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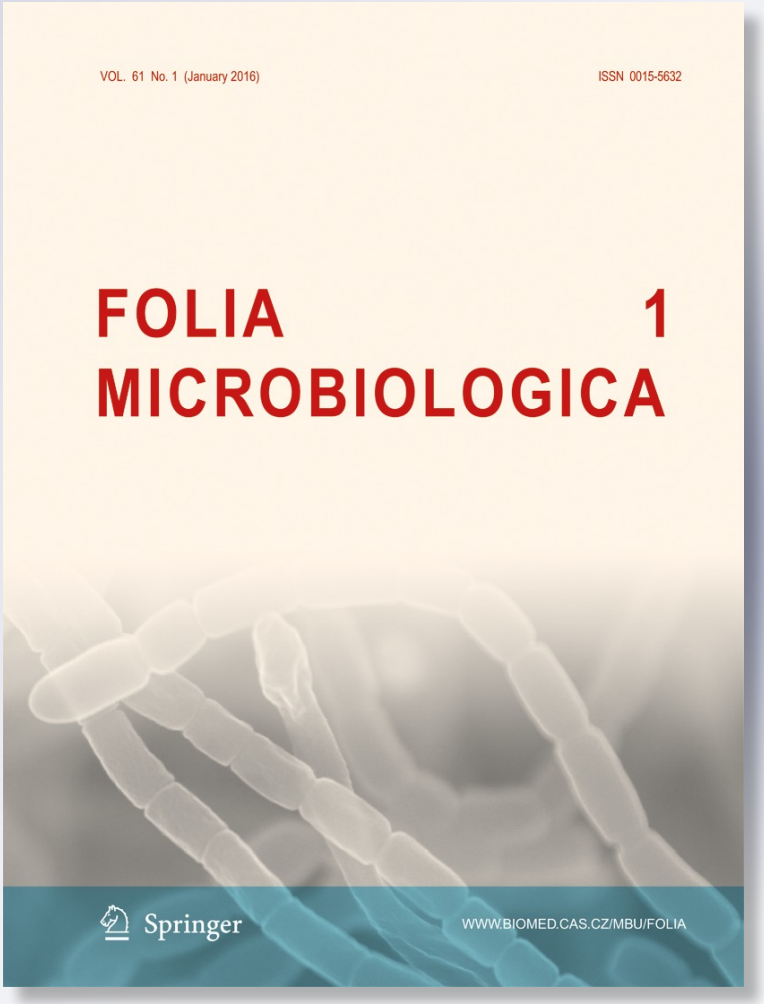

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Assessment of the genetic polymorphism and physiological characterization of indigenous *Oenococcus oeni* strains isolated from Aglianico del Vulture red wine

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Abstract The aim of this study was a reliable intra-species discrimination and strain biodiversity in *Oenococcus oeni* populations of two different Aglianico wineries by molecular, biochemical, and physiological characterization. Pulsed field gel electrophoresis (PFGE) analysis revealed a high polymorphism related to the origin (winery) of strains, while differential display PCR (DD-PCR) allowed a further discrimination of strains from the same winery. Moreover, the heterogeneity of these natural populations was investigated by capillary electrophoresis and enzymatic assays. A variability related to a different surface charge distribution was observed among strains, linked to their origin. Malolactic activity study evidenced strain-specific differences in malic acid degradation, and then, only the presence of L(-)-malic acid in the medium induced the *mle* gene. This study provided evidences on the importance of intra-species biodiversity of malolactic bacterial populations in wine ecosystems, as each wine possess peculiar winemaking conditions and physical–chemical properties which make specific the bacterial survival and growth. This study highlighted a great biodiversity among *O. oeni* strains that can be also winery specific. Such biodiversity within a certain winery and winemaking area is important for selecting malolactic starters, and strain-specific trait identification is especially important to match individual strains to specific industrial process.

Caterina Cafaro and Maria Grazia Bonomo contributed equally to this work.

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Introduction

Malolactic fermentation (MLF) is a natural process performed by lactic acid bacteria (LAB) resulting in deacidification and improvement of taste, flavor, and microbial stability of the wine (Henick-Kling 1995; Maicas et al. 1999). *Oenococcus oeni* is the main species identified during spontaneous MLF because it is well adapted to survive and grow in wine, so a large number of investigations on the role of *O. oeni* have been carried out (Bartowsky 2005; Ruiz et al. 2008; Sico et al. 2009; Bartowsky and Borneman 2011).

The positive contribution of MLF to wine characteristics and the need to achieve better control of the process have led wineries to use starter cultures as common oenological practice (Ruiz et al. 2012) tended to select from indigenous microorganisms, more competitive, well adapted to the particular product and to the specific production technology, preserving their typicity (Bonomo et al. 2008).

