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Short Communication

# Selection of indigenous *Saccharomyces cerevisiae* strains for Nero d'Avola wine and evaluation of selected starter implantation in pilot fermentation

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ABSTRACT

The present research studied *Saccharomyces cerevisiae* yeasts isolated from Nero d'Avola grapes, collected in different areas of the Sicily region. RAPD-PCR analysis with M13 primer was used for preliminary discrimination among 341 *S. cerevisiae* isolates. Inoculated fermentations with *S. cerevisiae* strains, exhibiting different RAPD-PCR fingerprinting, revealed the impact of selected strains on volatile compound concentration. Two selected strains were used in fermentation at cellar level and the restriction analysis of mtDNA on yeast colonies isolated during fermentation was used to control strain implantation. This study represents an important step to establish a collection of indigenous *S. cerevisiae* strains isolated from a unique environment, such as Nero d'Avola vineyards. Different starter implantation throughout inoculated fermentation represents an additional character, which might be considered during the selection program for wine starter cultures.



## 1. Introduction

As ancient tradition, the production of wine has been carried out for years by spontaneous fermentation of grape juice caused by indigenous yeasts, belonging to different genera and species (Heard and Fleet, 1988; Fleet, 2003; Lambrechts and Pretorius, 2000; Romano et al., 2003a). The number of species and their presence during fermentation depends on several factors (Longo et al., 1991; Pretorius et al., 1999), with subsequent wine quality variations from region to region but also from one year to another, and all this makes the outcome of spontaneous fermentation difficult to predict (Pretorius, 2000). In an attempt to address this problem, many winemakers use pure *Saccharomyces cerevisiae* cultures which are inoculated into the must after pressing (Pretorius, 2000; Querol et al., 1992a). However, there is some controversy about the use of these commercial wine yeasts, due to the lack of some desirable traits provided by spontaneous fermentation (Fleet and Heard, 1993). In recent years, there is increasing interest among wine-researchers and winemakers towards autochthonous strains with the aim to select starter cultures that are potentially better adapted to the growth in a specific grape must, and reflect the biodiversity of a given region (Lopes et al., 2002; van der Westhuizen et al., 2000; Versavaud et al., 1995; Torija et al., 2001; Sabate et al., 1998), which support the notion that specific native yeast strains can be associated with a *terroir*.

The maintenance of biological patrimony is essential both to obtain starter strains that are potentially able to develop the typical flavour and aroma of wines originating from different grapevine cultivars (Pretorius, 2000), and to ensure the conservation of gene pools of technological importance. Therefore, exploring the biodiversity of indigenous fermentative strains can be an important contribution towards the understanding and selection of strains with specific phenotypes.

On the other hand, the use of selected *S. cerevisiae* strains in the winemaking process requires the development of techniques that can clearly differentiate between the inoculated strain and the wild strains present in the grape must, also by considering that the dominance of the inoculated strain can be subordinated to the specific conditions of the vinification. It is generally assumed that indigenous yeasts are suppressed by the starter, however, studies show that indigenous yeasts can still participate in inoculated fermentation (Querol et al., 1992b). For these reasons, molecular methods, such as mitochondrial DNA (mtDNA) restriction analysis (Querol et al., 1992b; Versavaud et al., 1995) and comparison of chromosomal DNA profiles (Guillamón et al., 1996; Versavaud et al., 1995) would be useful to ensure that the fermentation is conducted by the inoculated yeast.

In the present investigation, data are presented on the indigenous yeast microflora, isolated from the Sicily region (southern Italy) vineyards planted with the Nero d'Avola grape variety. The Nero d'Avola grape gives a nice ruby red wine with berry, cherry, plum and spicy flavours depending on the type of vine, location and age.

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*S. cerevisiae* isolates were characterized for genetic and technological variability in order to create a strain collection contributing to the preservation of *S. cerevisiae* genetic resources of the peculiar *terroir* of Nero d'Avola.

Two selected *S. cerevisiae* Nero d'Avola strains were used to ferment Nero d'Avola grape must at cellar level and the restriction analysis of mtDNA on yeast colonies isolated during fermentation was used to monitor strain implantation.

