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Exploitation of autochthonous micro-organism potential to enhance the quality of Apulian wines

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Abstract A pressing necessity of the Apulian wine industry is to be able to pilot and to control wine production to obtain wines with peculiar characteristics and with the typicality guaranteed by the denominations of origin. The employment of selected autochthonous yeast strains would be a potent instrument to improve the organoleptic and sensory characteristics of typical regional wines. In fact, indigenous yeasts are better adapted to a specific must and therefore they are able to exalt the peculiarities of the derived wine. The present work describes the genetic diversity of autochthonous *Saccharomyces cerevisiae* strains derived from natural must fermentations of an important Apulian grape cultivar, denoted as "Primitivo". The yeast strains showing the best technological and oenological properties were selected, and their fermentative performances were assayed by either laboratory tests or industrial scale fermentations. Two autochthonous yeast strains were shown to be good candidates as industrial starter cultures, since they dominated the fermentation process and produced wines characterized by peculiar oenological and organoleptic features, that were judged very pleasant by a panel of winemakers.

Keywords Autochthonous yeast selection · Primitivo grape cultivar · *Saccharomyces cerevisiae* · Inoculated industrial fermentations · Wine yeast

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Introduction

Apulia is the second Italian wine-producing region and the most important producer of red-rosé wine. One peculiar characteristic of Apulian wines is that the podologic characteristics and the climatic conditions of this region contribute to enrich them with aromatic essences and to give, therefore, a characteristic and intense taste to the finished product. The Apulian wine industry is going through a period of great qualitative transformation, and the challenge of the market has therefore addressed the production methods, moving towards the use of innovative systems to guarantee and exalt the qualitative characteristics of regional wines. A heartfelt requirement is to be able to pilot and to control the production activity to obtain wines with peculiar characteristics, with respect of the typicality that is guaranteed by the denominations of origin.

The diversity of indigenous yeast strains can make wines denoted for their high quality and peculiar flavor (Pérez-Coello et al. 1999). However, to avoid the unpredictability of the must spontaneous fermentation, the winemakers add active dried yeast culture to initiate the fermentation process, thus impeding the development of non-*Saccharomyces* and favoring the full exhaustion of sugars and the homogeneity in the final product production. The use of commercial starters could affect the distinctive properties that characterize typical regional wines (Cappello et al. 2004). The

selection and the employment of new combinations of microorganisms, obtained from the native microflora, would be a powerful instrument to improve the organoleptic and sensory characteristics of the product (Rodríguez et al. 2009). Autochthonous yeasts are the micro-organisms better adapted to a specific must, which retain characteristics determined by the variety of the grapes and the terroir, and, therefore, they are able to exalt the peculiarities (aromas, structure and color) of a wine (Lopes et al. 2007).

In recent years, our research activity has been directed to the exploitation of autochthonous microbiota potential to enhance the quality of regional wines, and it has been conducted in collaboration with a large number of wine companies. The research activity has generated a virtuous circle, thus allowing the standardization of protocols for wine yeast enological selection and biomass production, the constitution of a Yeast Collection but, most of all, the transfer of technology to a large number of regional wineries. Natural fermentations of Primitivo musts have been performed and the *Saccharomyces cerevisiae* population has been analyzed and characterized by molecular, physiological, oenological and technological tests allowing the identification of four indigenous *S. cerevisiae* strains as candidates as autochthonous fermentation starters. This paper describes the genetic diversity of wild *S. cerevisiae* strains in spontaneous fermentations of a Primitivo wine. We selected the yeast strains endowed with remarkable technological and oenological properties, and then laboratory tests and semi-industrial scale fermentations were performed to confirm whether they might be used in industrial fermentations. Two of the selected autochthonous yeast strains were used in Primitivo wine industrial production in different wineries over several years. The above strains demonstrated that they were always able to dominate the fermentation process and to produce a final product characterized by oenological and organoleptic features, which were judged very satisfactory by a panel of winemakers.

Materials and methods

Must sampling from spontaneous fermentation

Four spontaneous fermentations from four different "Primitivo" cultivars were performed by sampling grapes (*Vitis vinifera*) in the Galatina, Torchiarolo, Manduria and Gioia del Colle areas, representing the most important areas for Primitivo wine production in Apulia. The fermentations were carried out from samples of 80–90 kg grape berries in 100-l tanks in an experimental cellar with a temperature between 16 and 18°C, and they were monitored by measuring the Babo grade (°Ba), in which one degree is equivalent to the presence of 10 g/l of fermentable sugars in the must. Samples were taken at the end of fermentation (0–1°Ba) and from the residual lees.