The oenological benefits of MLF continue to prompt research for the development of novel *O. oeni* starter cultures, tailored for specific wines (Ruiz et al. 2010; Torriani et al. 2011). Indeed, genetic and phenotypic surveys have revealed considerable strain diversity within natural *O. oeni* populations associated with different types of wine (Cappello et al. 2008; Vigentini et al. 2009; Solieri et al. 2010), and a correlation between such diversity and the peculiarity of certain oenological niches has also been supposed (Larisika et al. 2008; Canas et al. 2009; Vigentini et al. 2009). Recently, Pramateftaki et al. (2012) reported that native *O. oeni* isolates can even be winery specific. Such biodiversity within a certain winery and winemaking area is a valuable source for selecting malolactic starters, and the identification of strain-specific traits is especially important to match individual strains to a specific industrial process (Borneman et al. 2010; Torriani et al. 2011; Zapparoli et al. 2012).

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Previous studies reported that *O. oeni* was able to grow in the hostile environment of wine by several mechanisms of activation (G-Alegrìa et al. 2004; Beltramo et al. 2006). Moreover, in other studies (Carreté et al. 2002; da Silveira et al. 2003; Chu-Ky et al. 2005; Marcobal et al. 2008; Bon et al. 2009; Solieri et al. 2010), *O. oeni* responded to the harsh wine environment by specific cellular features, including genetic adjustments and modifications of surface structure and composition to allow bacterial survival and growth. This proved *O. oeni* as a highly heterogeneous species in terms of MLF performance because the resistance to wine conditions is strictly strain dependent.

"Aglianico del Vulture" is a traditional red wine, produced in the Vulture area of Basilicata region (Southern Italy) and bearing a Controlled Designation of Origin (CDO) in compliance with the legislation (EU 1971). The vineyards are located in the Vulture volcanogenic hilly grounds; the particular tuff composition of soil and the climate conditions give the unique features to the final product.

Owing to the specificity of the winemaking conditions, this wine represents a peculiar ecosystem still poorly investigated. The few studies carried out on the microbial community of Aglianico wine underlined high intra-species heterogeneity in bacterial populations (Sico et al. 2008).

Differentiation of several *O. oeni* strains isolated from Aglianico wine produced in different wineries has been reported (Lechiancole et al. 2006; Sico et al. 2008); however, information about the level and the meaning of the heterogeneity within a single *O. oeni* natural population has not yet been obtained.

To this aim, genomic and expressed DNA analyses were performed to achieve reliable intra-species discrimination and strain biodiversity in *O. oeni* populations of two different Aglianico wineries. Moreover, the heterogeneity of these natural populations was investigated by physiological characterization of the strains through capillary electrophoresis and enzymatic assays.

Materials and methods

Bacterial strains and growth conditions

Thirty-two *O. oeni* strains used in this study were previously isolated from Aglianico wines of two different wineries operating in the Vulture area of the Basilicata region (Southern Italy) and identified by molecular analysis and technological characterization (Sico et al. 2008). All strains (23 of winery D and 9 of winery S) were maintained as freeze-dried stocks in reconstituted (11 % *w*/*v*) skim milk, containing 0.1 % (*w*/*v*) ascorbic acid in the culture collection of the Dipartimento di Scienze, Università degli Studi della Basilicata (Potenza, Italy), and routinely propagated in de Man, Rogosa, and Sharpe (MRS) broth supplemented with 20 % (*v*/*v*) tomato juice (MRS-TJ) and adjusted to pH 4.8 at 30 °C under strict anaerobic conditions for 5–6 days before the analyses.

Strain typing

Pulsed field gel electrophoresis analysis

O. oeni strains were characterized at strain level by pulsed field gel electrophoresis (PFGE) of genomic digests. Genomic DNA of high molecular weight was prepared by the method of Mc Clelland et al. (1987). Inserts of intact DNA were digested in 200 mL of appropriate buffer supplemented with 40 U of restriction enzyme (*Apa* I, *Sfi* I) (Promega Corporation, Madison, USA) for 16 h. The separation of digested DNA and the running conditions were performed as described by Lechiancole et al. (2006).

Differential display PCR analysis

The intra-specific diversity in *O. oeni* strains was evaluated also by the expressed DNA analysis in order to discriminate them further at the strain level. Total RNA was extracted and cDNA was synthesized and used as template for differential display PCR (DD-PCR) amplification as described by Sico et al. (2009).

Two microliters of cDNA was subjected to DD-PCR analysis using two universal primers, M13 (5′-GAGGGTGGCG GTTCT-3′) and OPA9 (5′-GGGTAACGCC-3′). DD-PCR protocol was applied as previously described by Lechiancole et al. (2006).

Device settings of capillary electrophoresis

A series of laboratory tests was performed in order to optimize the work protocol and to standardize the method for our research. As separation in a capillary electrophoresis (CE) system depends on different features of analyte electrophoretic mobility, the change effects of some separation parameters were investigated.

Different buffer pH (7–10) of a selected TBE buffer electrolyte (Tris 4.5 mmol/L, Boric acid 4.5 mmol/L, EDTA 0.1 mmol/L, diluted 1:8) (Ebersole and McCormick 1993) were tested for the sample dilution and the electrophoretic separation. Moreover, two different capillaries were tested: the first one with a total length (L_{tot}) of 31.5 cm and an effective length (L_{eff}) of 21.0 cm, the latter with L_{tot} and L_{eff} of 49.5 and 38.5 cm, respectively. Other CE parameters, such as injection pressure and separation voltage, were also investigated. All the conditions were optimized by holding everything constant while varying only one parameter at a time. The electropherogram detection was performed at 214 nm

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(Armstrong et al. 1999). Data were collected and analyzed with a standard 32 Karat software (Version 4.0).

