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ORIGINAL ARTICLE

Technological properties of *Oenococcus oeni* strains isolated from typical southern Italian wines

V. Capozzi[1], P. Russo[1], L. Beneduce[1], S. Weidmann[2], F. Grieco[3], J. Guzzo[2] and G. Spano[1]

1 Dipartimento di Scienze degli Alimenti, Facoltà di Agraria, Foggia, Italy
2 Equipe de Recherche en vigne et vin (REVV), Institut Jules Guyot, Université de Bourgogne, Dijon, France
3 Istituto di Scienze delle Produzioni Alimentar; ISPA, via Provinciale Lecce-Monteroni, Lecce, Italy

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Correspondence
Giuseppe Spano, Dipartimento di Scienze degli Alimenti, Facoltà di Agraria, via Napoli 25, 71100 Foggia, Italy. E-mail: g.spano@unifg.it

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Abstract

Aims: To isolate indigenous *Oenococcus oeni* strains suitable as starters for malolactic fermentation (MLF), using a reliable polyphasic approach.
Methods and Results: *Oenococcus oeni* strains were isolated from Nero di Troia wines undergoing spontaneous MLF. Samples were taken at the end of alcoholic fermentation and during MLF. Wine samples were diluted in a sterile physiological solution and plated on MRS and on modified FT80. Identification of *O. oeni* strains was performed by a polymerase chain reaction (PCR) experiment using strain-specific primers. Strains were further grouped using a multiplex RAPD-PCR analysis. Then, six strains were inoculated in two wine-like media with two different ethanol concentrations (11 and 13% vol/vol) with a view to evaluate their capacity to grow and to perform MLF. In addition, a quantitative PCR (qRT-PCR) approach was adapted to monitor the physiological state of the strains selected.
Conclusion: A positive correlation between the malolactic activity performance and the ability to develop and tolerate stress conditions was observed for two selected *O. oeni* strains.
Significance and Impact of the Study: The results reported are useful for the selection of indigenous MLF starter cultures with desired oenological traits from typical regional wines. It should be the base for the improvement in organoleptic quality of typical red wine.

Introduction

Malolactic fermentation (MLF), the bacterial conversion of L-malic into L-lactic acid and carbon dioxide, is encouraged, especially for red wines, to eliminate the taste of malic acid, to decrease the acidity of the wine and to assure the biological stability of wines. However, indigenous lactic acid bacteria (LAB) can start spontaneous MLF after the alcoholic fermentation (AF), negatively affecting wine quality (Lonvaud-Funel 1999). A controlled MLF can be achieved with the inoculation of selected LAB (Ribéreau-Gayon *et al.* 2006).

Oenococcus oeni is the LAB that occurs naturally in wine. It is the dominant bacterial species found during the MLF, being well adapted to the harsh wine conditions, and it possesses the finest oenological malolactic characteristics (Bartowsky 2005). The key criteria to select *O. oeni* strains suitable for MLF are mainly related to (i) the ability to tolerate the harsh conditions encountered during wine fermentation (e.g. low pH, high ethanol concentration and sulfite tolerance, resistance to specific bacteriophages) (Guzzo *et al.* 2000; Spano and Massa 2006; Alexandre *et al.* 2008), (ii) physiological and biochemical properties (e.g. high malolactic activity, production of volatile compounds, growth rate, interaction with yeasts responsible for AF) and (iii) technological (e.g. resistance to freeze-drying) properties (Alexandre *et al.* 2008). Moreover, microbial starters in general, should be unable to produce undesirable compounds such as biogenic amines (BA) (Fiocco *et al.*

2007). Usually, oenological proprieties of *O. oeni* are strain-specific and this feature might influence differently the aromatic compounds of wine (Ugliano and Moio 2005; Boido *et al.* 2009).

In this article, we report a genetic and laboratory technological characterization of indigenous *O. oeni* strains isolated from two red wines derived from 'Nero di Troia' grapes, an autochthonous Apulian grape variety.