Selection of H_2S-low producer isolates

As a first selection step, yeast were screened for their ability to produce hydrogen sulfide. Decimal saline dilutions from each must sample were plated onto the surface of Biggy agar plates (Difco, USA) and incubated at 28°C for 3 days. Plates containing between 30 and 300 colonies were counted. All isolates were tested by colony color formation on Biggy agar plates (Difco), scoring the crowning degree associated with yeast growth, according to Sipiczki et al. (2001). About 100 white or light brown colonies from each sample were selected for isolation and identification. Isolates were stored frozen at −80°C until identification.

Identification of *S. cerevisiae* at species level

Yeast total genomic DNA was prepared according to the method used by Cappello et al. (2004). The isolates belonging to *Saccharomyces* genus (sensu strictu group) were identified according to the length of the rDNA region spanning the 5.8 S rRNA gene and flanking the internal transcribed spacers 1 and 2 (5.8-ITS; White et al. 1990). The PCR conditions were identical to those described by Esteve-Zarzoso et al. (1999). The amplified DNA products were visualized by agarose gel electrophoresis, as previously described (Bleve et al. 2006). The amplicon profiles obtained in the above described PCR reactions were analyzed with the Gel Compar 3.1 software (Applied Math, Kortrijk, Belgium).

Identification of *S. cerevisiae* at strain level

Thirty-six *S. cerevisiae* isolates from each sample were randomly selected for identification at strain level. The delta sequences that flank the TY1 retrotransposon region were analyzed according to Legras and Karst (2003). Amplification products were separated by gel electrophoresis. Agarose gels were scanned with a Gel Doc 1000 apparatus (Bio-Rad, USA) and analyzed with Molecular Analyst Fingerprinting (Bio-Rad). The obtained data were further confirmed by applying capillary electrophoresis (CE) for the analysis of strain-specific amplicon profiles as previously described (Tristezza et al. 2009). Cluster analysis of the pair wise values was generated by the UPGMA algorithm, using the NTSYS software (Applied Biostatistic, USA)

Microvinifications and wine analysis

To evaluate strain-specific fermentation performances, the identified strains were tested by micro-fermentations in Primitivo grape must. The must was clarified by centrifuga-

tion (10 min at 8,000 *g*), sterilized by filtration (0.45 μm membrane) and then supplemented with potassium metabisulphite (150 mg/l). One hundred milliliters of must were placed in sterile Erlenmeyer 150-ml flasks and then inoculated with 10^6 CFU/ml of yeast inoculum pre-cultured in the same must. The kinetics of the fermentations were monitored daily by gravimetric determinations, evaluating the loss of weight due to the production of CO_2. The samples were incubated at 25°C, and weighed daily to follow the weight loss caused by CO_2 production. When the CO_2 evolution stopped (i.e., at constant weight), the samples were stored at −20°C until required for analysis. Each fermentation experiment was carried out by performing three simultaneous independent repetitions.

Analytical determinations

Hydrogen sulfide production was evaluated by inserting a lead acetate strip between the plug and inner wall of the Erlenmeyer, above the level of the liquid. The qualitative hydrogen sulphyde production was detected by the blackening of the PbAcO paper (Martínez-Rodríguez et al. 2001). The main product components (ethanol, glycerol, pH, glucose, fructose, malic acid, tartaric acid, citric acid, total acid, brix, density, SO_2, total polyphenols, antocyans, CO_2, absorbance at 420, 520 and 620 nm, etc.) of wine and must under fermentation were evaluated by Fourier Transform Infrared Spectroscopy (FTIR) by employing the WineScan Flex (FOSS Analytical, DK). Samples were centrifuged at 8,000 *g* for 10 min and then analyzed following the supplier's instructions. The major volatile constituents [acetaldehyde, ethyl acetate, 2-methyl-1-propanol, higher alcohols (3-methyl- and 2-methyl-1-butanol), acetoin] were determined by gas-chromatography according to Mallouchos et al. (2003). The internal standard solution used was 4-methyl-1-pentanol.

