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Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region, Italy

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ABSTRACT

This work is the first large-scale study on vineyard-associated yeast strains from Apulia (Southern Italy). Yeasts were identified by Internal Transcribed Spacer (ITS) ribotyping and bioinformatic analysis. The polymorphism of interdelta elements was used to differentiate *Saccharomyces cerevisiae* strains. Twenty different species belonging to 9 genera were identified. Predominant on the grape surface were *Metschnikowia pulcherrima*, *Hanseniaspora uvarum* and *Aureobasidium pullulans*, whereas *M. pulcherrima* and *H. uvarum* were dominant in the early fermentation stage. A total of 692 *S. cerevisiae* isolates were identified and a number of *S. cerevisiae* strains, ranging from 26 to 55, was detected in each of the eight fermentations. The strains were tested for biogenic amines (BAs) production, either in synthetic media or grape must. Two *Pichia manshurica*, an *Issatchenkia terricola* and a *M. pulcherrima* strains were able to produce histamine and cadaverine, during must fermentation. The production of BAs in wine must was different than that observed in the synthetic medium. This feature indicate the importance of an "in grape must" assessment of BAs producing yeast. Overall, our results suggest the importance of microbiological control during wine-making to reduce the potential health risk for consumer represented by these spoilage yeasts.

1. Introduction

The conversion of grape must to wine is a complex biochemical process mediated by highly-specialized microorganisms, yeasts and bacteria (Fleet, 1999; Mortimer and Polsinelli, 1999). Non-*Saccharomyces* yeasts, including species of *Hanseniaspora*, *Candida*, *Metschnikowia* and *Pichia*, show lower fermentative activity than *Saccharomyces cerevisiae* and dominate the early stages of spontaneous fermentation (Bauer and Pretorius, 2000). Furthermore, the population of non-*Saccharomyces* rapidly decreases due to the strong selective pressure exerted by strains belonging to *S. cerevisiae*, the main species responsible for alcoholic fermentation (Fleet, 2008). However, it has been demonstrated that the fermentation process is carried out and completed by a limited number of dominant strains associated with a variable number of secondary strains (Versavaud et al., 1995; Redzepović et al., 2002).

Indigenous yeasts are present on the surfaces of grapes and their success in surviving and driving fermentation depends on the sum of various physical, chemical and biotic factors (Barata et al., 2012). The geographical distribution of *S. cerevisiae* strains within specific wine-producing regions has been analyzed (Versavaud et al., 1995; Pramateftaki et al., 2000; Redzepović et al., 2002; Martinez et al., 2004; Bisson, 2012) and changes in microflora composition in different vineyards have been observed. Over several consecutive years, predominant strains have also been observed in the same vineyard. The analysis of wine strain diversity and the relationship between genotype and phenotype can be used in the development and identification of specific strains well-tailored for specific production needs (Bisson, 2012). The fundamental role played by *S. cerevisiae* has induced many winemakers to use commercial yeast strains as fermentation starters, but their widespread utilization can lead to the progressive substitution of local microflora (Ganga and Martinez, 2004; Valero et al., 2005) and the flattening of the

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typical organoleptic features usually connected with indigenous yeast strains. The understanding of the yeast population dynamics during natural alcoholic fermentation could help to preserve the most representative native strains to use as autochthonous fermentative starters so as to enhance the organoleptic and sensory properties of the product (Capece et al., 2010; Comitini et al., 2011; Capozzi and Spano, 2011; Tristezza et al., 2012; Di Maio et al., 2012).

In this light, it is also increasingly important to evaluate microbial biodiversity in terms of wine safety (Pozo-Bayón et al., 2012). One of the emerging problems for wine consumers is caused by the presence of biogenic amines (BAs), low-molecular-weight organic bases formed in wine by the activity of microbial-specific amino acid decarboxylases (Capozzi et al., 2011).

Several studies have been conducted on the presence of biogenic amines in wines originating from different countries worldwide. Putrescine is the most abundant compound, followed by histamine, tyramine and cadaverine. Their concentrations range from a few hundreds of micrograms to a few tens of milligrams per litre (Landete et al., 2007; Spano et al., 2010; Beneduce et al., 2010). High concentrations of BAs in wine lead to adverse physiological consequences in sensitive consumers and these toxic effects are further enhanced by the presence of acetaldehyde and ethanol (Coton et al., 2010). BAs are thus considered a risk for human health and their toxicity has led to the universal concept that they should not be allowed to accumulate in food and beverages (Spano et al., 2010; Linares et al., 2012). This aspect is even testified by existing limits for histamine in wine in some European countries (Smit et al., 2008) and by the recently published Scientific Opinion of the Panel on Biological Hazards of the EFSA (European Food Safety Authority) on risk-based control of BA formation in fermented foods (EFSA Panel on Biological Hazards (BIOHAZ), 2011).

