., 2011). Parental haploid cell cultures are mixed to allow the cell fusion and subsequently to isolate the hybrid diploid cells (Figure 6). This technique has been described for the first time by Lindegren & Lindegren (1943). When the parental strains are homatallic, homozygous cultures are selected and the direct coupling is possible by placing with a micromanipulator two single spores, of the opposite conjugative type, on an agar surface and monitoring the hybridization event by microscopy. It is essential to monitor the zygote formation because the homotallism can give rise to secondary zygotes due to self-duplication. The use of the micromanipulator is preferred when both parental strains are homatallic or when the hybridization efficiency of the two parental strains is low compared to the hybridization efficiency of the same parental cells and hybrids with specific selection markers are difficult to isolate specifically.

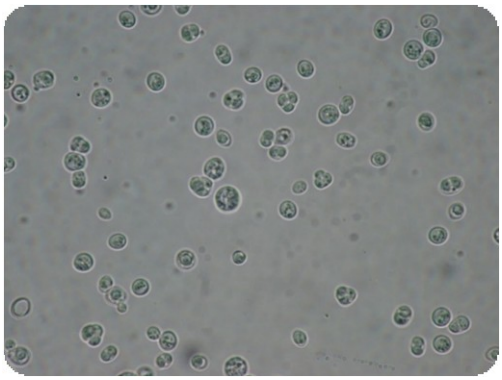

Figure 5. *S. cerevisiae* in sporification

Rare mating. Some diploid cells may become homozygous for the mating-type locus (a/a or α/α) and may be conjugated with a cell of the opposite mating type.

Typically, in a rare mating experiment, the cell suspensions of the parental strains are mixed together and the hybrids subsequently isolated (Figure 6). Generally, the selection takes place by exploiting an auxotrophic parental strain and another with respiratory deficit, producing hybrids easily selectable thanks to their prototype and their respiratory capacity (Pretorius, 2000). The rare conjugation was used both for studying interspecific hybridization events (de Barros Lopes et al., 2002), as well as for the improvement of different yeast traits. Recently, Bellon and collaborators (2011 and 2013) have used the rare conjugation to construct interspecific hybrids *S. cerevisiae* x *S. mikatae* with the aim of diversifying the aromatic profile of the wine.

Mass mating or genome shuffling. It is often used to generate, in a relatively short time, industrial strains with improved characteristics (Gong et al., 2009). A large number of haploid yeast cells from different parental strains are mixed and can be randomly conjugated (Figure 6). In this way, we have the advantage of fully exploiting the genetic diversity of a population and combine useful mutations from different individuals. This technique is particularly useful for homotallic strains, for strains that have low coupling efficiency or for the creation of interspecific hybrids with selective markers (Kunicka-Styczynska & Rajkowska, 2011). Genome shuffling is often applied for studies to improve stress factors tolerance, including fermentation. In this case, the variation is typically induced by the mutagenization of a single parental strain and the mutants then selected for the phenotype of interest. The latter will be used for multiple cycles of genome shuffling to increase the amount of stress and then select improved strains (Wang & Hou, 2010; Steensels et al., 2014).

Cytoinduction. It allows to transfer, from a donor strain to a receiving strain, non-*Mendelian* inheritance characters, such as some genes related to respiration present on mitochondrial DNA or plasmids (killer plasmids), without interrupting the nuclear integrity of the recipient strain (Pretorius, 2000). In particular, by crossing conjugated haploid cells mutated for the *Kar1* gene, and therefore not able to give rise to nuclear fusion (Conde & Fink, 1976; Georgieva & Rothstein, 2002), a cell is obtained (called transitory heterocarionte) containing the cytoplasm of both the parental strains, but the nucleus of only one of them. After subsequent mitotic divisions by budding, the heterocarionte will form heteroplasmic haploid cells containing only one genome but mixed cytoplasmic factors (Conde & Fink, 1976) determining the desired phenotype (Figure 6). Cytoinduction could be used to obtain industrial strains with a positive killer phenotype (Ouchi et al., 1979; Young, 1983; Seki et al., 1985; Yoshiuchi et al., 2000), to transfer flocculation characteristics (Barre et al., 1993), with specific nutrient assimilation capacity (Spencer et al., 1992) or in basic research to study the amyloids (Saifitdinova et al., 2010).

Protoplasts fusion. This technique can be used for strains that have not the requirements for sexual hybridization: they cannot sporulate or possess non-viable spores, they can show an unstable conjugative type or are unable to combine with each other (Pretorius, 2000; Attfield & Bell, 2003). It can also be used to increase ploidy of strains, which, in some cases, may increase cell productivity (Attfield & Bell, 2003). The generation of protoplasts involves the degradation of the yeast cell wall by breaking the β-1,3 glycosidic bonds by enzymes, the suspension of the cells

obtained in an isotonic medium, the hybridization and then the regeneration of the cell wall (Figure 6) (Curran & Bugeja, 1996). The success rate of hybrid formation mainly depends on the taxonomic closeness of the strains and the protocol applied for the fusion (Peberdy, 1980; Pina et al., 1986; Kavanagh & Whittaker, 1996; Attfield & Bell, 2003). Generally, it was observed a frequency of $10^{-3}/10^{-4}$ for intraspecific cells fusions. According to European Union regulations, the hybrids generated in this way, could be considered as GMOs. Hence, their use could be limited to industrial processes that do not involve food (Perez-Travéz et al., 2012).

In the oenological field, hybridization through protoplast fusion has been made by crossing *S. cerevisiae* x *Schizosaccharomyces pombe* and *S. cerevisiae* x *Saccharomyces kudravzevii* (Perez-Través et al., 2012).