Materials and methods

Sampling and isolation

Oenococcus oeni strains were isolated from Nero di Troia red wine (vintage 2007) undergoing spontaneous MLF during harvesting in 2007 and collected from two different vinifications. The first vinification (vinification VA) showed an alcoholic content of 13·9% (v/v), pH 3·87, and residual sugar of 1·43 g l^{-1}, whereas the second (vinification VB) was characterized by an alcoholic content of 12·5% (v/v), pH 3·96, and residual sugar of 2·02 g l^{-1}.

Samples were taken according to a fixed time schedule, at the end of AF (time 0) and after 7, 14, 21, 35, 42 days. Wine samples were diluted with sterile physiological solution (NaCl 8·5 g l^{-1}) and plated either onto MRS (pH 5·5) (De Man *et al.* 1960) or onto FT80 (pH 5·3) (Cavin *et al.* 1989), modified by the addition of meat extract instead of casamino acids. Plates, containing 100 mg l^{-1} cycloheximide (Sigma, USA) to prevent the growth of yeasts and other fungi, were incubated anaerobically at 30°C. Isolates were identified as putative LAB by positive Gram staining and negative catalase assay. All strains were stored at −80°C in MRS supplemented with glycerol (20% v/v).

Species-specific PCR

All the oligonucleotides used in this study are listed in Table 1. Genomic DNA of *O. oeni* strains was isolated using the Microbial DNA extraction kit (Cabru, Milan, Italy) according to manufacturer's procedure. Then, 20 ng of DNA was added to a 50-μl PCR mixture and amplified with the GoTaq® (Promega, Milan, Italy). The primer pairs Oo_smISRf/Oo_smISRr and On1/On2 were used for the identification of *O. oeni* using the GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Monza, Italy). Different reaction conditions were adapted when the Oo_smISRf/Oo_smISRr, (94°C for 30 s, 52°C for 30 s, 72°C for 20 s for 35 cycles) or the On1/On2, (94°C for 45 s, 64°C for 2 min, 72°C for 2 min for 30 cycles) primer pairs were used. PCR reactions were terminated by performing a final elongation step (72°C for 5 min) and then the amplicons were analysed by electrophoresis. A 1-kb ladder (Promega) was used as molecular weight marker.

Multiplex RAPD-PCR analysis

A multiplex RAPD-PCR analysis was performed as described by Reguant and Bordons (2003), with 0·5 μmol l^{-1} of primers Coc and On2, respectively. The 1-kb ladder (Promega) was used as molecular weight marker. After gel image acquisition, the amplification patterns were converted, normalized and further analysed with the Quantity One 4.1 software (Bio-Rad Laboratories, Hercules, CA, USA).

PCR-based method for the detection of genes involved in biogenic amines production in wine

To verify whether the *O. oeni* strains analysed in this study harbour genes involved in the production of BA

Table 1 List of primers used in this study

Name	Sequence (5′ → 3′)	Reference
Oo_smISRf	GAC TAG TAC TAA TAG GTC GA	Hirschhäuser *et al.* 2005
Oo_smISRr	TCA TCG GAA TTA ACA CGA CA	Hirschhäuser *et al.* 2005
Coc	AGC AGC GTG G	Cocconcelli *et al.*1995
On1	TAA TGT GGT TCT TGA GGA GAA AAT	Zapparoli *et al.* 1998
On2	ATC ATC GTC AAA CAA GAG GCC TT	Zapparoli *et al.* 1998
hsp18F	CGG TAT CAG GAG TTT TGA GTT C	Coucheney *et al.* 2005
hsp18R	CGT AGT AAC TGC GGG AGT AAT TC	Coucheney *et al.* 2005
Ldh1	GCC GCA GTA AAG AAC TTG ATG	Coucheney *et al.* 2005
Ldh2	TGC CGA CAA CAC CAA CTG TTT	Coucheney *et al.* 2005
TD2	ACA TAG TCA ACC ATR TTG AA	Coton *et al.*, 2004
TD5	CAA ATG GAA GAA GAA GTA GG	Coton *et al.*, 2004
HDC3	GAT GGT ATT GTT TCK TAT GA	Coton and Coton, 2005
HDC4	CAA ACA CCA GCA TCT TC	Coton and Coton, 2005
3	GTNTTYAAYGCNGAYAARACNTAYTTYGT	Marcobal *et al.*, 2004
16	TACRCARAATACTCCNGGNGGRTANGG	Marcobal *et al.*, 2004