Capillary electrophoresis analysis

Late-exponential phase cells grown in MRS-TJ pH 4.8 at 30 °C for 5–6 days were harvested by centrifugation (12, 000*g*, 10 min) at 4 °C and washed twice in sterile saline solution (0.85 % *w*/*v* NaCl). Cells were re-suspended in 2 mL of TBE buffer to a final A_{600nm}=1.0. All samples were vortexed for 1–2 min to prevent cell aggregation. Microscopic detection confirmed the absence of aggregates in the samples. All samples were shaken thoroughly before CE injection, and the analysis was performed in triplicate for each one with a Beckman Coulter P/ACE MDQ Capillary Electrophoresis System, equipped with a diode array detector (DAD). All experiments were carried out using an unmodified 100-μm i.d. fused silica capillary. The samples were introduced into the anodic end of the capillary by injection pressure at 1378.951458 Pa for 5 s. The electrophoretic separation was performed at 25 °C applying a constant voltage of 10 kV. Prior to each sample injection, the capillary was washed at 137, 895.1458 Pa for 1.5 min with 0.5 M H_3PO_4, 0.5 min with water, 1.5 min with 0.1 M KOH, 0.5 min with water, and at last, 1 min with running buffer (Armstrong et al. 1999). A mesityl oxide solution 1.6 mmol/L, diluted 1:4 in the running buffer, was used as an electroosmotic flow (EOF) marker.

Malic acid consumption and *mle* gene induction

Late-exponential phase cells grown in MRS-TJ pH 4.8 at 30 °C for 5–6 days were harvested and re-suspended in 50 mL of MRS-TJ medium added with L(-)-malic acid (5 g/L) to a final $A_{600\ nm}$=1.0 (~10^8 cfu/mL) and incubated at 30 °C for 6 days in order to evaluate the malic acid consumption. The amount of L(-)-malic acid was determined enzymatically using reagents and procedure kit (Boehringer Mannheim GmbH, Mannheim, Germany).

The induction of the *mle* gene was also assessed at different L(-)-malic acid concentrations. MRSb-TJ was supplied with L(-)-malic acid at final concentrations of 0.625, 1.25, 2.5, 5, 7.5, and 10 g/L. RNA extraction and cDNA synthesis were performed as described above.

Two microliters of cDNA were used for PCR analyses with primers On1 (5′TAATGTGGTTCTTGAGGAGAAAAT3′) and On2 (5′ATCATCGTCAAACAAGAGGCCTT3′) for amplification of the *mle* gene (1.025 bp) as described by Zapparoli et al. (1998).

Statistical analysis

The banding patterns were visualized by UV transillumination and captured with GelDoc™ XR+ System (Bio-Rad). Gel images were digitized in Diversity Database™ software (Bio-Rad Laboratories Ltd., Watford, Herts, UK) and processed for detection of the bands. Calculation of similarity in the profiles of bands was based on Pearson product–moment correlation coefficients. Dendrograms were obtained by means of the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm (Vauterin and Vauterin 1992). A reproducibility study for each of the techniques was carried out to determine the minimum percentage similarity necessary for strain discrimination. Repeated analysis of the same isolate in different gels was employed, and patterns were analyzed as described above and the level of similarity observed between repeats established a discrimination threshold below which patterns were deemed different.

The enzymatic test was carried out in triplicate for each strain and the values were analyzed statistically by the *t* test. Means of the values were considered significantly different when $P<0.05$. A calibration curve using known concentrations of L(-)-malic 1, 3, and 5 g/L was constructed to assess the amount of malic acid in terms of concentration (expressed in g/L). The equation obtained from the calibration line allowed to extrapolate from the absorbance values the residual concentrations of L(-)-malic acid and so to trace back, by difference, the concentrations of L(-)-malic acid metabolized by each strain.

Reagents and media

Unless otherwise specified, all reagents were obtained from Sigma-Aldrich (Milan, Italy), while bacteriological media and ingredients were obtained from Oxoid (Basingstoke, Hampshire, England). Deionized and ultra-filtered water, obtained by a combined system of Elix-5/Milli-Q (Millipore, S.p.A. Milan), was used throughout the CE experiments and to prepare solutions and running buffer.

Results

Genotyping of *O. oeni* strains

In this study, we used two molecular techniques to assess the intra-specific biodiversity in *O. oeni* populations of two different Aglianico wineries.

PFGE analysis of genomic DNA from thirty-two strains showed that the *Apa* I enzyme yielded the most discriminating restriction fragments producing a total of 8 different patterns consisting of 12–18 bands in the range of 30–600 kb (data not shown). As shown in Table 1, profile 1 was the most frequent, detected in 17 strains, while profiles 3 and 8 were found in 3 and 6 strains, respectively. Two strains showed profiles 7 while the remaining profiles (2, 4, 5, and 6) were represented by only one strain.