An extensive literature exists on biogenic amine production by lactic acid bacteria (LAB) in different fermented foodstuffs (Landete et al., 2005; Fernández and Zúñiga, 2006; Smit et al., 2008; Coton et al., 2010). However, the studies on BAs produced by wine yeasts reported contrasting results. Landete et al. (2007) analyzed wine yeast strains for their ability to produce biogenic amines and none of the strains analysed was able to produce BAs. In contrast, BA production by yeast has been reported by several authors in strains belonging to *S. cerevisiae*, *Kloeckera apiculata*, *Candida stellata*, *Metschnikowia pulcherrima*, *Brettanomyces bruxellensis* and *Zygoascus hellenicus* species (Caruso et al., 2002; Granchi et al., 2005; Chang et al., 2009).

The objective of this work was to analyse the biodiversity of indigenous yeast populations associated with grape and with spontaneous alcoholic fermentations in Apulia (Southern Italy) and to evaluate their ability to produce BAs. To the best of our knowledge, this is the first report concerning the vineyard-associated yeast strains in this important wine-producing area of Southern Italy. The data obtained shed further light on the contribution of undesired native yeasts on BA accumulation during wine fermentation.

2. Materials and methods

2.1. Grape sampling and fermentation

Eight spontaneous alcoholic fermentations were performed by sampling Primitivo grape cultivars in the Galatina, Torchiarolo, Manduria and Gioia del Colle areas and Negroamaro grape cultivars (*Vitis vinifera*) from four vineyards located in Torchiarolo, Copertino, Cutrofiano and Melissano, the most significant production areas for these cultivars and related wines in Apulia. The fermentations were carried out using samples from 80 to 90 kg grape berries in 100-L tanks in an experimental cellar at a temperature between 22 and 25 °C, with daily monitoring of sugar consumption. Samples were taken at three different stages: on the first day of fermentation; in the middle of fermentation (density 1050–1040 g/L) and at the end of fermentation (density 995–993 g/L). Isolation of the microorganisms associated with the grape surface was carried out according to Prakitchaiwattana et al. (2004).

2.2. Yeast molecular identification

Ten-fold dilutions of must samples were spread onto plates containing YPD medium (yeast extract 1% w/v, meat peptone 2% w/v, glucose 2% w/v and agar 2% w/v) and incubated at 28 °C for 24–48 h. Thus, 35 colonies were randomly selected, according to their different morphological aspects, from each stage of the eight spontaneous fermentations. The isolates were identified according to the length of the rDNA region spanning the 5.8S rRNA gene and flanking the internal transcribed spacers 1 and 2 (De Benedictis et al., 2011). To determine the species identity of isolates, amplicons were subjected to sequence analysis and compared with the sequences in the GenBank database. All isolates identified as *S. cerevisiae* were characterized at strain level by interdelta typing as previously described (Tristezza et al., 2009).

2.3. Statistical analysis

Binary matrices built with data from interdelta amplification were used for cluster analysis of the pairwise values based on the Dice coefficient by the UPGMA algorithm, using the NTSYS software (Applied Biostatistics, USA). Classical ecology indices, such as the Shannon–Wiener index of general diversity (H), the richness (S) of the microbial community, Simpson's diversity indices (D and $1 - D$) and Evenness (eH/S) were calculated using the free software package PAST (Palaeontology Statistics, http://folk.uio.no/ohammer/past/). Moreover, the frequency of the species (F') was calculated as the number of samples from which each species was isolated among the total number of samples and incidence of the species (I') as the percentage of occurrence of the species in the whole yeast population in the study. The ratio between the number of isolates belonging to the specie *S. cerevisiae* and the number of molecular patterns was also calculated as a polymorphism index (Schuller et al., 2005).

2.4. Amino acids decarboxylation assay

The identification of yeast strains possessing an amino acid decarboxylation activity was carried out by a plate assay method (Nikolaou et al. 2006). The amino acids histidine, tyrosine, phenylalanine, tryptophan, lysine, leucine and arginine were utilized. Briefly, ten microliters of a saturated yeast culture were applied onto YPD agar plates, added with 1% (w/v) of one of the chosen amino acids and 0.006% (w/v) bromocresol. After incubation at 25 °C for 5–7 days, the plates were analyzed for the presence/absence of a purple halo around the yeast colony: amino acid decarboxylation was considered positive when a purple halo surrounded the yeast colony. The isolates were screened according to the halo size, thus making it possible to recognize distinct groups of isolates identified by a numerical code: 1, purple halo 0–1 mm in width; 2, purple halo 1–3 mm in width; 3, purple halo 3–5 mm in width; 4, purple halo more than 5 mm in width. *Hanseniaspora uvarum* strain 8795 (De Benedictis et al., 2011) and *Lactobacillus brevis* IOEB 9809 (Arena et al., 2011) were used as negative and positive control, respectively.