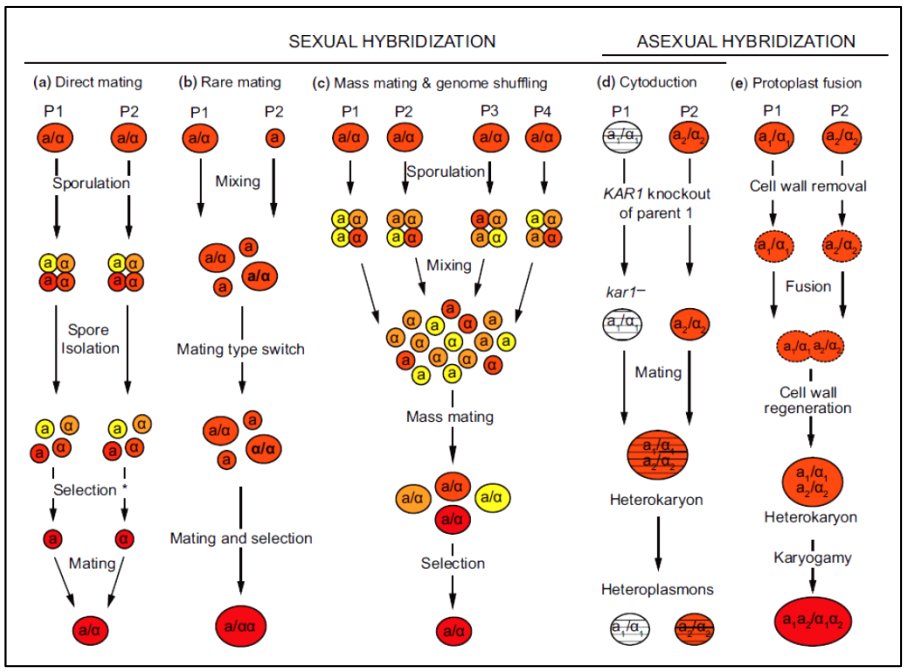

Figure 6. Hybridization techniques

1.3 Hybrid yeast applications in winemaking

The industrial strains belonging to the genus *Saccharomyces* have been highly specialized and well adapted to specific environments and ecological niches where they can fully express their activity. This process can be defined as "domestication" and it is responsible for the peculiar genetic characteristics found in industrial yeasts, such as the high level of chromosomal length polymorphism (Bidenne et al., 1992; Rachidi et al., 1999; Fay and Benavides, 2005; Liti et al., 2009; Almeida et al., 2015) and the presence of aneuploidy or polypidias (Codón & Benítez, 1995; Naumov et al., 2000). Many researchers have focused their attention on the study of the molecular mechanisms involved in the adaptation of yeasts to industrial processes and one of the most interesting mechanisms is the interspecific hybridization (de Barros Lopes et al., 2002; Sipiczki, 2008; Pérez-Través et al., 2012; Da Silva et al., 2015). This technique was designed to improve genetic flexibility and promote adaptive change (Greig et al., 2002). The best example described in the field of hybrid yeasts is the *Saccharomyces pastorianus* yeast (also called *Saccharomyces carlsbergensis*), widely used in lager beers production. This hybrid derived from two species belonging to *Saccharomyces sensu stricto* group: *S. cerevisiae* and *Saccharomyces eubayanus* (Nguyen et al., 2000; Casaregola et al., 2001; Libkind et al., 2011). In particular, *Saccharomyces pastorianus* is characterized by chromosomes of both parental species (Yamagishi & Ogata, 1999) and mtDNA only from *S. cerevisiae* (Piskur et al., 1998). Hybrid strains have also been described in wine and in cider (Masneuf et al., 1998; Naumova et al., 2005). In winemaking, the hybrid yeasts are mainly selected for their higher temperature tolerance and their unique aromatic compounds production (Bisson, 2017). These aromatic compounds could be *de novo* synthesized or derived by metabolic modification of varietal

aromas from grapes (Gamero et al., 2011). Moreover, the use of a hybrid strain rather than the use of two different strains, prevents microbial competition phenomena during the fermentation process. The first hybrid yeast obtained from two different sub-species of *S. cerevisiae* and denominated VIN 13, was marketed in 1991. This was created by Department of Microbiology of the University of Stellenbosch in South Africa (IWBT), in collaboration with Anchor Yeasts company (Lallemand group).

1.3.1 GMO in winemaking

The genetic improvement based on the use of recombinant DNA techniques, can be exploited to obtain yeasts with improved technological characteristics (Pretorius & Van der Westhuizen, 1991). In Europe, according to the legislative decree n.91 of 3/03/1993, it is not allowed the use of genetically modified strains in winemaking (Rainieri & Pretorius, 2000). Furthermore, the employment of genetically modified yeasts is influenced by winemakers and consumers ethical imprint, which is strongly linked to the tradition and naturalness of the process.

Hybridization is a spontaneous process that occurs very frequently in nature and is adopted by yeasts in case of adverse environmental conditions, such as evolutionary and adaptation processes. The application of this technique in the laboratory is relatively simple and does not involve the use of genetic manipulation procedure, therefore hybrid strains obtained by hybridization are not considered genetically modified organisms (OGM). For this reason, they can be used in the wine industry to obtain wines with innovative characteristics (Marullo et al., 2007; Berlese-Noble et al., 2014).

1.4 The main sulfur compounds in wine: hydrogen sulphide and sulfur dioxide

Sulfur is an abundant element present in nature and in many compounds, it is necessary for biological life, such as a component of the cysteine and methionine aminoacids, and a part of vital co-factors. It can occur in an oxidized state (sulphate) or in a reduced form (sulphide).

The main wine yeast *S. cerevisiae* is responsible for the production of more volatile sulfur compounds, which influence the aromatic quality of wines. The main volatile sulfur compounds found in wine are hydrogen sulphide (rotten eggs aroma), metantiol (rotten cabbage flavor), dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide (cabbage, cauliflower and garlic aroma), methyl-thioesters (cooked cauliflowers, cheeses and aromatic herbs) and fruity volatile thiols (passion fruit, grapefruit, gooseberry) (Swiegers & Pretorius 2005; Swiegers et al., 2006). It is known that *S. cerevisiae* is responsible for wine defect due to its hydrogen sulphide (H_2S) production. The amount of H_2S produced by yeasts is strain-dependent (Acree et al., 1972; Mendes-Ferreira et al., 2002; Mendes-Ferreira et al., 2004) and influenced by the sulfur compounds availability in the grape juice (70 to 3.000 mg/l), the fermentation conditions and the nutritional status of the environment (Rauhut, 1993; Mendes-Ferreira et al., 2010; Butzke & Park , 2011). Most of the H_2S produced during vinification is due to the biosynthesis of aminoacids containing sulfur, through the sulfate reduction metabolic pathway (Figure 7). In the first step of sulfate reduction pathway, sulfate is transported into the cell, reduced to sulphite and

then to sulphide by sulphite reductase. At this stage, the sulfide is combined with a nitrogen precursor, O-acetyl-serine or O-acetyl-homoserine, to eventually produce cysteine or methionine respectively. In yeast, the sulfite reductase enzyme is composed by two α and two β subunits (α2β2). The α subunit is encoded by *MET10* gene, while the β subunit by *MET5* gene (Thomas & Surdin-Kerjan, 1997). During sulfite reductase activity, if the quantity of available nitrogen is too low in grape must, the action of the enzyme stops with sulfide accumulation as a consequence. Sulfide can be converted into the volatile gas H_2S (Jiranek et al., 1995; Vos & Gray, 1979). For this reason, the nitrogen availability during wine fermentation becomes very important, indeed many wine producers regularly add ammonium phosphate to grape juice as nitrogen source.

Regarding sulfur dioxide (SO_2), this has been used since Roman times for cellars disinfection and cleaning. Its use in pre-fermentative, fermentative and wine storage phases occurred only relatively recently. In wines, this compound has multiple properties, it can be used as antiseptic agent against yeast and bacteria, as antioxidant, as solvent and clarifying agent. The addition of SO_2 to wine is strictly regulated because high doses can cause organoleptic changes in the final product and risks to human health, as allergic reactions in sensitive individuals (Koch et al., 2010; Aberl & Coelhan, 2013).

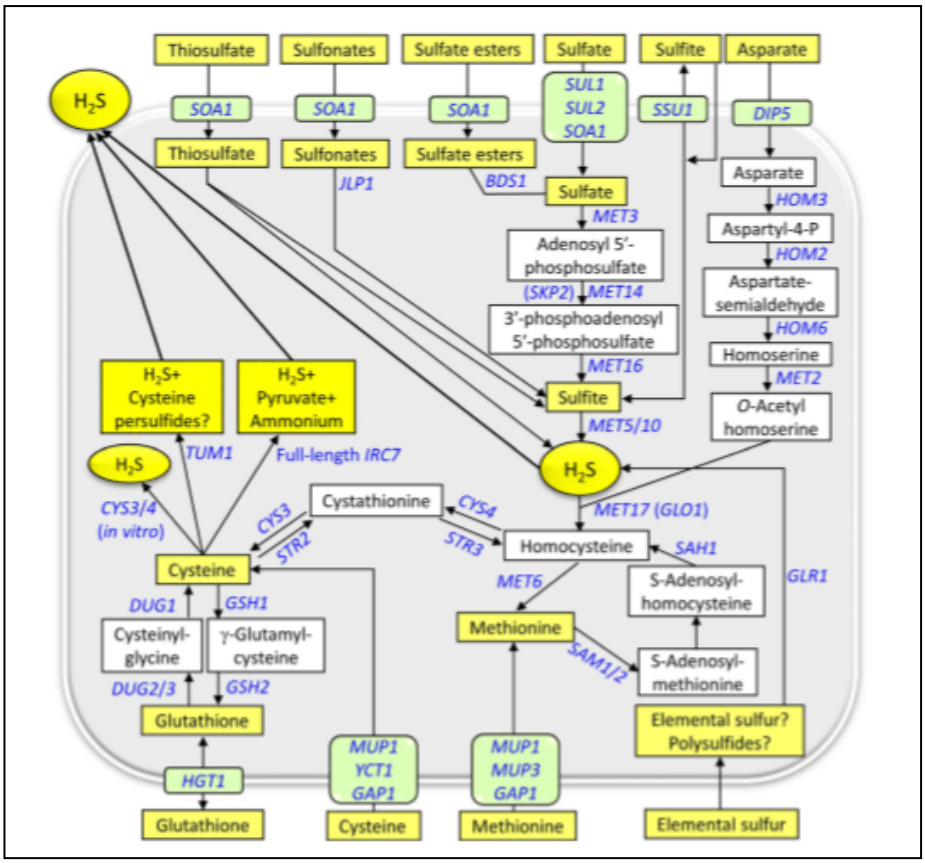

Figure 7. Sulfur metabolism in *S. cerevisiae* yeast (Huang et al., 2017)

