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ORIGINAL ARTICLE

Non-*Saccharomyces* biodiversity in wine and the 'microbial *terroir*': a survey on Nero di Troia wine from the Apulian region, Italy

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Abstract The aim of this work was to study the biodiversity of cultivable non-*Saccharomyces* yeasts isolated from the autochthonous wine variety 'Uva di Troia' from the North-Apulian region during vintages 2012 and 2013. Grapes were collected in vineyards from four different geographical areas on which four different wines with the status of geographical indication are produced. Different restriction profiles of ITS–5.8S rDNA regions, corresponding to those of *Candida boidinii*, *Candida zemplinina*, *Hanseniaspora guilliermondii*, *Issatchenkia terricola*, *Zygosaccharomyces bailii*, *Hanseniaspora uvarum*, *Zygoascus hellenicus* and *Hanseniaspora opuntiae*, were observed. The most abundant genera were represented by *Hanseniaspora guilliermondii* and *Candida zemplinina*. Significant differences among locations and vintages were observed. This first report on non-*Saccharomyces* diversity during the early steps of spontaneous alcoholic fermentation of Nero di Troia wines provides the basis for an improved management of non-*Saccharomyces* in typical Apulian wines, which will be important for the development of the local wine industry and to achieve an enhanced standard of safety in the final production.

Keywords Non-*Saccharomyces* · Biodiversity · Wine · Alcoholic fermentation · *Hanseniaspora* · *Candida*

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Introduction

Grape juice is a non-sterile mixture with several types of microorganisms belonging to the microbiota naturally present on grape berries. This microbial consortium is important for wine production. In particular, yeasts can ferment the substrate, promoting the transformation of grape sugars into ethanol, carbon dioxide and hundreds of other metabolites (alcoholic fermentation), while lactic acid bacteria (LAB) are responsible for the decarboxylation of L-malic acid into L-lactic acid and CO_2 (malolactic fermentation). Spontaneous fermentation is carried out through a sequence of different yeast species, according to their metabolic aptitudes and alcohol tolerance: non-*Saccharomyces* yeasts are the most abundant at the beginning of alcoholic fermentation (AF), replaced, after 3–4 days, by *Saccharomyces cerevisiae* (Pretorius 2000; Ciani et al. 2009). *S. cerevisiae* is considered the principal microorganism responsible for vinification, since it completes the fermentation of available sugars. However, during spontaneous AF several yeast genera, such as *Hanseniaspora*, *Kloeckera*, *Candida*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Kluyveromyces* and *Metschnikowia*, have been isolated (Fleet 2003, 2008; Jolly et al. 2014).

Non-*Saccharomyces* yeasts, generally considered spoilage yeasts, in some cases display physiological characteristics that lead them to be considered as potential starter cultures. Indeed, some strains may produce compounds that exert a positive influence on the quality of the wine (Fleet 2003) and/or may be used with a specific technological purpose (e.g., to decrease volatile acidity, decrease alcohol content) (Bely et al. 2008; Contreras et al. 2013). Moreover, several authors have recently proposed the direct application of non-*Saccharomyces* as biocontrol agents against molds or spoilage microorganisms, including spoilage lactic acid bacteria (LAB) or yeast belong to *Brettanomyces bruxellensis* species (Úbeda et al. 2014; Oro

et al. 2014). For this reason, in order to improve the aroma and flavor of wine, several studies suggest the inclusion of non-*Saccharomyces* wine yeasts, together with *Saccharomyces* strains as part of mixed and multi-starter fermentations (Rojas et al. 2003; Romano et al. 2003a; Ciani et al. 2006; Jolly et al. 2006). From this point of view, the study of non-*Saccharomyces* microbial biodiversity represents a subject of increasing interest in wine regions. This attention is also attested by studies highlighting the importance of 'virtuous' microbial diversity during fermentation of artisanal/typical fermented foods and geographical indications (Capozzi and Spano 2011; Capozzi et al. 2012a, b). Additionally, in the grape/wine environment, recent studies investigating the microbial biogeography—the so-called "microbial terroir" (Gilbert et al. 2014)—have revealed a close relationship between production region, climate and microbial patterns (Bokulich et al. 2013). Such evidence sheds new light also on the selection and characterization of autochthonous microbes from regional wines and autochthonous grapevine varieties, such as microbial resources isolated from the Northern Apulian region (Capozzi et al. 2010, 2014; Lamontanara et al. 2014; Di Toro et al. 2014). It is crucial to stress that non-*Saccharomyces* yeast are a very heterogeneous group of microbes, representing not only a vast source of direct applications (Jolly et al. 2013), but also posing risks to wine quality. Among the potential risks due to the non-*Saccharomyces* presence in wine are the production of biogenic amines (Tristezza et al. 2013), generation of off-flavors (acetic acid, esters, acetaldehydes, H_2S) (Fleet 2003, 2008) and competition for the availability of nutrients during fermentation with *S. cerevisiae* (Taillandier et al. 2014).