such as tyramine, histamine and putrescine, a PCR-based assay was carried out to detect the presence of tyrosine decarboxylase (*tdc*; Coton *et al.* 2004), histidine decarboxylase (*hdc*; Coton and Coton 2005) and ornithine decarboxylase (*odc*; Marcobal *et al.* 2004) genes. About 100 ng of genomic DNA was added to a 50-μl PCR mixture containing 1·25 U of Taq polymerase (Qiagen, Milan, Italy), 0·2 mmol l^{-1} of each dNTP, 10 mmol l^{-1} Tris–HCl pH 8·3, 50 mmol l^{-1} KCl, 1·5 mmol l^{-1} $MgCl_2$ and 0·4 μmol^{-1} of each primer.

The reaction mix was cycled through the following temperature profile: 94°C for 5 min; 15 cycles of 94°C for 1 min, 55°C for 1·3 min, 72°C for 1 min; 12 cycles of 94°C for 1 min, 58°C for 1·3 min, 72°C for 1 min; 72°C for 5 min.

Evaluation of sulfite tolerance

Bacteria were grown in the presence of sulfite (15 mg l^{-1}) in FT80 (pH 3·5) (Guzzo *et al.* 2000) and then incubated in the same *medium* added with 30 mg l^{-1} sulfite. Bacterial survival rate was monitored during 132 h (0, 12, 24, 60, 132 h) by counting cells (CFU ml^{-1}) spread on agar plates of FT80 agar medium, pH 5·3 and incubated at 30°C for 5 days.

Synthetic must, wine-like medium and malolactic fermentation

The composition of the synthetic must and wine-making conditions were based on the protocol described by Beltramo *et al.* (2006). Two different synthetic musts were formulated to achieve two different ethanol concentrations, i.e. 11 and 13% (v/v). The synthetic musts contained glucose (88 g l^{-1}), fructose (99 g l^{-1}), DL-malic acid (10 g l^{-1}), L-tartaric acid (2 g l^{-1}), ammonium chloride (0·2 g l^{-1}) and yeast carbon base (11·7 g l^{-1}); pH was adjusted to 3·5 with NaOH. The wine was clarified and sterilized by filtration through a 0·22-μm-pore size filter (Millipore, Molsheim, France). Must sugar content was increased (105 g l^{-1} of glucose and 118 g l^{-1} of fructose) to obtain the desired 13% ethanol content in corresponding wine-like *medium*. The must was inoculated at the concentration of 10^6 CFU ml^{-1} with a 24-h-old *Saccharomyces cerevisiae* strain Lalvin Bourgoblanc CY3079 culture, and AF was performed at 22°C for 22–30 days. Then, the wine was clarified by filtration through a 0·22-μm-pore size filter (Millipore, France) to remove yeasts. After filtration, the physico-chemical properties of the two wine-like media were as follows: (i) 11% (v/v) ethanol, 4·6 g of L-malic acid per litre, pH 3·5 and (ii) 13% (v/v) ethanol, 4·6 g of L-malic acid per litre, pH 3·5. Wine was stored at 4°C until required for further experiments. MLF was initiated by direct inoculation with *O. oeni* strains grown at pH 3·5 for 16 h ($OD_{600\ nm}$ = 0·6) to a final concentration of 2×10^6 CFU ml^{-1} (1 unit $OD_{600\ nm} = 5 \times 10^8$ CFU ml^{-1}) in 50 ml of wine. A control without inoculation was performed to verify any spontaneous MLF. Thereafter, the containers were incubated at 18°C.

