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ORIGINAL PAPER

Influence of organic viticulture on non-*Saccharomyces* wine yeast populations

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Abstract This study evaluated the population dynamics of non-*Saccharomyces* biota during spontaneous fermentation of organic musts. Non-*Saccharomyces* yeasts were found to be present at high levels during all fermentations. A total of 543 yeast colonies were isolated, 190 from Lysine-Medium (LM) agar, 254 from Wallerstein Laboratory Nutrient (WLN) agar and 99 from YPD agar. To estimate yeast population dynamics during spontaneous fermentation a genotypic approach was applied. PCR-RFLP of the ITS1-5.8S rDNA-ITS2 region and sequence determination of the D1/D2 region of the 26S rRNA gene enabled identification of the yeast isolates at the species level. *Hanseniaspora uvarum*, *Metschnikowia fructicola* and *Candida zemplinina* predominated, while *Issatchenkia terricola*, *Issatchenkia orientalis* and *Pichia* sp. were identified with a lower frequency. *Hanseniaspora uvarum*, *M. fructicola* and *C. zemplinina* represented 43%, 31% and 11% of the total non-*Saccharomyces* population isolated, respectively. Some yeast isolates were shown to be closely related to *Hanseniaspora* spp. and *Candida* spp. on the basis of the D1/D2 sequences. Based on those results, the coexistence of different *Hanseniaspora* and *Pichia* species in grape musts was supposed, and their complete identification was achieved using additional molecular markers. Moreover, strain typing and differentiation was carried out by RAPD-PCR. High strain polymorphisms were observed in the different species. For some strains, appreciable properties were demonstrated both *in vitro* by the API-ZYM test and in must. In must microvinification some strains showed good fermentation performances, low production of acetic acid and a partial capability to degrade malic acid.

Keywords Organic musts · Yeasts · D1/D2 domain · Organic acids

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Introduction

Organic grapes come from vineyards grown under organic farming methods, as defined at European level by the European Council (EC) Regulation No 834/2007 and No 889/2008 on organic production. In particular, the International Federation of Organic Agriculture Movement (IFOAM) defines organic agriculture, including viticulture and wine-making, as a "holistic production management system which promotes and enhances agro-ecosystem health, including biodiversity, biological cycles, and soil biological activity. It emphasizes the use of management practices in preference to the use of off-farm inputs, taking into account that regional conditions require locally adapted systems" (IFOAM 2005; Trioli and Hofmann 2009). Indeed, the yeasts present on grape berries and must from organic vineyards could have a unique composition and these indigenous yeasts impart distinct regional and desired characteristics to wines. In fact, traditionally, wine has been produced by the spontaneous fermentation of grape juice by the yeasts that originate from grapes and winery equipment. The variety and proportion of different yeasts in grape berries and in musts depends on many different factors, including the geographic location of the vineyard (Longo et

al. 1991), climatic conditions (Fleet et al. 1984) grape variety (Pretorius et al. 1999), vinification technology (Charoenchai et al. 1997) and grape integrity (i.e. damaged vs undamaged; Prakitchaiwattana et al. 2004). A study on yeast populations residing on healthy or *Botrytis*-infected grapes from a vineyard showed a larger population and greater diversity of non-*Saccharomyces* (NS) yeasts (Nisiotou and Nychas 2007). In recent years, numerous studies have evaluated the NS species present in wine ecosystem, and have demonstrated the impact of grape conditions on NS populations (Fernández et al. 2000; Raspor et al. 2006; González et al. 2007). The role of NS yeasts in wine production has been debated extensively and several researchers have shown that NS yeasts survive during fermentation and could reach cell concentrations similar to those reached by *Saccharomyces cerevisiae* (10^6–10^8 cells/ml) (Fleet et al. 1984; Gafner and Schultz 1996). In fact, as suggested by several authors (Zironi et al. 1993; Toro and Vazquez 2002; Gil et al. 1996; Lema et al. 1996; Ciani et al. 2006; Viana et al. 2008), there is growing evidence that NS yeasts play an important role in wine quality.

The aims of the present study were: (1) to monitor, by means of traditional and molecular methods, the "indigenous" yeast communities of grapes and musts from an organic vineyard of red Montepulciano d'Abruzzo and white Trebbiano cultivars. The producer cellar of organic wine from its own vineyard is located in Abruzzo region (Italy). (2) To screen different NS strains for their suitability for must fermentation.

Materials and methods

Trebbiano and Montepulciano vinifications

Grapes of Montepulciano d'Abruzzo and Trebbiano varieties were collected from the organic vineyard of the winery "Azienda BioVitivinicola Pepe" located in Torano Nuovo (Teramo, Italy), during the vintage 2008–2009. After hand destemming, the grape berries of two varieties were separately crushed by foot, in accordance with the traditional procedure of the cellar, and poured into (200 l) vessels, in which spontaneous fermentations were carried out according to traditional vinification methods. No sulphur was added. A vessel containing Montepulciano d'Abruzzo must inoculated with a *S. cerevisiae* indigenous starter strain (Suzzi et al. 2008) was used as a control. All vinifications were performed at room temperature and fermentations were monitored by analysing the sugar consumption (°Brix) at different stage of fermentation (0, 24 h, 48 h, 7 days, 14 days and 30 days).