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Table 1 PFGE and DD-PCR patterns shown by 32 *O. oeni* strains analyzed in this study

Strain	PFGE	DD-PCR		Cluster DD-PCR (95 % similarity)*
	Apa I	M13	OPA9	
D12	1	A	a	Y4
D10	1	B	b	Y5
D9	1	C	b	Y5
D11	1	D	a	Y4
D14	1	E	a	Y3
D17	1	F	a	Y2
D15	1	F	c	Y1
D16	1	F	a	Y1
D2	1	B	d	X13
D6	1	G	e	X5
D22	1	H	f	X4
D26	1	H	f	X4
D29	2	I	g	X4
D21	1	H	a	X1
D24	1	H	i	X1
D30	1	L	g	X1
D18	1	F	a	X11
D5	3	M	l	X10
D4	3	B	l	X9
D3	3	N	l	X8
D8	1	O	l	X7
D7	4	P	d	X12
D20	5	H	a	X1
S1	6	L	h	X1
S10	7	Q	h	Z2
S11	7	Q	h	Z2
S12	8	R	h	X6
S13	8	L	h	X2
S14	8	L	h	X3
S3	8	I	h	Z1
S5	8	L	h	X1
S8	8	R	m	Z1

*Strain subdivision in clusters on the basis of 95 % of similarity in the combined DD-PCR analysis with both primers

In the case of DNA digests with *Sfi* I, the number of bands decreased to 8–9 and was considered as less discriminatory for strain differentiation (data not shown).

It has been possible to observe that the high polymorphism, caused by *Apa* I digestion, was related to the origin (winery) of strains, although some similarities were detected among all strains, such as the number of fragments, the size of the smallest and the largest fragments, and the band distribution along DNA profile.

Moreover, in order to discriminate further strains of this species, analysis of expressed DNA polymorphism by DD-PCR was also performed. This technique allowed to obtain an additional differentiation of strains isolated from the same winery by using two primers, M13 and OPA9.

DD-PCR analysis of the 32 strains using M13 primer discriminated 16 different patterns; seven major clusters grouping more than one strain were obtained (with 2–5 strains), while nine strains displayed single patterns (Table 1). Strains showing profiles A–H, M, N, O, P were isolated from the same winery (D), while strains showing profiles Q and R were isolated from the S winery; the remaining two profiles (I and L) were displayed by two and five strains, respectively, isolated from both wineries. A lower discrimination was achieved when primer OPA9 was used (Table 1); a unique profile (h) was displayed by all strains of S winery, except for S8 (profile m), while other nine different patterns were widely distributed among strains isolated from D winery. When the results from DD-PCR with both primers were combined, the discriminating power was greater than with each primer separately, as shown in the consensus dendrogram (Fig. 1).

The different banding patterns obtained for the strains were analyzed by selecting arbitrarily a coefficient of correlation to distinguish the clusters (reported in Table 1). In the numerical elaboration of the different strain profiles, as shown in Fig. 1, the lowest similarity level was 45 %; hence, only clusters with a correlation coefficient above 69 % were considered different. The data obtained by the combined DD-PCR were statistically analyzed by UPGMA cluster analysis, and the

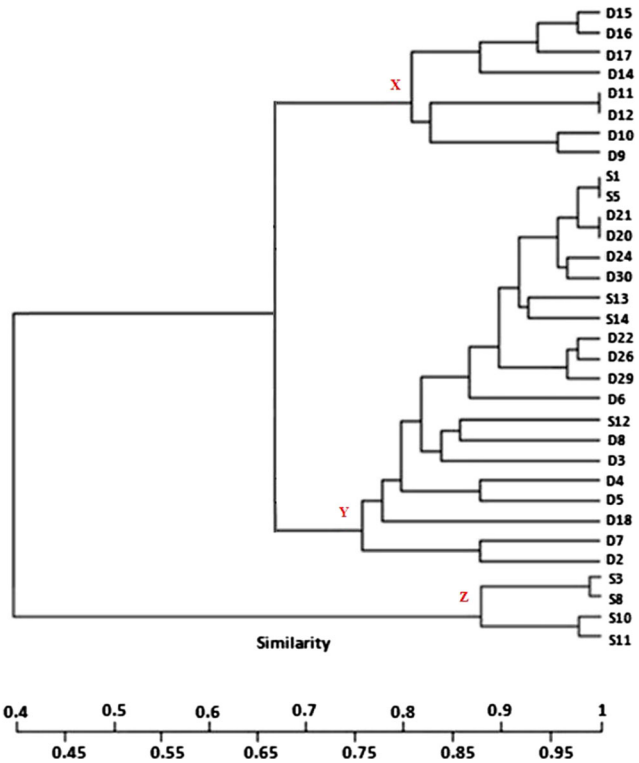

Fig. 1 Consensus dendrogram obtained combining M13 and OPA9 DD-PCR patterns

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produced dendrogram showed the genetic distance among each single genotype (Fig. 1). The strains were divided into three different principal clusters (X, Y, and Z).