Bacterial numeration and consumption of l-malic acid in wine

Bacterial numeration was performed by counting cells (CFU ml^{-1}) spread on agar plates of FT80 agar medium, pH 5·3 and incubated at 30°C for 5 days. The concentration of L-malic acid was determined with the Boehringer enzymatic kit (Mannheim, Germany) according to the manufacturer.

Real-time RT- PCR experiments

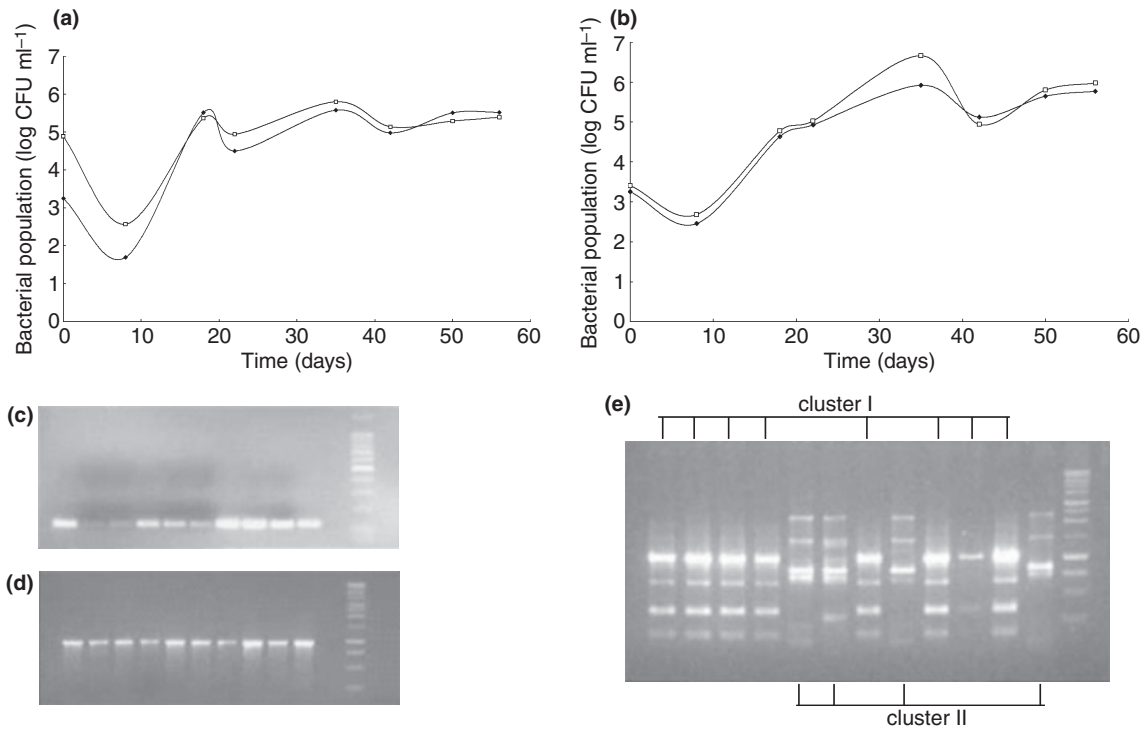
The molecular-based selection of *O. oeni* strains to produce malolactic starters was performed following the work of Coucheney *et al.* (2005). Total RNA was extracted, as described by Jobin *et al.* (1997), under stationary phase in FT80 at pH 5·3, and cDNAs were synthesized using 0·5 μg of RNA template using the Quantitect Reverse Trascription kit (Qiagen). The constitutive *ldhD* gene, chosen as an internal control for these experiments (Desroche *et al.* 2005), was amplified with primers Ldh1 and Ldh2. The *hsp18*-specific cDNA amplification was carried out by real-time PCR with 100 nmol l^{-1} of hsp18F and hsp18R primers using the Power SYBR Green PCR Master Mix in the Applied Biosystems 7300 PCR System (Applied Biosystems, Foster City CA). Real-time PCRs were performed in triplicate for each cDNA sample. For each measurement, a threshold cycle (CT) value was determined. The results were calculated by the comparative critical threshold ($\Delta\Delta$CT) method, in which the amount of target RNA is adjusted to a reference relative to an internal-calibrated target RNA.