2.5. Biogenic amine detection

The ability of the selected yeast strains to produce biogenic amine was investigated by a recently developed analytical method (Romano et al., 2012), for determining the four main biogenic amines of wine (i.e. histamine, tyramine, putrescine and cadaverine). Yeast strains were grown in YEPG broth (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L) supplemented with a single amino acid (histidine, lysine and arginine at 1% w/v concentration or tyrosine at 0.1% w/v concentration) at 25 °C for 24 h and then samples were collected for further analysis. Yeast were also grown in Negroamaro must (sterilized by autoclaving at 121 °C for 15 min) supplemented with a single amino acid as described above, incubated at 25 °C for 14 days, and samples were taken at 0, 7 and 14 days post inoculation. All collected samples were centrifuged and supernatant aliquots (200 μL) were mixed with 200 μL of saturated $NaHCO_3$ solution and 400 μL dansyl chloride solution (5 mg/mL in acetone) and then incubated at 55 °C for 1 h. Vials were incubated at room temperature for a few minutes and supplemented with 100 μL saturated NaCl solution and 200 μL iso-hexane. Extraction was performed on a tabletop shaker for 5 min. Finally, 20 μL aliquots of organic phase were deposed on the TLC plates to detect histamine, tyramine, cadaverine and putrescine. Separation of analytes was performed on silicagel 60 TLC glass plates, without fluorescent indicator (Merck, Darmstadt, Germany). TLC plates were illuminated at a wavelength of 312 nm and images were captured and then analyzed using the BIO-1D software (Vilber-Lourmat, France). A semi-quantitative determination was performed by comparing the fluorescence intensity of the samples with 2 reference samples containing 50 and 0.5 mg/L of each biogenic amine.

3. Results

3.1. Molecular identification of indigenous yeasts

In Table 1 the distribution of different yeast species identified in samples of spontaneous fermentations of Negroamaro and Primitivo grapes is summarized. The predominant yeast species on the grape surfaces were *M. pulcherrima*, *H. uvarum*, *Aureobasidium pullulans*, *C. stellata* and *Candida zemplinina*. However, additional species such as *Issatchenkia terricola*, *Cladosporium* sp., *Penicillium* spp., *Cryptococcus* sp., *Z. hellenicus* and *Kluyveromyces thermotolerans* were identified. The species *M. pulcherrima* and *H. uvarum* were dominant in the initial phases of fermentation, although *C. stellata*, *Kluyveromyces* spp., *Pichia* spp. and *Issatchenkia* spp. were also detected. In contrast, in the Copertino sample, *S. cerevisiae* was already detectable from the early phase of fermentation onwards (Table 1). Furthermore, in all the fermentation steps analysed, all winemaking trials showed a sequential replacement of non-*Saccharomyces* species with populations of yeasts belonging to the species *S. cerevisiae*, with the exception of the Melissano sample, for which the genus *Saccharomyces* appears and dominates only at the end of fermentation. In Table 1 is also reported the frequency (F') and incidence (I') of each species. The most frequent species was *S. cerevisiae* ($F' = 0.60$) which was present from the early phase of fermentation and was also the most abundant ($I' = 48.33$), followed by *H. uvarum* ($F' = 0.45$; $I' = 14.08$) and *M. pulcherrima* ($F' = 0.33$; $I' = 9.90$). *C. stellata* ($F' = 0.18$; $I' = 5.55$) and *C. zemplinina* ($F' = 0.13$; $I' = 4.55$) that were more abundant though less frequent than *Hanseniaspora guilliermondii* ($F' = 0.25$; $I' = 3.78$) and *I. terricola* ($F' = 0.30$; $I' = 4.40$). *A. pullulans* was found in only four samples ($F' = 0.10$) but had a relative high incidence ($I' = 3.58$). As reported in Table 2, statistical analysis revealed that species richness was highest in the sample from Galatina ($S = 14$) followed by Melissano

Table 1
Distribution of the yeast species associated with eight spontaneous fermentation of Negroamaro and Primitivo grapes.