The cluster Y resulted the most variable but also the most numerous (including both D and S strains) with the lowest correlation coefficient among 20 strains grouped (76 %). These strains formed two subgroups (named Y1 and Y2) and a single-strain cluster (D18), increasing the similarity level to 80 %; the larger one included the most of strains revealing a similarity ranging from 82 to 98 %, whereas the smaller showed a similarity level of 88 % clustering only two strains. Cluster X (eight strains of winery D) clustered with a similarity of 81 %, forming a major subgroup of four strains (X1) with a similarity level of 88 %, and two more homogeneous subgroups (X2 and X3, each including two strains) showed a similarity of 100 and 97 %, respectively. Moreover, the homogeneous cluster Z showed a high similarity forming two groups of S strains with a level of 98 and 99 %.

We reported (Tables 1 and 2) the strain subdivision in clusters at the similarity level of 95 % that allowed to obtain a fine intra-specific discrimination, with 13 single-strain clusters (ten belonging to winery D and three to winery S), besides seven small clusters including closely related strains (96–100 %) especially in the major cluster X and Z; all of these clusters were formed by S/D winery strains, except for cluster X1 (Table 2) in which four D strains clustered with two S strains. So, this analysis underlined a raised strain biodiversity in *O. oeni* populations of the two different Aglianico wineries in accordance with their origin.

Table 2 Malic acid consumption ability of *O. oeni* strains after growth at 30 °C for 6 days in the presence of 5 g/L L(-)-malic acid

Strains	Cluster number*	ΔA	Residual c [g/L]	Consumed c[g/L]	% Consumed malic acid
D15	X1	0.41	0.783	4.217	84.34
D16	X1	0.33	0.633	4.367	87.34
D17	X2	0.03	0.070	4.930	98.60
D14	X3	1.81	3.395	1.605	32.09
D11	X4	0.39	0.753	4.247	84.94
D12	X4	0.46	0.881	4.119	82.38
D10	X5	0.58	1.106	3.894	77.88
D9	X5	0.74	1.398	3.601	72.03
S1	Y1	0.99	1.864	3.136	62.72
S5	Y1	0.74	1.406	3.594	71.88
D21	Y1	0.56	1.053	3.946	78.93
D20	Y1	0.67	1.274	3.725	74.50
D24	Y1	0.39	0.745	4.254	85.09
D30	Y1	0.00	0.018	4.982	99.65
S13	Y2	0.87	1.646	3.353	67.07
S14	Y3	0.80	1.511	3.489	69.77
D22	Y4	0.64	1.218	3.781	75.63
D26	Y4	0.63	1.196	3.803	76.08
D29	Y4	0.28	0.543	4.457	89.14
D6	Y5	0.31	0.580	4.419	88.39
S12	Y6	0.03	0.055	4.945	98.90
D8	Y7	0.15	0.295	4.704	94.09
D3	Y8	1.89	3.553	1.447	28.94
D4	Y9	0.96	1.819	3.181	63.62
D5	Y10	0.58	1.106	3.894	77.88
D18	Y11	1.34	2.532	2.468	49.36
D7	Y12	0.59	1.121	3.879	77.58
D2	Y13	0.89	1.684	3.316	66.32
S3	Z1	0.83	1.571	3.428	68.57
S8	Z1	0.92	1.736	3.263	65.27
S10	Z2	0.66	1.248	3.751	75.03
S11	Z2	0.39	0.745	4.254	85.09

*Cluster number at 95 % of similarity, in the combined DD-PCR analysis with both primers

Physiological characterization

The heterogeneity of these natural *O. oeni* populations was investigated by physiological characterization of the strains through capillary electrophoresis and enzymatic assays. The variability related to bacterial size and surface charge changes due to different compositions was investigated. It has been necessary to first perform a series of laboratory tests in order to optimize the CE work protocol and to standardize the method for our research, since a wide variability of size and surface charge of single cells obtained in early electrophoretic patterns. Some modifications of different electrophoretic parameters for the cell separation were carried out to achieve good-quality electropherograms, reproducible analyses with well resolved peaks and intact cell stability.

First of all, different pH of TBE running buffer were tested on the cells' mobility to overcome the problem of short mobility and unresolved migration peaks. Moving along the pH scale, the electropherograms of *O. oeni* cells showed changes in cell charges and in resolution peaks from physiological to alkaline values; cells lost the charge in the alkaline environment and moved trailed by EOF, so annulling the cell heterogeneity under a single peak (data not shown). According to these observations, we chose the pH 8.4 of TBE running buffer for the following analyses. Then, two different capillaries were examined, and the next CE analyses were performed by using the long capillary (L_t 49.5 and L_{eff} 38.5 cm) with i.d. 100 μm that allowed a better separation efficiency.

Moreover, the investigation of other electrophoretic parameters led to the application of a constant separation voltage of 10 kV and of an injection pressure of 1378.951458 Pa for 5 s. So, this optimized and reproducible method was used to investigate the biochemical variations of *O. oeni* strains from the different wineries.

