Yeast distribution in Grignolino grapes growing in a new vineyard in Piedmont and the technological characterization of indigenous Saccharomyces spp. strains

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Yeast distribution in Grignolino grapes growing in a new vineyard in Piedmont and the technological characterization of indigenous Saccharomyces spp. strains

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ABSTRACT

The aim of this study was to characterize the yeast consortium isolated from Grignolino grapes in a newly planted vineyard in Piedmont (Italy) via analysis of the intra-vineyard yeast distribution of grape samples from single rows. A two-phase approach allowed the identification of culturable yeasts present on grape skins and, through an enriching procedure via grape fermentation, the isolation of low frequency non-Saccharomyces and Saccharomyces spp. fermentative species, including S. paradoxus, which is highly unusual during grape fermentation, along with the intra-specific characterization of S. cerevisiae isolates. Culture-based molecular techniques revealed a grape yeast microbiota formed by (in order of abundance) Hanseniaspora uvarum, the yeast-like fungus Aerobasidium pullulans, Candida zemplinina, Pichia kluyveri, Candida californica, Curvibasidium cygneicollum, Meyerozima caribbica, Rhodotorula babjevae, Metschnikowia pulcherrima and Cryptococcus flavescens.

Technological properties of isolated Saccharomyces spp. strains were analysed, identifying strains, including S. paradoxus, potentially suitable as an ecotypical starter for territorial wines.

Introduction

Wine production is one of the most ancient biotechnological processes. The transformation of grape juice into wine is due to microorganism-mediated alcoholic fermentation carried out by yeast, although other microorganisms also contribute to the final organoleptic properties of the wine. Oenological microorganisms, such as fungi, yeasts and bacteria, have been widely studied, particularly yeast species involved in alcoholic fermentation, among the most important of which are species belonging to the *Saccharomyces* genus, in particular, *Saccharomyces cerevisiae* (Pretorius, 2000).

Even though most studies have focused on searching for new *S. cerevisiae* strains with improved performance in wine fermentation, more recently, the contribution of numerous non-*Saccharomyces* yeasts to wine quality has also been explored (Ciani et al., 2010; Jolly et al., 2014).

Moreover, in recent years, the relationship between the wine microbial community and "terroir" has been highlighted (Capozzi et al., 2015; Combina et al., 2005; Di Maio et al., 2012; Di Maro et al., 2007; Tristezza et al., 2013). The term "terroir" describes a clearly delimited area where the natural environment, the physical and chemical features of the soil, and climate conditions allow the achievement of specific grape characteristics, so that, the obtained wine can be identified by means of the unique traits of its territoriality. In this sense, different studies have highlighted the important role of the microbiota associated with the "terroir" from which the grapes are grown. Actually, the terroir-associated microbiota is able to impart a unique quality to the wine (Csoma et al., 2010), although the influence of the grape microbiota on the overall regional characteristics of wines is yet to be elucidated (Bokulich et al., 2014).

Focusing on yeasts, after *véraison* (the onset of ripening) the microbiota of healthy and intact berries is dominated by basidiomycetous yeasts (e.g., *Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp.) and the yeast-like fungus *Aureobasidium pullulans* (see the review of Barata et al., 2012). Approaching harvest time there is an increase of oxidative or weakly fermentative ascomycetous populations (e.g., *Candida* spp., *Hanseniaspora* spp., *Metschnikowia* spp. and *Pichia* spp). In fact, the health of the grapes affects the accessibility of microorganisms to the nutrients of the juice, thus determining the yeast cell number on the surface. Other factors also influence the development of yeasts on the grape surface, with climatic conditions such as rainfall (and consequently dew and moisture), solar irradiation and temperature among the most influential (Barata et al., 2012; Pretorius et al., 1999; Renouf et al., 2005). However, data often differ probably due to the spatial fluctuation of yeast populations (Fonseca and Inácio, 2006; Setati et al., 2012), which makes is difficult to achieve a reliable sampling procedure.

The microbial population (fungi, yeasts and bacteria) of grapes contributes to the final characteristics of wine (Fleet, 2003). Since fungi are unable to grow in wine, their effect on wine quality is due to grape damage and the production of specific metabolites; contrarily, yeasts and bacteria are able to survive and grow in wine. However, it is well known that *Saccharomyces* spp. yeasts are present at very low abundance on healthy berries (Mortimer and Polsinelli, 1999) and thus, they can only be detected during fermentation, when they prevail over other yeasts due to their better resistance to wine harsh conditions, in particular, the ethanol content.

The selection and use of "autochthonous yeasts" (i.e., yeasts present in a certain territory, appellation or vineyard) belonging to the *S. cerevisiae* species has been a reality for many years and this approach is rapidly spreading (Capece et al., 2010; Grieco et al., 2011; Ilieva et al., 2017; Tofalo et al., 2013; Vigentini et al., 2017). The use of autochthonous yeasts is in response to the ever-increasing need for wine personalization, and it represents a compromise between spontaneous fermentation, unacceptable by modern oenology, and practical problems arising from a sensory standardization resulting from the use of selected industrial starter cultures (Ciani et al., 2010). These approaches, based on the accurate selection of

territorial strains, could represent the final step in achieving wines belonging entirely to their own "terroir" (Lopes et al., 2007; Šuranská et al., 2016; Tristezza et al., 2014).

All these data prompted us to comprehensively characterize the yeast microbial consortium on Grignolino wine grapes in an experimental newly planted vineyard in Piedmont (Italy). Grignolino is an ancient red Italian wine grape variety with increasing economic relevance, commonly cultivated in south Piedmont. The aim was to determine the distribution of the different yeasts within a single vineyard using culture-dependent methodologies by isolating the yeasts directly from grape skins. On the other hand, the presence and distribution of fermentative species, which are present in a low number on the grapes, were determined after the spontaneous alcoholic fermentation of grape must. Moreover, the main technological features of isolated *Saccharomyces* spp. strains were assessed in order to identify possible candidates as an indigenous starter for Grignolino grape fermentation.