Samples were taken in duplicate and collected at beginning and middle of fermentation, and at the end of wine production. The sugar content, total acidity (g/l) and pH of the collected samples are summarised in Table 1. Three replicates were analysed in each case.

Yeast sampling, enumeration and isolation

To sample the yeast populations of grape berries, after destemming and before crushing, 150 g berries were collected randomly, crushed in sterile bags and blended in a stomacher (Laboratory Blender Stomacher 400, Seward Medical, London, UK) for 5 min at high speed. Samples of Trebbiano and Montepulciano musts, as well as control samples, were taken aseptically at different stage of fermentation (0, 24 h, 48 h, 7 days, 14 days and 30 days).

Decimal dilutions in Ringer's solution were prepared and 100 μl spread in triplicate on three different media: a non-selective YPD agar (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose and 2% w/v agar), lysine-medium agar (LM, Morris and Eddy 1957) and Wallerstein laboratory nutrient agar (WLN, Oxoid, Hampshire, UK). All media were supplemented with chloramphenicol (150 ppm) to inhibit bacterial growth. LM was used in order to estimate the number of NS yeasts since it is a synthetic medium containing glucose, vitamins, inorganic salts, and lysine as the sole nitrogen source; *Saccharomyces* spp. are unable to grow on this medium (Di Maro et al. 2007). According to Pallmann et al. (2001), WLN medium can be used to monitor yeast population diversity during fermentations. This medium has proved useful for wine yeast identification since colonies of different species grow on it with a distinguishable morphology and colour (Cavazza et al. 1992; Pallmann et al. 2001). Colony forming units (CFU) were determined after incubation on the three different media for 5 days at 28°C. After determining viable counts, morphologically different colonies from each substrate were purified by repetitive streaking on YPD agar, and the isolates were stored at −20°C in YPD broth supplemented with glycerol (25% final concentration) and selected according to colony shape, colour, surface features and frequency.

Molecular yeast identification

Yeast cells were grown aerobically in YPD at 28°C. Total genomic DNA was extracted and purified from 7 ml cultures as described by Querol et al. (1992). Identification of the isolates was performed by PCR-RFLP of ITS1-5.8 S rDNA-ITS2 region as described by Esteve-Zarzoso et al. (1999). For amplification of the ITS1-5.8S rDNA-ITS2 region, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers were used. The reactions were performed in an automatic thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer, Norwalk, CT)

Table 1 Viable counts of yeast populations during fermentation of Trebbiano, Montepulciano and Montepulciano control grapes

Grape cultivar	Sample	Stage	Brix (°)	pH	Total acidity (g/l)	CFU/ml[a]	
						WLN agar[b]	LM agar
Trebbiano	T1	Grape	22.0	3.42	4.10	3.1×10^4	3.3×10^4
	T2	Grape must after pumping-over	21.8	3.40	4.15	9.0×10^2	6.0×10^2
	T3	24 h	21.5	3.74	4.70	1.8×10^2	1.6×10^2
	T4	48 h	21.5	3.72	5.76	6.0×10	8.0×10
	T5	7 days	19.0	3.63	10.27	2.06×10^5	2.13×10^5
	T6	14 days	14.5	3.63	8.36	1.58×10^7	1.52×10^7
	T7	30 days	0.76	3.71	5.79	6.8×10^6	6.6×10^6
Montepulciano	M1	Grape	25.0	3.58	7.53	8.3×10^6	9.4×10^6
	M2	Grape must after pumping-over	24.8	3.56	7.29	1.0×10^5	9.5×10^4
	M3	24 h	23.7	3.59	9.13	7.5×10^5	1.03×10^6
	M4	48 h	23.4	3.61	9.79	3.5×10^4	6.1×10^4
	M5	7 days	16.4	3.71	8.98	1.5×10^6	1.0×10^6
	M6	14 days	10.3	3.56	7.29	8.3×10^6	9.4×10^6
	M7	30 days	7.10	3.59	9.13	1.0×10^5	9.5×10^4
Montepulciano (control)	M1	Grape	25.0	3.58	7.53	8.3×10^6	9.4×10^6
	αM2	Grape must after pumping-over	24.8	3.56	7.32	1.06×10^7	7.4×10^6
	αM3	24 h	14.0	3.62	9.04	9.0×10^7	1.3×10^7
	αM4	48 h	13.6	3.6	10.6	4.0×10^7	4.0×10^7
	αM5	7 days	6.20	3.51	7.49	5.5×10^7	2.48×10^7
	αM6	14 days	0.91	3.56	7.32	4.4×10^5	3.1×10^5
	αM7	30 days	0.53	3.62	9.04	8.3×10^6	9.4×10^6

[a] Data are the means based on three replicates; standard deviations were less than 20% of the means

[b] *CFU* Colony forming units, *WLN* Wallestein laboratory nutrient, *LM* Lysine medium

under the following conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, final extension at 72°C for 10 min, holding at 4°C. The PCR products were digested with 1U of *Cfo* I, *Hae* III and *Hinf* I endonucleases (Roche Diagnostics, Mannheim, Germany) at 37°C for 2 h. PCR products and their restriction fragments were analysed on 1.5% and 2% agarose gels, respectively, in 1×TBE (89 mM Tris-borate, 2 mM EDTA pH 8) buffer. Gels were stained with ethidium bromide. Fragment lengths were estimated by comparing them with a 1-kb plus DNA ladder (Invitrogen, Carlsbad, CA) as size marker. Thr program Webcutter 2.0, available at http://rna.lundberg.gu.se/cutter2/, was used to predict restriction sites in ITS molecular sequences.