Sensory analysis

At the end of fermentation, the wines were stored at 4°C. After 10 days, the deposited solids were discarded and the wines stored at 4°C for another 10 days. Then, the deposited solids were discarded and the wines were bottled. Twenty-five days after the end of fermentation, a sample of 50–70 ml of each fermented must at a temperature of 16–19°C was poured into a tasting glass immediately before analysis. The wines were evaluated by each judge according to the following criteria: visual profile: each wine is noted between 1 and 4 for the red hue and between 0 and 3 for blue and brown shades; flavor profile: each wine has been noted between 0 and 3 for each characteristic aroma. The aromas "wood", "fruity", "spicy", "mineral" are generally positive. The aromas "sulphide", "chemical", "herbal" and "animal" are generally negative.

Large-scale fermentation for biomass production

Fed-batch experiments were carried out by employing the Biostat C fermenting system with the capacity of 30 l (Sartorius, Germany). The temperature was regulated at 30°C and the pH at 5 with the addition of 15% (v/v) NH_4OH solution. The parameters (stirring rate, pH, temperature, partial pressure of dissolved oxygen, NH_4OH and antifoam addition) were monitored and regulated on-line by a connected computer. The fermenter was supplied with a sterile feed and the mass of glucose added to the fermenter was estimated instantly and submitted to pH regulation. Bioreactor conditions and feeding strategy during fermentations were performed according to Alfenore et al. (2004).

Inoculation of the industrial fermentations with selected wine yeast strains

Industrial fermentations were carried out in 200,000-l stainless steel vessels. To start must fermentations on large scale, a 30-l initial yeast inoculum, corresponding to 1.5 x 10^{12} CFU/ton was used. These cultures were transported to the winery and used as starters. Once in the winery, the yeast suspension was mixed with 300 Kg of Primitivo must and then let stand for 6 h at room temperature. After this period, the yeast–must mixture was added to 15 tons of Primitivo must (sugars 226 g/l, 19.7° Babo, pH 3.4, assimilable nitrogen concentration 156.5 g/l). The winemaking was conducted at 25°C and its kinetics was followed by measuring the Babo degrees. At the end of alcoholic fermentation (0–1°Babo), wine and residual lees were collected and yeast population isolated for further molecular analyses.

Results and discussion

The present study was aimed at the individuation of autochthonous yeast strains useful for the improvement of oenological production of Apulia, which is a very important wine-producing Italian Region. Grapes were sampled from the most representative Apulian areas [Gioia del Colle (Bari), Manduria (Taranto), Torchiarolo (Brindisi), Galatina (Lecce)] for "Primitivo" production and separately subjected to natural fermentation on an experimental scale. The identification of microbiota present during the last steps of wine fermentation (>1°Ba) of Primitivo grapes was carried out to select autochthonous yeast strains for industrial wine production.

The first step of this protocol for the selection of oenological autochthonous yeasts associated with natural fermentations of Primitivo grapes was concerned with the selection of isolates low-producer of hydrogen sulfide (H_2S). Hydrogen sulfide, which is produced by almost all

yeasts from the enzymatic reduction of inorganic sulfur and organic compounds, is an undesired compound conferring unpleasant off-flavor to wine. Nine hundred yeast colonies from each one of the four must samples in the study were analyzed by selective plate test and four populations of H_2S–no producers were obtained, composed of 114 (Gioia del Colle), 104 (Manduria), 121 (Galatina) and 125 isolates (Torchiarolo), respectively.

The molecular analysis of yeast isolates rDNA allowed to confirm that all isolates belonged to the species *Saccharomyces cerevisiae*. The molecular analysis performed on the yeast isolate population present at the end of fermentation showed a high intraspecies polymorphism, comparable to that found in other wine fermentations (Pulvirenti et al. 2009; Rodríguez et al. 2009). The absence of a dominant strain in each of the four performed natural fermentation is in agreement with Sabate et al. (1998) who reported an unusually high number of different strains of *S. cerevisiae* in spontaneous fermentation of grapes in Spain. Thirty-six isolates for each of the four populations were characterized at strain level using a rapid PCR-based protocol, relying on the amplification of interdelta regions. The statistical analysis of the obtained data allowed the identification in each population of distinct strain clusters with a similarity value within 92%: 13 distinct strains among the population of Manduria, 20 distinct strains among the population of Torchiarolo, 16 different strains in the population of Gioia del Colle, and 17 different strains in the population of Galatina (Fig. 1).