2. Materials and methods

2.1. Sample collection and recovery

The study was conducted in an experimental vineyard located in Portacomaro (Asti, Italy, Latitude 44°57'23.6" N, Longitude 8° 15' 09.6" E, altitude: from 195 to 205 m.a.s.l. (metres above sea level), exposition: south-east). The vineyard is composed of 15 rows with a total of 1600 Grignolino vines, which was planted in 2013. Grignolino grape samples were collected at maturity in September 2016 (the first year of industrial production) from the odd rows (1, 3, 5, 7, 9, 11, 13, 15) starting from the bottom of the vineyard, row 1 being the lower one and row 15 the upper one in terms of altitude. The vineyard is characterized by a different level of solar radiation, decreasing from the upper to the lower rows due to the presence of tall trees close to the vines in the south border.

Two types of samples were analysed: one from grape skins, to monitor the culturable yeast population on the berry surface; and one from the end of grape fermentation, used as an enriching medium to identify low frequency fermenting yeasts and, in particular, to isolate *Saccharomyces* spp. strains.

To isolate yeasts from grape skins, 50 grape berries for each row (95.2 ± 4.8 g of berries, average ± standard deviation), randomly sampled, using a sterilized scissors and withdrawing the berries with the pedicel avoiding must release. Berries were put into a sterile flask with 200 ml of autoclaved physiologic solution and incubated at 25 °C overnight with agitation in a shaker at 75 rpm. These washing solutions were pelleted at 5000 rpm for 15 min, and then pellets were re-suspended in 5 ml of new physiologic solution and were immediately used for yeast isolation and count.

In order to isolate *Saccharomyces* spp., samples of around 400 berries from each row (310 g on average) were crushed, and the musts were placed in sterilized flasks for spontaneous fermentation. Flasks were maintained at 22 °C during the fermentation process, which was monitored by daily weight loss measurement. When fermentation stopped (weight remained unchanged), lees were separated by centrifugation at 2000 *g* for 5 min and immediately used for isolation.

2.2. Yeast count and isolation in pure culture

To isolate and enumerate grape yeast populations, WLD (Wallerstein Laboratory Differential) agar was used (MERCK, Darmstadt, Germany). Ampicillin sodium salt, 100mg/l, and biphenyl, 400mg/l, (Sigma-Aldrich), previously dissolved in water and ethanol, respectively, were added to the medium to prevent mould and bacterial growth. Washing suspensions were diluted (10^{-1} to 10^{-4}) and 100µl of each dilutionwere spread in duplicate onto WLD and plates were incubated at 25°C for eight days to allow adequate growth. Then, single colonies from plates with 100–200 colonies, and showing different morphologies, were sampled and grown in WL agar for eight days at 25°C. To reduce the risk of bias (different species with the same morphology), at least five random selected colonies belonging to the single morphologies were isolated. In

addition, unique and rare morphologies present on plates were also isolated for a total of 81 different colonies. After growth, morphology characteristics were recorded, including dimension, colour and appearance. For long-term conservation, isolates were inoculated in YEPG (Yeast Extract, Peptone, Glucose) medium for eight days at 25 °C, and the suspension was diluted in 50% v/v glycerol and stored at −80 °C.

For the isolation of yeasts present at the end of fermentation, samples were diluted and spread on WL agar. After growth, 15 colonies from each plate were randomly collected from plates containing 200–300 colonies and conserved at −80 °C as described above.

2.3. DNA extraction from liquid cultures

DNA was extracted from cultures obtained after inoculation in YEPG of the frozen glycerol stocks and incubated overnight, as described previously (Querol et al., 1992) with some modifications (Vaudano et al., 2016). DNA was dissolved in 50µl of ultrapure sterile water and stored at −20°C.

2.4. Identification

Yeast identification was achieved using a "clustering and sequencing" approach. For this, amplified ribosomal DNA restriction analysis (ARDRA) of the 5.8S-ITS region was applied on DNA extracted from yeast colonies using primers ITS1 and ITS4 as previously reported (Esteve-Zarzoso et al., 1999), and *Cfo*I, *Hae*III and *Hinf*I were used as restriction enzymes. Data obtained were analysed using Bionumerics software (Applied Maths, Keistraat, Belgium). Clustering was performed using the option "average of experiments", including ITS amplicons and restriction fragmentpatterns. Dendrograms were built with the UPGMA method considering 90% similarity as grouping cut-off. Cophenetic correlation was applied to determine reliable and unreliable clusters, as described by Rossetti and Giraffa (2005).

After grouping, for unequivocal yeast species identification, the 26S region of rDNA from 1 to 3 samples/group (depending to group dimension) was sequenced using the D1–D2 domain as target, and amplified with primers NL1–NL4 (Kurtzman and Robnett, 1998). PCR products were visualized by electrophoresis at 80 V for 60 min on a 1.2% (w/v) agarose gel. Amplicons were purified using an Illustra CFX kit (GE Healthcare, Little Chalfont, United Kingdom) and bidirectionally sequenced with primers NL1-NL4 (Kurtzman and Robnett, 1998) using The BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Monza, Italy) according to the manufacturer's instructions and the ABI 310 Genetic Analyzer (Applied Biosystem).

Sequences were deposited in NCBI Genbank with accession number reported in Table S1, Table S2 and compared using Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and confirmed by alignment with D1-D2 type strain sequences from the CBS database (http://www.westerdijkinstitute.nl/Collections/Biolomics.aspx?Table=Yeasts%202011) or, when possible, with the D1–D2 sequence of the type strains collected in CREA-VE (CREA-Centro di Ricerca Viticoltura ed Enologia). All identified microorganismswere stored in triplicate at −80°C in 50% (v/v) glycerol.