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The different surface charge distribution has been observed among strains. Figure 2 shows electropherograms of a representative strain isolated from winery S (A) and of a representative strain from winery D (B), in which a different surface charge distribution reflects differences in the cell surface composition. The electrophoretic pattern of each strain was characterized by three peaks: the first one, at the EOF marker left, represented the positively charged species in the sample; the halfway peak characterized neutral species migrated with EOF; the last one represented all species with negative surface charges. However, the electrophoretic patterns of strains isolated from both wineries showed a difference in the sub-

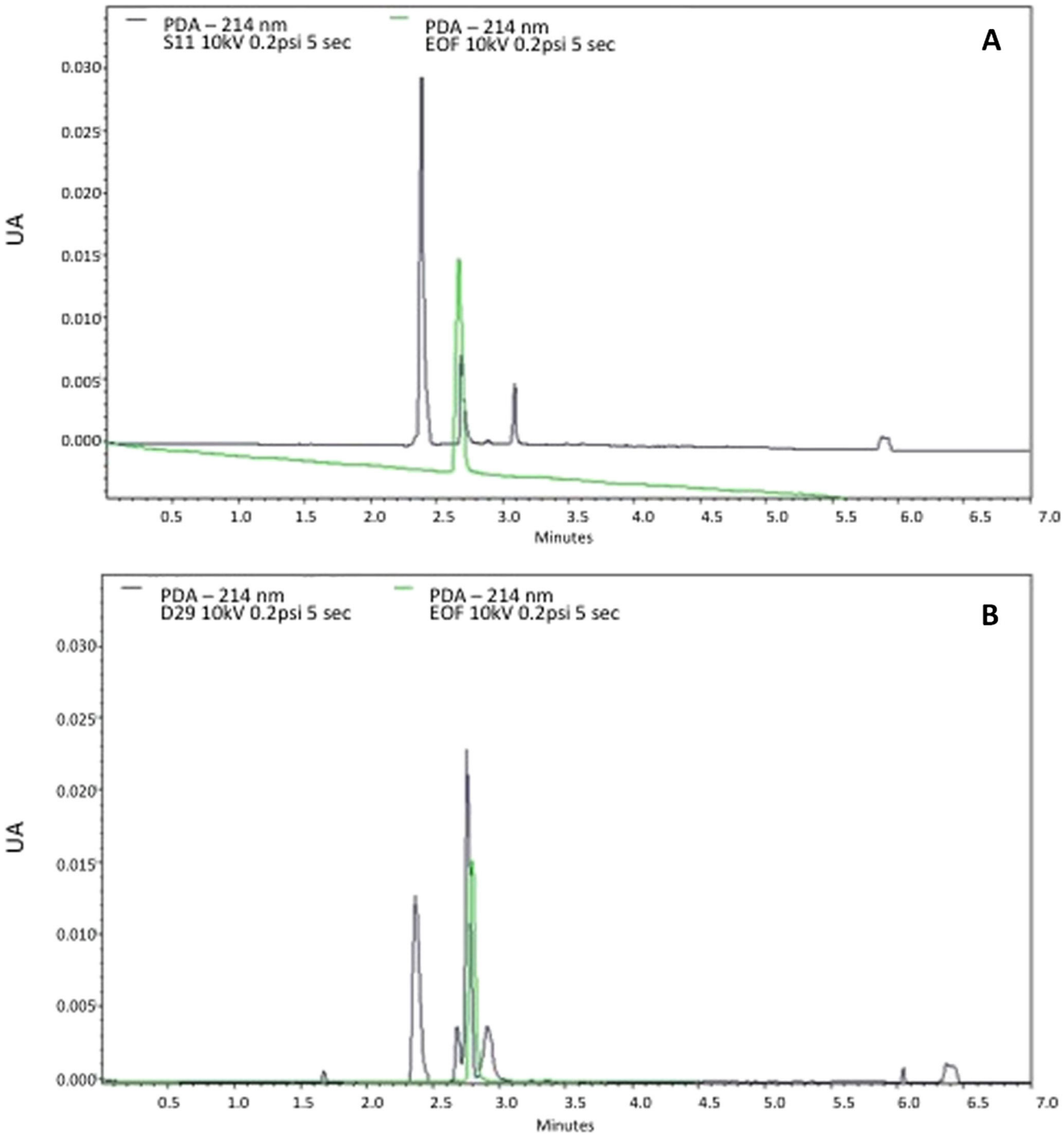

Fig. 2 CE electropherograms of a representative strain isolated from winery S (**a**) and of a representative strain from winery D (**b**). *Green pattern* represents the EOF marker; *black pattern* represents the strain

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population composition, in terms of charge/dimension ratio of cells and peak number linked to strains' origin.

The migration time of the negative peak of S winery strain was higher than the D winery strain (3.1 and 2.9 min for S and D strains, respectively), suggesting that negative cells of S strains are smaller and/or more charged (Fig. 2), while D strains are characterized by a greater number of neutral cells as shown by the high peak which migrates with the EOF marker. In addition to the three cell sub-populations common to all strains, D winery strains produced a further fourth sub-population, closer to the neutral peak (Fig. 2b), that is absent in the profile of S strains (Fig. 2a). These preliminary CE results suggested that the different compositions of cell populations in *O. oeni* strains associated with the variability of surface charges were the evidence of the heterogeneity of the *O. oeni* species linked to their origin. The CE method optimized and adapted for bacterial analyses allowed to observe the heterogeneity of bacterial strains in a rapid and easy way.