The aim of this work was to study, for the first time, the biodiversity of cultivable non-*Saccharomyces* yeasts associated with grapes collected from North-Apulian region on the autochthonous vine variety 'Uva di Troia' ('Nero di Troia' is the corresponding wine obtained with 'Uva di Troia' as unique variety) during the vintages '2012' and '2013'. We collected grapes in vineyards from four different geographical areas (San Severo, Barletta, Lucera, Ascoli Satriano) that produce four different wines with the status of geographical indication (wine with appellation of origin) ('San Severo Rosso DOC, 'Rosso Barletta DOC', 'Cacc'e Mmitte DOC', 'Tavoliere delle Puglie DOC'). The common denominator of these wines is the autochthonous grape variety 'Uva di Troia', which can be used at different percentages for specific product formulations.

Materials and methods

Spontaneous fermentation and yeast isolation

Spontaneous alcoholic fermentations were performed by sampling Nero di Troia grape cultivars in the North Apulia area from four vineyards located in the geographical areas of San Severo, Barletta, Lucera, and Ascoli Satriano, during vintages 2012 and 2013. The fermentations were carried out using samples from 1 kg to 5 kg grape berries in 1-L tanks, then spontaneous AF was carried out in the laboratory at 25 °C temperature without further inoculation of starter culture and monitored for 1 month. Yeasts were sampled at the beginning of AF, which was determined on the basis of alcohol content (about 1 %). Decimal saline dilutions were plated on Wallerstein Laboratory (WL) nutrient agar (Oxoid, Basingstoke, UK) and Lysine medium (Oxoid), supplemented with 10 mg/L chloramphenicol to inhibit bacterial growth, according to Lopandic et al. (2008). About 25 green colonies, with different morphologies, were selected for isolation and identification from every fermentation stage and stored at −80 °C in YPD medium supplemented with glycerol (30 % v/v). All assays were conducted in duplicate.

Molecular characterization of non-*Saccharomyces* yeast

The isolates were identified by PCR-RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers (ITS), performed according to Esteve-Zarzoso et al. (1999), with some modifications. The amplification reactions were performed using a PCR reaction mix containing 0.5 μM of each primer (ITS1 and ITS4), 200 μM dNTP, buffer 10X, solution Q and 1.25 units of Qiagen *Taq* DNA Polymerase (Taq PCR Core, Qiagen, Hilden, Germany). PCR was performed in a thermocycler (I-Cycler, Bio-Rad, Richmond, CA), using the following program: initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min; samples were then conserved at 4 °C. Products of amplification were verified on 2 % agarose gels run in 1X TBE buffer and stained with ethidium bromide. After electrophoresis, gels were visualized under UV light and photographed (Versa Doc, Bio-Rad). Sizes were estimated by comparison against a DNA length standard (50 bp ladder, Promega) with Quantity One Software (Bio-Rad). PCR products were then digested without further purification with the Fast Digest® restriction endonucleases *Hae*III, *Hha*I (*Cfo*I) and *Hinf*I (Fermentas, M-Medical, Milan, Italy), although in some cases endonuclease *Dde*I was used.

Restriction analysis was performed following the manufacturer's instructions, using a mix containing 10 μL (about 0.2 μg) PCR product, 2 μL 10X Fast Digest® Green buffer, 1 μL of endonuclease and 17 μL bi-distilled water. The mix was then incubated at 37 °C for 20 min using a thermo cycler (I-Cycler, Bio-Rad). The restriction fragments were separated on a 3 % agarose gel with 1X TBE buffer and stained with ethidium bromide. After electrophoresis, gels were visualized under UV light and photographed (Versa Doc, Bio-Rad).

Sizes of the PCR products obtained were estimated by comparison against a DNA length standard (1 kb ladder, Promega). Two randomly selected PCR fragments for each restriction pattern obtained with RFLP-PCR were purified using the QIAquick PCR purification kit (Qiagen) and sent to Primm Biotech (Milano, Italy) for sequencing. Strains were identified by comparison with sequences available at the NCBI database (GenBank) using the standard nucleotide_nucleotide homology search Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST).