As it is not easy to isolate *Brettanomyces bruxellensis* cells, it was detected using a molecular, culture-independent method according to Phister and Mills (2003). Grape must samples (1 ml) were placed in enrichment medium (YPD) for 5 days at 28°C. After incubation, nucleic acid samples were extracted according to Querol et al. (1992). BRUXR (5′-GAAGGGCCACATTCACGAACCCCG-3′) BRUXF (5′-GGATGGGTGCACCTGGTTTACAC-3′) specific primers were used for PCR amplification. Real-time amplifications were carried out in a 25 μl reaction mixture, using 1× Platinum SYBR Green Supermix-UDG reaction mixture (Invitrogen, Milan, Italy), 0.2 μl of each primer (10 μM) and 5 μl DNA. The reactions were performed in *Cycle* IQ (Biorad, Milan, Italy) under the following conditions: initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 60 s, annealing at 69°C for 45 s, extension at 72°C for 30 s, final extension at 72°C for 7 min, holding at 4°C. A semiquantitative qPCR method was performed. As positive controls for qPCR amplification, nucleic acids from must and wine samples that were artificially inoculated with the strain type *B. bruxellensis* MUCL 27700^T were used. The correlation coefficient between the qPCR threshold cycle (C_T) and count values was analysed and interpreted using the appropriate Excel function. Each C_T was the average of three measures obtained by amplifying three DNA extracts from the same artificially inoculated sample.

Sequencing of the D1/D2 domain of the large subunit (26S) ribosomal DNA

Sequencing of the D1/D2 domain of the large subunit (26S) ribosomal DNA was performed for 64 isolates, selected as

described above. The analysis was performed according to Kurtzman and Robnett (1998). NL-1(5′-GCATATCAA TAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGT GTTTCAAGACGG-3′) primers were used for amplification of the D1/D2 domain. The reactions were performed in an automatic thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer) under the following conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, extension at 72°C for 1 min, final extension at 72°C for 10 min, holding at 4°C. To confirm the identification at species level, the D1/D2 domain was sequenced after PCR amplification. PCR products were purified by the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Uppsala, Sweden), following the manufacturer's instructions, and delivered to Centro Ricerca Interdipartimentale Biotecnologie Innovative (Padua University, Padua, Italy) for sequencing. The sequences were assembled and compared with sequences reported in GenBank using the basic local alignment search tool (BLAST) algorithm.

Alignment of sequences obtained for the isolates and reference sequences retrieved from the database was performed with CLUSTALX (Thompson et al. 1997). Phylogenetic analysis was conducted with MEGA version 4 (Tamura et al. 2007), with maximum likelihood estimation of distances, neighbour joining as the algorithm for tree reconstruction, and complete deletion of positions of ambiguous alignment. Bootstrap analysis was performed on 1,000 replicates.

Strain typing

Strain typing was assessed by RAPD-PCR. Two random primers, R5 (5′-AACGCGCAAC-3′) and M13 (5′-GAGGGTGGCGGTTCT-3′), were used for RAPD analysis according to Martín et al. (2006) and Huey and Hall (1989), respectively. Each reaction was carried out in 25 μL reaction mix containing 2.5 μL 10× PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol primer, 1 U *Taq* polymerase and 20 ng extracted DNA. When primer R5 was used, the amplification process consisted of 5 min of initial denaturation at 94°C and 40 cycles of denaturation at 94°C for 1 min, annealing at 29°C for 1.5 min and elongation at 72°C for 2 min, followed by a final elongation for 5 min at 72°C. With primer M13, the thermal cycle was performed according to Huey and Hall (1989). RAPD-PCR products were visualized on a 1.5% agarose gel after staining with ethidium bromide and acquired using Gel Doc 2000 (Biorad). Conversion, normalisation and analysis of RAPD-PCR profiles were carried out using the software Fingerprinting II Informatix™ Software (Bio-Rad). Similarity between patterns was evaluated using the Pearson coefficient. Clustering analysis was performed by means of the UPGMA (unweighted pair group method with arithmetic mean) method.

Microvinification

Fermentation of 95 ml aliquots of pasteurised white grape must from Trebbiano cultivar [180 g/l fermentable sugars, 7.4 g/l titratable acidity, pH 3.2] was carried out in 130 ml Erlenmeyer-flasks. The must samples, after treatment at 70°C for 30 min, were inoculated with 5 ml of a pre-culture grown for 48 h in the same must, as described by Romano et al. (1998). Alcoholic fermentations were carried out at 25°C for 15 days and the weight loss due to CO_2 production was evaluated. When CO_2 release ceased, fermentation was considered completed and the samples were refrigerated for 2 days at 4°C, racked and stored at −20°C until analysis. Two fermentations were carried out with each strain. Non-inoculated must was used as a control.