The significance of the differences was determined by a two-tailed Student *t*-test. The confidence interval for a difference in the means was set at 95% ($P \leq 0{\cdot}05$) for all comparisons.

Results

Molecular characterization of *Oenococcus oeni* strains

Bacterial populations from two different vinifications of Nero di Troia musts were monitored by plating wine either onto MRS (pH 5·5) or onto FT80 (pH 5·3) plates. In both vinifications, indigenous LAB consortium carried



(f)

Strain	Vinification	Sampling time (days)	Amplification RAPD profile
TA5F8	a)	22	Cluster I
TA1M10	a)	0	Cluster I
TA3F8	a)	7	Cluster II
MA4F31	b)	22	Cluster II
MA1M77F	b)	0	Cluster II
MA3M10-25	b)	7	Cluster I

Figure 1 (a,b) Lactic acid bacteria counts on de Man Rogosa Sharpe agar (□) and on FT80 agar (♦) of samples from Nero di Troia grapewines vinifications. Samples were taken at the end of alcoholic fermentation (time 0) and after 7, 14, 21, 35, 42 days. The data presented are mean values from two separate counts. (c, d) Amplifications obtained with selective primers Oo_smISRf and Oo_smISRr and On1 and On2. (e) Example of randomly amplified polymorphic DNA-PCR profiles (using primers Coc + On2) using genomic DNA from isolates of *Oenococcus oeni* strains. The two main clusters profile are reported. (f) Vinifications and sampling times of the selected strains.

out a spontaneous MLF, which was considered concluded after 3 weeks starting from the very end of the AF. This event was confirmed by the consumption of malic acid and by a classical increase of 0·2 units in wine pH. Figures 1a and 1b shows the dynamics of LAB populations. In both vinifications, a slight growth decrease in LAB was recorded after a week, then followed by a rapid increase in LAB concentration (up to 10^5–10^6 CFU ml^{-1}). A total of 320 strains were randomly isolated from the six sampling points. Gram-positive, catalase-negative and coccoid-shaped strains were stored for further analysis.

For rapid identification of *O. oeni* strains, isolates were screened with selective primers, Oo_smISRf and Oo_smISRr, based on the *O. oeni*-specific ITS-2 region, and selective primers, On1 and On2, based on a region of *O. oeni* gene encoding malolactic enzyme (Table 1). Primers amplified regions of 125 bp (Fig. 1c) and 1025 bp (Fig. 1d), respectively. The selected strains were TA1M10 (vinification VA, time 0, cluster I), TA3F8 (vinification VA, 7 days, cluster II), TA5F8 (vinification VA, 22 days, cluster I), MA1M77F (vinification VB, time 0, cluster II), MA3M10-25 (vinification VB, 7 days, cluster I) and MA4F31 (vinification VB, 22 days, cluster II) (see Fig. 1e,f). The criterion was to select the strains to obtain a probable heterogeneity. The presence of key genes involved in BA production in the isolated *O. oeni* strains was also ascertained and none of them demonstrated to carry any genes involved in the BA biosynthetic pathway.