Species	Negroamaro																Primitivo																					
	Copertino					Cutrofiano					Melissano					Torchiarolo					Galatina					Gioia del Colle					Manduria					Torchiarolo						
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	F′	I′
A. pullulans											2										29										9					10					0.10	3.55
C. cladosporioides																					2																					0.03	0.15
C. incommunis							1															1																				0.03	0.10
C. oeirensis																																										0.03	0.08
C. stellata							1										12	6								9	11				15		24								0.18	5.55
C. thermophila																					1																					0.03	0.08
C. zemplinina																		9					10					1								19		25			0.13	4.55
H. guilliermondii											2	2	2			2	4						3									26	3				8	1			0.25	3.78
H. uvarum	16	12	1			9	11				11	21	18	4		21	9	4					28	4			3	5			3						19			0.45	14.08
Lorientalis		3										2											2				15	1									2			0.15	1.83
I. terricola								3			1	5	8			12	11						2	1							5	6				7	1			0.30	4.40
K. marxianus																							3																		0.03	0.20
K. thermotolerans						1																	2			5	2														0.08	0.58
M. pulcherrima	19	17	3			25	18	3			16	2												1		21					3	4									0.33	9.90
P. manshurica																								5																	0.03	0.33
Pichia sp.																											2	14													0.05	1.13
P.glabrum											3																														0.03	0.20
P. nalgiovense																					1																					0.03	0.08
S. cerevisiae	3	31	35	35				32	35	35				31	35			16	35	35		15	35	35			14	35	35					2	35	35		8	35	35	0.60	48.30
Z. hellenicus											3	8									1										5									0.10	1.18

1 = berry surface, 2 = start of fermentation, 3 = mid-fermentation, 4 = end of fermentation, 5 = residual lees; F′ = frequency of the species, i.e. the proportion of samples from which the species was isolated; I′ = incidence of the species, i.e. the percentage occurrence in the whole yeast population in the study.

Table 2
Biodiversity indices of the eight spontaneous fermentations of Negroamaro and Primitivo grapes.

	Negroamaro				Primitivo			
	Copertino	Cutrofiano	Melissano	Torchiarolo	Galatina	Gioia del Colle	Manduria	Torchiarolo
Species richness (S)	4[a]	7[a,b]	9[a]	6[a]	14[c]	9[b]	8[b]	8[b]
Dominance (D)	0.4306[a]	0.4236[a]	0.2549[b]	0.3074[b]	0.2985[b]	0.2754[b]	0.2557[b]	0.2798[b]
Simpson ($1-D$)	0.5694[a]	0.5764[a]	0.7451[b]	0.6926[b]	0.7015[b]	0.7246[b]	0.7443[b]	0.7202[b]
Shannon (H)	1.011[a]	1.079[a]	1.631[b]	1.434[b]	1.61[b]	1.657[b]	1.624[b]	1.569[b]
Evenness_(e^H/S)	0.6874[a]	0.42[b]	0.5674[c]	0.6992[a]	0.3574[b]	0.5828[a,c]	0.6344[a,c]	0.6003[a,c]

Different letters indicates statistically significant differences ($p < 0.05$) of values in the same row.

and Gioia del Colle ($S = 9$). However, biodiversity does not depend only on the number of species found, but also on its relative dominance and abundance. The general index of biodiversity (H) for samples of Primitivo appeared to be higher ($1.569 < H < 1.657$) than for Negroamaro microbial communities ($1.011 < H < 1.631$). Moreover, among the fermentations of Negroamaro grapes, the Simpson's indices effectively distinguished the two samples with the highest dominance index, Copertino and Cutrofiano ($D = 0.43$ and $D = 0.42$, respectively), from the sample with the highest diversity, i.e. Melissano ($1 - D = 0.75$).

3.2. Intraspecific analysis of S. cerevisiae population

A total of 692 *S. cerevisiae* isolates were identified among the grape surfaces and the eight samples of spontaneous fermentation. The molecular patterns obtained by interdelta analysis enabled the differentiation of a variable number of *S. cerevisiae* strains per vineyard, ranging from 26 to 55 (Table 3). The *S. cerevisiae* population from Cutrofiano showed the lowest polymorphism (28%) calculated as the ratio between the number of molecular patterns and the number of isolates, while the population from Gioia del Colle was the most polymorphic (variability 63%). Despite the elevated number of distinct molecular patterns, the Negroamaro grape samples from Cutrofiano, Melissano and Torchiarolo showed low intraspecific diversity indices due to relative high dominance indices. In fact, in these samples, up to 52% of the population was grouped into one or two patterns at most (Table 3).

