MOLECULAR AND TECHNOLOGICAL CHARACTERIZATION OF INDIGENOUS *OENOCOCCUS OENI* STRAINS IN NEGRO AMARO, TYPICAL SALENTO WINE (APULIA, ITALY), PRODUCED IN TWO DIFFERENT AREAS.

L. FANTASTICO, S. DE DOMENICO and M. S. CAPPELLO
Institute of Science of Food Production, CNR, Prov.le Lecce-Monteroni, 73100 Lecce, Italy
Corresponding author e-mail: maristella.cappello@ispa.cnr.it Tel +39 (0)832 422607- Fax +39 (0)832 422620

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INTRODUCTION

Malolactic fermentation (MLF) is a fundamental biochemical reaction occurring in wine at the end of alcoholic fermentation. During this spontaneous process, bacteria convert malic acid into lactic acid and carbon dioxide (Ribéreau-Gayon et al., 2006) contributing to the organoleptic quality of wines through the production of flavour compounds (Lonvaud-Funel, 1999).

MLF is induced by the indigenous flora or could be started artificially through injection of starter cultures. The last process is a commonly practice used in the cellar fermentations and consists in the injection of bacterial strains selected to ensure the quality of the product. Various bacterial genera have been reported to be able to conclude the MLF and, among them, *Oenococcus oeni* is the most important species involved.

Many strains of this species are well adapted to survive and grow in wine (Bartowsky, 2005). The spontaneous development of *O. oeni* strains in wine is related to their different ability to use such energy sources as sugars, L-malate and amino acids. (Ribéreau-Gayon et al., 2006).

Molecular tools were widely used to study microbial diversity of grapes and wine and they have proved to be particularly useful in identifying strains within species that are homogeneous at genome level, such as for *O. oeni* species. Genotyping diversity among strains of this species was performed using DNA fingerprinting based on Restriction analysis of genomic DNA, as Restriction Endonucleases Analysis-Pulsed-Field Gel Electrophoresis, Polymerase Chain Reaction-based methods, as Random Amplified Polymorphic DNA, *16S-23S rDNA* intergenic spacer region analysis-PCR (Kelly et al., 1993; Viti et al., 1996; Zavaleta et al., 1997; Sato et al., 2000; Zapparoli et al., 2000; Bartowsky et al., 2003). It has been demonstrated that genotyping, besides having a taxonomic value, has practice relevance for the individuation of strains of interest for biotechnological processes (Rodas et al., 2005). Several strains of *O. oeni* were identified by distinct genomic fingerprinting according to the geographical origin (Zapparoli et al., 2000, Guerrini et al., 2003).

In addition, a high genotypic heterogeneity of wild *O. oeni* strains, isolated in wines undergoing spontaneous MLF, was demonstrated (Lòpez et al., 2007). Strains discriminated by molecular tools can be characterized by biochemical and technological assays in order to verify their aptitude to represent a possible fermentation starter (Coucheney et al., 2005). Thus, the availability of a reliable tool able to explore genetic source represented by malolactic microflora harbouring in grapes and wines, would be recommended.

In this study was to use Amplified Fragment Length Polymorphism (AFLP) analysis as tool for molecular characterization of *O. oeni* population, with the perspectives to select strains for using as a MLF starter in wine. This technique which has proved to be the most useful tool so far for

discriminating *O. oeni* among strains of bacteria, *Lactobacillus* (LAB) included (Gancheva et al., 1999; Rico et al., 2004; Hong et al., 2005; Cappello et al. 2008; Cappello et al. 2010).