Characterization of the physiological state of selected *Oenococcus oeni* strains

Initially, we determine the levels of a specific biomarker that has been reported to be correlated with the MLF

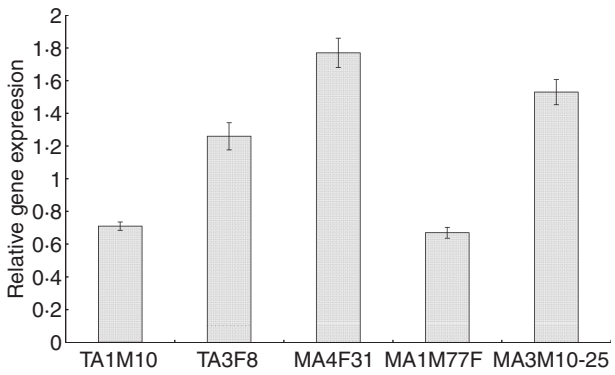


Figure 2 Relative level of the *hsp18* gene expression determined by quantitative real-time RT-PCR. The relative levels of expression were calculated with the comparative critical threshold (ΔΔCT) method by normalizing to the expression of *hsp18* in the strain TA5F8. The *ldh*D gene was used as internal control.

performances of *O. oeni* strains (Coucheney *et al.* 2005). Using a quantitative real-time PCR approach, we have monitored the relative gene expression of the *hsp18* gene, which codes for the small heat shock protein Lo18 to compare the physiological state of the strains analysed (Fig. 2). Lo18 synthesis in *O. oeni* is induced by several stresses such as ethanol, low pH and sulfite usually characteristic of wine conditions and stationary growth phase and heat shock (Guzzo *et al.* 1998). Experiment was performed in stationary growth phase that represents one of the conditions of gene expression induction. Differences were observed between strains, with strains MA4F31 and MA3M10-25 showing the highest relative gene expression (1·8 and 1·5 respectively) (see Fig. 2). All the relative genes expression were normalised to that of strain TA5F8 that present a mean cycle threshold in comparison to those of other strains.

Furthermore, *O. oeni* strains were investigated for their tolerance to sulfite by culturing the *O. oeni* strains in FT80 medium supplemented with sodium metabisulfite (30 mg l^{-1}). Strains TA5F8 and MA1M77F showed the lowest tolerance to sulfite, while the remanent strains were observed to be much more tolerant (data not shown).

Evaluation of malolactic performances in wine-like media

Two synthetic musts containing different concentration of sugar (see Materials and method) were subjected to fermentation by a commercial oenological yeast (see Materials and method) to achieve two different contents of ethanol. In two independent experiments, MLF was conducted with *O. oeni* cells previously adapted to pH 3·5, and this procedure was performed either for the selected strains analysed or for the wine-like media used. The results obtained by the quantification of LAB biomass and L-malic acid degradation are reported in Figs 3 and 4. The sampling times were 0, 2, 7 and 21 days after strain inoculation. Neither degradation of L-malic acid nor spontaneous growth was observed in the control wine. Most of the strains were unable to grow in a wine-like medium containing 13% v/v of ethanol, except the strains MA4F31 and MA3M10-25, which showed a slight increase in cell viability. In a wine-like medium containing 11% v/v of ethanol, we observed a constant increase in LAB biomass concentration up to the 7th day post inoculation. In this case, the growth level and the L-malic acid consumption rates showed to be strain dependent (Figs 4 and 5). Strain TA1M10 and MA1M77F showed a still high concentration of L-malic acid until the 21st day, in both wine-like *media*, whereas strain TA5F8 was able to powerfully degrade L-malic acid in synthetic wine containing 11% v/v of ethanol, but not in that containing ethanol at 13% concentration. Strains TA3F8, MA4F31 and MA3M10-25 efficiently conducted MLF in both wine-like media. Moreover, after a week, only strains MA4F31 and MA3M10-25 showed a possible satisfactory degradation of L-malic acid.