2.5. *Saccharomyces cerevisiae* strain genetic characterization

Microsatellite multiplex PCR (MM-PCR) was applied to distinguish *S. cerevisiae*strains by using three highly polymorphic microsatellite loci (SC8132×, YOR267C and SCPTSY7) (Vaudano and Garcia-Moruno, 2008). Minor modifications of the method consist of the use of the QIAGEN® Multiplex PCR Master Mix (2×) and 0.5µM individual primer concentrations. PCR conditions were also slightly modified as follows: initial denaturation at 95 °C for 15 min; 28 cycles of denaturing at 94 °C for 30 s, annealing at 57 °C for 1 min and 30 s, and extension at 72 °C for 1 min; and a final extension step at 60 °C for 30 min. Amplifications were performed in a BioRad T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, USA).

PCR products were separated on 2.5% (w/v) agarose (Bio-Rad) gels with 1× TBE buffer using 80 ml gel at 100 V for 75 min. A 100 bp low ladder (Sigma) was used to estimate band sizes. MM-PCR data were processed using Bionumerics software. Strain differentiation was performed via clustering using binary Dice as the similarity coefficient and the UPGMA dendrogram construction method, considering 80% similarity as the cut-off for determination of strain identity. Cophenetic correlation was evaluated as described above.

2.6. Technological properties of isolated *Saccharomyces* spp. strains

2.6.1. Fermentation tests

To evaluate fermentative power, vigour and purity (the ratio between volatile acidityg/l and ethanol % v/v), monoculture fermentation trials using the isolated *S. cerevisiae* strains were carried out as reported by Zambonelli (1988). In addition, an isolated *S. paradoxus* strain was included with the aim of testing its potentialoenological interest. To achieve this, must with excess sugar (300 g/l) was prepared from sterilized grape juice with the addition of 0.4 g/l of ammonium salts (50/50 w/w ammonium sulphate, di-ammonium hydrogen phosphate, Merck). Musts (200 ml) were distributed in pre-sterilized conical flasks capped with a rubber cap with a Müller valve. Pre-inocula were prepared in sterile 100 ml conical flasks with 50 ml of liquid YEPG medium and solid samples from slant tubes. After one day of incubation at 25 °C under stirring conditions, the inoculum was done at a concentration of 10^6 cells/ml. Each strain was tested in triplicate. The fermentation process was monitored by recording CO_2 loss via weight change.
When weight loss stopped, fermentation was considered to be finished and the evaluation of alcohol content, volatile acidity and total acidity was performed using official methodologies (EUR-Lex, 1990). Residual sugar concentrations and glycerol were quantified using an HPLC equipped with a refractometric detector using a Rezex RCM-Monosaccharide column (dimension: 300×7.8mm; particle size 8µm; Phenomenex, Torrance, USA). Conditions were as follows: eluent: water; column temperature: 85°C; flow: 0.35ml/min; injection volume: 20µl.

2.6.2. SO_2 inhibition, H_2S production and Killer character

To evaluate the inhibition of *Saccharomyces* spp. strains by SO_2 during fermentation, 200 ml flasks were prepared for each strain with 100 ml of sterile must (Zambonelli, 1988). SO_2 (100 mg/l) in the form of a 30% (w/w) potassium metabisulfite solution ($K_2S_2O_5$, Merck) was added. Pre-inocula were prepared as described above. The fermentation process was monitored by weight loss, comparing the duration of the lag phase with that of fermentation without SO_2. An arbitrary scale was used from 0 to 8 on the basis of lag phase duration.

Semi-quantitative evaluation of H_2S production was carried out on Biggy Agar (Candida elective agar, Merck) medium (Nickerson, 1953). Using a sterile loop, a dense (10^8 cells/ml) liquid culture was streaked onto the agar surface. After incubation at 25 °C for eight days, the presence of brown to black pigmented colonies, a result of sulphide combining with bismuth, was recorded. The arbitrary scale used was: 0, white (no H_2S production); 1, light brown; 2, brown; 3, dark brown. A non-producing *S. cerevisiae* strain ISE36 (CREA-VE yeast collection) was used as a negative control.

The identification of a killer character was performed using Malt Agar buffered at pH 4.6, onto which was poured 1 ml of physiologic solution containing a known sensible *S. cerevisiae* strain (ISE 1, CREA-VE yeast collection). Yeast strains to be tested for their killer activity were streaked on the agar surface and the plates incubated at 20 °C for eight days. The presence of an inhibition zone due to the death of sensible cells was evaluated. A positive control (*S. cerevisiae* strain ISE 987, CREA-VE yeast collection), known to be a toxin producer and belonging to the CREA-VE yeast culture collection, was tested in each Petri dish.

2.7. Statistical analysis

The Shannon diversity index (Shannon, 1948) and Margalef Richness index (Margalef, 1958) were used to determine grape yeast biodiversity. For this the relative abundance of a single species, with respect to the total isolates in the samples, expressed in CFU/g of grape, was considered.

Analysis of variance (ANOVA) (XLStat, Addinsoft SARL, Paris, France) was performed to evaluate the significance of the variation of fermentation parameters and metabolites produced at the end of fermentation. The Tukey test, to evaluate group (strain) differences in metabolite synthesis, was carried out and the significance level was set at $p \leq 0.05$.

The Pearson correlation (R) (XLStat) was used to observe the relationship among data obtained from fermentation tests.

3. Results and discussion

3.1. Identification and quantification of yeasts from grape skins

The vineyard studied was planted in 2013 with a Grignolino variety of *Vitis vinifera*thanks to a project for territory recovery. Being separated from other cultivated areas, data obtained will not be conditioned by the microbiota of neighbouring vineyards.