The clones identified in this study were subjected to biochemical and technological assays commonly used for the characterization of yeasts belonging to the genus *Saccharomyces* (Caridi et al. 2002; Nikolaou et al. 2006). Micro-fermentation assays allowed to evaluate the technological properties, which directly affect the progress of the fermentation (Romano 2005), in addition to enological and aromatic properties, which affect the quality of wines (Vincenzini et al. 2005).

The following enological and technological parameters were primarily considered and their values were employed as discriminatants for the selection of autochthonous yeast strains for their use in controlled fermentations: acetic acid production (<0.6 g/l), because the formation of this volatile acid produced by *S. cerevisiae*, generally in high concen-

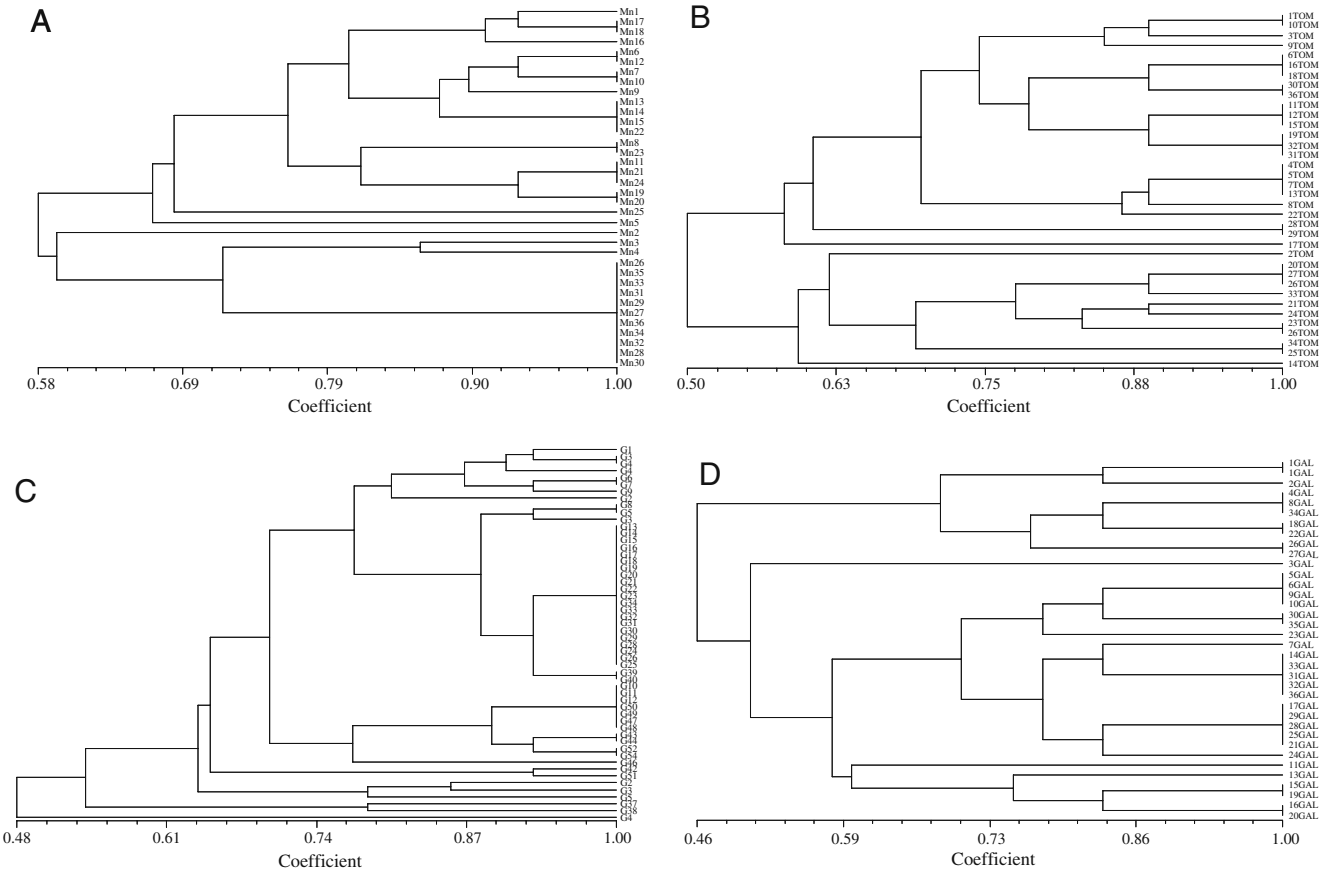

Fig. 1 UPGMA dendrograms generated by cluster analysis of inter-δ region patterns obtained from the *Saccharomyces cerevisiae* strains isolated during the later stages of spontaneous fermentation of Primitivo grapes from **A** Manduria, **B** Torchiarolo, **C** Gioia del Colle, **D** Galatina. Calculated percentages of homology are given on the bottom of each dendrogram