Moreover, all *O. oeni* strains were screened by evaluating their malic acid consumption ability and the *mle* gene induction since these traits may be affected by environmental factors. Table 2 showed the malic acid consumption ability of the thirty-two strains after growth at 30 °C for 6 days in the presence of 5 g/L L(-)-malic acid. Almost all strains possessed a good ability with a consumed malic acid percentage greater than 60 %; among these, 53.1 % of strains was able to degrade L(-)-malic acid at a percentage ranging from 60 to 80 %, while 9.4 % of strains was able to degrade more than 95 % of the L(-)-malic acid. The comparison between the consuming capacity of each strain pointed out strain-specific differences in malic acid degradation.

The relationship between the presence of L(-)-malic acid in the medium and the induction of the *mle* gene was studied. In this work, we analyzed the *mle* transcript in the presence and in the absence of L(-)-malic acid added to the medium and we carried out the species-specific PCR on the cDNA to observe when the transcript was expressed. Results demonstrated that only the presence of L(-)-malic acid in the growth medium induced the *mle* gene, as, in growth conditions without L(-)-malic acid, the *mle* transcript was not detected (data not shown).

We have also displayed that different concentrations of L(-)-malic acid ranging for 0.625 to 10 g/L can induce the *mle* gene and not only 5 g/L. Figure 3 shows the effect of different concentrations of L(-)-malic acid on *mle* gene induction in a representative strain. The presence of L(-)-malic acid in the growth medium induced the *mle* gene (Fig. 3, lanes 2–7), as, in growth conditions without L(-)-malic acid, the *mle* transcript was not detected (Fig. 3, lane 1). The results revealed that the presence of L(-)-malic acid in the medium was able to induce the *mle* gene ever since from low quantities.

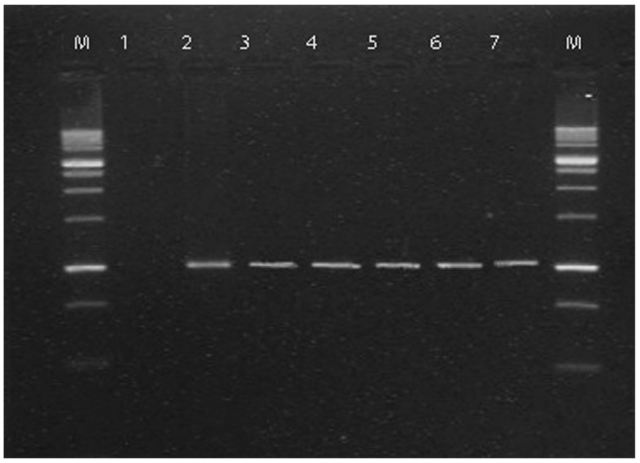

Fig. 3 Effect of different concentrations of L(-)-malic acid on *mle* gene induction in a representative strain. *M* marker 1 kb, *1* 0 g/L L(-)-malic acid, *2* 0.625 g/L L(-)-malic acid, *3* 1.25 g/L L(-)-malic acid, *4* 2.5 g/L L(-)-malic acid, *5* 5 g/L L(-)-malic acid, *6* 7.5 g/L L(-)-malic acid, *7* 10 g/L L(-)-malic acid

Discussion

The development of starter cultures for food fermentations is a multidisciplinary endeavor requiring not only an ecological study of the spontaneous process but also characterization of useful technological and physiological features of the predominant strains in order to select those with the highest potential for industrial applications. Intra-specific differentiation is therefore a required preliminary step for the selection of strains because technological characteristics, such as survival in wines with the relative adjustment changes, the rate of L-malic acid consumption, or the production of enzymatic activities, are reported to be strain dependent (Zapparoli et al. 2000; G-Alegrìa et al. 2004; Coucheney et al. 2005; Ruiz et al. 2008).

In this study, the discrimination of *O. oeni* strains from two different Aglianico wineries was carried out, highlighting a rich biodiversity in accordance with the variety of sample origins. The data obtained by two powerful methods used for strain typing evidenced a high level of genetic heterogeneity within each single *O. oeni* natural population. As regards the usefulness of the methods assayed in this study, there were important discrepancies among authors. So far, most authors agree that PFGE typing by using appropriate restriction endonucleases gives the greatest discriminatory power and the highest reproducibility (Lopez et al. 2007, 2008; Gonzalez-Arenzana et al. 2011; Zapparoli et al. 2012; Pramateftaki et al. 2012); on the contrary, Ruiz et al. (2008) reported PFGE analysis using restriction enzyme *Apa*I or *Sfi*I showed poorer discriminant capacity than RAPD-PCR, and also evaluated DD-PCR method not suitable for their typing purposes. This is in disagreement with Lechiancole et al. (2006) that evidenced the reliability of the DD-PCR in the differentiation of closely related strains suggesting that this method can represent an

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alternative and/or additional tool for fine characterization of oenococcal strains. The same authors, however, had also different opinions on the use of the endonuclease more suitable for the discriminating analysis.