GenBank accession number

The 5.8 ITS sequences obtained were deposited with the GenBank data library under the accession numbers listed in Table 1.

Statistical data analysis

Data generated were analyzed by one-way ANOVA, Turkey test ($P<0.005$). All statistical analyses were performed using Past, version 3.05 (Hammer et al. 2001).

Results

Yeast species identification

A total of 200 purified colonies isolated from grape juice from the Uva di Troia variety during AF was subjected to PCR-RFLP analysis of the 5.8SITS rDNA region. Samples were collected from four different vineyards located in the north Apulia region (Fig. 1) during two consecutive vintages: 2012 and 2013. The yeast species identified and the isolation frequencies obtained during the spontaneous fermentations are shown in Table 1. A wide variety of non-*Saccharomyces* yeast was found. PCR products varying in length from 450 to 880 bp were digested with *Hha*I (*Cfo*I), *Hae*III, *Hinf*I and *Dde*I enzymes, and the molecular mass of the restriction products obtained was compared with those described previously in the literature (Esteve-Zarzoso et al. 1999; Pham et al. 2011). In general, we observed eight different restriction profiles of the ITS–5.8S rDNA region, corresponding to *Candida boidinii*, *Candida zemplinina*, *Hanseniaspora guilliermondii*, *Issatchenkia terricola*, *Zygosaccharomyces bailii*, *Hanseniaspora uvarum*, *Zygoascus hellenicus* and *Hanseniaspora opuntiae* (Table 1). Two randomly selected strains for each pattern obtained were sequenced to confirm species assignation, performed by comparison with sequences available at the NCBI database (GenBank) using the standard nucleotide_nucleotide homology search Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST) (corresponding gene accession numbers are reported in Table 1).

Several yeast species, e.g., *C. boidinii*, *C. zemplinina*, *H. guilliermondii* and *I. terricola*, represented a common denominator of all the vineyards studied. Other species were isolated from one vineyard only (for example, *Zygoascus hellenicus* from San Severo and *H. opuntiae* from Lucera) (Table 1). The predominance of non-*Saccharomyces* yeasts during the first step of the fermentation was observed for all the grape juice analyzed. Figure 2 shows the frequencies of strains identified from the four different vineyards, respectively, during the vintages 2012 and 2013, while Fig. 3 reports the frequencies of yeast isolated during vintages 2012 and 2013 in Nero di Troia vineyards. Among the non-*Saccharomyces* yeasts characterized in this study, the most abundant genera were *Hanseniaspora* (about 58 %, *H. guilliermondii* 53 %, *H. uvarum* 4 % and *H. opuntiae* 1 %) and *Candida* (about 32 %, *C. zemplinina* 19 % and *C. boidinii* 16 %) (Fig. 3). Analysis of non-*Saccharomyces* diversity in the four different areas revealed a great variability, showing, in several cases, statistically significant differences among locations (Fig. 2) and vintages (Fig. 3). The presence of *H. guilliermondii* is different in San Severo (46 and 49 %, respectively, for vintage 2012 and 2013) and Ascoli Satriano (59 and 60 %, respectively, for vintage 2012 and 2013) vineyards, while in Barletta and Lucera vineyards we found a similar frequency of *H. guilliermondii* (about 50 %). *H. uvarum* ecotypes were isolated only from San Severo and Lucera. In addition, *H. uvarum* was detected at higher frequency during vintage 2013 than 2012, (from 2 % to 16 %) (Fig. 2). *C. boidinii* shows a higher presence in Barletta and Ascoli Satriano vineyards, with respect to Lucera and San Severo. Considering the genus *Candida*, significant differences in *C. boidinii* selected ecotypes were found between the two vintages studied, with a frequency decrease from 21 % for the vintage 2012 to 11 % for the vintage 2013 (Fig. 3). During the vintage 2012, *C. zemplinina* exhibited the highest presence in San Severo vineyards (25 %), with lower frequencies in Barletta, Lucera and Ascoli Satriano. In contrast, during vintage 2013, the frequency of the same non-*Saccharomyces* species analyzed was higher in the Barletta area (23 %) and lower (about 20 %) in the other areas studied. Comparing the frequency of strains analyzed for *C. boidinii*, *C. zemplinina*, *H. guilliermondii* and *H. uvarum*, they also showed significant differences between the two vintages. Considering minor yeast genera isolated, *I. terricola* isolates were found to be lower in Barletta, Lucera and the Ascoli Satriano area (about 4 %) and higher in the San Severo area (about 10 %), although no significant differences were observed in the frequency of any of the *I. terricola* strains analyzed during vintage 2012 and 2013 (Fig. 3). *Zygosaccharomyces bailii* and *Zygoascus hellenicus* species were isolated only from specific