Sampling was conducted in September 2016, during the grape harvest, and it was preceded by an unusual rainy August (https://www.arpa.piemonte.gov.it/rischinaturali/accesso-ai-dati/annali_meteoidrologici/annali-meteo-idro/banca-dati-meteorologica.html). These climatic conditions are known to affect the microbiota, since rainfall near or during harvesting increases the yeast concentration on the grapes, and its negative effects on grape integrity lead to a major availability of nutrients on the berrysurface (Renouf et al., 2005). On the contrary, a lower precipitation degree is correlated with a lower yeast density on grape berries, hence microbial populations decrease in number following a dry period (Combina et al., 2005).

The yeast population ranged between 6.4 and 2.8 log CFU/g of berries with an evident tendency to decrease from row 1 to 15 (Table 1). The three lower sampled rows (1, 3, 5) comprise 95% of the yeast population of the whole vineyard, showing around 6.0 log CFU/g cell, which is greater with respect to data reported for undamaged grapes, comprising between approximately 2 and 4 log cells/g (Barata et al., 2012; Prakitchaiwattana et al., 2004). The three upper rows represent 0.23% of the population (Table 1) with a yeast abundance comprised between 2.8 and 4.0 log CFU/g, which is more consistent with previous cited reports. The data shown reflect the influence of the row position on yeast abundance considering that at a lower altitude, due to the presence of tall trees in the south border of the vineyard, solar irradiation was probably lower and humidity was higher during the period of grape maturation. In agreement with these data, it has previously been reported that relative humidity influences yeast populations (Brilli et al., 2014). Moreover, wet conditions favour mould growth that can cause non-visible microcracks in the berry surface (Becker and Knoche, 2015). This damage increases juice nutrient availabilityfor yeast proliferation (Barata et al., 2008, Barata et al., 2012; Prakitchaiwattana et al., 2004).

Table 1. Yeasts count in each row of Grignolino grape vineyard at harvest.

Row	Log CFU/g of berries[a]	Percent respect to whole vineyard[b]
1	6.2	28
3	6.4	48

Row	Log CFU/g of berries[a]	Percent respect to whole vineyard[b]
5	6.0	19
7	5.2	3
9	5.0	2
11	4.0	<1
13	3.3	<1
15	2.8	<1

a

Mean of two replicas that differ by no more than 15%.

b

Values were calculated considering grapes harvested for each row.

Eighty-one colonies of yeast were identified based on ARDRA analysis and clustering with BioNumerics™ (Fig. S1). For each evidenced group (identity percentage>90%), a representative sample was sequenced and identified using the Blast and CBS database (Table S1). The identified colonies belong to 10 species: *Cryptococcus* flavescens, *Candida* californica, *Candida zemplinina*, *Curvibasidium cygneicollum*, *Hanseniaspora uvarum*, *Metschnikowia* pulcherrima, *Meyerozima caribbica*, Pichia kluyveri, *Rhodotorula* babjevae and the yeast-like fungus, *Aerobasidium pullulans*.

Yeast species present on grapes differed among different rows, evidencing the heterogeneity in terms of species diversity in the vineyard (Table 2). The Shannon diversity index, calculated for each row, showed a variation among rows ranging from 0.16 to 1.10, evidencing how yeast diversity drastically changes in the same vineyard within a few metres. Data also highlighted a tendency of reduced richness, expressed as the Margalef index, consistent with the reduction of grape microbial load. In fact, as shown in Table 3, the number of yeast species decreased from rows 1 and 3 (5 species) to row 15 (only one species). Single species distribution was also variable: *Aureobasidium pullulans* is present in all rows, except 9, *Hanseniaspora uvarum* in six rows out of the eight, *Pichia kluyveri* in rows 1, 3, 5 and 9, and *Candida zemplinina* in rows 3 and 9. The rest of the identified microorganisms exhibited lower frequencies: *Curvibasidium cygneicollum*, *Cryptococcus flavescens* and *Meyerozima caribbica* were isolated only in row 1, *Rhodotorula babjevae* only in row 7, and *Candida californica* in rows 5 and 9 (Table 3). Intra-vineyard differences have been attributed to the myriad of microclimates created within the vineyard (Setati et al., 2012), which, as shown in this study, are probably influenced by the different growth conditions, the environment, and in particular, humidity which differs among rows and decreases with altitude.

Table 2. Yeast Ecological indices in single rows and the whole Grignolino vineyard.

Ecological indices	Row 1	Row 3	Row 5	Row 7	Row 9	Row 11	Row 13	Row 15	Whole vineyard
Shannon index (H')	0.44	0.70	0.80	0.99	0.16	1.10	0.66	–	1.33
Margalef's richness index	0.28	0.27	0.22	0.17	0.26	0.22	0.13	–	0.67

Table 3. Percentage distribution of yeasts associated with grapes of single rows in Grignolino vineyard.

Specie name	Row 1	Row3	Row 5	Row 7	Row 9	Row 11	Row 13	Row 15
Aureobasidium pullulans	89%	<1%	8%	51%	–	33%	62%	100%
Candida californica	–		8%		<1%	–		–
Candida zemplinina	–	39%	–	–	97%	–	–	–
Cryptococcus flavescens	<1%	–	–	–	–	–	–	–
Curvibasidium cygneicollum	5%	–	–	–	–	–	–	
Hanseniaspora uvarum	–	60%	76%	34%	2%	33%	38%	–
Metschnikowia pulcherrima	–	<1%	–	–		34%	–	–
Meyerozyma caribbica	5%	–	–	–		–	–	
Pichia kluyveri	1%	<1%	8%	–	1%	–	–	–
Rhodotorula babjevae	–	–	–	15%	–	–	–	–

Three yeast species, *Hanseniaspora uvarum*, *Aureobasidium pullulans* and *Candida zemplinina* dominate the culturable microbiota, representing 93% of the yeast population (Fig. 1). This scenario is consistent with a heterogeneous sanitary status of grapes at maturity, where healthy and micro-damaged berries were present at the same time, promoting the growth of weakly fermentative species such as *H. uvarum*and *C. zemplinina*, together with ubiquitous, oligotrophic *A. pullulans* (Barata et al., 2012; Fonseca and Inácio, 2006).