All of the other five samples not only showed a higher number of distinct molecular patterns of *S. cerevisiae* but most of these patterns were unique, which gave rise to higher indices of biodiversity ($H > 3$) with low concentration of dominance ($D < 0.15$).

3.3. Biogenic amine production

In order to identify the strains possessing a significant capacity for amino acid decarboxylation, halo dimension from the amino acid decarboxylation assay was used as a discriminative parameter in a preliminary *in vitro* screening (Romano et al., 2007). Three hundred and fifty isolates, randomly selected from the initial population made up of 1400 isolates were investigated for their ability to decarboxylate the amino acids histidine, tyrosine, phenylalanine, tryptophan, lysine, leucine and arginine. Sixty-nine isolates, representing 20% of the analyzed population, showed a positive reaction for at least one of the above amino acids. Among the tested genera, *Hanseniaspora* spp., *Kluyveromyces* spp. and *Candida* spp. isolates all failed to show significant capacity (code 1 or 2) to decarboxylate any of the amino acids used for the strain biotypization, showing behavior comparable to the totality of *S. cerevisiae* (score 0) and *A. pullulans* (code 0) isolates, analyzed at the same time. As reported in Table 3, eleven isolates, representing 3.1% of the analyzed population, showed a code ranging from 3 to 4 associated with the decarboxylation of at least one of the amino acids used (isolates scoring 1 and 2 were not reported). The biotypization assay showed that three isolates belonging to *Z. hellenicus* and one isolate of *M. pulcherrima* were able to decarboxylate all of the tested amino acids. The other isolates scoring a code >2 were all able to decarboxylate histidine (Table 4). Moreover, *I. terricola* (2KUT21) and *Pichia manshurica* (3KUT25 and 3KUT29) were also able to decarboxylate the amino acid lysine, whereas the isolates 2KUT12 (*Issatchenkia orientalis*), 2KUT31 and 3KUT26 (both *I. terricola*) showed analogous enzymatic activity against leucine. Furthermore, the eleven strains were tested for the production of the biogenic amines histamine, tyramine, cadaverine and putrescine in a synthetic medium (Table 5; Fig. 1). Concerning the *Z. hellenicus* isolates, strain variability in BAs formation was observed. Indeed, although strain 1KUT24 was able to produce all the BAs analyzed, only tyramine, cadaverine and putrescine or tyramine and cadaverine were detectable in strain 2M2 and 2M5, respectively. Histamine was also produced by strains 3KUT31 (*I. terricola*) and 3KUT27 (*M. pulcherrima*), which was also able to form tyramine and putrescine. Both *P. manshurica* isolates synthesized cadaverine (Table 5).

The eleven yeast strains were further tested for BAs production during grape must fermentation. Both 3KUT31 (*I. terricola*) and 3KUT27 (*M. pulcherrima*) strains confirmed that they were capable to produce histamine and cadaverine, but not tyramine and putrescine (Fig. 2). The ability to produce cadaverine was confirmed in the two strains of *P. manshurica*. Additionally, the production of

Table 3
Intraspecific diversity of *S. cerevisiae* yeasts isolated from the different spontaneous fermentations.

	S. cerevisiae isolates	Molecular patterns	Variability (%)[a]	Dominance (D)	Shannon's index (H)	Evenness (e^H/S)
Copertino	104	35	34	0.06	3.23	0.72
Cutrofiano	102	29	28	0.15	2.65	0.49
Torchiarolo (N)	90	31	35	0.28	2.24	0.30
Melissano	68	26	38	0.15	2.57	0.50
Manduria	80	36	45	0.06	3.20	0.68
Gioia del Colle	81	55	63	0.03	3.83	0.84
Torchiarolo (P)	80	40	50	0.10	3.07	0.54
Galatina	87	45	52	0.06	3.33	0.62

[a] ratio between the number of patterns and the number of isolates.

Table 4
Yeast isolates able to decarboxylate at least one of the amino acids used in agar plate test and showing a phenotype identified by code 3 or 4 (see Section 2.4). The phenotypes showed by the *Hanseniaspora uvarum* strain 8795 (negative control) and the *Lactobacillus brevis* IOEB 9809 (positive control) are indicated.