Organic acids determination

All chemicals were of analytical reagent grade and supplied by Carlo Erba (Milan, Italy). Ultrapure Water, obtained from a reverse osmosis system (Teknolabo, Milan, Italy), was used to prepare all solutions. Organic acids were purchased from Sigma (St. Louis, MO). The HPLC system consisted of an Alliance (Waters) equipped with a Waters 2695 separation module connected to a Waters 2996 photodiode array detector. Analysis was performed under isocratic conditions at 25°C with a mobile phase consisting of a 22.5 mM H_2SO_4 solution delivered at a rate of 0.4 ml/min to a Biorad Aminex HPX-87H ion exclusion column (300 mm×7.8 mm I.D.). Organic acids were detected at 210 nm. After centrifugation for 10 min at 3,000 *g*, the samples were diluted 5-fold with mobile phase prior to injection of 10 μl into HPLC. Quantification of organic acids was carried out by the external standard method. Linear regression curves (r>0,9990) based on peak area were calculated for each organic acid after injection of six standard solutions covering the sample range of concentrations.

Enzymatic activities

Enzymatic activities of the isolates were evaluated by the API-ZYM (BioMérieux, Marcy l'Etoile, France) system according to the instructions of the manufacturer. Results were expressed on a scale from 0 (no activity) to 5 (maximum activity).

Results

Yeast dynamics during organic must fermentation

To study the dynamics of yeast populations during fermentations of organic musts of Montepulciano and Trebbiano cultivars, yeasts were enumerated at different

Table 2 Sizes of the 5.8S ITS rRNA gene amplicons and restriction fragments of the yeast isolates

Profile	No. of isolates	PCR product (bp)	Restriction fragments (bp) with:			Species
			Cfo I	*Hae* III	*Hinf* I	
I	35	750	320+110	750	320+190+170	*Hanseniaspora uvarum*
II	8	400	298+100	300+100	200+200	*Metschnikowia fructicola*
III	8	473	208+110+77	448	215+215	*Candida zemplinina*
IV	2	450	140+100	298+107	245+100	*Issatchenkia terricola*
V	1	450	200+170+70	380+90	232+139+75	*Issatchenkia orientalis*
VI	7[a]	750	320+110	750	350	*Hanseniaspora* spp.
VII	3	450	200+104	300+150	298+100	*Pichia* sp.

[a] The RFLP pattern of these isolates did not match the data reported in literature (Esteve-Zarzoso et al. 1999). Identification of these isolates was based on sequence analysis of the D1/D2 domain that revealed low (between 83-94%) identity with the species *Hanseniaspora* spp.

times and at the end of the fermentation process. The size of the yeast populations in grapes was greater than the size of yeast population in must samples. Spontaneous fermentation of Montepulciano and Trebbiano organic musts started after 7 days and proceeded slowly for 30 days. At this time point, Trebbiano fermentation was concluded and was characterised by a low content of residual sugars, whereas Montepulciano fermentation continued, probably due to the high content of initial sugars without the help of a starter (Table 1, Montepulciano grapes). In fact, fermentation of the same Montepulciano must inoculated with the selected autochthonous yeast (Suzzi et al. 2008) started quickly and proceeded for 15 days with low residual sugars (Table 1). Total counts of organic grape berries of Trebbiano resulted in about 3.1×10^4 CFU/ml, whereas the fresh grape juice exhibited a total yeast count on WLN