Table 1 Concentration of major chemical compounds in wines obtained by the selected autochthonous *S. cerevisiae* strains and a commercial starter. Values are the mean of three injections of each replicate (n=9); the standard deviations (±) are indicated

Strain	Malic[a]	Acetic[a]	Glucose[a]	Fructose[a]	Glycerol[a]	Ethanol[a]
8743	5.48±0.25	0.37±0.04	n.d.	0.96±0.06	5.36±0.19	89.46±1.52
8750	5.84±0.11	0.40±0.88	n.d.	1.75±0.41	4.70±0.14	90.51±13.75
8752	5.18±0.83	0.13±0.02	n.d.	1.19±0.05	5.30±0.12	90.55±1.27
8759	5.57±0.62	0.39±0.44	n.d.	3.82±0.20	9.07±0.13	85.47±1.98
8760	5.36±0.14	0.24±0.17	n.d.	1.95±0.16	9.05±0.41	91.36±1.08
8763	5.71±0.47	0.46±0.04	n.d.	2.88±0.42	8.56±0.63	88.49±1.56
8766	5.76±0.07	0.30±0.25	n.d.	0.96±0.52	6.90±0.77	90.21±0.13
8777	5.64±0.16	0.48±0.14	n.d.	1.20±0.02	6.43±0.24	88.57±0.33
8783	5.94±0.18	0.33±0.01	n.d.	3.20±0.76	6.45±0.12	85.99±2.55
8792	5.46±0.16	0.47±0.06	n.d.	2.41±0.16	6.83±0.47	86.79±1.20
Commercial starter	5.96±0.15	0.49±0.01	n.d.	3.20±0.27	6.96±0.11	88.10±3.18

[a] In g/l; n.d. not detectable

trations, is definitely undesirable (Fleet and Heard 1993); total sugar consumption, since the presence of residual sugar in the must (>4 g/l) is indicative of an incomplete fermentation (Pérez-Coello et al. 1999); lack of H_2S production, perceptible on a sensory level with as strong and unpleasant odor of rotten eggs, also called "reduced odor" (Vincenzini et al. 2005).

The results obtained indicated that the indigenous yeast strains 8759, 8760, 8763, 8783, 8792, 8743, 8752, 8750, 8766 and 8777 satisfied the above requested parameters. These strains were selected to be further characterized, and the results of quantitative analysis of the main chemical compounds present in fermented must by them are shown in Table 1. The glycerol, formed by yeast during fermentation, is one of the main components of wine, where it is usually found in concentrations ranging from 5 to 8 g/l. The glycerol has a key role in the formation of the bouquet of wine as it helps improve balance and structure of wine (Noble and Bursick 1984).

In order to quantify the presence of higher alcohols produced by fermentation in must, the concentrations of 2-methyl-1-propanol (isobutyl alcohol) and the summation of amyl alcohols (2-methyl-1-butanol and 3-methyl-1-butanol) were evaluated. The concentrations of isobutyl alcohol varied from 10.57 mg/l (8759) to 20.04 mg/l (8777), being concentrations that add complexity to the bouquet without overpowering the wine fragrance (Jackson 2008). The summation of amyl alcohols (Table 2) ranged from 66.30 mg/l (strain 8743) to 84.24 mg/l (strain 8777), indicating that all strains were involved in the positive aromatic complexity of wine (Ribéreau-Gayon et al. 1998). Another very important constituent of the major polar compound is ethyl acetate. The values found for this ester (Table 2) ranged between 3.65 mg/l for 8766 and 8.15 mg/l for 8743, lower than the commercial strain (8.62 mg/l), concentrations for which the contribution of this ester to the aroma of wines is positive (Lopes et al. 2007). Acetaldehyde is the most important carbonyl compound produced during fermentation and its concentrations ranged from a minimum of 6.46 mg/l (strain 8763) to a maximum of 20.09 mg/l (strain 8743). The amount of acetoin, produced by the tested strains, ranged from 2.80 mg/l for 8766 to 6.41 mg/l for 8792 (Table 2) and these concentrations were consistent with those described in a similar study by Romano and Suzzi (1993).