Discussion

The aim of this study was to apply a polyphasic approach to select indigenous starters suitable for MLF in wines produced in Apulia region. Six putative *O. oeni* strains were randomly selected, considering differences in sampling time and RAPD-PCR profile. To evaluate the ability of *O. oeni* strains to perform MLF, the level of expression of stress gene marker was estimated together with the sulfite tolerance. Stress genes are molecular markers for the fitness of starter cultures and could be used as positive indicators for a culture that is fully adapted to resist an upcoming stress condition (Beltramo *et al.* 2006). Recently, a good correlation between the expression level of the *hsp* 18 gene encoding the small heat shock protein (Lo18) of *O. oeni* and its ability to grow and perform MLF has been observed (Coucheney *et al.* 2005). The authors proposed the *hsp18* gene of *O. oeni* as molecular markers to select good MLF starters. In this work, the expression level of the *hsp18* gene of the selected *O. oeni* strains was also evaluated using a quantitative PCR approach. The highest expression level of the *hsp18* gene was observed in strains MA4F31 and MA3M10-25, the strains presenting the best MLF performances. Therefore, we confirm that *hsp18* is a useful tool to evaluate the ability of *O. oeni* strains to survive in wine after direct inoculation and to perform MLF. The results

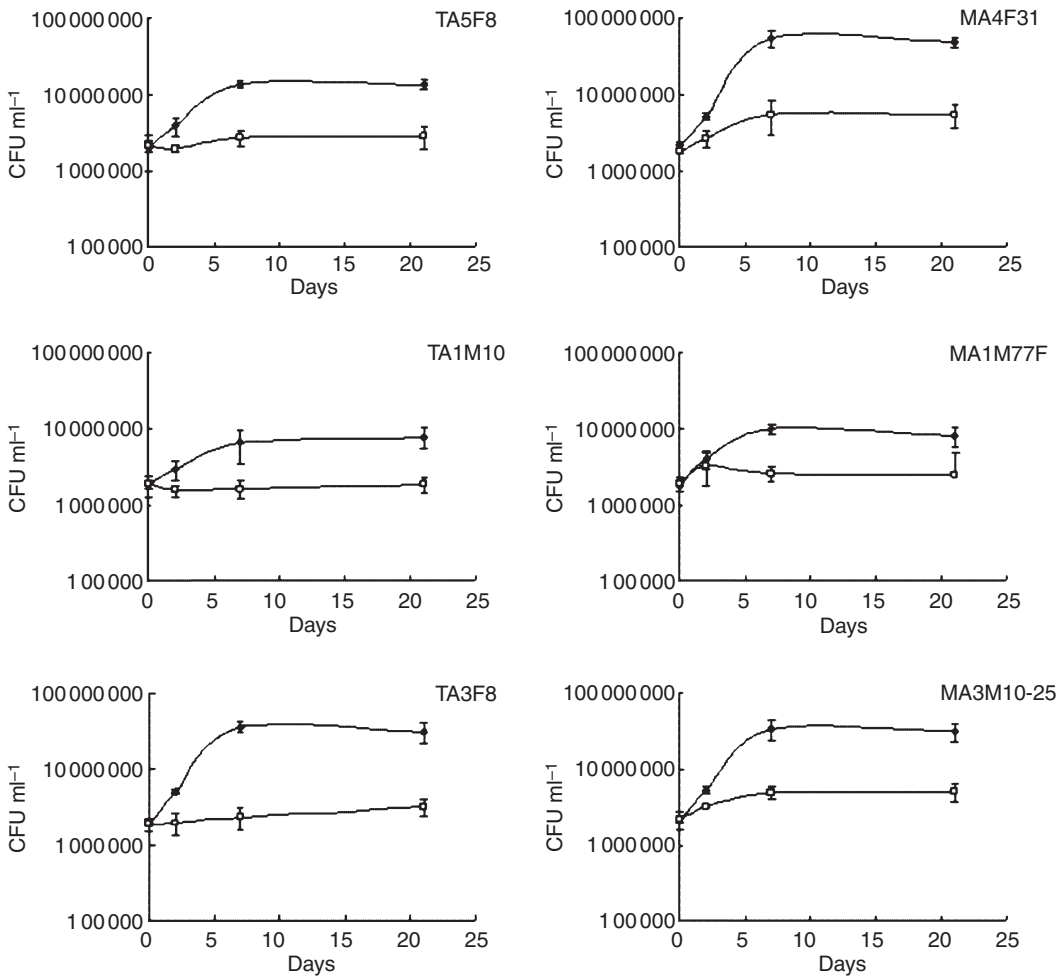


Figure 3 Evolution of the bacterial population during malolactic fermentation, in wine-like *media* with 11% (♦) and 13% v/v (□) of ethanol, carried out by the six selected *Oenococcus oeni* strains.

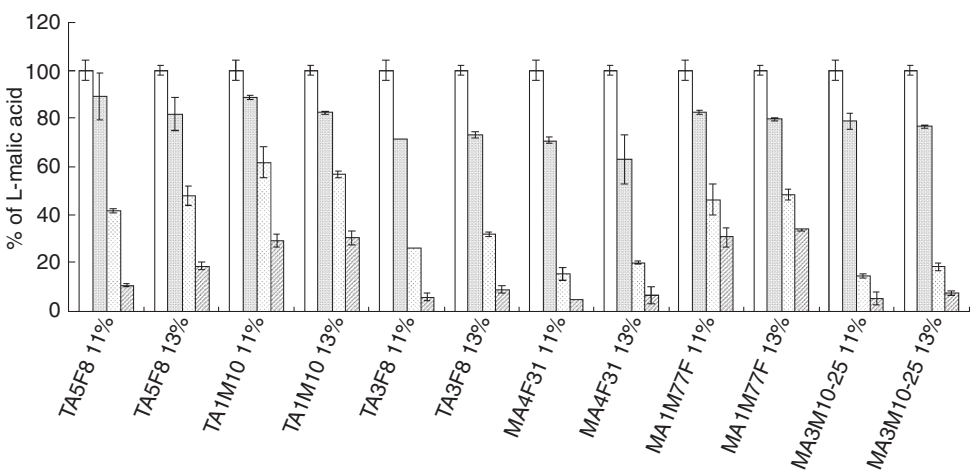


Figure 4 Evolution of L-malic acid content during malolactic fermentation in wine-like *medium* carried out by the six selected *Oenococcus oeni* strains. Percentages of L-malic acid after 0 (□), 2 (▨), 7 (▨) and 21 (▨) days are reported.