2. Aim of the work

During wine fermentation, most winemakers use commercially *S. cerevisiae* active dried yeast to inoculate grape must and to quickly start the process (Bauer et al., 2004). Yeasts from the *Saccharomyces* genus exhibit important oenological properties, such as an excellent ability to ferment sugars vigorously, to produce alcohol under both aerobic and anaerobic conditions and to strongly compete with other yeasts belonging to common microflora present in the fresh grape juice (Goddard, 2008; Salvadó et al., 2011; Dashko et al.,2014). Furthermore, inoculation of selected *S. cerevisiae* as starter yeasts allows to reduce the risks of stuck fermentations and microbial contaminations. It also allows an even better control of the fermentation process and to obtain wines with specific desired aromatic characteristics (Bauer et al., 2004). In recent years, the attention of some researchers has been focused on the improvement of *S. cerevisiae* strains with the aim of obtaining yeasts characterized by a high efficiency of fermentation performance, high sensory quality and healthy for humans (Steensels et al., 2014; Bisson, 2017); all these characteristics are unlikely to be found together in native yeasts. Molecular approaches, like intraspecific hybridization, have been proposed and described for the first time by Pretorius and Van der Westhuizen (1991) for wine yeasts improvement. Hybridization between yeasts occurs spontaneously in nature and for this reason, the new yeasts obtained in this way is not considered genetically modified and can be widely used in winemaking.

The aim of this study was to improve genetically and phenotypically *S. cerevisiae* oenological strains and to use them in winemaking. Two parental strains, *S. cerevisiae* 151 and *S. cerevisiae* P300, belonging to the Department of Life and

Environmental Sciences (Polytechnic University of Marche, Italy) collection, are chosen on the basis of their phenotypic characteristics. In particular, the first parental strain is a native yeast isolated from Verdicchio vineyard (one of the typical vines of Marche region) and selected for its interesting oenological properties, such as the capacity to confer a typical aroma complexity to the wine. The second parental strain is a selected strain with the characteristic of non-producer of H_2S, a compound responsible of off-flavor in winemaking. The goal was to obtain new *S. cerevisiae* strains characterized by good fermentation performance, typical aromatic complexity and low or no H_2S production and at the same time low SO_2 and acetaldehyde production.

3. References

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Article: “New *Saccharomyces cerevisiae* wine strains characterized by reduced SO_2 and H_2S production”

The present Article is in progress for submission

Introduction

Wine and other fermented beverages production have been started from thousands of years and play an important role in human societies (Hornsey, 2012). Recently, Bailey (2015) revealed that wine industries, together with beer productions, represent 64.8% of the alcoholic beverage market. *Saccharomyces cerevisiae* represents the yeast mainly used as starter culture in winemaking for its physiological properties not commonly found in other yeasts (Vaughan-Martini and Martini, 2011). In particular, it is characterized by high capacity to ferment sugars vigorously, to produce alcohol under both aerobic and anaerobic conditions (Piškur et al., 2006; Dashko et al., 2014), to compete with other yeasts that commonly colonize fresh grape juice and releases in wines an important range of compounds (Pinu et al., 2015) that can positively or negatively affect wines quality. The main compounds produced by yeasts during alcoholic fermentation are alcohol and carbon dioxide, with a series of metabolites such as organic acids, amino acids, monosaccharide sugars, fatty acids, esters and higher alcohols (Chambers et al., 2009). Moreover, wine yeasts can also convert grape derived aroma-inactive precursors in active compounds, which contribute to enhance the aroma complexity of wines (King, 2010; Bovo et al., 2015). On the other hand, these yeasts could be involved in the overproduction of undesirable metabolites such as acetic acid or sulfur compounds that led sensory defect in wine (Swiegers et al., 2007; Ugliano et al., 2009; Noble et al., 2015). In particular, acetic acid is responsible of vinegar-like aroma (Du Toit & Pretorius, 2000;) while the level of sulfur compounds (sulfites and sulfide) in the finished product is one the most important parameter determining the acceptability of the wine for its marketing (Linderholm et al., 2008). Sulfites (SO_2) and sulfide (H_2S)

are produced by yeasts during sulfur assimilation pathway and an excessive production can confer negative rotten egg aroma, inhibit malolactic fermentation and could represent a source of health concerns (Carrete et al., 2002; Komarnisky et al., 2003; Mendes-Ferreira et al., 2009).

Nowadays, in the market, exists hundreds of "domesticated" *S. cerevisiae* yeast strains that can be used as fermentative starter, but the widely use of them could bring a wine standardization about analytical and sensory properties (Vigentini et al., 2014; Guillamón and Barrio, 2017). Numerous studies, based on molecular approach, described the genetic differences within indigenous *S. cerevisiae* species, at strain level. These variability, translated in phenotypic differences, seems to be in relationship with their geographical distribution (Liti et al., 2009; Mercado et al., 2011; Scacco et al., 2012; Capece et al., 2013; Tofalo et al., 2013; Tofalo et al., 2014). The native and unknown yeasts could represent an important pool of yeast biodiversity, characterized by specific and unique properties, that could be exploited in winemaking as valorization of wines with a representative imprinting of the viticultural area of production (Romano et al., 2003; Aa et al., 2006; Callejon et al., 2010; Orli´c et al., 2010; Camarasa et al., 2011; Capozzi et al., 2015). On the other hand, it is known that to found indigenous *S. cerevisiae* yeasts, characterized by a combination of phenotypic traits required for their application in winemaking, could become difficult for the extremely conditions of industrial fermentations.

Recently, molecular approaches have been applied to increase yeasts biodiversity and to obtain novel yeasts useful to optimize the production process and to improve wine quality (Liu et al., 2017). Hybridization represents the first technique used for yeast improvement (Winge and Lausten 1938). Romano and co-workers (1985) obtained intraspecific hybrids of *S. cerevisiae* characterized by non-H_2S production, like one

parental strain and flocculation ability like the other parent. In the same way, Shinohara and colleagues (1994) created *S. cerevisiae* hybrid strains with improved properties about fermentation rate, aroma productivity and wine quality. De Vero et al. (2011) combined the sexual spores recombination and specific selection pressure to generate and select wine *S. cerevisiae* strains characterized by low sulfite and impaired H_2S production.

The aim of the current study was to obtain new *S. cerevisiae* strains by intraspecific hybridization technique. The parental strains were chosen for their interesting oenological properties, the first one was selected because H_2S^-. The second one was selected for its complex aromatic imprinting, associated to the geographical area of its isolation, as valorization of viticultural area of wine production. The new *S. cerevisiae* strains obtained were evaluated for their fermentation performance and their aromatic tribute to the final wine.

Materials and methods

Parental strains characteristics

Two parental strains were chosen for their interesting oenological properties. The first yeast was *S. cerevisiae P300* characterized by H_2S^-, the second parental strain, *S. cerevisiae 151* isolated from Verdicchio grapes, one of the typical vineyard of Marche region (Italy), was selected for its oenological aptitude, associated to the geographical area of its isolation. Both parental strains belong to the *Department of Life and Environmental Sciences* (Polytechnic University of Marche, Italy). The

yeasts were maintained at 4 °C for short-term storage on YPD (1% yeast extract – 2% peptone – 2% dextrose) agar medium (Oxoid, Basingstoke, UK) and for long-term storage in YPD broth supplemented with 80% (w/v) glycerol at −80 °C.