## 2. Materials and methods

### 2.1. Yeast isolation of Saccharomyces-type colonies

Yeast isolation was carried out from Nero d'Avola grapes. The grapes were collected in fifteen vineyards, located in three areas of the Sicily region: Trapani (T), Ragusa (R) and Caltanissetta (C). Five different vineyards (A–E) were selected in each area and, in each vineyard, ten sampling points were defined. A total of 150 grape samples have been taken (three sampling areas × five vineyards × ten sampling points in each vineyard). From each sampling point, approximately 2 kg grapes were harvested, by choosing grape clusters healthy and bird-damaged alike.

At the laboratory, the samples were crushed under aseptic conditions in the original plastic bags. The juice obtained (volume about 1 L) was transferred into sterile flasks and underwent spontaneous fermentation at a controlled temperature of 25 °C. The determination of weight loss was used as a parameter to follow the fermentation process. Samples were taken aseptically at the middle and end of alcoholic fermentation. Must samples were diluted and spread on plates with Wallerstein laboratory (WL) nutrient agar (Oxoid CM 309; Oxoid, Basingstoke, UK) (Pallmann et al., 2001), and incubated at 28 °C for 2–6 days. From each sample and each fermentation stage, 5 colonies showing *Saccharomyces* colony morphology were selected and purified on YEPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar). The isolates were stored in glycerol (30%, v/v) at −80 °C.

### 2.2. Molecular identification of Saccharomyces isolates

Yeast isolates were identified by PCR amplification of the region spanning ITS1 and ITS2 and the 5.8S rRNA gene (5.8 S-ITS region) and subsequent restriction analysis according to the work by Granchi et al. (1999).

PCR products were digested without further purification with the restriction endonucleases *Hae*III, *Hin*fI and *Scr*fI (Promega).

### 2.3. Genotypic characterization of S. cerevisiae isolates

Differentiation between the 341 indigenous *S. cerevisiae* isolates was performed by RAPD-PCR with primer M13 (5′-GAGGGTGGCGGTTCT-3′), using the DNA extraction and methods described by Capece and Romano (2009) and Romano et al. (2008).

### 2.4. Technological characterization of S. cerevisiae isolates

Resistance to sulphur dioxide, ethanol and copper and production of hydrogen sulphide were chosen as parameters for technological characterization of *S. cerevisiae* isolates, following the method described in Mauriello et al. (2009).

### 2.5. Inoculated fermentations at laboratory-scale with selected S. cerevisiae isolates

One hundred-thirty strains of *S. cerevisiae*, selected on the basis of M13 fingerprinting and technological traits were tested in inoculated fermentations.

Inoculated fermentation assay was performed in 130-mL Erlenmeyer flasks filled with 100 mL of "Nero d'Avola" pasteurized grape must (100 °C for 20 min). Grape must characteristics were: 19% (w/v) fermentable sugar, pH 3.18. Each strain was inoculated in grape must at a concentration of $10^6$ cells/mL, from a pre-culture grown for 48 h in the same must. The fermentation was performed at 26 °C and the fermentative course was monitored by measuring weight loss, determined by carbon dioxide evolution during the process. At the end of the process, the wine samples were refrigerated at 4 °C to clarify the wine, racked and stored at −20 °C until required for analysis. All the experiments were performed in duplicate.

### 2.6. Analysis of volatile compounds by gas-chromatography

Higher alcohols (*n*-propanol, isobutanol, amyl alcohols), acetaldehyde, ethyl acetate and acetic acid were analysed by direct injection gas-chromatography using the procedure described by Romano et al. (2003b). Levels of secondary compound determined in the experimental wines were submitted to statistical analysis by descriptive Box plots and whiskers, using the software 'Statistica for Windows', version 6.0 (Statsoft Inc.).

Data relating to the volatile compounds were converted into adimensional values in function of mean and Standard Deviation (SD) values, by elaborating a matrix, as reported by Mauriello et al. (2009).

The data matrix was subjected to cluster analysis by using the software 'Statistica for Windows', version 6.0 (Statsoft Inc.).