Isolate	Species	AA decarboxylation						
		His	Tyr	Phe	Trp	Lys	Leu	Arg
1KUT24	*Z. hellenicus*	4	4	3	3	3	4	4
2M2	*Z. hellenicus*	3	4	3	3	4	4	4
2M5	*Z. hellenicus*	3	4	4	3	3	3	4
2KUT12	*I. orientalis*	3	0	1	2	0	3	1
2KUT21	*I .terricola*	3	1	0	0	3	1	1
2KUT31	*I .terricola*	4	1	1	0	0	3	1
3KUT26	*I .terricola*	3	0	0	0	0	3	1
3KUT25	*P. manshurica*	3	1	1	0	4	2	1
3KUT29	*P. manshurica*	3	1	1	2	4	2	1
3KUT27	*M. pulcherrima*	4	3	3	3	3	3	4
ITEM 8795	*H. uvarum*	0	0	0	0	0	0	0
IOEB 9809	*L. brevis*	0	4	0	0	0	0	0

histamine during must fermentation, was observed in the analysed *P. manshurica* strains (Fig. 2). The remaining strains, including the strains belonging to the *Z. hellenicus* species, were confirmed only as cadaverine producer strains when inoculated in grape must (data not shown).

4. Discussion

The aims of this work were to provide data on the composition of vineyard-associated microflora and to evaluate possible risks to consumers caused by the presence of the analysed microorganisms during the wine production process.

Even though numerous examples of protocols for laboratory experimental vinification have been described in the literature (Romano et al., 1998; Esteve-Zarzoso et al., 2000; Cordero-Bueso et al., 2011), we decided to carry out the winemaking process in a tailor-designed experimental winery and to reproduce the must fermentation on a significant scale, maintaining microbiological control of the process. The number of colonies selected at each stage of yeast fermentation, as determined in 35 individual colonies, represented a statistically significant sample of the biomass present in each of these phases (Brežná et al., 2010; Cordero-Bueso et al., 2011).

The data obtained by the molecular identification of isolated yeasts were in agreement with previous ecological studies carried out in Spain, France, Greece, Slovenia and Italy, concerning the microflora present in natural fermentations of typical grape varieties (Versavaud et al. 1995; Pramateftaki et al., 2000; Povhe Jemec et al., 2001; Torija et al., 2001; Clemente-Jiménez et al. 2004; Di Maro et al. 2007).

M. pulcherrima and *H. uvarum* were the predominant yeast species on the surface of the berries of Negroamaro. Additionally, strains of *K. thermotolerans*, *I. terricola* and *H. guilliermondii* were isolated.

The Primitivo grapes showed a microbial population with *C. stellata*, *M. pulcherrima* and *C. zemplinina* as the predominant species. The different population composition between the two cultivars can be attributed to several factors, such as geographical location and climatic conditions (Fleet et al., 1984; Parish and Carroll, 1987), age of the vineyard, grape variety (Rosini et al., 1982) and farming system (Comitini and Ciani, 2008). The presence of *C. stellata* on Primitivo berries may be justified by the higher sugar concentration of this grape variety (Lafon-Lafourcade, 1983). The species *Z. hellenicus* (teleomorph of *Candida steatolytica*) has been described as a contaminant and has been often associated with damaged grapes (Barata et al., 2008).

Many species, such as *H. uvarum*, *Issatchenkia* sp., *Candida* sp. and *M. pulcherrima*, already present on the berry surface were also present in the must at the beginning of the fermentation process (Lambrechts and Pretorius, 2000). Of great interest is the detection of isolates of *S. cerevisiae* (8% of the population examined) in the initial phase of the fermentation of grapes from Copertino. Although quite unusual, the consistent presence of this species of yeast in the early stages of fermentation has already been reported from natural fermentation in Greece (Pramateftaki et al., 2000) and Spain (Torija et al., 2001).

The data concerning the population dynamics of *S. cerevisiae* strains showed a higher intraspecific variability (between 28% and 63%) compared to that found in other indigenous populations associated with the fermentation of musts in France (Versavaud et al., 1995), Spain (Sabate et al., 2002; Cordero-Bueso et al., 2011), and Argentina (Sangorrín et al., 2002). This evidence can be explained by the fact that most of these analyses have regarded natural fermentations conducted in an industrial cellar or in laboratory. A cellar is in itself an ecosystem that can affect the population dynamics of strains of wine yeast with the so-called "cellar effect", which would lead to the creation of a self-selected and stable microflora in the cellar over the years (Vezinhet et al., 1992; Ciani et al., 2004; Li et al., 2012). In contrast, fermentations performed on a small laboratory scale do not represent a real winemaking process. The vinification system used in this study was not influenced by the microflora already present in the cellar and it has proved to be an excellent tool for the implementation of a protocol for native microflora identification.

The formation of biogenic amines in wine is mainly due to the metabolism of lactic acid bacteria involved in malolactic fermentation

Table 5
Production of the biogenic amines histamine, tyramine, cadaverine and putrescine in synthetic medium by TLC by the eleven strains positive to the AA decarboxylation plate test. The nucleotide sequence of their ITS1-5.8S-ITS2 region were deposited in the EMBL database and the corresponding accession numbers are given.