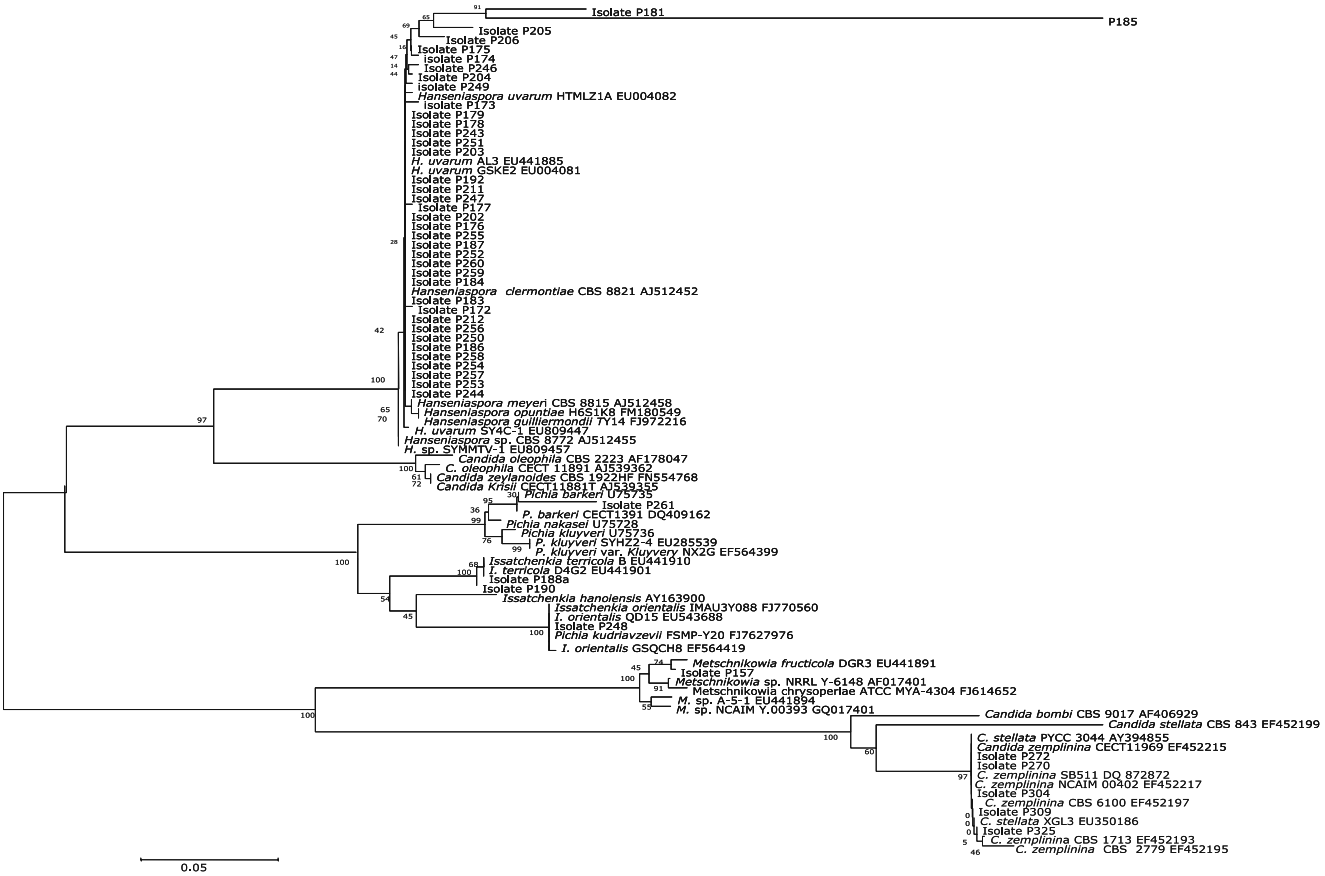

Fig. 1 Phylogenetic tree depicting evolutionary relationships among isolates and related reference species as determined by partial 26S rRNA gene sequence analysis. Bootstrap values reported at the nodes in percentages indicate support of the analysis. In this representation of the tree, distance between taxa is given by the sum of horizontal branch lengths. *Bar* Number of nucleotide substitutions per site

Table 3 Quantification by qPCR of *Brettanomyces bruxellensis* in grape and must samples

Sample	Ct	Corresponding log CFU/ml
Fermentation Montepulciano grapes		
M1	24.0	4.5
M2	35.2	1.19
M3	30.0	2.72
M4	24.6	4.44
M5	28.8	3.07
Fermentation Montepulciano control grapes		
M1	24.0	4.5
αM2	28.8	3.07
αM3	28.0	3.31
αM4	33.8	1.60

medium of 9.0×10^2 CFU/ml and a maximum number of 1.58×10^7 CFU/ml on day 14. Viable counts on LM showed a similar general trend as that detected on WLN medium. In fresh grape juice, NS yeasts were present at 6.0×10^2 CFU/ml and reached a maximum on day 14 (1.52×10^7 CFU/ml).

Total yeasts of Montepulciano organic fresh must on WLN medium (Table 1, Montepulciano grapes) were 1.0×10^5 CFU/ml, reaching a maximum number of 8.3×10^6 CFU/ml after day 14.

On grapes, NS yeasts were present at 9.4×10^6 CFU/ml, decreased during the first phase of fermentation and then increased up to 9.4×10^6 CFU/ml on day 14. This behaviour could be explained by the presence of ethanol-resistant NS strains, such as *Candida zemplinina* (Mills et al. 2002). In the control sample (Table 1, Montepulciano control grapes) yeasts reached a maximum number of 9.0×10^7 CFU/ml at 24 h fermentation on WLN with a NS yeast presence on LM of 1.3×10^7 CFU/ml. At the end of fermentation, NS yeasts were always detected at high levels.

Identification and molecular characterization of NS yeasts

A total of 543 yeast colonies were isolated, 190 from LM, 254 from WLN and 99 from YPD medium that were selected according to colony shape, colour, surface features and frequency. A total of 64 isolates representative of all the morphologies detected were selected for identification by RFLP analysis. Seven different 5.8S-ITS PCR-RFLP patterns were detected among all the yeast isolated (Table 2). Moreover, to attribute species assignation correctly, the D1/D2 domain of the 26S rRNA gene from 51 isolates was sequenced and subsequently compared with those available in the EMBL nucleotide sequence database. Most of the sequences obtained displayed similarity values ranging from 99 to 100% to reference sequences (Fig. 1). The D1/D2 26S rDNA