Table 1 Identification of yeasts isolated from grape must collected from four wineries (Barletta, San Severo, Lucera, Ascoli Satriano) by internal transcribed spacer–restriction fragment length polymorphism (ITS-RFLP) analysis and sequencing

Species [a]	ITS	Restriction fragments				Number of isolates		Origin	Accession number
		*Hae*III	*Hinf*I	*Cfo*I	*Dde*I	2012	2013		
Candida boidinii	750	700	390+190+160	350+310+90	/	12	8	Barletta	KP241860-KP241861
Candida zemplinina	475	475	235+235	215+110+80+60	/	9	12	Barletta	KP241864-KP241865
Hanseniaspora guilliermondii	750	750	350+200+180	320+310+105	380+180+95+80	25	30	Barletta	KP241874-KP241875
Issatchenkia terricola	450	290+125	240+105+105	130+100+90+85+45	/	2	/	Barletta	KP241882-KP241883
Zygosaccharomyces bailii	790	690+90	340+225+160+55	320+270+95+95	/	2	/	Barletta	KP241898-KP241899
Candida boidinii	750	700	390+190+160	350+310+90	/	7	2	San Severo	KP2281424-KP281425
Candida zemplinina	475	475	235+235	215+110+80+60	/	12	10	San Severo	KP241866-KP241867
Hanseniaspora guilliermondii	750	750	350+200+180	320+310+105	380+180+95+80	22	24	San Severo	KP241876-KP241877
Issatchenkia terricola	450	290+125	240+105+105	130+100+90+85+45	/	6	5	San Severo	KP241884-KP241885
Hanseniaspora uvarum	750	750	350+200+180	320+310+105	300+180+95+90+85	1	8	San Severo	KP241872-KP241873
Zygoascus hellenicus	650	625	350+170+130	325+325	/	2	1	San Severo	KP241902-KP241903
Candida boidinii	750	700	390+190+160	350+310+90	/	10	4	Lucera	KP2281426-KP281427
Candida zemplinina	475	475	235+235	215+110+80+60	/	8	10	Lucera	KP2281428-KP281429
Hanseniaspora guilliermondii	750	750	350+200+180	320+310+105	380+180+95+80	26	26	Lucera	KP241878-KP241879
Issatchenkia terricola	450	290+125	240+105+105	130+100+90+85+45	/	2	1	Lucera	KP241886-KP241887
Zygosaccharomyces bailii	790	690+90	340+225+160+55	320+270+95+95	/	2	1	Lucera	KP241900-KP241901
Hanseniaspora uvarum	750	750	350+200+180	320+310+105	300+180+95+90+85	1	6	Lucera	KP241870-KP241871
Hanseniaspora opuntiae	750	750	350+200+180	320+310+105	/	1	2	Lucera	KP241868-KP241869
Candida boidinii	750	700	390+190+160	350+310+90	/	12	8	Ascoli Satriano	KP241862-KP241863
Candida zemplinina	475	475	235+235	215+110+80+60	/	6	10	Ascoli Satriano	KP2281430-KP281431
Hanseniaspora guilliermondii	750	750	350+200+180	320+310+105	380+180+95+80	30	30	Ascoli Satriano	KP241880-KP241881
Issatchenkia terricola	450	290+125	240+105+105	130+100+90+85+45	/	2	2	Ascoli Satriano	KP241888-KP241889

[a] Species assignation according to Esteve-Zarzoso et al. (1999)

Fig. 1 Schematic representation of the geographical vineyard sampling location

vineyards: San Severo and Lucera for *Zygosaccharomyces bailii* and San Severo for *Zygoascus hellenicus*, respectively. However, even in this case, no significant differences in frequency were observed (Fig. 3).