Moreover, to select bacterial strains, for specific winemaking conditions, several criteria you need to consider; these include resistance to ethanol, SO_2, growth at low pH, assessment the malate metabolisms and the absence of negative traits to the health of consumers (biogenic amines [BA], ethyl carbamate) Guzzoni R. et al. (2009). In the present work, these selection criteria were applied to 14 strains, chosen from different subgroups of the cluster obtained by AFLP carried out on isolates from wines produced by grapes of two different Salento areas (Apulia, Italy): "*Negro amaro"* (Salice Salentino area) (6 strains) and "*Negro amaro"* (Guagnano area) (8 strains), undergoing MLF wine. The aim of this study was to select some of them which could have a good fermentation performance and could be used as a malolactic fermentation starter for the enhancement and improvement of the wines from Puglia.

MATERIAL AND METHODS

Bacteria DNA extraction

Lactic acid bacteria cells at the exponential growth phase were harvested by centrifugation and suspended in 1ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) solution containing 10mg/ml of lysozyme (Sigma, Germany). Cells were then lysed by adding 100µl of 10% SDS (w/v) and 10µl of proteinase K (20 mg/ml) (Sigma). Crude DNA preparation was purified by performing two phenol/chloroform/isoamyl alcohol (25:24:1) and one chloroform/isoamyl alcohol (25:1) extractions. Genomic DNA was precipitated by adding two volume of cold ethanol. The precipitated DNA was washed with 70% ethanol and left to air dry. The DNA pellet was dissolved in an appropriate volume of TE buffer. Concentration of genomic DNA was spectrophotometrically quantified using the Nanodrop ND-1000 Apparatus (Celbio, Italy).

Fluorescent AFLP analysis

The AFLP fingerprinting (Vos et al., 1995) was performed using the AFLP Microbial Fingerprinting Kit (Applied Biosystems-PE Corporation, Foster City, California U.S.A) according to the manufacturer's instructions. Six separate primer combinations were used for the selective amplification: E1-M1 Fam (*EcoRI*CA - *MseI*AA); E2-M1 Fam (*EcoRI*CC - *MseI*CA); E3-M3 Fam (*EcoRI*CG - *MseI*AG); E1-M2 Joe (*EcoRI*CA – *MseI*AC); E2-M3 Joe (*EcoRI*CC – *MseI*AG); E2-M3 Joe (*EcoRI*CG - *MseI*AC). The Eco*RI* primers were labelled with fluorescent dye (Applied Biosystems-PE Corporation). The PCR cycling condition for these reactions was done according to the AFLP microbial fingerprinting protocol by using a model PCR Express CELBIO Italy. Gene Mapper version 4.0 software (PE Applied Biosystems) was used to automatically size and quantify individual fragments by using the internal lane standard. For clustering, fragments between 50 and 500 bp were analysed with NTSYS software by using the Dice similarity coefficient based on presence/absence of the bands and clustered by the un weighed pair group method with arithmetic Mean (UPGMA).

Malate metabolism assays

To determine the malic acid consumption rate, strains were cultured in MRS–tj, to which were added 2.5 g/l L-malic acid, monitoring cell growth by a turbimetric assay. Concentration of L-malic acid was determined enzymatically using Boehringer kit L-Malic acid (Mannheim, Germany). Cultures were sampled and, after removing bacterial cells by centrifugation, malic acid depletion was enzymatically monitored.

Malolactic activity (MLA) was determined in whole *O. oeni* cells according to (Zapparoli et al. 2004). MLA was expressed as μmol L-malic acid decarboxylated per hour and per mg dry weight. One unit $O.D._{600}$ was equivalent to 0.4 g dry weight (dry litre)$^{-1}$. (Cavin et al., 1998)
Malate metabolism assays were carried out at least in triplicate and values were analysed statistically by Tukey's Multiple Comparison Test. Mean values were considered significantly different when $p < 0.05$.

Histamine analysis

This analysis was carried out by ELISA - enzyme immunoassay (R-Biopharm Kit), according to AFLP clusters. The concentration of the substance under investigation (Histamine), was measured by spectrophotometer, which assessed the amount detected by the intensity of color.