### 2.7. Inoculated fermentations at pilot scale

Fermentations were carried out in a cellar of the Sicily region during the 2008 vintage. Two indigenous strains of *S. cerevisiae*, selected on the basis of the laboratory experiments, were tested in sulphited (50 mg/L) Nero d'Avola grape must by inoculating $10^6$ cells/mL in 100-L-vats and the fermentation processes were monitored daily by determining sugar and temperature.

### 2.8. Implantation of indigenous strains of S. cerevisiae

For yeast isolation and characterization, samples were taken from each vinification at final stages of fermentation. The sampling for determination of viable counts was determined in duplicate. Each sample was adequately diluted in sterile water and spread on plates of WL nutrient agar. The plates were incubated at 28 °C for five days. After viable yeast counting, from each sample 20 colonies showing *Saccharomyces* morphology were randomly selected and isolated on YEPD for further characterization. Yeast identification was performed, as previously reported. Yeast isolates belonging to *S. cerevisiae* species were characterized by using mtDNA-RFLP analysis according to Querol et al. (1992c) using the restriction endonuclease *Hin*fI and *Rsa*I (Promega).

## 3. Results

### 3.1. Isolation and identification of Saccharomyces yeasts

In the present work, fifteen vineyards, located in the Sicily region (Southern Italy) and cultivated with Nero d'Avola grape variety, were sampled during the 2004–2005 harvest season. During middle and later phases of grape spontaneous fermentation, yeast colonies showing *Saccharomyces*-type morphology were selected and identified by restriction analysis of ITS region. By comparing the molecular sizes of the restriction fragments of selected isolates with those described previously (Fernandez-Espinar et al., 2000; Granchi et al., 1999) 341 *S. cerevisiae* isolates were identified.

### 3.2. Genotypic characterisation of S. cerevisiae isolates

The 341 *S. cerevisiae* isolates were characterized by using RAPD-PCR analysis. This technique has proved to be informative and suitable for the study of a large number of strains in short time (Andrighetto et al., 2000). Primer M13 generated RAPD-PCR fingerprints composed of a number of well distributed bands (4–10) ranging from approximately 2000 to 350 bp. The analysis of molecular fingerprintings obtained with this primer allowed us to distinguish numerous strains within our isolates. Among the 341 *S. cerevisiae* colonies isolated in this study, only 130 different M13 patterns were found.

### 3.3. Technological characterisation of S. cerevisiae isolates

The screening of *S. cerevisiae* isolates for parameters of technological interest revealed a similar behaviour among all the isolates analyzed (data not shown). In fact, the main percentage (98%) of isolates exhibited a high resistance to all the compounds tested (98% of isolates tolerated 300 mg/l of $SO_2$ and 18% v/v of ethanol, whereas 80% of isolates grew in presence of 500 μmol/$CuSO_4$). As regards hydrogen sulphide production, the isolates exhibited a medium production level of this compound, forming colonies characterized by a color ranging between hazelnut and brown.

### 3.4. Inoculated fermentations with selected S. cerevisiae isolates

The 130 *S. cerevisiae* isolates, chosen in function of M13 profile and high resistance to antimicrobial compounds, were subjected to further characterization. These strains were tested in inoculated fermentations of Nero d'Avola grape must. The fermentation course was monitored by evaluating carbon dioxide ($CO_2$) evolution and the process was completed after 12–14 days. All the isolates exhibited similar fermentative performance, with values comprised between 9.2 and 11 g $CO_2$/100 mL grape must (data not shown). The experimental wines obtained were analyzed for the content of some by-products correlated to the organoleptic quality of the wine. The metabolites determined by gas-chromatographic analysis were: higher alcohols (*n*-propanol, isobutanol, amyl alcohols), acetaldehyde, ethyl acetate and acetic acid.