Isolate	EMBL acc. no.	Closest match (NCBI accession no.)	% identity[a]	Histamine	Tyramine	Cadaverine	Putrescine
1KUT24	HE965021	*Zygoascus hellenicus* (AY447022.1)	100	+	+	+	+
2M2	HE965022	*Zygoascus hellenicus* (AY447022.1)	100	−	+	+	+
2M5	HE965023	*Zygoascus hellenicus* (AY447029.1)	100	−	+	−	+
2KUT12	HE965025	*Issatchenkia orientalis* (FM199958.1)	100	−	nt	nt	nt
2KUT21	HE965026	*Issatchenkia terricola* (GU943495.1)	97	−	nt	−	nt
2KUT31	HE965027	*Issatchenkia terricola* (AY235808.1)	99	+	nt	nt	nt
3KUT26	HE965028	*Issatchenkia terricola* (EF648009.1)	99	−	nt	nt	nt
3KUT25	HE965029	*Pichia manshurica* (FM199959.1)	99	−	nt	+	nt
3KUT29	HE965030	*Pichia manshurica* (FM199959.1)	99	−	nt	+	nt
3KUT27	HE965031	*Metschnikowia pulcherrima* (EU137672.1)	98	+	+	−	+

nt = not tested.
[a] Identity represents the % identity shared with the sequences in the GenBank databases.

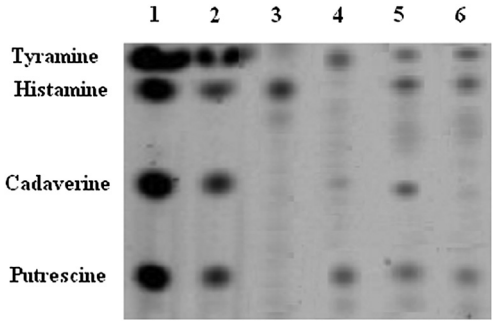

Fig. 1. Examples of biogenic amines (BA) production by non-*Saccharomyces* yeasts isolated from Apulian grapes. TLC plate of extracts from supernatants of synthetic medium supplemented with histidine, lysine and arginine at 1% w/v concentration or tyrosine at 0.1% w/v concentration tyrosine 0.1% and inoculated with different yeast strains, Lanes 1 and 2, biogenic amine standard (50 mg/L and 0.5 mg/l, respectively); lane 3, *I. terricola* strain 3KUT31; lane 4, *Z. hellenicus* strain 2M2; lane 5, *Z. hellenicus* strain 1KUT24; lane 6: *M. pulcherrima* strain 3KUT27. The position of different BAs is indicated to the left of the panel.

(Smit et al., 2008) and little is known about the production of non-volatile amines by yeast during alcoholic fermentation.

Wine yeasts unable to produce BAs have been reported by several authors (Herbert et al., 2005; Marcobal et al., 2006; Landete et al., 2007) and a decrease in BAs is usually observed during both spontaneous and induced commercial must fermentations (Granchi et al. 2005).

However, several studies have reported a direct role of wine yeasts in increasing BA contents during vinification experiments on laboratory- and large-scale (Goñi and Ancín Azpilicueta, 2001; Torrea and Ancín, 2002). Furthermore, the analysis conducted in two independent studies by Caruso et al. (2002) and by Granchi et al. (2005) demonstrated that different strains belonging to the species *S. cerevisiae*, *K. apiculata*, *C. stellata*, *M. pulcherrima* and *B. bruxellensis* were able to significantly produce putrescine, phenylethylamine and ethanolamine.

The data presented herein shed new light on the role of non-*Saccharomyces* yeasts in the production of biogenic amines. For the first time, we experimentally demonstrated the ability of a yeast species of enological provenience in producing histamine during grape must fermentation. Indeed, among the yeast species tested, an isolate of *I. terricola* (2KUT31) and *M. pulcherrima* (3KUT27) as well as two isolates (3KUT25 and 3KUT29) of *P. manshurica* were capable to synthesize histamine. In strains belonging to these species isolated from wine, histamine production had never been reported (Caruso et al., 2002; Granchi et al., 2005; Landete et al., 2007). We also demonstrated that all the eleven characterized strains were able to form cadaverine, showing similar behaviour to that previously described for *C. stellata* strains (Caruso et al., 2002). The data obtained on *P. manshurica* confirmed its capacity for spoilage not only due to the production of volatile phenols (Saez et al., 2011) but also due to its capacity to produce cadaverine, thus representing a possible source of hazard for amine production in wine, given its ability to survive in wine (Saez et al., 2011).