Hybridization protocol

The parental strains were cultivated in YPD broth for 24h at 25 °C, then 20µl of each cell suspension was spread on Sporulation Medium (SM) (Sebastiani et al., 2004) and incubate at 23 °C for at least 5 days. When tetrads were observed, each spot of culture was resuspended separately in 45µl of sterile distilled water containing 5µl of Zymolyase 100-T (ICN Biomedicals, Inc., Irvine, California, United States) solution (4mg/ml of sorbitol 2M) and incubated at 37 °C for 30 minutes to allow the cell wall destruction. The solutions were vortex briefly, mixed and incubated at room temperature for 30 minutes to allow the restoration of diploid state by the conjugation of gametes. Subsequently, 10µl of this suspension was spread on YPD agar medium and incubated at 25 °C for 24h. The colonies obtained were randomly selected as potential new strains and analyzed for specific characteristics of selection.

New strains selection

The potential new strains were analyzed for specific selective characteristics related of the two parental strains: H_2S^- like *P300,* and enable to grow in medium containing galactose as unique carbon source, like *151* parental.

Each potential hybrid was spread on Biggy agar medium (Oxoid Ltd, Cheshire, England) for the evaluation of hydrogen sulfide production, and incubated at 25 °C for 48h. In this medium, the H_2S^- strains show white colonies while the strains H_2S^+ exhibit black colonies. The ability to metabolize galactose was evaluated culturing each potential hybrid H_2S^- on YP-Gal broth (0.5% yeast extract – 0.5% peptone – 2% galactose), considering an initial inoculum of 1×10^6 CFU/ml, and incubated at 25 °C for 48h. The growth was monitored spectrophotometrically (OD_{600nm}) by Spectrophotometer (UV-1800 Shimadzu, UV Spectrophotometer, SHIMADZU CORPORATION, Kyoto, Japan) and only the strains galactose$^-$ were selected.

The new (H_2S^- and galactose$^-$) strains were also tested for their respiratory activity. GLY medium, containing non-fermentable glycerol as carbon source, was used (Mannazzu et al., 2002). The strains able to grow in this medium, after 48h at 25 °C of incubation, were selected.

Molecular fingerprinting

To assess the real hybridization, selected strains were submitted to molecular fingerprinting comparing the electrophoretic profile with the parental strains. For each strain the whole genome DNA extraction was performed as follow: 700µl of YPD broth has been inoculated with one colony and let it grow overnight at 30 °C in shaker (200 rpm). The overnight cultures were centrifuged 3' at 3000 rpm and supernatant removed. The cell pellets were resuspended in 200µl of TE-buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0), 250µl of glass beads (0.45 mm diameter) and 200µl of PCI (25:24:1, phenol pH 8:chlorophorm:isoamyl alcohol). The cells were lysed for 2:30' at 30 Hz (twice) and centrifugated (20', 3000 rpm, 4 °C) to spin

down cellular detritus. 100-150µl from the upper aqueous phase (DNA) were collected in a new tube and 800µl of diethylether were added. After 20'' of vortex (30 Hz), the tubes were centrifugated 20' at 3000 rpm, 4 °C and the diethylether completely removed leaving the tubes uncapped under laminar flow hood. The quality and concentration of DNA obtained was checked by Nanodrop (ND-8000, 8-Sample Spectrophotometer, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and conserved at -20 °C.

Molecular characterizations of interdelta sequences were performed using two primer pairs: delta 2/12 and delta 12/21 as described by Legras and Karst (2003). Moreover, length and sequence polymorphisms of *SED1* and *AGA1* genes, both involved in cell wall proteins production, were analyzed as described by Mannazzu et al. (2002) and Mariangeli et al. (2004) respectively. RAPD (Random Amplified Polymorphic DNA) analyses were performed using primers R3 (5'-ATGCAGCCAC-3') and OPA11 (5'-CAATCGCCGT-3') as described by Corte et al. (2005) and Couto et al. (1996) respectively. Hypervariable microsatellite loci were also analyzed using primers SCAAT1, C4 and SCYOR267c as described by Legras et al. (2005).

PCR-delta 2/12 products were separated by automated capillary electrophoresis QIAxcel Advanced system (Qiagen, Venlo, Netherlands) with a Screening Gel Cartridge (Qiagen, Venlo, Netherlands) under the following parameters: sample injection voltage 5 kV, sample injection time 10 s, separation voltage 5 kV and separation time 420 s. The QX Size Marker 50bp/5kb (Qiagen, Venlo, Netherlands) was used for fragment size and the QX Alignment Marker for 50bp/5kb (Qiagen, Venlo, Netherlands) was used to align the resulting restriction fragments. RAPD amplicons were separated by electrophoresis on 1.0% (w/v) agarose gels submitted to 120 V for 2 h in 1×TAE buffer. SmartLadder MW-1700-02 (Eurogentec, Liegi,

Belgium) was used for fragment size. Others amplicons were separated by electrophoresis on 1.5% (w/v) agarose gels submitted to 100 V for 1 h in 0.5×TBE buffer. Gene ruler 100 bp (Fermentas, Waltham, Massachusetts, United States) was used for fragment size.

Parental strains tetrads dissection and mating type characterization

The parental strains tetrads, after sporulation and wall cell disruption as previously described, were dissected using a micromanipulator (Singer SMS Manual, Somerset, UK) on YPD-agar and incubated at 25 °C for 48h to allow the growth of spores. The heterothallic or homothallic nature of the parental strains was evaluated by mating type analyses of viable spores (10 tetrads of each parental strain were chosen), as reported by Steensels et al. (2014). Furthermore, each viable spore was also tested for its H_2S production, as previously described.

Genomic stabilization

To ensure genetic stability over time, the new strains were subjected to genomic stabilization protocol using synthetic grape juice (Ciani and Ferraro, 1996) as growth medium. All strains were pre-cultured in modified YPD broth for 24h at 25 °C under stirrer condition (200 rpm) and used to inoculate 750µl of synthetic grape juice with an inoculum ratio of 1:100. Initial yeast concentrations were measured spectrophotometrically (OD_{600}) and each culture left to grow up at 25 °C for 1 week.

Optical density (OD_{600}) of each culture was measured again to obtain the yeast generation number achieved. Then, the yeast cultures were transferred in fresh synthetic grape juice with the same inoculum ratio (1:100), OD_{600} measured and left to grow up at the same conditions. This procedure was repeated once a week until to reach approximately 20 yeast generations. To check the population homogeneity of each stabilized culture, ten and two isolates of new strains and parental yeasts were isolated respectively. The isolates were subjected to genetic fingerprinting using primer pair delta 12/21 as previously described, with the aim to compare their genotype before and after stabilization. The stabilized isolates were also tested for H_2S production as previously described.

Microvinification in synthetic grape juice

Some isolates of new stabilized strains were tested for their fermentative aptitudes using synthetic grape juice (Ciani and Ferraro, 1996) and compared with parental strains and two commercial strains commonly used in winemaking: Lalvin ICV OKAY (Lallemand Inc., Toulouse, France) and VIN13 (Anchor Wine Yeast, Cape Town, South Africa), no and moderate H_2S producers respectively. Each isolate was pre-cultured in modified YPD broth for 24h at 25 °C, 200 rpm and used to inoculate the must (1×10^6 cells/ml of final concentration). The flasks containing 150ml of synthetic must were locked with a Müller valve containing distilled water to allow CO_2 to escape from the system and placed at 20 °C under static condition. Each trial was conducted in duplicate. H_2S production was evaluated during the fermentation

by acetate strip (CARLO ERBA Reagents S.r.l., Milan, Italy). The fermentation kinetics were followed measuring daily weight loss until the end of fermentation.

The resulted wines were analyzed for ethanol content using DMA 4500 M density meter and Alcolyzer Beer ME (Anton Paar, Graz, Austria) (http://www.anton-paar.com/). While sugar residual, acetic acid, total SO_2 and glycerol were quantified using Gallery™ Plus Beermaster (ThermoFisher, Finland) discrete photometric analyzer. Acetaldehyde, ethylacetate, n-propanol, isobutanol, amyl and isoamyl alcohols were detected using by direct injection into a gas–liquid chromatography system as reported by Canonico et al. 2014.

Statistical analyses

The data related to wines were elaborated by one-way ANOVA. STATISTICA 7 (Statsoft, Tulsa, OK, USA) was used to the means analyses. The significant differences were obtained considering the associated p values <0.05 by Duncan test. Mean values of volatile compounds were analyzed by Principal Component Analysis (PCA), carried out using JMP 11 statistical software. The mean data were normalized to eliminate influence of hidden factors.