The strains exhibited a significant variability in the production levels for all determined compounds (Fig. 1), in particular for acetaldehyde, isobutanol, amyl alcohols and acetic acid. On the other hand, a very low variability was found for ethyl acetate and *n*-propanol. Considerable amounts of isoamyl alcohol (mean values of 170–300 mg/L) were formed by yeast isolates in the experimental fermentations of Nero d'Avola. In fact, the total higher alcohols may have a positive impact on wine aroma at levels up to 300 mg/L, while over this value they can exert a negative effect on wine organoleptic quality because their penetrating smell masks the other aromas.

In order to individuate a potential correlation between the origin of isolates and their metabolic behaviour, the levels of secondary compounds determined in the experimental wines were statistically elaborated. The obtained data matrix was subjected to a hierarchical agglomerative cluster analysis of cases, taking the Euclidean distance as metric and the Ward's method as amalgamation rule. The dendrogram obtained, reported in Fig. 2, shows the presence of two main clusters (A and B). By comparing experimental wines clustering with raw data (Table 1), it seems that wine clustering is related with secondary compound levels: group A includes experimental wines characterized by lower amounts of the main secondary compounds than wines clustering in B. In particular, compounds determining the highest differences were acetic acid, isobutanol, D-amyl and isoamyl alcohols. As expected, the wine clustering is also correlated to the isolation area of inoculated *S. cerevisiae* strains. In fact, group A includes the major percentage of experimental wines produced with *S. cerevisiae* isolated from Ragusa (81%) and Trapani (74%) areas,

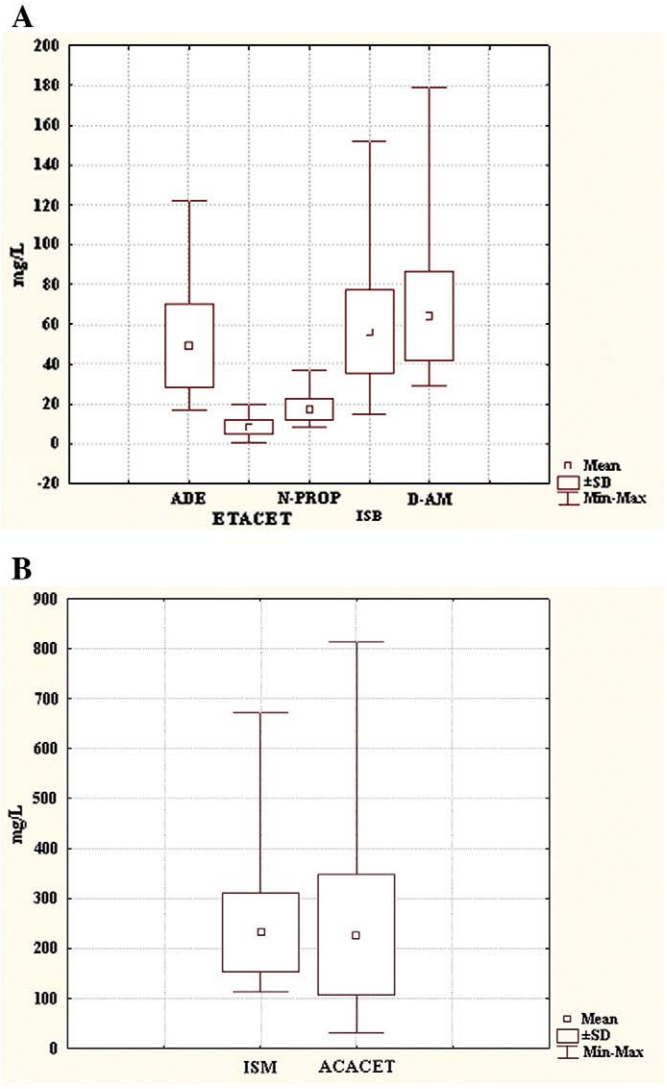


**Fig. 1.** Box plot representing the variability of secondary compounds determined in the experimental wines produced by inoculating 130 wild *S. cerevisiae* strains. ADE = acetaldehyde; ETACET = ethyl acetate; N-PROP = n-propanol; ISB = isobutanol; D-AM = amyl alcohol; ISM = isoamyl alcohol; ACACET = acetic acid.

whereas 63% of wines derived by fermentation with *S. cerevisiae* isolated from the Caltanissetta area fall into group B.