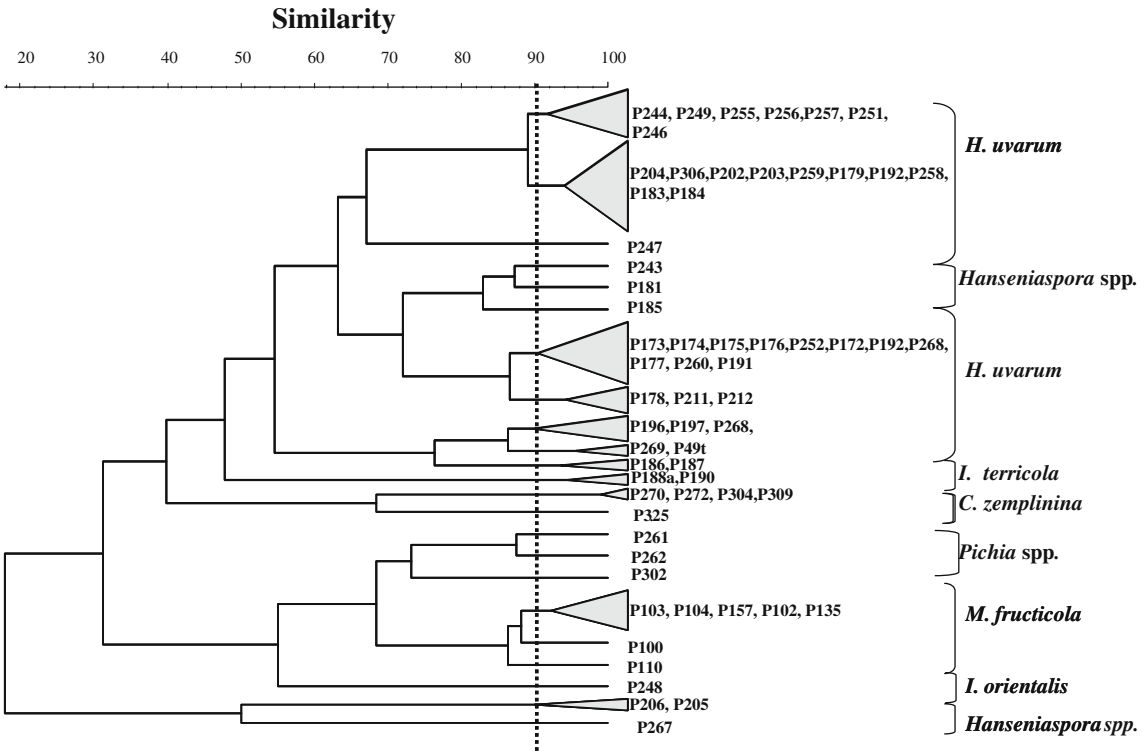

Fig. 2 Unweighted pair group method with arithmetic mean (UPGMA) dendrogram derived from comparison of the RAPD-PCR patterns of the yeast isolates obtained with primers R5 and M13

Table 4 Organic acid contents in fermented wines by non-*Saccharomyces* (NS) selected strains (means±SD). Fisher's F-value and probability *P*-value, obtained from ANOVA, are also listed. Means in rows without common letters (a–l) are significantly different ($P \leq 0.05$). The limits of detection (LOD) for organic acids were set on the basis of the signal to noise ratio (S/N) of 3: citric acid, 0.06 g/l; tartaric acid, 0.04 g/l; malic acid: 0.07 g/l; aconitic acid, 0.003 g/l; lactic acid, 0.10 g/l; acetic acid, 0.05 g/l. Lactic acid was absent in all fermented wines

Species	Strain	Organic acids (g/l)			
		Citric acid	Tartaric acid	Malic acid	Acetic acid
Control	-	0.31±0.007 g	6.95±0.07 l	1.97±0.007 k	NR (<0.05) a
Hanseniaspora uvarum	P172	0.30±0.01 fg	5.38±0.01 f	1.59±0.01 ghi	0.85±0.01 hi
	P176	0.26±0.007 c	5.19±0.007 c	1.33±0.007 d	0.86±0.007 hij
	P179	0.30±0.007 fg	5.12±0.007 b	1.52±0.007 f	0.84±0.007 h
	P184	0.31±0.007 g	5.35±0.007 f	1.57±0.007 ghi	0.86±0.007 hij
	P191	0.28±0.007 de	5.50±0.007 h	1.55±0.007 fgh	0.88±0.007 ij
	P202	0.29±0.007 ef	5.52±0.007 hi	1.38±0.007 e	0.83±0.007 h
	P244	0.31±0.007 g	5.55±0.007 i	1.57±0.007 ghi	0.91±0.007 k
	P249	0.27±0.007 cd	5.44±0.007 g	1.15±0.007 b	0.75±0.007 g
	P252	0.29±0.007 ef	5.45±0.007 g	1.39±0.007 e	0.68±0.014 f
	P253	0.30±0.007 fg	5.72±0.014 j	1.55±0.007 fghi	0.70±0.014 f
	P256	0.30±0.007 fg	5.31±0.007 e	1.72±0.007 j	0.63±0.014 e
	P259	0.27±0.007 cd	5.24±0.007 d	1.25±0.014 c	0.70±0.007 f
Hanseniaspora sp.	P185	0.29±0.007 ef	5.21±0.007 cd	1.53±0.007 fg	0.75±0.007 g
	P267	0.21±0.007 b	4.85±0.007 a	1.39±0.007 e	0.89±0.007 jk
Metschnikowia fructicola	P110	0.30±0.007 fg	5.51±0.007 h	1.60±0.007 i	0,16±0.007 c
Pichia sp.	P261	0.30±0.007 fg	5.09±0.007 b	1.02±0.007 a	0.46±0.007 d
Issatchenkia orientalis	P248	0.30±0.007 fg	6.92±0.021 l	1.93±0.014 k	NR (<0.05) a
Issatchenkia terricola	P190	0.19±0.007 a	6.19±0.007 k	1.41±0.007 e	0.09±0.0 b
Candida zemplinina	P309	0.35±0.007 h	5.17±0.014 c	1.25±0.007 c	0.63±0.007 e
	P272	0.29±0.007 ef	4.84±0.014 a	1.11±0.007 b	0.64±0.007 e
F		46.93	1,870.0	211.0	596.18
P		<0.001	<0.001	<0.001	<0.001