The product of micro-fermentations were also subjected to sensory analysis (not shown). In all fermented musts, a *fruity* aroma has been identified with scores ranging from a minimum of 1.3 (8750) to a maximum of 3.0 (8766). Except for strains 8752 and 8750, in all wines a *spicy*

Table 2 Concentration of major volatile compounds in wines obtained by the selected *S. cerevisiae* autochthonous strains and one commercial starter. Values expressed in mg/l are the mean of three injections of each replicate (n=9); the standard deviations (±) are indicated

Compound	Strain										
	8743	8750	8752	8759	8760	8763	8766	8777	8783	8792	Commercial starter
Acetaldehyde	20.09±2.58	7.52±0.57	11.82±0.16	6.70±0.22	6.69±0.12	6.46±0.22	10.30±0.03	12.70±0.23	17.25±0.21	16.65±0.26	14.02±0.06
Ethyl acetate	8.15±0.37	5.23±0.63	3.77±0.11	5.72±0.80	4.79±0.34	4.36±0.25	3.65±0.23	6.45±0.97	6.81±0.03	4.95±0.06	8.62±2.17
2-methyl-1-propanol	13.98±0.17	14.67±0.20	15.22±0.12	10.57±0.15	17.26±0.31	15.32±0.22	18.61±0.14	20.04±0.13	15.81±0.06	10.80±0.25	17.78±0.14
Amyl alcohols	66.30±0.26	66.82±0.64	69.10±0.35	69.44±0.47	69.03±0.71	68.36±0.20	76.59±0.20	84.24±0.21	71.38±0.21	75.06±0.40	77.06±0.19
Acetoin	3.25±0.30	3.50±0.28	3.42±0.32	4.13±0.50	3.21±0.59	3.34±0.38	2.80±0.08	4.81±0.29	3.45±0.21	6.41±0.14	5.46±0.82

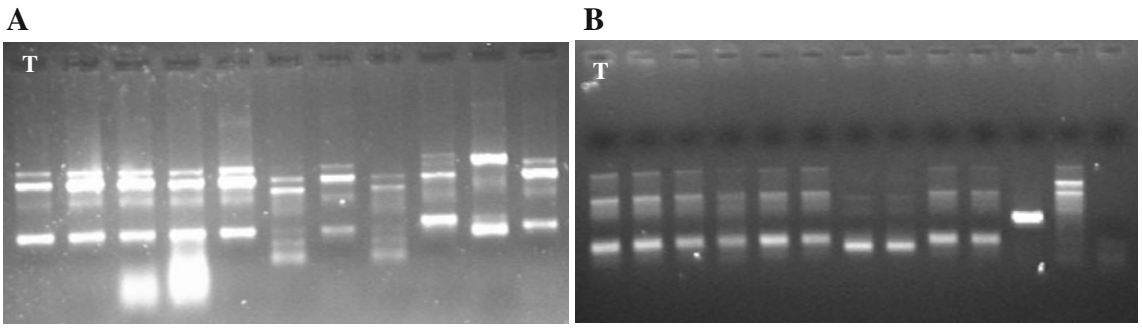

Fig. 2 Electrophoretic profiles of inter-δ region patterns obtained from ten *Saccharomyces cerevisiae* stains randomly isolated at the end of the two large-scale fermentations, respectively inoculated with the 8766 (**A**) and the 8760 (**B**) strains. The strain-specific profile for the 8766 and 8760 strains is reported in each panel (*T*)

aroma has been detected. The aromatic negative descriptors *sulphide, chemical* and *herbaceous* were identified only in samples 8759, 8763, 8783 and 8752.

Taken together, the results of this analysis have revealed that, among the 10 *S. cerevisiae* analyzed strains, the 8760 (native from Torchiarolo) and 8766 (native from Manduria) retained enological and technological properties optimal for their possible use as indigenous fermentation starters for Primitivo wine production.

These two strains were used in fermentation on a large scale in two industrial wineries. Yeast biomasses were produced by performing a fed-batch fermentation, thus obtaining a quantity of inoculum sufficient to directly inoculate 15 tons of Primitivo must each.