### 3.5. Implantation of indigenous strains of S. cerevisiae

On the basis of the previous results, two strains, representative of the two different metabolic groups (Fig. 2), were chosen and subjected to further study. The selected strains were:

- CB1-7Sr3, metabolic group B, high fermentative power (10.7 gr $CO_2$/100 mL grape must);
- RB3-7Sc2, metabolic group A, high fermentative power (10.2 gr $CO_2$/100 mL grape must);

These two strains were used as starter cultures in inoculated fermentations in a Sicilian cellar producing Nero d'Avola wine.

In order to evaluate the capacity of implantation of the selected native strains, the mtDNA-RFLP analysis was used to characterize the isolates from the inoculated fermentations.

Some mtDNA-RFLP patterns different from the profiles of inoculated strains were found. By analyzing the distribution of the different patterns among the isolated colonies, the indigenous strain RB3-7Sc2 exhibited a significant higher implantation ability than CB1-

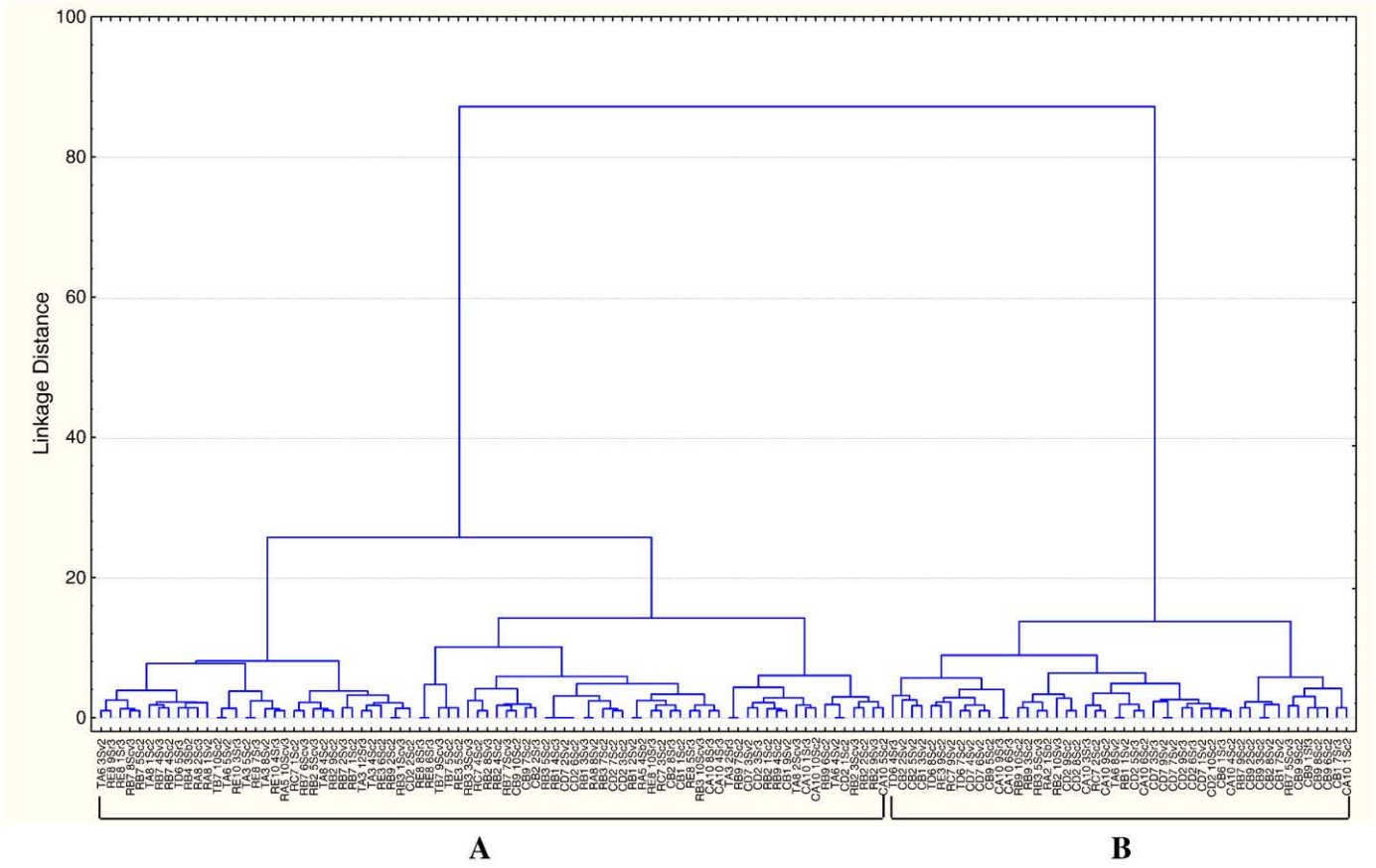


**Fig. 2.** Dendrogram obtained by Ward's hierarchical clustering method performed on volatile compounds determined in 130 the experimental wines.

7Sr3 (Table 2). In fact, at the end of the pilot scale fermentation inoculated with the *S. cerevisiae* strain CB1-7Sr3 the isolated strains of *S. cerevisiae* gave 7 different mtDNA profiles. The profile corresponding to the inoculated strain CB1-7Sr3 represented only 15% of the population, suggesting that it had not implanted and dominated the fermentation. In addition, among the colonies isolated from the pilot fermentation with this strain, the predominant profile (A) was exhibited by 50% of the analyzed isolates (Table 2).

As regards the *S. cerevisiae* colonies isolated from the pilot fermentation inoculated with strain RB3-7Sc2, 7 different profiles were found, but 40% of the isolates showed the same restriction pattern of the inoculated strain, which, in this case, resulted the dominant one. The profiles L and N (Table 2) were exhibited from 25% and 15% of the colonies, respectively, whereas the other patterns were present in a low percentage of colonies.