sequence of some strains could not be ascribed to any known species. Strains NS181 and NS185 showed the highest similarity value (94 and 83%, respectively) with the strain *Hanseniaspora* spp. HTLMLZ1A. According to the phylogenetic results, determined by partial 26S rRNA gene sequence analysis, as reported in Fig. 1, the unidentified strains of *Hanseniaspora* were not assigned to the species of this genus frequently isolated in must and wine, such as *Hanseniaspora osmophila* or *Hanseniaspora guilliermondii*.

The presence of *B. bruxellensis* in grape berries and in the organic musts was determined by semiquantitative qPCR (Table 3). *Brettanomyces bruxellensis* was not detected in the Trebbiano cultivar, whereas it was found in Montepulciano grapes. During vinification, it was revealed by qPCR in Montepulciano wine control at 48 h and in Montepulciano spontaneous fermentation up to 7 days after the start of fermentation. After this time, *B. bruxellensis* remained undetected using the above method; nevertheless, due to its presence in other phases of the fermentation, the possibility that viable cells of this microorganism can grow during storage was not excluded.

Strain typing

RAPD-PCR analyses were used to discriminate among strains. Primers R5 and M13 generated reproducible RAPD-PCR fingerprints composed of a number of well distributed bands of different intensity. The reproducibility of RAPD assays and running conditions was higher than 90%. Different patterns were generated for the species tested. Also, at the strain level, RAPD-PCR assays gave different polymorphism in the different species. Numerical analysis (UPGMA) of combined RAPD-PCR patterns with the two primers resulted in the dendrogram shown in Fig. 2. By considering 90% similarity as the arbitrary threshold to define biotypes, one can observe that ten biotypes of *Hanseniaspora uvarum*, four of *Metschnikowia fructicola*, two of *C. zemplinina*, and two of *Issatchenkia terricola* were distinguishable. Interestingly, *Hanseniaspora* spp. strains were well discriminated from *H. uvarum* strains.

Table 5 Results of the API-ZYM tests applied to selected yeast strains

Species	Strain	Enzyme activity[a]				
		Alkaline phosphate	Esterase	Esterase lipase	α-glucosidase	β-glucosidase
H. uvarum	P173	2	0	0	0	0
	P178	2	2	2	0	0
	P179	1	0	0	5	5
	P250	5	0	3	0	0
	P257	3	2	2	0	0
Hanseniaspora sp.	P205	3	0	0	0	0
M. fructicola	P101	1	1	1	0	5
	P102	0	1	2	0	5
	P135	0	1	1	0	5
	P157	0	3	3	0	5
Pichia sp.	P261	3	1	3	0	0
I. orientalis	P248	2	2	2	0	0
C. zemplinina	P270	2	0	2	0	0
	P315	2	0	1	0	0
	P325	2	2	1	0	0
Candida sp.	P302	5	3	3	0	0

[a] Expressed on a scale of 0 (no activity) to 5 (maximum activity)

Enological characterisation of non-*Saccharomyces* yeasts

All the strains evaluated in the present study, belonging to the species *H. uvarum*, *M. fructicola*, *C. zemplinina*, *I. terricola* and *I. orientalis*, were tested for fermentation power and organic acid consumption in Trebbiano must, and for in vitro enzymatic activities as reported in Materials and methods. Although the fermentations were carried out in a small volume, significant differences were found.

Among the 37 strains of *H. uvarum*, two different fermentation performances were observed. The values of residual sugars ranged from 4.9 to 10.9 °Brix. The percentage of *H. uvarum* strains and other NS yeasts varied according to the residual sugar content of Trebbiano wine. The 4–7% of NS population were able to ferment sugars with a residual content less than 5 °Brix, whereas most of them (56–72%) showed a fermentative capability with residual sugar content ranging from 8 to 10 °Brix. Among the different species, 20 strains were selected on the basis of fermentation performance. By considering the effect of NS yeasts on organic acid wine content (Table 4), 16 of the 20 strains studied have more than 0.60 g/l acetic acid, with a mean of 0.77 g/l , ranging from 0.09 g/l for *I. orientalis* P110 to 0.91 g/l for *H. uvarum* P244. Furthermore, some strains, such as *Pichia* sp or *C. zemplinina* P 272, were able to partially degrade malic acid. Regarding enzymatic activity, API-ZYM tests were performed on 16 yeast strains, selected from among the 64 isolates on the basis of RAPD-PCR pattern (Table 5). A strong α-glucosidase activity was detected in *H. uvarum* P179, and all the *M. fructicola* strains exhibited β-glucosidase, esterase and esterase-lipase activities. All *C. zemplinina* strains displayed alkaline phosphatase, and only strain P325 showed esterase activity.