The data corresponding to the fermentation performance of the two isolates used and their ability to dominate the fermentative event have indicated that these two autochthonous yeast strains possess the fundamental properties required for starter cultures (Pulvirenti et al. 2009). Indeed, the fermentations had progressed regularly and sugar depletion was completed in 6 days. The dominance of inoculated strains was confirmed by the analysis of the inter-δ region polymorphism (Fig. 2). Data show that strain 8760 and 8766 were able to overwhelm the yeasts naturally present in the must, with a high predominance (60–70%) in total yeast population at the end of fermentation. The results of chemical and gas chromatographic analysis of the two wines produced are shown in Table 3. Both strains produced low concentrations of acetic acid (volatile acidity) ranging from 0.15 g/l for 8766 to 0.34 g/l for 8760. The total acidity was higher in must fermented by strain 8766 (6.77 g/l), in fact in this sample the pH value was lowest (3.4). As can be seen in both samples, tartaric acid was the most abundant organic acid detected (Table 3). Both strains were able to metabolize sugars at a final concentration below the value of 3.0 g/l. Furthermore, the two strains showed a high alcohol-power [actual alcoholic content (g/100 ml)], with values of 13.22% (strain 8766) and 13.71% (strain 8760).

The color of a wine can be considered as the sum of the three color components: yellow (420 nm), red (520 nm) and blue (620 nm). For the wine made with strain 8766, the total color intensity was minimum (1.93), although the percentage of red was the maximum for this strain (58.12%). For wine made with strain 8760, color intensity was 2,26, with 57.78% of red.

The concentrations of the main volatile components of wine were determined by gas chromatographic analysis (Table 3). The values of acetaldehyde ranged from a

Table 3 Chemical properties of the two wines obtained by using the 8760 and 8766 *S. cerevisiae* selected strains in a large-scale Primitivo must fermentation. Values are the mean of three injections; the standard deviations (±) are indicated

	Strain 8760	Strain 8766
Alcohol (g/100 ml)	13.71±0.01	13.22±0.00
Reducing sugars ((g/l)	2.66±0.18	2.4±0.07
Total acidity (g/l)	6.19±0.03	6.77±0.04
Volatile acidity (g/l)	0.34±0.04	0.15±0.01
Glycerol (g/l)	11.54±0.01	9.3±0.08
Methanol (ml/100 ml)	0.37±0.01	0.33±0.00
Malic (g/l)	0.09±0.01	0.63±0.01
Lactic (g/l)	1.65±0.05	0.76±0.05
Tartaric (g/l)	2.81±0.05	3.40±0.05
Citric (g/l)	0.21±0.05	0.29±0.00
pH	3.74±0.00	3.40±0.01
Total polyphenols (mg/l)	3843	3164
Anthocyanins (mg/l)	52	76
Absorbance at 420 nm	0.686	0.586
Absorbance at 520 nm	1.307	1.12
Absorbance at 620 nm	0.269	0.221
Acetaldehyde (mg/l)	2.56±0.3	9.20±0.4
Ethylacetate (mg/l)	62.15±2.6	41.68±1.5
2-methyl-1-propanol (mg/l)	15.65±0.2	17.65±0.3
Amyl alcohols (mg/l)	93.59±0.2	98.89±0.0
Acetoin (mg/l)	6.89±0.5	6.73±0.6

minimum of 2.56 mg/l (strain 8760) to a maximum of 9.20 mg/l (strain 8766). The ethyl acetate was detected in quantities in the range of 41.68 mg/l (strain 8766) and 62.15 mg/l (strain 8760), while as regards the amyl alcohol products, the amounts detected ranged from 93.59 mg/L (8760) to 98.89 mg/l (strain 8766). The concentrations of acetoin proved to be very similar for both strains with values of 6.89 mg/l and 6.73 mg/l.

In conclusion, this research aimed to be a preliminary study on the possibilities to improve the quality of Apulian wine by the use of indigenous starter cultures. Large-scale experimental vinifications were conducted in two winery cellars of Salento, where the two selected indigenous strains efficiently performed the fermentation process, showing them to be technologically comparable to a commercial starter culture, and produced Primitivo wines with exquisite and peculiar organoleptic characteristics. The outcome of this research suggests a possible use of selected autochthonous strains for the production of Primitivo wines, thereby determining a positive contribution to enhance the peculiar features of this typical Apulian wine.

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