It has to be underlined that only the profile F was found among the colonies isolated from both the fermentation tests. Consequently this profile could correspond to an indigenous strain, which probably derived from the grape must used in this study and resulted more competitive against both the starters.

**Table 1**
Levels of secondary compounds determined in the 130 experimental wines. The groups are the same reported in Fig. 2.

| Compounds | Group A | Group B |
|---|---|---|
| | Range (mg/l) | Range (mg/l) |
| Acetaldehyde | 17.23–111.05 | 27.5–122.24 |
| Ethylacetate | 0.91–12.78 | 4.33–19.74 |
| n-Propanol | 8.41–28.30 | 12.97–36.93 |
| Isobutanol | 14.72–74.22 | 47.07–151.88 |
| Acetic acid | 81.07–610.27 | 32.65–813.68 |
| D-amyl alcohol | 29.06–82.06 | 61.93–179.11 |
| Isoamyl alcohol | 113.27–293.26 | 223.71–671.55 |

## 4. Discussion

The present study has been carried out in a traditional Italian viticulture region, Sicily, which produces very high amounts of wine, mainly by inoculated fermentation with commercial yeast strains. In fact, oenological use of selected strains of *S. cerevisiae* was widespread in Sicily until the 1990s. The population of indigenous *S. cerevisiae* yeasts present on Nero d'Avola grapes, collected in the vineyard, has never been characterized before.

**Table 2**
Frequency of mtDNA-RFLP patterns of colonies isolated from inoculated fermentations with CB1-7Sr3 and RB3-7Sc2 *S. cerevisiae* strains.

| mtDNA-RFLP patterns | Colonies (%) corresponding to mtDNA-RFLP patterns | |
|---|---|---|
| | CB1-7Sr3 | RB3-7Sc2 |
| IN.[a] | 15 | 40 |
| A | 50 | |
| B | 15 | |
| C | 5 | |
| D | 5 | |
| E | 5 | |
| F | 5 | 5 |
| G | | 5 |
| H | | 5 |
| L | | 25 |
| M | | 5 |
| N | | 15 |

[a] mtDNA-RFLP patterns identical to the profiles of the inoculated strains.