Statistical treatment of data

Assays of enzymatic activity were carried out at least in triplicate and values were analysed statistically by Tukey's multiple comparison test. Mean values were considered significantly different when $p < 0.05$. Cluster analysis of strains was carried out using data of enzymatic activities, growth parameters and metabolite consumption rates by paired group algorithm and Euclidean similarity measure.

RESULTS

A total of 75 *O. oeni* strains out of 210 lactic acid bacteria isolates from *Negro amaro* wine (Salice Salentino area) undergoing MLF and a indigenous population of *O. oeni,* (80 from 220 lactic acid bacteria isolates) responsible of spontaneous MLF in *Negro amaro* wine, (Guagnano area) were analyzed by AFLP technique. The data obtained were statistically analyzed by UPGMA cluster analysis using the Dice similarity coefficient. The dendrogram was produced, showing the genetic distance among each single genomic fingerprinting.

The results showed that, in *Negro amaro* (Salice Salentino area) isolates, three main clusters were distinguished (A, B and C). Moreover, the homogeneous clusters A showed intraspecific homology of 73% (Fig. 1); two biotypes (named a1 and a2) comprising 21 and 12 isolates respectively were identified. Also the group B (35 isolates), clustered with an AFLP similarity of 73%; these isolates formed three different subgroups named b1, b2 and b3 and the type strain clustered together with the subgroup b1.

The cluster C showed the lowest AFLP similarity among the 7 isolates grouped (56%). These isolates formed a single AFLP subgroup.

Fig.1. *Genotyping analysis performed by molecular assay based of the AFLP technique* ***NA*** *represent Negro amaro grapes must of (Salice Salentino area) of Apulia wine.*

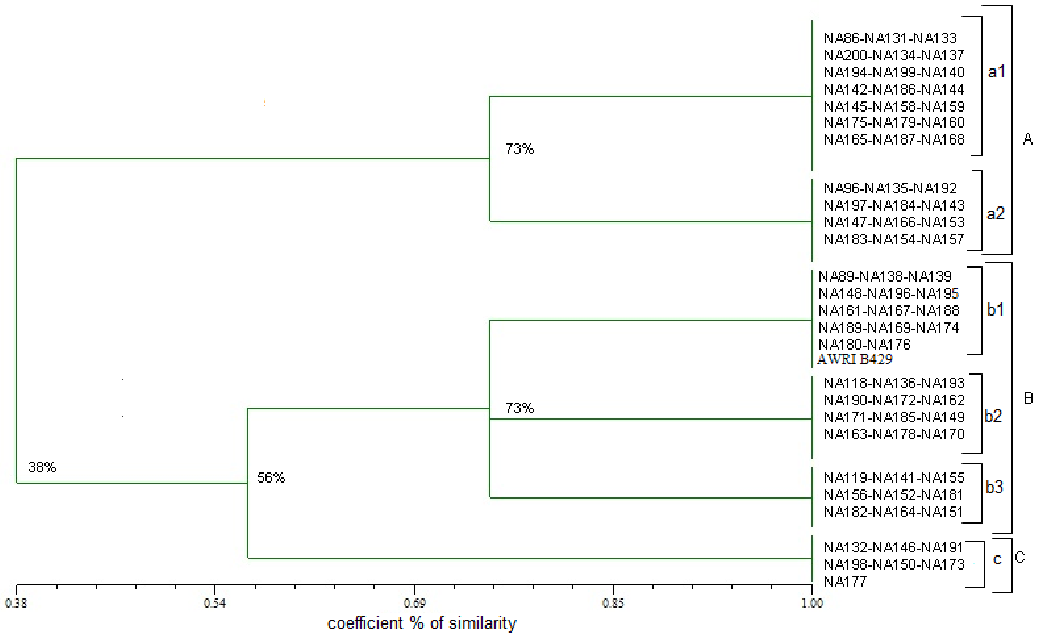
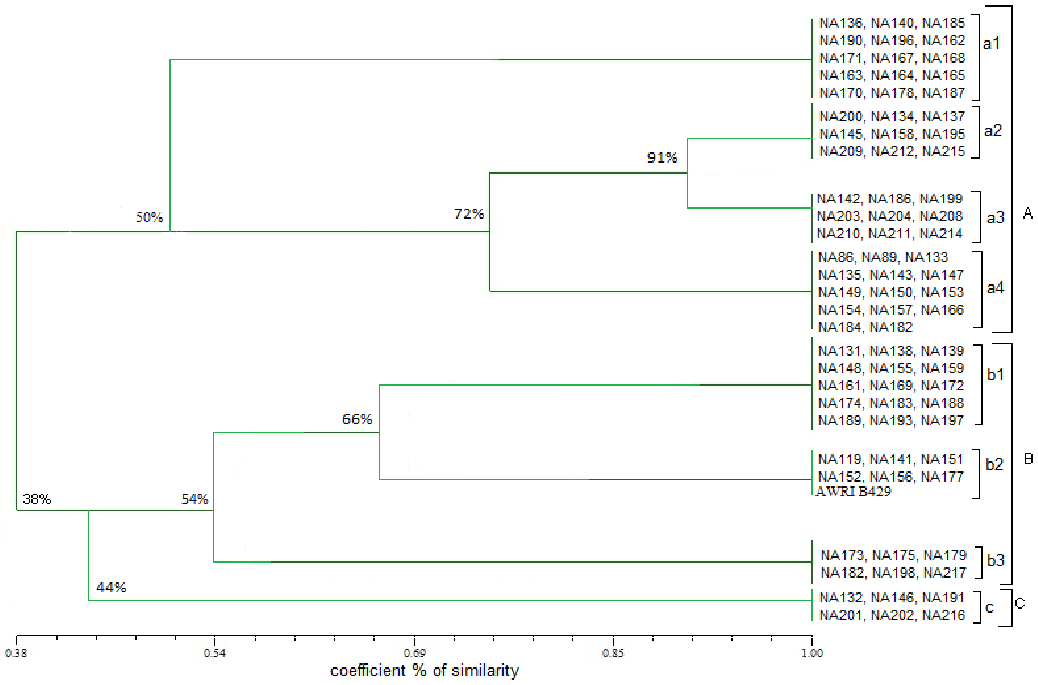