obtained were correlated with the capacity to conduct MLF in microvinification experiments.

In this article, all the strains were analysed using two different ethanol concentrations, either for a classical experimental trial (lower concentration) or to mime alcoholic content of typical south Italian red wines (higher concentration). Strains TA1M10 and MA1M77F do not represent an economic and valid alternative to spontaneous MLF, because strains TA1M10 and MA1M77F are able to perform the MLF like spontaneous MLF. In addition, strain MA1M77F was sulfite sensitive, exhibiting poor technological properties. Strain TA5F8 was able to carry out MLF in a wine-like medium with 11% (v/v) ethanol, but failed to grow in a media containing a 13% (v/v) of ethanol and to perform MLF efficiently. Yet, this strain was unable to tolerate sulfite. Strain TA3F8 performed MLF efficiently in wine-like media containing two different ethanol concentrations. However, after 1 week, malic acid degradation was inadequate to be considered concluded, and therefore, this strain could be judged economically unsatisfactory. In contrast, strains MA4F31 and MA3M10-25 showed a profitable performance, with a malic acid consumption probably efficient also after 1 week. These strains will be subjected to further analysis, for a final winery technological characterization.

To our knowledge, this is the first report that focuses on both genotypic and technological characterization of *O. oeni* strains in Apulia region, the second south Italian region considering protected designation of origin wines production. Moreover, our results confirm the ability of molecular stress markers as suitable tools to positively correlate the stress tolerance and the malolactic activity performance in selected *O. oeni* strains.

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