The only other study of these grapes was reported by Romancino et al. (2008) and it was aimed to study the microbiota present in grape musts collected during the grape-pressing step from different areas of Sicily.

In recent years, with the aim to produce wines with distinctive quality, some Sicilian winemakers revealed an increasing interest toward the practice of spontaneous fermentations. The connection of a typical wine to a specific *terroir* seems to involve also the microbial component, meaning that the transformation activity by indigenous yeasts can give peculiar aromatic notes to the final product. This association between grape must and starter yeast of the same origin, seems to communicate a positive image to the consumer, thus promoting potential commercial benefits to wine-producer. Therefore, the use of indigenous selected yeasts, isolated from grapes of specific region, could represent a useful alternative to spontaneous fermentation in order to optimize the typical attributes of the vine variety (Clemente-Jimenez et al., 2004; Rementeria et al., 2003; Romano et al., 2008).

With the aim to select indigenous *S. cerevisiae* strains from Nero d'Avola grapes, strain genetic and technological diversity was evaluated.

Laboratory-scale fermentations with *S. cerevisiae* isolates, selected on the basis of biotype, revealed a significant impact of these strains on wine aroma. Considerable differences in the volatile compound levels were found in Nero d'Avola experimental wines, in the function of the inoculated strain. In our research, *S. cerevisiae* isolation area and wine aromatic composition were correlated. In particular, experimental wines produced with strains isolated from the Caltanissetta area were characterized by presence of higher amounts of secondary compounds than the wines obtained from fermentation with Trapani and Ragusa strains. These strains may be responsible for distinctive wine properties. Therefore, in order to avoid a loss of wine typicality, it is necessary to highlight the importance of selecting wild vineyard strains from each area that represents, not only a reservoir of yeast biodiversity, but also the potential expression of the typical sensory properties of wine produced in that region. Recent results (Mauriello et al., 2009) have demonstrated that the production of volatile organic compounds differentiated *S. cerevisiae* strains in function of the different geographic isolation zones.

On the basis of the technological characteristics tested among the indigenous *S. cerevisiae* isolates, two strains, CB1-7Sr3 and RB3-7Sc2, were selected and used as starter in a cellar producing Nero d'Avola wine in the Sicily region. This test was performed with the main purpose to evaluate the implantation ability of yeast starters, which represents an important parameter to be evaluated in all wine yeast selection programs. In fact, several works have evidenced that the dominance of the starter is not always guaranteed (Beltran et al., 2002) and that the growth of non-starter yeasts can significantly affect the effectiveness of the dominance process (Cocolin et al., 2000; Santamaria et al., 2005). Several factors, such as those related to the cellar operations, grape must type, geographical characteristics, among others, can affect the diversity of the indigenous biota also influencing the implantation capacity of a wine yeast starter (Barrajón et al., 2009). Our work shows that implantation of both the starters was quite low, and was lower for the CB1-7Sr3 strain than the RB3-7Sc2 strain. The results obtained in this study underline that the inoculation of fermentation tanks with selected starters does not always guarantee their implantation during alcoholic fermentation. In the sampled tanks, low-implantation of the starter native yeasts may be due to a number of different factors, including lower-than-recommended doses and unsuitable fermentation conditions. By considering that the two Nero d'Avola indigenous strains were inoculated in two different tanks of the same cellar and in the same grape must, the different strain behaviour could be related to a different strain ability to dominate the natural microflora present in the grape must, and not from extension and diversity of the indigenous biota at the beginning of the fermentation or from different winemaking practices.

The low strain implantation could be due to a strong competition between wild yeasts and starter cultures. The results of this part of our work suggest that yeast competition can be considered as a specific character which confers to a selected *S. cerevisiae* strain the capacity to compete with wild strains of the same species, thus defending its domain and becoming the main actor of that grape must transformation.

Therefore, this work has shown more light on the strain implantation during alcoholic fermentation, showing that in the selection program of wine starter cultures, the aptitude of candidate strains to predominate and maintain the dominance on the natural microflora along the whole process has to be strongly evaluated on the specific grape must.

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