Fig.2. *Genotyping analysis performed by molecular assay based of the AFLP technique* ***NA*** *represent Negro amaro grapes must of (Guagnano area) of Apulia wine.*



The data, obtained by means of the AFLP assay of 80 O. oeni isolates, in *Negro amaro* (Guagnano area) showed three different principal clusters (A, B, and C) (Fig. 2). The cluster A displayed a total of four subgroups, named a1, a2, a3, a4, with an AFLP similarity, from 50% to 91%, and comprise 47 isolates. The subgroups a2 and a3 clustering with highest similarity (91%).

In the group B (27 isolates) was comprised three subgroups named b1, b2 and b3; b1 and b2 clustered with an AFLP similarity of 66% while b3 of 54%. The reference strain clustered in the biotype b2.
Cluster C, consisting of 6 isolates (with 100% similarity), showed the lowest AFLP similarity (44%) among the O. oeni population.

The isolates will be deposited at the bank of microorganisms ITEM ISPA, Bari, relevant bank for the collection of Agro-food cultures of toxigenic fungi, yeasts and malolactic bacteria, (http://www . ispa.cnr.it / Collection).

According to the results of AFLP analysis, an isolate was selected randomly to represent each subgroup for technological characterization. The strains were evaluated about the resistance to ethanol, SO_2, growth at low pH and malate metabolism.

All the strains were not able to grow and degrade the malic acid at a pH equal to 3; considerable activity was observed, however, a more alkaline pH such as 4.5. Moreover, all the selected strains of O. oeni were able to grow on solid media and to tolerate different concentrations of SO_2 (50, 80 and 150 mg / ml), demonstrating a remarkable capacity to adapt to the presence of sulfur dioxide.