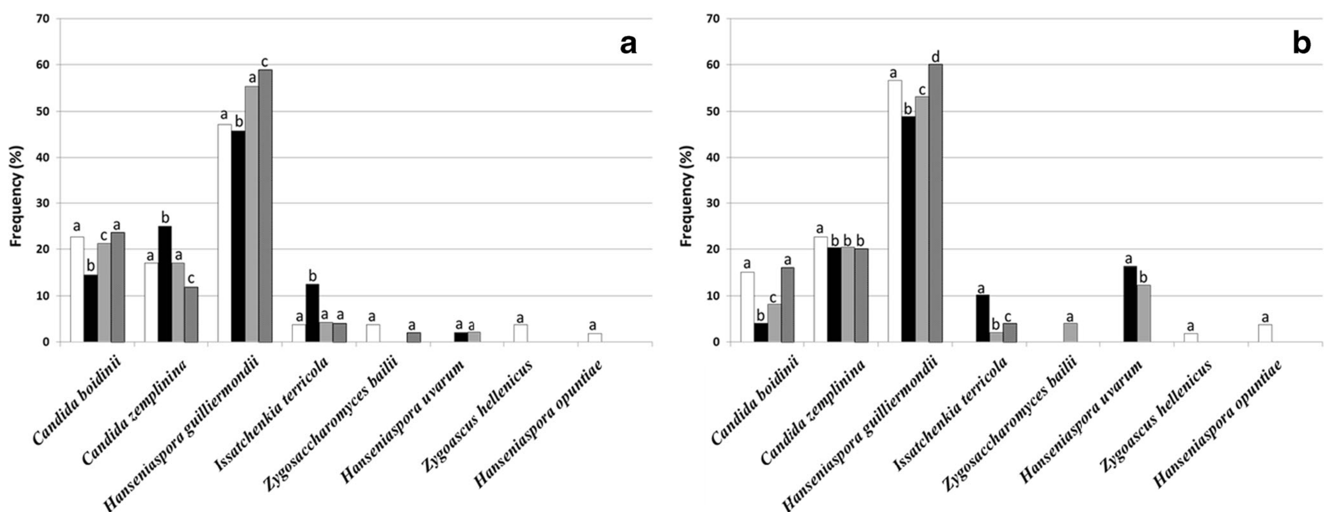

Fig. 2 **a**,**b** Percentage frequencies of non-*Saccharomyces* yeasts isolated from spontaneous fermentation of vine variety "Uva di Troia", during vintages 2012 (**a**) and 2013 (**b**). Vineyard: *Open bars* Ascoli Satriano; *black bars*, San Severo; *light grey bars*, Lucera; *dark grey bars*, Barletta. Different letters above bars indicate statistical significance (one-way ANOVA, Turkey test $P<0.005$)

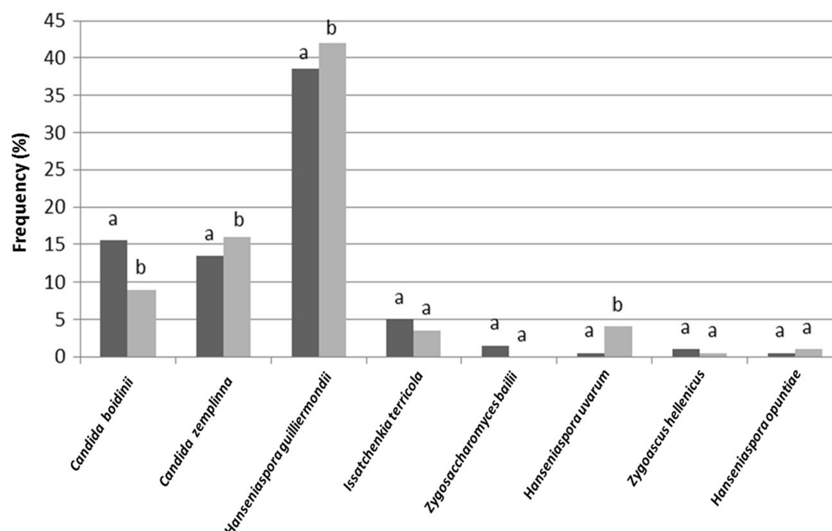

Fig. 3 Percentage frequencies of non-*Saccharomyces* yeast during vintage 2012 (*dark grey bars*) and vintage 2013 (*light grey bars*). Different letters above bars indicate statistical significance (one-way ANOVA, Turkey test $P<0.005$)