Discussion

Organic wine is wine made from organically growing grapes without the help of or need for synthetic fertilisers, synthetic plant treatments or herbicides. Accurate studies have been carried out on soil and vineyard management in organic wine-making (Trioli and Hofmann 2009), whereas, as far as we know, no or few data are available on microbial populations of grape berries from organic vineyard as well as of those from organic wines. The main objective of the present work was to analyse native NS populations of the red Montepulciano d'Abruzzo and white Trebbiano musts obtained from organic grapes, fermented in 200 l vessels and processed by applying old traditional methods without SO_2 addition, in order to elucidate the impact of these populations on organic must spontaneous fermentation. The presence of yeasts on grapes ranged from 10^4–10^5 CFU/ml (Trebbiano cultivar) to 10^5–10^6 CFU/ml (Montepulciano cultivar). A significant decrease in the yeast population was observed in fresh juice of Trebbiano grapes, probably because the skin of Trebbiano cultivar grapes is more affected by hand destemming and crushing by foot than the Montepulciano cultivar. As

expected, fermentation of must inoculated with a starter culture followed a normal trend and finished quickly with a low content of residual sugars. In addition, the presence of a starter seemed to inhibit the growth of *B. bruxellensis*, as showed in Table 3. Non-*Saccharomyces* yeasts were always present throughout the entire fermentation (10^6–10^7 CFU/ml) in all three batches. These results are of particular interest since NS yeasts generally decrease or die off after a few days of fermentation (González et al. 2006; Lopandic et al. 2008). In our study, NS yeasts were also detected at the end of fermentation in wine inoculated with starter. Whether these yeasts are still present at the end of the fermentation process seems quite variable (Fleet et al. 1984; Lema et al. 1996). Similar results have been reported by Jemec et al. (2001) during five spontaneous fermentations of Malvasia must. Torija et al. (2001) found NS populations of 10^6 CFU/ml at later stages of winemaking. In our study, the spontaneous fermentation of organic musts took longer than that carried out with starter culture, but the high number of NS yeast did not seem to affect the *Saccharomyces* fermentation, according to residual sugar content. Residual sugars in spontaneously fermented Montepulciano red wine were higher than in the other two fermentations, but this was probably related to the high initial content of sugars or to the presence of *S. cerevisiae* strains with low alcoholic performance.

To estimate yeast population dynamics during spontaneous fermentation a genotypic approach comprising RFLP-PCR of 5.8S-ITS, sequence determination of D1/D2 regions of the 26 S rRNA gene and RAPD fingerprinting was applied. Over the last 10 years, a large amount of information on yeast D1/D2 26S rDNA sequences has been accumulated. The public databases have inestimable value for identification purposes since they can be used for comparison against the sequence of any known or unknown yeast (Fell et al. 2000; Kurtzman and Robnett 2003). In this work, a number of yeast isolates were shown to be closely related to *Hanseniaspora* spp., *Pichia* spp. and *Candida* sp. on the basis of the above genotypic information. The unidentified *Hanseniaspora and Pichia* strains isolated from organic musts will require additional molecular markers for their complete identification. Among the species identified, *H. uvarum*, *M. fructicola* and *C. zemplinina* predominated, while *I. terricola*, *I. orientalis* and *Pichia* sp. were identified with a lower frequency. *Hanseniaspora uvarum*, *M. fructicola* and *C. zemplinina* species represented 43%, 31% and 11%, respectively, of the total NS population isolated. Among these species, *C. zemplinina* has been found generally in musts with high sugar content or in botrytized musts (Sipiczki 2003; Mills et al. 2002; Tofalo et al. 2009). *Metshnikowia fructicola*, a related sister species of *Metshnikowia pulcherrima*, is known to be active in biological control of postharvest fruit rots (Kurtzman and Droby 2001). Despite it being known that NS yeasts are good producers of extracellular enzymes, only a few strains exhibit determinable enzymatic activities. In conclusion, the results of the present study provide an overview of the NS community of organic musts, which are shown to be complex and rich in different microbial species and strains. Some of these strains present unique and interesting characteristics from an oenological point of view, as also found by Ciani et al. (2006, 2010) and Viana et al. (2008).

The present work represents a first approach to NS yeast characterization as well as adding to the knowledge of population dynamics in spontaneous fermentation based on organic musts. Further investigation is needed in order to elucidate the actual contribution of the above NS populations to the typical characteristics of organic wines. In this context, autochthonous NS strains could be selected to co-ferment organic musts alongside *S. cerevisiae*.

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