The strains selected by AFLP method were assayed for malate metabolisms. The utilization of this acid was widely demonstrated as being beneficial for bacterial survival and growth in wine (Cox and Henich Kling, 1995).

Different capabilities to metabolize the malate in MRS-tj were observed according to the statistical analysis (Table 1 and Table 2). In Table 1 a total of three strains (N1, N3 and N6) consumed L-malate in an amount comparable to that consumed by the Type strain AWRIB429^T used as reference strain, and deposited in the culture collection of the Australian Wine Research Institute, (AWRI) of (Adelaide, Australian).

Lower consumption rates were measured in the remaining strains when compared to that of Type strains. N2 strain was characterized by the lowest rate with a range of 0.21 ± 0.04 mol/g dry weight. Similarly to the quantity of malate consumed per biomass produced, the specific L-malate consumption rate, determined in whole cells in phosphate buffer at pH 3.5, was different among the strains (Table 1). In this case, different rates were recognized.

The N1 strain displayed a specific L-malate comparable to that measured for the Type strain (8.3 mol h^{-1}[mg dry weight] $^{-1}$) consumption rate. A group of strains (N3, N4, N5 and N6) showed a specific L-malate consumption rate, which varying from 7.2 to 7.8 mol h^{-1} [mg dry weight] $^{-1}$. The lowest rate (6.8 ± 1.1 mol h^{-1} [mg dry weight] $^{-1}$) was displayed by the N2 strain. In general, strains that consumed more malic acid in MRS-tj showed a higher specific L-malate consumption rate. Also the *O. oeni* strains, isolates

from *Negro amaro* wine (Guagnano area), showed a different capabilities to metabolize the malate in MRS-tj. In particular the N2 and N5 strains consumed L-malate in an amount comparable to that consumed by the reference type strain (0.26 ± 0.04 mol/g dry weight).

The strains N2 and N5 that consumed more malic acid in MRS-tj showed a higher specific L-malate consumption rate (8.3 ± 1.3 mol h^{-1}[mg dry weight]$^{-1}$ and 8.4 ± 1.4 mol h^{-1}[mg dry weight]$^{-1}$.

Table 1. *Malate Metabolism assessed in Oenococcus oeni strains isolated from Negro amaro wine (Salice Salentino area).*

***O.oeni* strains**	**L-malate consumed per biomass produced mol (g dry weigtht)$^{-1}$**	**Specific L-malate consumption rate µmol h^{-1} (mg dry weigth)$^{-1}$**	**Cluster AFLP**
NA86 (N1)	0.25 ± 0.03 a	8.3 ± 1.5 a	a1
NA96 (N2)	0.21 ± 0.04 ab	6.8 ± 1.1 a	a2
NA148 (N3)	0.24 ± 0.03 a	7.8 ± 2.0 a	b1
NA149 (N4)	0.22 ± 0.03 a	7.2 ± 1.7 a	b2
NA152 (N5)	0.23 ± 0.03 a	7.5 ± 2.0 a	b3
NA177 (N6)	0.24 ± 0.04 a	7.4 ± 1.4 a	C
AWRIB429 ^{T}	0.27 ± 0.04 a	8.5 ± 1.9 a	Type

The values are mean ± standard deviation of three independent experiments; differents letters (a and b) following the values denote significant differences in $p<0.05$.

Table 2. *Malate Metabolism assessed in Oenococcus oeni strains isolated from Negro amaro wine (Guagnano area).*