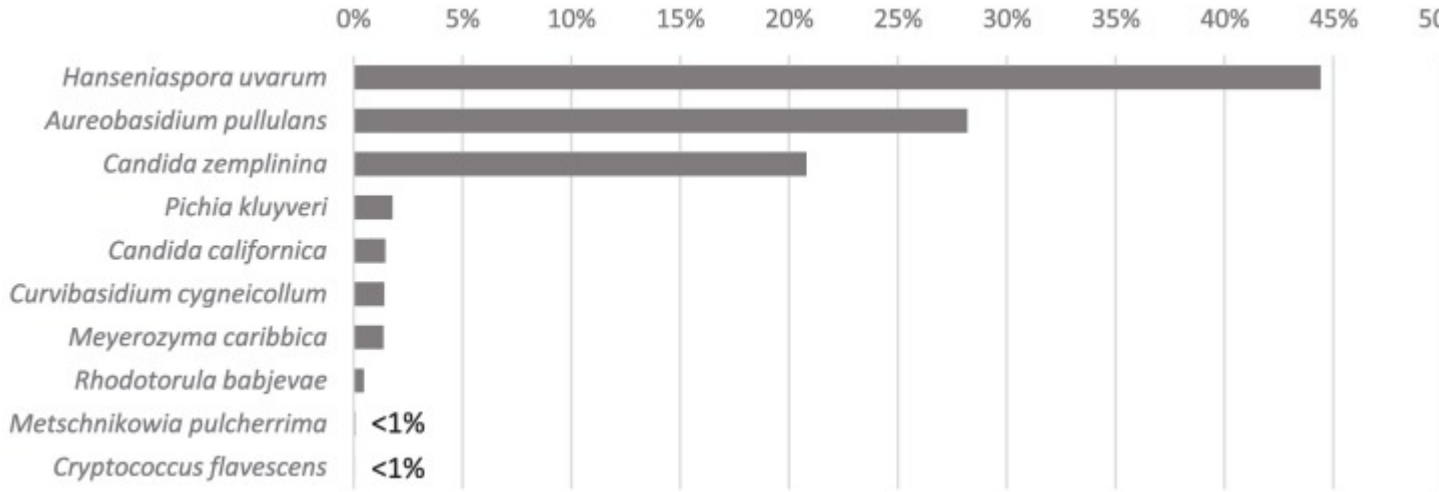

Fig. 1. Yeast species abundancy in the whole Grignolino vineyard. Values were calculated considering grape harvested for each row.

Saccharomyces cerevisiae was not isolated directly from grape skin, as their number is too low when compared with other microorganisms (Capozzi et al., 2015; Guimarães et al., 2006; Mortimer and Polsinelli, 1999). Conversely, *S. cerevisiae* can be easily detected during fermentation, when it prevails over other yeasts due to its better fermentative performance and ethanol tolerance (Fleet and Heard, 1993; Martini, 1993).

3.2. Isolation and characterization of yeasts at the end of alcoholic fermentation

To isolate yeasts belonging to the *Saccharomyces* genus, the identification of yeasts present at the end of fermentation of Grignolino wine grapes was carried out from fermentations performed using grapes harvested in each single row.

ARDRA analysis and D1/D2 26S sequencing showed that almost all isolated microorganisms at the end of fermentation belong to a species of *S. cerevisiae*, except in the row 7 grape must fermentation, where Zygosaccharomyces bailii and *S. paradoxus* represent 30% and 50% of the isolates, respectively, and in row 13 where *Candida zemplinina* stands for 30% (data not shown). The presence of *S. paradoxus*and *S. cerevisiae*, with equal ARDRA profiles, were differentiated by MM-PCR and subsequently confirmed by sequencing (Table S2).

As mentioned earlier, *S. cerevisiae* is generally absent on grape skin isolations, but it is always the predominant yeast at the end of fermentation. The presence of weakly fermentative *Candida zemplinina* and *Zygosaccharomyces* spp. is not a surprise because they are able to survive at a medium-high ethanol concentration. However, it is important to note that the fermentations with grape from rows 7 and 13 were no able to reach the complete consumption of sugars and had less ethanol content (10.30 and 8.75% v/v respectively) respect to the other fermentations (average 12.45% v/v, data not shown); this ethanol content is compatible with the presence of non-*Saccharomyces* species. A remarkable event was the detection of *S. paradoxus*. This yeast is frequently found in association with oak trees, but rarely isolated from fruitsor fermentations (Johnson et al., 2004; Sniegowski et al., 2002), and has only sporadically been associated with wine production (Redzepović et al., 2002).

Regarding *S. cerevisiae* species, 10 different strains were discriminated by MM-PCR analysis (Fig. 2), with 1 out of 10 colonies analysed showing a different MM-PCR profile. This ratio is similar to that reported in

Northeast Italy (Viel et al., 2017), but lower than reported in Portugal and Spain (Schuller et al., 2005, Schuller et al., 2012; Valero et al., 2007). However, biodiversity data can be underestimated, as the methodology applied favours the growth of the best fermentation performer strains. Moreover, as the vineyard studied was newly planted, the observed biodiversity degree has not been influenced by age-affecting aspects, such as colonization of the vineyard's yeast via the soil, insects or residue of unharvested grapes after successive vintages (Sipiczki, 2016; Stefanini et al., 2012) that could increase biodiversity.