Moreover, our preliminary discrimination by PFGE associated with a further analysis of strain discrimination level by DD-PCR revealed a better estimation of strain heterogeneity of *O. oeni* populations as previously demonstrated by Lechiancole et al. (2006) confirming that this species is characterized by high intra-species diversity (Sato et al. 2001; Cappello et al. 2008, 2010; Vigentini et al. 2009; Solieri et al. 2010; Zapparoli et al. 2012). These results are supported by the peculiarity of Aglianico del Vulture wine vinification where low temperatures, pH generally below 3.5, and high ethanol content are selective features for the indigenous microflora; and at the same time, it is possible that the typical characteristics of the products are due, at least partially, to the high degree of heterogeneity, confirmed by the clusters obtained. The use of the DD-PCR method for genetic biodiversity study of closely related strains revealed an agreement with a natural *O. oeni* winery-specific population, associated with a certain type of wine and winemaking area. So, a high level of similarity was used to discriminate among strains noticing a raised variability in *O. oeni* populations of two different Aglianico wineries.

The physiological strain characterization carried out in this study allowed to determine the presence of high winery-specific heterogeneity within *O. oeni* populations also at a phenotypic level, with a great variability on malic acid consumption ability among strains and interesting differences in morphological and surface composition of the cells. In this work, the CE analysis was extended, in addition to organic and inorganic ion analysis, to study small particles such as bacterial cells, that were characterized by a wide distribution of size, shapes, and charge which reflect on a distribution of electrophoretic mobility in a single sample (Desai and Armstrong 2003; Horká et al. 2010; Petr and Maier 2012). This effect, well known as electrophoretic heterogeneity (EH) (Radko and Chrambach 2002), on one hand determinates broad and often irregular peak shapes, which make more difficult the data analysis, but on the other hand allows to obtain information on both size and surface characteristics of intact cells in a very rapid and easy manner (Armstrong et al. 2001; Rodriguez and Armstrong 2004). The versatility of CE, in addition to its characteristics of speed, facility, and automation of the separation, allowed to analyze and characterize intact and viable microbial cells with small-volume consumption of sample and buffer (Moon et al. 2003; Kłodzińska and Buszewski 2009). The results obtained from electropherogram analysis, showing different electrophoretic profiles, suggested a wide variability of size and surface charge of single cells in each strain in accordance with the variety of strain origins (different wineries) strongly linked to traditional vinification procedures. This underlines the importance to characterize *O. oeni* strains that are unique to particular geographical wine regions in order to enhance regionality in the wines (Li et al. 2006; Sico et al. 2008; Yanagida et al. 2008; Canas et al. 2009; Vigentini et al. 2009; Solieri et al. 2010; Ruiz et al. 2010; Capozzi et al. 2010).

Moreover, these results provided the evidence of the potential role of the CE technique as an alternative cell-whole method to characterize *O. oeni* diversity at the strain level and to give further information, added to genotyping, about autochthonous bacteria for the enhancement of specific physico-chemical conditions of wine.

An interesting point of discussion was offered by the results obtained from the evaluation of L(-)-malic acid consumption and the study of *mle* gene induction in the presence of L(-)-malic acid in the medium. The comparison between MLA of each strain pointed out strain-specific differences in malic acid degradation. These differences might be due to the different viability of the cells or to the different activities of malic acid transporting and degrading systems.

In the past, contradictory data about the role of L(-)-malic acid in the induction of the *mle* gene have been reported; some studies demonstrated that, in the absence of L(-)-malic acid, *O. oeni* was able to perform the MLF indicating that *mle* gene was constitutively present (Henick-Kling et al. 1989; Labarre et al. 1998); some works (Lonvaud-Funel and Strasser De Saad 1982; Spettoli et al. 1984; Lonvaud-Funel 1995) proposed that the gene was inducible by L(-)-malic acid added to the medium, as in the absence of L(-)-malic acid the MLA was not detected, and then, a study of Pardo et al. (1992) suggested that, since the presence of L(-)-malic acid promoted the synthesis of the malolactic enzyme and the values of malolactic activity had a great heterogeneity, the *mle* gene induction is strain dependent. Recently, the *mle* gene has been reported as being inducible by L(-)-malic acid in *Lactobacillus plantarum* (Miller et al. 2011), and our study supported this finding also in *O. oeni* at different concentrations of L(-)-malic acid, ranging from 0.625 to 10 g/L, apart from the different winery origins.

In conclusion, this study provided some evidences on the importance of intra-species biodiversity of malolactic bacterial populations in wine ecosystems. Although the mechanisms involved in the strain evolution and diversification may be similar in all wines, each wine possesses peculiar winemaking conditions and physical–chemical properties which make specific the survival and growth of bacterial cells. This study pointed out a great biodiversity among *O. oeni* strains, underlining that native *O. oeni* strain variability can be also winery specific. Such biodiversity within a certain winery and winemaking area is a valuable source for selecting malolactic starters, and the identification of strain-specific traits is especially important to match individual strains to a specific industrial process. This efficient assessment of the genetic

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polymorphism and physiological strain characterization and differentiation contributed to increase the collection of fully characterized Aglianico wine indigenous strains with potential use in winemaking, and in the future, it could contribute to prevent homogenization of the natural microbiota of wines by the use of commercial starters as well as to maintain the current natural biodiversity.

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