Results

Phenotypic selection of the new *S. cerevisiae* strains

The selection of the potential hybrid strains was carried out evaluating the use of galactose as unique carbon source, the H_2S production and the respiratory activity. Out of 100 colonies randomly selected, four were chosen as potential new *S. cerevisiae* strains (G4, I1, I4, B4) for their desired phenotypic characteristics: H_2S^- (like P300 parental strain), galactose$^-$ (like 151 parental strain), respiratory activity$^+$ (like both parental strains), excluding eventual "petite mutant" strains.

Molecular characterization of the potential hybrids

The results of molecular fingerprinting carried out using interdelta primers (delta 12-21) are reported in Figure 1. The electrophoretic gel showed that each new strains selected for the desired phenotypic characteristics (G4, I1, I4, B4), exhibited an unique profile in comparison with those of the parental strains and each other; it was confirmed by delta 2-12 profiles (data not shown). On the other hand, the results obtained by RAPD R3 and OPA11, SED1 and AGA1 minisatellite analyses and C4, SCAAT1 and SCYOR267c hyper variable microsatellite analyses showed any significant difference among the strains evaluated (data not shown).

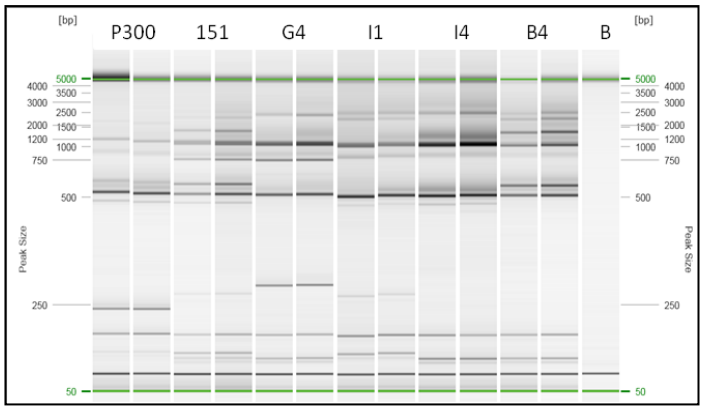


Figure 1. Delta 2-12 molecular fingerprinting of the new *S. cerevisiae* and parental strains. Each electrophoretic profile was reported in duplicate as results confirmation. The QX Size Marker 50bp/5kb (Qiagen) was used for fragment size. Lane B was indicated as negative control.

Mating-type and H_2S production of parental strains spores

After the sporulation of *P300* and *151* yeasts, ten tetrads of each strains were selected, dissected and each spore let it grow on YPD agar medium at 25 °C for 48h. Both strains showed 62.5 % of spores's mortality, the viable ones were evaluated for the mating-type and H_2S production (Table 1). The tetrads have been named from one to ten and the spores of each tetrad have been named from "a" to "d". The results showed a/α mating-type for all spores of *P300*, defining *P300* strain as homothallic. On the contrary, the spores of *151* exhibited mating-type variability within the same tetrad. A clear example was observed for the ninth tetrad: "a" spore exhibited mating-type α, "b" and "c" spores exhibited mating-type a/α, while "d" spore exhibited mating-type a. The same trend was also observed for the H_2S production. All P300's spores showed the same H_2S^- phenotype, while 151's spores exhibited great variability within the same tetrad, as observed for the eighth tetrad: "a" spore

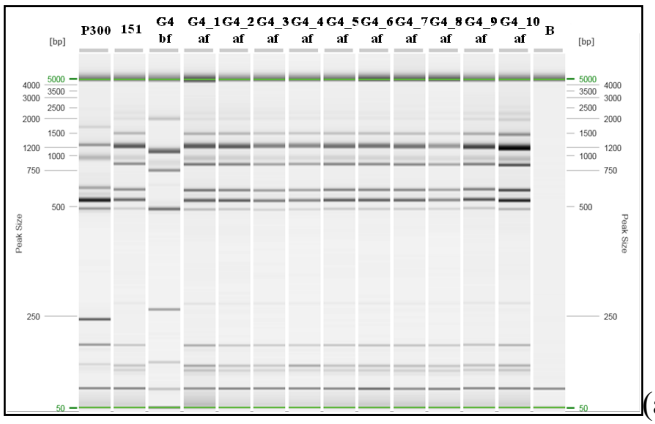
showed the maximum expression of hydrogen sulfide, an intermediate production of this compound was detected for "b" spore, while "c" spore was H_2S^-.

Spores name	Mating-type	H_2S production	Spores name	Mating-type	H_2S production
P300_1_a	a/α	-	151_1_a	a/α	++
P300_1_b	a/α	-	151_1_b	α	-
P300_1_c	a/α	-	151_1_c	α	++
P300_1_d	a/α	-	151_2_b	a	++
P300_2_a	a/α	-	151_3_c	a/α	++
P300_2_b	a/α	-	151_4_b	a/α	+++
P300_2_c	a/α	-	151_8_a	a	+++
P300_2_d	a/α	-	151_8_b	a/α	++
P300_4_a	a/α	-	151_8_c	α	-
P300_4_b	a/α	-	151_9_a	α	+
P300_4_c	a/α	-	151_9_b	a/α	-
P300_4_d	a/α	-	151_9_c	a/α	-
P300_6_a	a/α	-	151_9_d	a	++
P300_6_b	a/α	-	151_10_a	a/α	+++
P300_6_c	a/α	-	151_10_b	α	++

Table 1. Mating-type and H_2S production of viable spores of *P300* and *151* strains. Each spore within the tetrad was named from "a" to "d" preceded by tetrad's isolation number and strain's name. The amount of H_2S produced by spores was indicated with "+++", "++", "+" and "–" for the maximum, medium, low and absent production, respectively.

Genomic fingerprinting of the stabilized new *S. cerevisiae* strains

Genomic stabilization of the four new *S. cerevisiae* strains was carried out using synthetic grape juice as medium to growth. The *P300* and *151* strains were used as controls. After stabilization, ten colonies of each new strain and two colonies of *P300* and *151* strains were isolated to check the population homogeneity within each culture and the genome stabilization's effects. The electrophoretic profiles of the stabilized isolates belonging to the same group, were compared each other and with that obtained before of the stabilization's procedure. *P300* and *151* yeasts showed identical electrophoretic profiles comparing before and after stabilization results (data not showed). Regarding the new *S. cerevisiae* strains, all stabilized G4 isolates showed the same electophoretic profile, indicating population homogeneity. At the same time, they exhibited electrophoretic profiles equal to that of 151 strain and different from that of unstabilized G4 strain (Figure 2a). Also stabilized I1 and B4 isolates showed population homogeneity. The other hand, no genomic difference was observed comparing the electophoretic profiles obtained before and after stabilization (Figure 2b and 2c). Regarding stabilized I4 isolates did not exhibit population homogeneity (Figure 2d). Concerning H_2S production, all stabilized isolates maintained the characteristic observed before stabilization (absent or low H_2S production). The unique exception was observed for stabilized G4 isolates, which exhibited level of H_2S similar to the *151* strain (data not showed).