The comparison of *in vitro* and *in vivo* decarboxylation activity and/or biogenic amines production data revealed significant differences. As first, a detected decarboxylase activity in a synthetic media did not result in BA-production in wine must. This is likely to be due to limits of the analytical techniques with false positive that may arise from the *in vitro* assay or, as previously suggested for lactic acid bacteria, to the lack of expression of the BA pathway in wine must (Coton et al., 2010). Furthermore, in several cases, BAs production differs from synthetic media to wine must, indicating that environmental conditions may influence these biological phenomena. Moreover, we demonstrated BA production such as cadaverine in wine must, in the absence of detection of the corresponding decarboxylase activity in a synthetic media. This finding could be explained by the presence of novel BA pathways still uncovered in wine yeast. For these reasons, we underline the importance of performing a test of biogenic amine formation in wine yeast, directly in grape must and in the framework of a complete technological characterization of non-*Saccharomyces* isolates for starter culture design in enology.

This investigation is the first large-scale study of the vineyard-associated strains from the Apulia Region in Italy and represents a helpful approach to gaining further knowledge about the ecology and biogeography of non-*Saccharomyces* and *S. cerevisiae* strains. The results of our screening for amine production show that yeast of wine origin can produce biogenic amines, thus resulting a potential risk for wine quality and for consumer health (EFSA Panel on Biological Hazards (BIOHAZ), 2011). The possible risk of formation

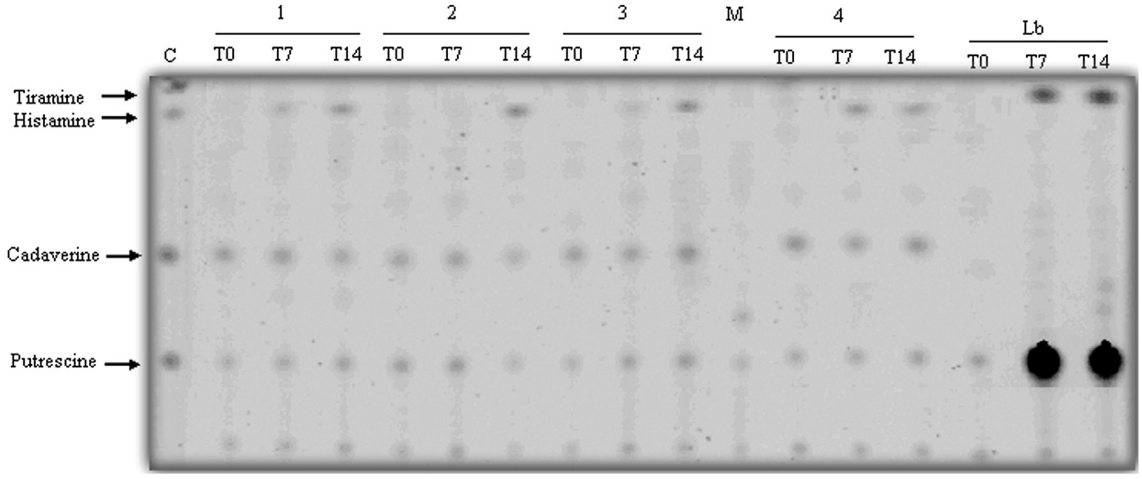

Fig. 2. Biogenic amines (BAs) production by the non-*Saccharomyces* yeasts isolated from Apulian grapes during must fermentation. TLC plate of extracts from supernatants of sterilized Negroamaro must supplemented with histidine, lysine and arginine at 1% w/v concentration or tyrosine at 0.1% w/v. The must was inoculated with the following yeast strains and samples collected at 0 (T0), 7 (T7) and 14 (T14) days post inoculation: 1, *I. terricola* strain 2KUT31; 2, *P. manshurica* strain 3KUT25; 3, *M. pulcherrima* strain 3KUT27; 4, *P. manshurica* strain 3KUT25; Lb, *L. brevis* strain IOEB 9809; C, biogenic amine standard (0.5 mg/L); M, non-inoculated must. The position of different BAs is indicated to the left of the panel.

of biogenic amines, even during the first steps of the vinification process, emphasizes the importance of correct microbiological control throughout wine-making to eradicate or to reduce the action of these spoilage yeasts, even though the use of non-*Saccharomyces* yeasts has been receiving increasing attention in winemaking (Ciani et al., 2010; De Benedictis et al., 2011).

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