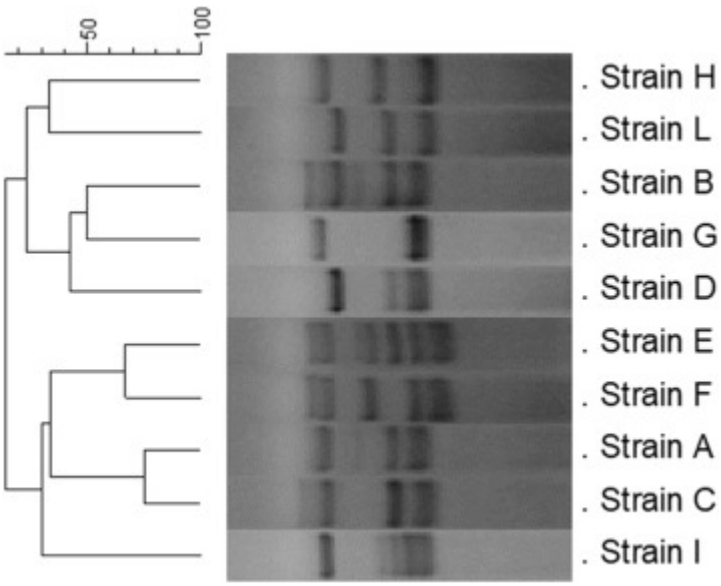

Fig. 2. MM-PCR electrophoresis patterns and cluster analysis of different *Saccharomyces cerevisiae* strains isolated in Grignolino vineyard with similarity above 80%, obtained with 2.5% agarose gel using Dice similarity coefficient. Dendrogram was built with UPGMA method.

Single fermentations from grapes harvested in each single row further confirmed that yeast distribution in the vineyard differs among rows, even at the *S. cerevisiae*strain level (Fig. 3). At the end of fermentation, a great variability was found, with rows with only one strain compared with rows with four different strains. However, this variability was not directly linked to row altitude, being more probably randomly determined by vectors, such as birds, wasps and fruit flies, which feed on the juice of damaged berries (Francesca et al., 2012; Lam and Howell, 2015; Stefanini et al., 2012).

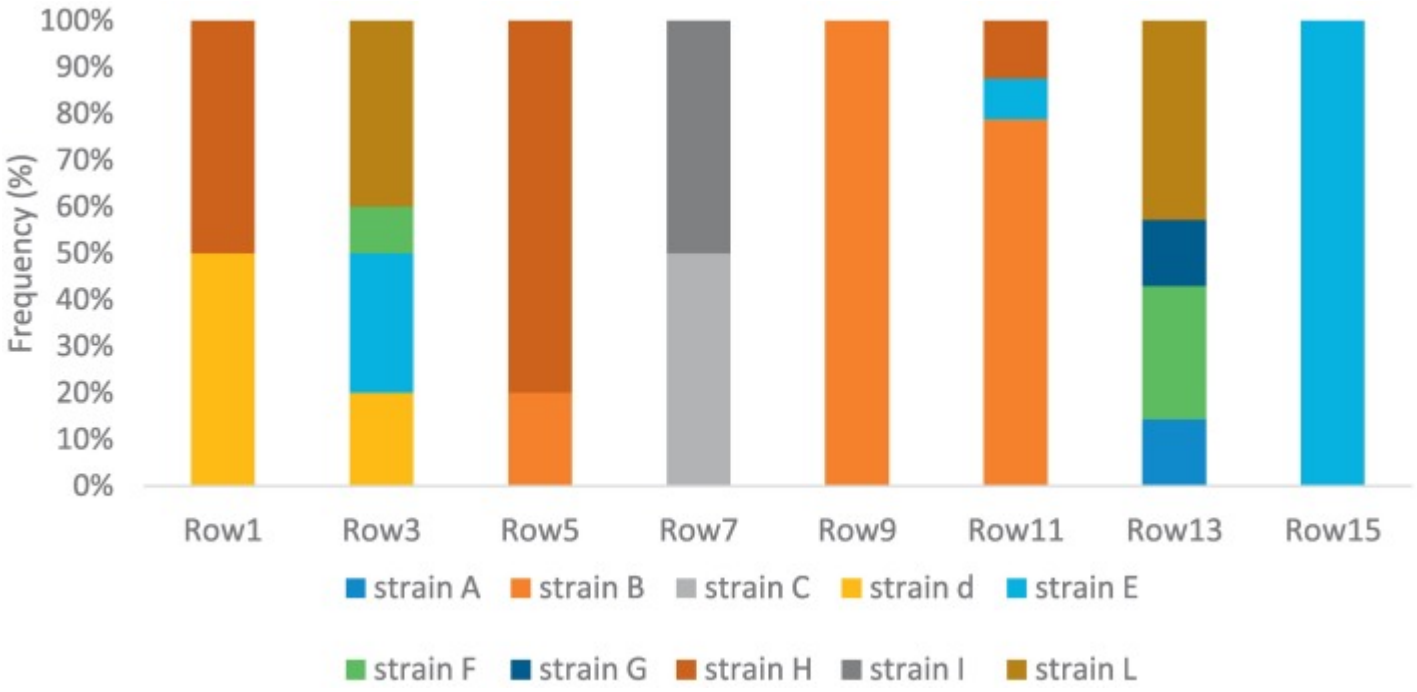

Fig. 3. Frequency of different *Saccharomyces cerevisiae* strains isolated in fermentation of single rows Grignolino grapes.