P300
151
G4 bf
G4_1 af
G4_2 af
G4_3 af
G4_4 af
G4_5 af
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G4_10 af
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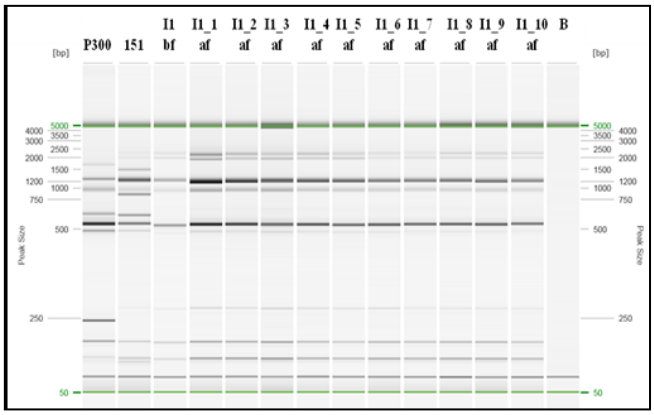
(a)


P300
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I1 bf
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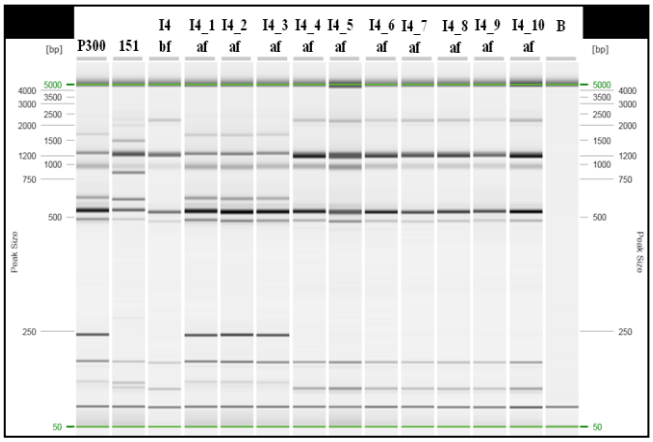
P300
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I4 bf
I4_1 af
I4_2 af
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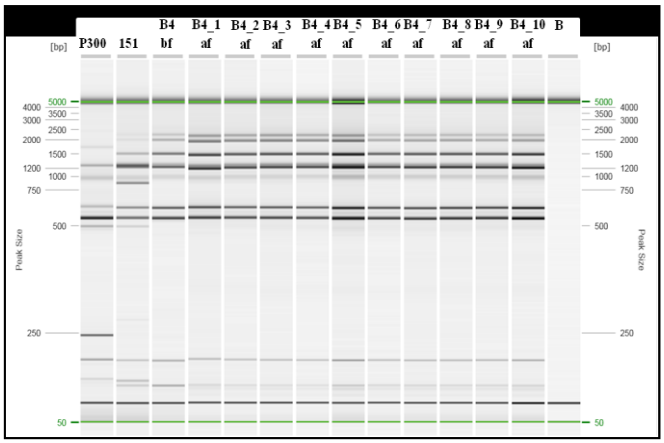

(d)

Figure 2. Electrophoretic profiles of interdelta sequences obtained by primer pair delta 12-21. Each stabilized isolate, belonging to each group, was named from 1 to 10 preceded by strain's name and compared with the correspondent unstabilized strain and *P300* and *151* yeasts. The acronym "bf" and "af" used to indicate before and after genome stabilization respectively. Lane B: indicated as negative control. The QX Size Marker 50bp/5kb (Qiagen) was used for fragment size. a) Electrophoretic profiles of unstabilized and stabilized G4 isolates. b) Electrophoretic profiles of unstabilized and stabilized I1 isolates. c) Electrophoretic profiles of unstabilized and stabilized I4 isolates. d) Electrophoretic profiles of unstabilized and stabilized B4 isolates.

Microvinification trials

Fermentation kinetics

On the basis of the results obtained by interdelta analyses of stabilized strains, three isolates of each new strain were chosen to conduct lab-scale fermentation trials in

comparison with unstabilized strains, *P300* and *151* and with two common commercial strains. The results of fermentation kinetics are reported in Figure 3. The data showed similar or greater fermentation kinetics of the stabilized and unstabilized strains, compared with the best controls represented by 151 and OKAY strains. On the contrary, the other two controls (P300 and VIN13) showed the slowest fermentation kinetics. All the strains completed the fermentation process after 27 days from the beginning.

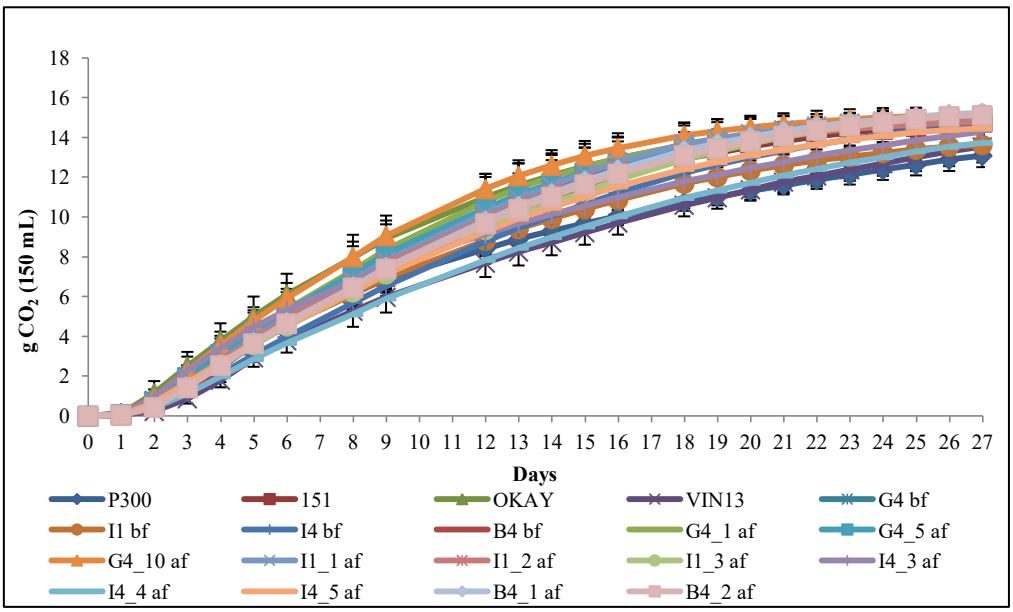

Figure 3. Fermentation kinetics of the new stabilized and unstabilized strains compared with P300, 151 OKAY and VIN13 strains, used as controls. The number near the new strain's name represented the number of isolate and the acronym "bf" and "af" indicated before and after genome stabilization, respectively.

Main wine analytical compounds

The main analytical compounds of the wines were analyzed and the results are showed in Table 2. *P300*, VIN13 and unstabilized I1 strains did not complete the fermentation, indeed the resulted wines showed the higher residual sugars (26.6±0.9 g l^{-1}, 15.4±2.7 g l^{-1} and 27.1±0.8 g l^{-1}, respectively) and the lower ethanol content (11.8±0.0 % v/v , 12.0±0.4 % v/v and 11.5±0.3 % v/v for P300, VIN13 and I1 bf strains, respectively). The other strains completed the fermentations, indeed the resulted wines showed low sugar residual (< 10 g l^{-1}) and about 13 % v/v of ethanol content. Significant differences in volatile acidity production were observed for *P300* and VIN13 strains. They exhibited the lowest and the highest acetic acid production, 0.5±0.0 g l^{-1} and 1.3±0.0 g l^{-1}, respectively. Regarding the total SO_2 production, great variability was observed among the strains. OKAY and B4 (before and after stabilization) strains exhibited the lowest SO_{2tot} production (< 1 mg l^{-1}), while P300 and stabilized G4_1 and G4_10 strains showed the highest values (9.6±0.5 mg l^{-1}, 9.1±0.6 mg l^{-1} and 9.0±0.2 mg l^{-1}, respectively).