***O.oeni* strains**	**L-malate consumed per biomass produced mol (g dry weigtht)$^{-1}$**	**Specific L-malate consumption rate µmol h^{-1} (mg dry weigth)$^{-1}$**	**Cluster AFLP**
NA136 (N1)	0.20 ± 0.03ab	5.6 ± 2.2 a	a1
NA200 (N2)	0.26 ± 0.04 a	8.3 ± 1.4 a	a2
NA142 (N3)	0.23 ± 0.03 a	7.9 ± 1.2 a	a3
NA86 (N4)	0.24 ± 0.04 a	8.0 ± 1.2 a	a4
NA131 (N5)	0.26 ± 0.04 a	8.4 ± 1.5 a	b1
NA119 (N6)	0.22 ± 0.03 ab	7.4 ± 1.1 a	b2
NA173 (N7)	0.24 ± 0.02 a	8.1 ± 2.0 a	b3
NA132 (N8)	0.23 ± 0.03 ab	7.2 ± 1.7 a	C
AWRIB429 ^{T} (N9)	0.27 ± 0.03 a	8.6 ± 2.2 a	Type

The values are mean ± standard deviation of three independent experiments; differents letters (a and b) following the values denote significant differences in $p<0.05$.

Finally, the results obtained, on six, *O. oeni* strains isolated from *Negro amaro* (Salice Salentino area) and eight *O. oeni* strains isolated from *Negro amaro"* (Guagnano area), chosen from different subgroups of the cluster obtained by AFLP analysis, have evidenced a mean value of histamina in un range of 1.470 and 2.377 mg/L in the strains examined. Also the content of ethyl carbamate obtained showed a range values low, comprising of 0.20 mg/mL.

CONCLUSION

The present study evidenced the existence of genetic variability characterizing indigenous strains from wines produced by grapes of two different Salento areas (Apulia, Italy): *Negro amaro* (Salice Salentino area) and *Negro amaro* (Guagnano area) undergoing MLF wine. The obtained results prove how complex a natural *O. oeni* population in a single MLF. Each component of this population could be contribute differently to the final aroma depending on its ability to interact with the environment.

By AFLP analysis the main clusters were distinguished and was valuated the homology of them. Data of the L-malic acid consumption linked to the specific L-malate consumption rate, furnished relevant information concerning the fermenting capability of the selected strains.

In order to select bacteria with high malate metabolism performances and genomically different, representative strains for each AFLP cluster were individuated as suitable candidates for further selection assays. By the analysis of genotyping and malate metabolism data good candidates were identified, which could be destined to a further selection program, after evaluating their performance by microvinification experiments.

The content of biogenic amines in the studied wines is negligible. These values are within the full legal limit. Also the content of ethyl carbamate obtained is not significant with a range values low.

This study represents a valid approach to individuate *O. oeni* strains destined for a selection program for malolactic starter. The selected strains could be an interesting ecological source that can be useful for techonological purposes. Apart from its applicative aim this study, also represents a contribution in understanding the population dynamics of *O. oeni* during wine malolactic fermentation.

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ABSTRACT

The purpose of this study was to perform the molecular and technological characterization of indigenous strains of Oenococcus oeni, with excellent winemaking skills, isolated from wines produced by grapes of two different Salento areas (Apulia, Italy), in order to select strains from using as a malolactic fermentation (MLF) starter in wine. Sequences discriminating the different species were obtained by species-specific PCR with primers specific for the MLE gene and subsequent sequence analysis of PCR products on RNA containing the region for the 16S gene. In this way were identified as *O. oeni*, 75 lactic acid bacteria from a total of 210, isolated from *Negro amaro* wine (Salice Salentino area) and 80 from a total of 220 lactic acid bacteria, isolated from *Negro amaro* wine (Guagnano area), during malolactic fermentation. Genotypic analysis was performed by molecular technique (AFLP) based on amplified fragment length polymorphism, in order to develop molecular profiles that differentiate and characterize several *O. oeni* strains.

To design specific bacterial strains for the different characteristics of a wine, it is necessary to consider various criteria as resistance to ethanol, SO_2, growth at low pH, assessment of the malate metabolism and absence of negative traits to the health of consumers (biogenic amines [BA], ethyl carbamate). These selection criteria were applied to several strains (chosen from different subgroups of the cluster obtained by AFLP). The results allowed to select some of these strains that may have a good fermentation performance.