3.3. Main technological features of isolated *Saccharomyces* spp. strains

In order to obtain information regarding the main technological features of isolated *S. cerevisiae* strains for the possible use in actual wine production, an initial evaluation of their oenological traits was undertaken. In addition, we include *S. paradoxus* strain for the previously reported potential oenological interest of this yeast species (Orlic et al., 2007; Orlić et al., 2010). For this purpose, some characteristics are essential, such as the ability to ferment up to 14.5–15.0% (v/v) of ethanol, fermentative vigour, allowing the strain to compete with natural microflora, and the low production of volatile acidity. Other features can be defined as ancillary characters, but they can significantly affect the quality of the produced wine. Table 4 shows technological and metabolic characteristics of the isolated *Saccharomyces* spp. strains. The production of ethanol in conditions of sugar excess (fermentative power) ranged from 10.9% v/v to 16.5% but does not show statistically significant differences among the *S. cerevisiae* strains, while the *S. paradoxus* strain M differs showing low fermentative power. No statistically significant differences in glycerol production
(ranging from 7.3 to 8.7 g/l) at the end of fermentation were observed. Significant differences among the strains were found in terms of volatile acidity ($p < 0.01$), total acidity ($p < 0.01$), fermentative vigour ($p < 0.05$) and fermentative purity ($p < 0.01$). In particular, strong differences in volatile acidity were found among the isolated strains. *S. cerevisiae* strains H and E were found to be low volatile acidity producers (with concentrations of 0.29 g/l and 0.36 g/l) compared with high producer strains, C and I, generating 0.80 and 0.83 g/l at the end of fermentation, respectively.

Table 4. Main technological and metabolic features of the isolated *S. cerevisiae* strains (A–L) and *S. paradoxus* (strain M).

Strain	Ethanol (% v/v)	Residual sugar (g/L)	Volatile acidity (g/L)	Total acidity (g/L)	Glycerol (g/L)	Fermentation purity	Fermentative vigour (g/L CO_2)*	H_2S** production	SO_2** inhibitio
A	15.32^{a}	42.6^{a}	0.79^{ab}	7.30^{a}	7.9	0.05^{a}	37.7^{b}	0	0
B	14.73^{a}	59.2^{a}	0.44^{cd}	6.90^{d}	7.6	0.03^{ab}	51.5^{ab}	3	5
C	15.70^{a}	43.5^{a}	0.80^{ab}	7.40^{a}	8.8	0.05^{a}	56.6^{ab}	2	1
D	15.13^{a}	51.3^{a}	0.41^{cd}	6.96^{cd}	6.7	0.03^{b}	56.3^{ab}	3	1
E	15.27^{a}	48.8^{a}	0.36^{cd}	7.00^{bcd}	7.9	0.02^{b}	63.4^{a}	2	3
F	15.89^{a}	41.8^{a}	0.43^{cd}	7.00^{bcd}	7.8	0.03^{b}	60.9^{ab}	1	3
G	14.70^{a}	58.6^{a}	0.56^{bc}	7.20^{abcd}	7.6	0.04^{ab}	50.1^{ab}	1	1
H	15.10^{a}	50.8^{a}	0.29^{d}	7.12^{abcd}	6.9	0.02^{b}	58.8^{ab}	2	1
I	16.49^{a}	29.5^{a}	0.83^{a}	7.25^{ab}	8.7	0.05^{a}	55.7^{ab}	1	1
L	15.05^{a}	49.8^{a}	0.58^{abc}	7.23^{abc}	7.9	0.04^{ab}	45.5^{ab}	1	0
M	10.92^{b}	108.9^{b}	0.41^{cd}	6.50^{d}	7.3	0.04^{ab}	36.4^{b}	1	8

Data are the average of three replicas.

When reported, superscript letters indicate statistically significant differences among strains.

*

After three days of fermentation.

**

H_2S and SO_2 inhibition arbitrary scales are described in Materials and methods section.

It is important to highlight that the production of acetic acid, which is the predominant portion of volatile acid production during wine fermentation, is related to the synthesis of glycerol, the main cellular osmolite in *S. cerevisiae*(Hohmann, 2002). In fact, at the beginning of fermentation, glycerol is produced in response to the high sugar concentration of the must, while acetate production is needed to recover the redox balance by reducing NAD^{+} produced during the synthesis of glycerol (Hohmann, 2002; Pigeau and Inglis, 2007). Even if the glycerol content did not show significant differences among strains, there is a positive relationship between glycerol and acetate ($R = 0.420$, $p < 0.05$); therefore, in our tests, the large differences among the isolated yeasts in terms of acetate production were related to the diverse sensibility and response to the hyperosmotic environment (Noti et al., 2018).

S. paradoxus strain M was not comparable with *S. cerevisiae* strains in terms of fermentative power under conditions of excess of sugars probably due to osmotic sensibility, while the other characteristics are similar and the strain proved to not be a high volatile acidity producer (Table 4). To test how the sugar content

could affect strain M performance, fermentation was carried out under less restrictive conditions (23.5°Brix), i.e., closer to the levels of sugar normally found in grapes. Under these conditions, *S. paradoxus* strain M is able to complete fermentation (Fig. 4) confirming the oenological interest of this species (Orlic et al., 2007; Orlić et al., 2010).

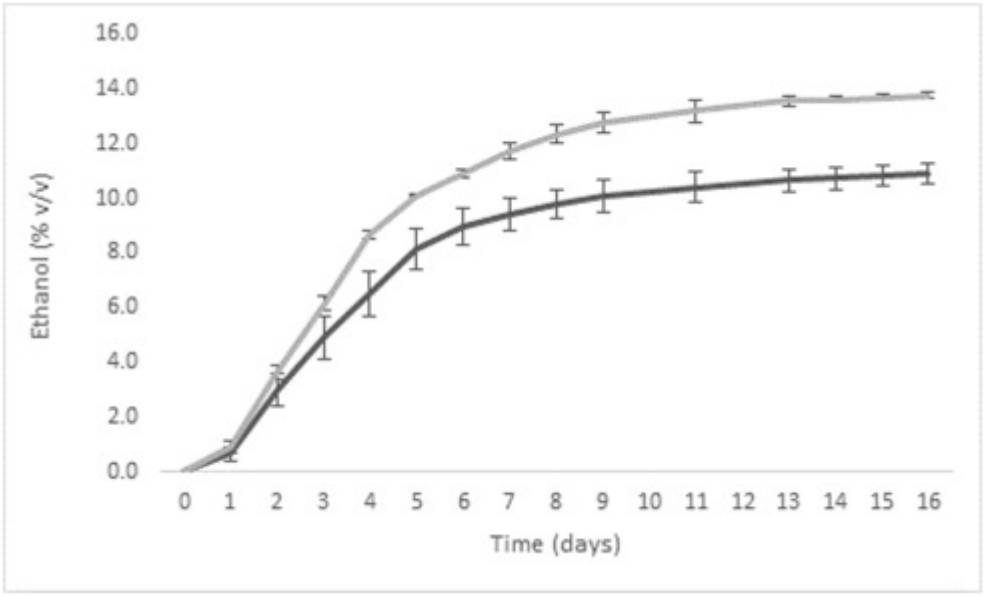

Fig. 4. Fermentation performances of *S. paradoxus* strain M in grape must at 23.5° Brix (gray line) and at 27.0° Brix (black line). Ethanol (% v/v) was indirectly calculated via CO_2production monitored by weight loss.