Strains	Ethanol (% v/v)	Residual sugar (g l^{-1})	Volatile acidity (g l^{-1} of acetic acid)	$SO_{2\ tot}$ (mg l^{-1})	Glycerol (g l^{-1})
P300	11.8±0.0^{de}	26.6±0.9^{a}	0.5±0.0^{e}	9.6±0.5^{a}	3.7±0.1^{n}
151	13.2±0.0^{a}	2.1±0.3^{ef}	0.8±0.0^{bc}	3.8±0.6^{g}	5.0±0.1^{e}
OKAY	13.0±0.2^{a}	2.1±0.5^{ef}	0.8±0.1^{b}	0.3±0.0^{h}	5.6±0.0^{f}
VIN13	12.0±0.4^{cde}	15.4±2.7^{b}	1.3±0.0^{a}	4.7±0.4^{def}	6.0±0.0^{d}
G4 bf	13.1±0.1^{a}	1.4±0.7^{f}	0.7±0.0^{bcd}	4.9±0.5^{de}	6.6±0.0^{b}
I1 bf	11.5±0.3^{e}	27.1±0.8^{a}	0.8±0.1^{bc}	3.6±0.5^{g}	7.7±0.3^{a}
I4 bf	12.8±0.6^{ab}	4.1±1.5^{d}	0.8±0.1^{bcd}	4.6±0.4^{def}	5.3±0.0^{ghi}
B4 bf	13.2±0.0^{a}	1.3±0.0^{f}	0.8±0.0^{b}	0.4±0.1^{h}	5.7±0.1^{ef}
G4_1 af	13.2±0.0^{a}	1.8±0.1^{ef}	0.7±0.0^{bc}	9.1±0.6^{a}	5.3±0.0^{gh}
G4_5 af	13.3±0.0^{a}	1.7±0.1^{ef}	0.8±0.0^{bcd}	8.0±0.3^{b}	5.2±0.^{hi}
G4_10 af	13.2±0.0^{a}	1.4±0.1^{ef}	0.8±0.1^{bc}	9.0±0.2^{a}	5.4±0.1^{gh}
I1_1 af	13.2±0.0^{a}	1.3±0.0^{ef}	0.7±0.0^{bcd}	4.3±0.2^{efg}	6.2±0.0^{c}
I1_2 af	13.1±0.2^{a}	1.9±0.2^{ef}	0.7±0.1^{cd}	4.7±0.0^{def}	6.2±0.0^{c}
I1_3 af	13.2±0.0^{a}	1.7±0.2^{ef}	0.8±0.0^{bc}	7.0±0.2^{c}	5.0±0.0^{l}
I4_3 af	12.3±0.2^{bc}	8.7±0.2^{c}	0.6±0.0^{de}	4.0±0.5^{fg}	4.4±0.1^{m}
I4_4 af	12.1±0.2^{cd}	8.4±1.3^{c}	0.7±0.0^{bcd}	0.5±0.1^{h}	5.1±0.2^{il}
I4_5 af	13.0±0.2^{a}	3.6±0.9^{de}	0.7±0.0^{bcd}	5.2±0.3^{d}	5.4±0.0^{g}
B4_1 af	13.2±0.0^{a}	1.2±0.2^{f}	0.8±0.0^{bcd}	1.0±0.0^{h}	5.7±0.0^{ef}
B4_2 af	13.2±0.1^{a}	1.9±0.1^{ef}	0.8±0.0^{bcd}	0.3±0.0^{h}	5.9±0.1^{de}

Table 2. The main wine analytical characteristics of each *S. cerevisiae* strains. Data are means ± standard deviations. Data with different superscript letters (a, b, c, d, e, f, g, h, i, l, m, n) within each column are significant (Duncan test; $p < 0.05$). The number near the new strain's name represented the number of isolate and the acronym "bf" and "af" indicated before and after genome stabilization, respectively.

Main fermentation by-products

The main fermentation by-products produced by *S. cerevisiae* strains were subjected to Principal Component Analysis (PCA) (Figure 4). The total variance explained was 74.90% (PC1 56.40%; PC2 18.50%). In the left quadrant was reported the distribution of strains in function of by-products (right quadrant). The plots grouped the new strains's isolates, before and after stabilization, indicating no relevant differences in the by-products formation. On the other hand the new strains could be distinguished each other for the volatile compounds. The autochthonous 151 strain showed an intermediate production of these compounds compared with the new strains.

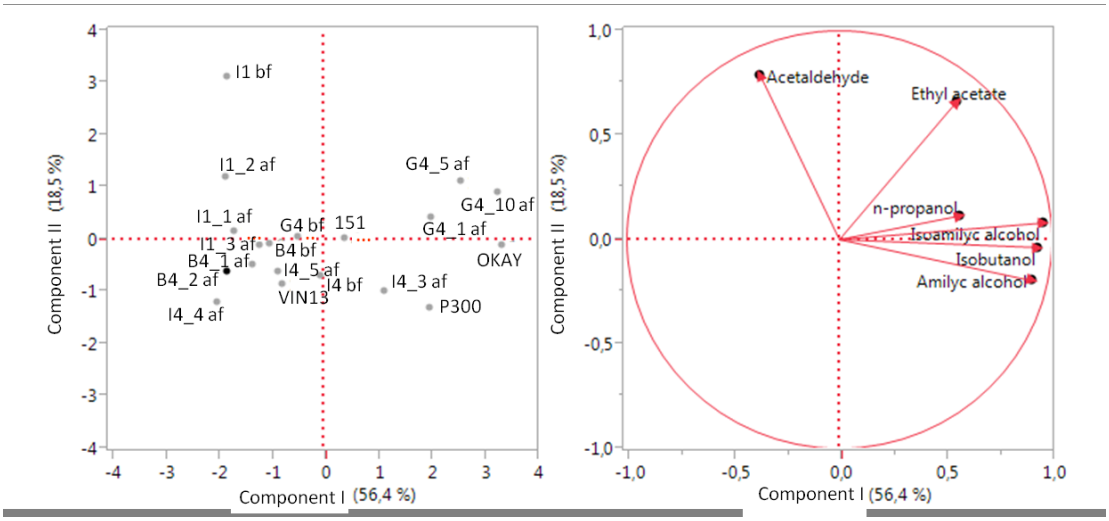

Figure 4. Principal component analysis based on the data for the main fermentation by-products in the wines produced by different *S. cerevisiae* strains. The number near the new strain's name represented the number of isolate and the acronym "bf" and "af" indicated before and after genome stabilization, respectively.

Discussion

Metabolites released by yeasts during alcoholic fermentation strongly influence the aromatic characteristics and consequently the quality of wines (Pretorius, 2000; Cebollero et al., 2007). In particular, compounds derived from sulfur metabolism, such as sulfite (SO_2) and sulfide (H_2S) could inhibit malolactic fermentation, could be responsible of health concerns, could led off-flavors or mask the aromatic profile of the final wine (Mendes-Ferreira et al., 2009; Noble et al., 2015). The absence or the reduced presence of these sulfur compounds are particularly required in selected starter strains used to produce organic wines. In these wines the absence of SO_2 requires a more strict limitation of oxygen contact. In this condition, the strict reduced environment increases the negative effect of H_2S on aromatic profile of wines. To data, the selection of *S. cerevisiae* starter strains able to produce low concentration of these undesirable compounds become one of the main researchers interest to meet winemakers needs. The ideal combination of desirable oenological properties in natural yeasts is highly improbable to find (Pérez-Través et al., 2012), then the use of hybridization methods, could allow to generate new yeast strains with selected desirable features (Sipiczki, 2008).

Numerous authors described the use of hybridization method to obtain improved oenological yeasts (Romano et al., 1985; Masneuf-Pomarède et al., 2002; Pérez-Través et al., 2012; Bellon et al., 2013; Steensels et al. 2014; Pérez-Través et al., 2015; Bonciani et al., 2016).

In the present work, we obtained new no-genetically modified *S. cerevisiae* strains exploiting mass-mating hybridization approach, despite molecular fingerprinting results highlighted that the new strains are not hybrid yeasts. Indeed, interdelta

profiles analyses evidenced that the new strains exhibithed new or characteristic bands of 151 strain and no characteristic bands of P300 strain. Probably the new strains originated from self-mating of only 151's spores, with the consequence of shuffling of its genetic traits and allowing the formation of strains H_2S^-. This hypothesis was confirmed by analyses of 151's spores for the H_2S production. Although these new yeasts are not hybrids, they are characterized by the absence of H_2S production and by the low SO_2 and acetaldehyde production and with a specific aromatic imprinting of autochthonous strain, all aspects particularly desired in organic wine production and in winemaking in general. A previous work exploited the sexual recombination of spores and specific selective pressure to generate non-genetically modified *S. cerevisiae* with desired oenological characteristics. In particular, they used toxic analogues of sulfate to select strains unable to assimilate sulfates (De Vero et al., 2011). Also Bizaj et al. (2012) used mass-mating hybridization procedure to obtain industrial yeast strains characterized by low hydrogen sulfide production and high esters production.