Discussion

The presence and variability of non-*Saccharomyces* on grapes, musts and wines are studied in order to determine their potential effects on the organoleptic qualities of the final products (González et al. 2006). For this reason, a greater understanding of non-*Saccharomyces* biodiversity in fermenting wines is an essential criterion for quality improvement programs in oenological production and, more specifically, in the sector of typical wine and oenological geographical indications. All samples analyzed in this study show the predominance of non-*Saccharomyces* yeasts in the first steps of a spontaneous AF (Ganga and Martínez 2003; Clavijo et al. 2010; Bezerra-Bussoli et al. 2013). In terms of yeast diversity, our results are similar to those found in other wine-producing areas. In fact, several studies have already reported a dominance of *Candida* and *Hanseniaspora* genera at the beginning of spontaneous AF in wine (Beltran et al. 2002; van Keulen et al. 2003; Combina et al. 2005; Romancino et al. 2008; Pramateftaki et al. 2012; Bezerra-Bussoli et al. 2013) although non-*Saccharomyces* yeast such as *Lachancea*, *Wickerhamomyces* and *Torulaspora* were sometimes reported as the main non-*Saccharomyces* dominant species (Cordero-Bueso et al. 2012). Furthermore, it is important to stress that, within *Candida* and *Hanseniaspora* genera, the species with the highest frequencies are usually different. For example, in this work, *H. guilliermondii* and *C. zemplinina* were found as dominant species, while Ocón et al. (2010), investigating the yeast population present in four spontaneous alcoholic fermentations in the Rioja appellation (D.O.Ca. Rioja, Spain), found *C. stellata* and *H. uvarum* in major proportions during fermentation. Our results can be correlated to specific pedoclimatic conditions. Indeed, both vintages analyzed in this study were characterized by high levels of precipitation.

Non-*Saccharomyces* yeast species play relevant roles in determining wine flavor and complexity. In particular, they can improve the chemical composition of wines due to several aromatic compounds, besides they are often isolated from wines with anomalous sensorial profiles and associated with the production of compounds toxic to human health (Tristezza et al. 2013). Several studies have proposed non-*Saccharomyces* as a tool to mimic natural biodiversity and to enhance the complexity and the particular characteristics of a wine, avoiding the risk of sluggish or stuck fermentation, as part of mixed/multi-strains starter cultures (Romano et al. 2003b; Ciani et al. 2006, 2009; Jolly et al. 2006, 2014; Ciani and Comitini 2011). The most represented species isolated in this study, in both vintages and in all locations, are *H. guilliermondii* and *C. zemplinina*, non-*Saccharomyces* yeasts of oenological interest. These findings lead us to suggest these two species as possible candidates for the design of mixed/multistrains autochthonous starter cultures for 'San Severo Rosso DOC, 'Rosso Barletta DOC', 'Cacc'e Mmitte DOC', 'Tavoliere delle Puglie DOC', as well as for 'Nero di Troia' wines, with the final aim of achieving a product via a representation of autochthonous virtuous microbial diversity. Moreira et al. (2008) reported that wines inoculated with *H. guilliermondii* show higher levels of 2-phenylethyl acetate, 1-propanol and 3-(methylthio)propionic acid, highlighting that certain apiculate yeasts have the capacity to influence, in a positive way, the aromatic profile of wines. Moreover, *C. zemplinina* can be exploited advantageously in sweet wine production due to the lower amount of acetic acid produced and elevated concentration of glycerol (Sipiczki 2004; Rantsiou et al. 2012; Tofalo et al. 2012; Magyar et al. 2014). Whereas not present at higher concentrations, *H. uvarum* and *C. boidii* species are also representative of the non-*Saccharomyces* diversity observed. Concerning their oenological significance, with mixed fermentation, *H. uvarum* increased the content of isoamyl acetate in wines (Moreira et al. 2008), while *C. boidinii* is a species frequently isolated in cellar surfaces from spoiled wines (Saez et al. 2011).

With regard to non-*Saccharomyces* genera isolated at lower concentrations, it is crucial to highlight how they were often connected with spoilage and unwonted phenomena (Loureiro and Malfeito-Ferreira 2003; Pretorius 2000). Among yeasts with lower frequency, spoilage yeast, such as *Zygosaccharomyces bailii*, *Zygoascus hellenicus*, and *I. terricola*, were isolated. *Zygosaccharomyces bailii* is one of the main spoilage yeasts in the wine industry (Zuehlke et al. 2013), while several strains belonging to *Zygoascus hellenicus* and *I. terricola* have been characterized as producers of biogenic amines in wine (Tristezza et al. 2013).

In conclusion, this is the first report on yeast microbiota during the early steps of spontaneous AF from Apulian Nero di Troia wines. Our findings provide the basis for improved management of non-*Saccharomyces* in typical Apulian wines that will be important for the development of the local wine industry and to achieve an enhanced standard of safety in the final product.

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Conflicts of interests The authors declare no conflicts of interest

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