Semi-quantitative analysis confirms the high intra-specific genetic variability of *S. cerevisiae*, reflecting metabolic differences in the production, assimilation and transformation of certain metabolites (Mortimer, 2000), and detoxification ability toward added preservatives. Data (Table 4) showed differences in H_2S production among strains as previously reported (Kumar et al., 2010): strain A was the only one to not display H_2S synthesis, while strains B and D exhibited the greatest H_2S production among the 10 strains. Only two strains, E and H, showed the ability to produce killer toxins. A large variability was also observed with respect to SO_2sensitivity. Almost all strains of *S. cerevisiae* are able to complete fermentation (data not shown), with the exception of strain B which was completely inhibited by sulfite. The fermentation lag phase with added SO_2 varies from 0 (strains A and L which do not demonstrate inhibition) to eight days in the *S. paradoxus* strain M fermentation.

Finally, *S. cerevisiae* strains E and H appeared to be the most suitable for wine production as they possess the overall characteristics of fermentative vigour, good fermentative purity, low acetic acid production and high ethanol and glycerol production, even though they weren't completely SO_2 resistant. However, it has to be considered that, when performing the SO_2 inhibition test, a high concentration of this compound was used, which is rarely used during winemaking fermentation. Moreover, the *S. paradoxus* strain M result is interesting especially for its good fermentative purity and low H_2S synthesis and it deserves further study and analysis. Further assessments are needed to test strains in Grignolino grape fermentation in a winery trial with final sensorial evaluation.

4. Conclusion

To the best of our knowledge this is the first ecological study performed on Grignolino grapes in Piedmont (Italy), one of the most ancient and relevant grape varieties grown in this viticultural area.

Moreover, it should be highlighted that studies on yeasts isolated from a new implantation vineyard have not been performed before.

In our study, a comprehensive evaluation of the cultivable yeast microbiota present in the vineyard was performed during harvest, evidencing a high intra-vineyard variability in terms of microbial load, species frequencies and distribution. Notably, the very unusually found *S. paradoxus* yeast was isolated from fermenting grapes in addition to 10 different *S. cerevisiae* strains. The technological characterization of the isolated *Saccharomyces* spp. strain revealed the potential oenological interest of some of them, including *S. paradoxus*, for the possible use in actual Grignolino grape winemaking in a "terroir" vision that includes, in addition to climatic, viticultural and pedological features, the contribution of territorial microflora.

The following are the supplementary data related to this article.

table S1: yeasts isolated on grape skins sequenced

mple id.	Accession No.	Row origin	% similarity and closest CBS accession		% similarity and closest BLAST accession		Species name
/1	MH681735	1	100	CBS 8264	100	KY107295.1	*Curvibasidium cygneicollum*
/5	MH681736	1	100	KX067798	99	MH485393.1	*Cryptococcus flavescens*
/6	MH681737	1	100	CBS 8264	100	KY107295.1	*Curvibasidium cygneicollum*
/8	MH681738	1	100	CBS 12034	100	MF101739.1	*Meyerozyma caribbica*
/2	MH681739	3	100	CBS 140243	100	MF420364.1	*Aureobasidium pullulans*
/6	MH681740	3	100	CBS 2579	100	KT922893.1	*Hanseniaspora uvarum*
/9	MH681741	3	100	CBS 7907	100	KY108823.1	*Pichia kluyveri*
/10	MH681742	3	99	CBS 4729	99	KY296077.1	*Candida zemplinina*
/11	MH681755	3	100	CBS 6101	100	KY946978.1	*Candida zemplinina*
/5	MH681743	5	100	CBS 989	100	KY106378.1	*Candida californica*
/1	MH681744	7	100	CBS 9477	100	KY108988.1	*Rhodotorula babjevae*
/2	MH681745	7	100	CBS 10325	99	KT923037.1	*Hanseniaspora uvarum*
/8	MH681746	7	100	CBS 5934	99	KY992079.1	*Hanseniaspora uvarum*
/11	MH681754	7	100	CBS 10325	100	JN214494.1	*Hanseniaspora uvarum*
/2	MH681747	9	100	CBS 9494	100	KY109779.1	*Candida zemplinina*
/7	MH681756	9	100	CBS 989	99	KY106378.1	*Candida californica*
1/13	MH682166	11	99	KT029787	99	KT029787.1	*Metschnikowia pulcherrima*
1/4	MH681748	11	99	HE572532	98	KY108498.1	*Metschnikowia pulcherrima*
1/6	MH681749	11	100	CBS 140243	100	KX958050.1	*Aureobasidium pullulans*

3/2	MH681750	13	100	CBS 702.76	99	KX958050.1	*Aureobasidium pullulans*
3/3	MH681751	13	100	CBS 2580	100	KT922432.1	*Hanseniaspora uvarum*
5/1	MH681753	15	100	KX958048	100	JF278561.1	*Aureobasidium pullulans*
5/4	MH681752	15	100	CBS 702.76	99	KX958050.1	*Aureobasidium pullulans*

Tab. S1

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