The fermentation performance of the new *S. cerevisiae* strains resulted comparable to the control strains (151, P300, OKAY and VIN13). Regarding to the fermentation by-products, a general reduction of acetic acid, acetaldehyde and ethyl acetate was observed in the new strains than the controls. These compounds, when present in excess can negatively affect the analytical and sensorial profile of the final wines (Zoecklein et al., 1995; Eglinton et al., 2002; Bely et al., 2008).

In conclusion, our results emphasizes the potential use of hybridization approach to obtain no-genetically modified yeasts with desiderate characteristics, although we have not been able to get real hybrid yeasts. However, the genetic reassortment of the autochthonous 151 strain allowed to obtain stabilized *S. cerevisiae* strains with

interesting oenological properties that coul be proposed as a new starter strains, especially for organic wines production, where the absence or low presence of sulfite and sulfide compounds is highly desirable. This strategy could be used to influence the aroma profile of the wines in relationship to the low production of undesirable compounds and the specific aromatic imprinting, also related to the geographical area of wines production.

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Supplementary material: Main fermentation by-products (mg l^{-1})

S. cerevisiae strains	Carbonyl compounds	Esters	Alcohols			
	Acetaldehyde	Ethyl acetate	n-propanol	Isobutanol	Amylic alcohol	Isoamylic alcohol
OKAY	15.2±0.2^{efg}	18.7±0.6^{cde}	41.6±0.4^{a}	19.7±0.3^{a}	18.9±0.4^{ab}	47.5±0.1^{c}
VIN13	16.5±0.2^{def}	12.7±0.1^{hi}	21.1±0.4^{def}	9.8±0.2^{bcd}	13.0±0.1^{e}	30.7±0.3^{fgh}
P300	14.8±0.6^{fg}	12.2±0.2^{hi}	21.4±0.0^{def}	20.1±0.1^{a}	19.7±0.1^{a}	46.7±0.3^{c}
151	13.9±0.1^{g}	22.3±0.3^{d}	19.2±0.4^{f}	14.8±0.1^{b}	12.2±0.2^{f}	35.9±0.1^{f}
G4 bf	17.6±0.3^{d}	19.1±0.2^{cde}	18.8±0.6^{f}	13.1±0.2^{b}	10.2±0.3^{fgh}	32.8±0.3^{efg}
I1 bf	50.8±0.0^{a}	20.6±2.9^{c}	25.3±9.6^{bcd}	8.3±0.7^{cd}	8.0±0.5^{l}	27.6±5.3^{il}
I4 bf	9.0±0.7^{i}	17.3±0.4^{def}	28.0±0.5^{b}	9.0±0.2^{cd}	11.2±0.3^{f}	35.4±0.2^{e}
B4 bf	19.3±0.3^{c}	16.3±0.1^{efg}	20.5±0.6^{def}	8.9±0.3^{cd}	10.1±0.5^{fgh}	33.0±0.2^{ef}
G4_1 af	11.7±0.3^{h}	26.5±0.5^{b}	24.6±0.1^{bcde}	17.2±0.2^{a}	13.7±0.5^{e}	47.8±0.0^{c}
G4_5 af	16.7±0.1^{de}	30.4±0.5^{a}	22.5±0.2^{cdef}	16.8±0.3^{a}	16.6±0.2^{cd}	52.4±0.6^{b}
G4_10 af	14.8±0.4^{fg}	28.1±0.4^{ab}	27.4±0.3^{bc}	18.1±0.1^{a}	15.9±0.4^{d}	61.6±0.6^{a}
I1_1 af	20.1±0.3^{c}	17.3±0.5^{def}	20.4±0.6^{def}	6.8±0.1^{d}	8.3±0.5^{il}	28.2±0.3^{il}
I1_2 af	32.6±0.0^{b}	18.4±0.4^{cde}	17.2±0.0^{f}	7.6±0.4^{d}	9.1±0.3^{ghil}	28.8±0.4^{ghi}
I1_3 af	17.0±0.3^{efg}	17.5±0.4^{cd}	20.8±0.0^{ef}	8.2±0.2^{bc}	9.3±0.4^{fg}	30.1±0.1^{ef}
I4_3 af	17.5±0.6^{e}	13.0±0.0^{hi}	19.4±0.3^{ef}	17.6±0.2^{a}	17.9±0.5^{bc}	42.3±0.3^{d}
I4_4 af	13.9±0.2^{g}	10.1±0.5^{i}	19.2±0.2^{f}	6.4±0.4^{d}	8.7±0.2^{hil}	28.0±0.4^{il}
I4_5 af	13.9±0.2^{g}	14.0±0.5^{gh}	27.3±0.4^{bc}	7.4±0.5^{d}	9.8±0.0^{fghi}	32.7±0.4^{efg}
B4_1 af	14.5±0.3^{g}	14.6±0.2^{fgh}	18.7±0.0^{f}	8.8±0.0^{cd}	7.5±0.2^{l}	25.7±0.8^{l}
B4_2 af	16.6±0.2^{de}	14.7±0.7^{fgh}	19.6±0.1^{ef}	8.2±0.1^{cd}	9.7±0.5^{fghi}	30.8±0.2^{fgh}

Main wine fermentation by-products obtained in the trials conducted by each *S. cerevisiae* strains. Data are means ± standard deviations. Data with different superscript letters (a, b, c, d, e, f, g, h, i, l) within each column are significantly different (Duncan test; $p < 0.05$). The number near the new strain's name represented the number of isolate and the acronym "bf" and "af" indicated before and after genome stabilization, respectively.

Conclusions

Nowadays, there is a growing request for organic wine by consumers due to the link with health concerns, environmental awareness, interest for the peculiarity of the region ("*terroir*") and the general perception of higher value of organic wines than conventional ones. Organic wine production represents about 5% of total wine market and in Italy 37% of viticulture areas are represented by organic grapes cultivar. To meet market requirements, the main objectives of organic wine producers are to obtain high standard quality wines, stable over the time and characterized by absence or low presence of sulfide and sulfite having specific and recognizable aromatic imprinting. In this regard, it is possible to set in different processing stages of wine production, which together contribute to the quality of the final wine. In the first part of the present PhD thesis, it was analyzed the yeast community composition associated with the grape surface of organic and conventional farming system and untreated grapes belong to two different grape varieties monitoring its evolution during the spontaneous fermentation. The data obtained highlighted the impact of both grape varieties and agricultural treatments on the yeast colonization of grape surface, influencing the fermenting yeasts responsible to drive the spontaneous fermentation process. In particular, organic treatment revealed great fungal biodiversity than conventional ones. Moreover, differences in fermenting yeasts present at the end of spontaneous fermentation were also found. These differences may strongly influence the analytical and aromatic profile of wines giving a specific yeast imprinting to organic or conventional wines. In this regard, to detect and exploit the biodiversity of a specific wine region, in the second part of the thesis was evaluated the enological aptitude of indigenous *Saccharomyces cerevisiae*

yeasts, chosen for their promising oenological properties. These new starter strains may especially used for the production of organic wines characterized by aromatic complexity recognizable to the geographical area of winemaking. On the other hand, it is rare to find indigenous strains with a desired combination of all oenological properties for organic wine production. In particular, strains characterized by low sulfide and sulfite production and with desired aromatic complexity are required. Indeed, the absence of H_2S production allows to maintain more strictly reduced conditions during the vinification process avoiding the production of sulfur off-flavors. Moreover, in organic wines the SO_2 content is strictly maintained at very low level and should not be produced by yeasts. In the third part of the research, a selected indigenous *S. cerevisiae* strain was improved using classical genetic methods by mean sporification and self-mating of segregants. The improved strains obtained are characterized by low or no H_2S and SO_2 production maintaining, at the same time, the peculiar aromatic imprinting. Therefore, this technique could represent a natural strategy to achieve new oenological strains with desired characteristics. In conclusion the results obtained highlighted that the production of organic wine needs to modify the microbiological approach during the whole winemaking process. The microbial community of grapes was influenced by organic farming system, determining the fermenting yeasts involved in the fermentation process, while new strains tailored on the technological market requests to product high quality organic wines able to satisfy the consumer's requests.