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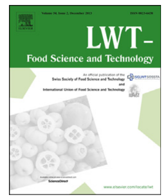

Assessment of competition in wine fermentation among wild *Saccharomyces cerevisiae* strains isolated from Sangiovese grapes in Tuscany region

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ABSTRACT

In this study we analyzed *Saccharomyces cerevisiae* isolates obtained from spontaneous fermentations of grapes collected in one vineyard (same rows), during four harvest seasons (2006–2010). A total of 160 isolates were characterized by PCR amplification of inter-δ region. Six strains, representative of the different inter-δ profiles obtained, were tested in single and mixed fermentations at laboratory scale. In all the mixed fermentations, the strain possessing profile A (the only biotype found for two consecutive years) was tested in co-cultures with each of strains showing the other profiles. The strain "A" dominated in almost all the mixed fermentations. The experimental wines obtained from single and co-culture fermentations were analyzed for the content of main secondary compounds. The wines produced by mono-cultures were very different from the wines obtained by co-fermentations, except the wine by strain "A", which grouped together with mixed-culture wines. Furthermore, the comparison between volatile components determined in mixed culture and blended wines, obtained by mixing monoculture wines, can suggest the existence of yeast metabolic interactions during mixed fermentations. The strain exhibiting the profile A resulted a good "competitor" not only among natural yeast population, but it was also capable to dominate mixed fermentations at lab scale.

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1. Introduction

Yeasts are part of the natural microbial communities of grapes (Fleet, Prakitchaiwattana, Beh, & Heard, 2002), which are, understandably, considered a potential source of autochthonous *Saccharomyces cerevisiae* strains associated with specific production areas. Although the low frequency reported for *Saccharomyces* species on the grapes, the predominance of *S. cerevisiae* in fermentations has led to its recognition as the principal wine yeast, and various strains of *S. cerevisiae* are commercialized as starter cultures for wine production (Fleet, 2003). Any way, the inoculated starter culture must compete with indigenous yeasts (Barrajón, Arévalo-Villena, Rodrìguez-Aragòn, & Briones, 2009; Capece et al., 2011) and, consequently, also wines produced by addition of starter cultures are the products of mixed fermentation. The sequential evolution of strains and species throughout fermentation is largely determined by their different susceptibilities to the increasing concentration of ethanol – the non-*Saccharomyces* species dying off earlier in the process due to their high sensitivity to ethanol. In addition, other phenomena such as fermentation temperature, dissolved oxygen content, killer factors, quorum-sensing molecules and spatial density influences are known to affect the competitive interaction between yeast species and strains in wine fermentations (Fleet, 2003; Hogan, 2006; Nissen, Nielsen, & Arneborg, 2003; Perez-Nevado, Albergaria, Hogg, & Girio, 2006; Yap, Lopes, Langridge, & Henschke, 2000), but little is known regarding how these factors might affect the dominance and succession of individual species and strains within the total population (Bisson, 1999; Fleet, 2003; Fleet & Heard, 1993; Zott, Miot-Sertier, Claisse, Lonvaud-Funel, & Masneuf-Pomarede, 2008). An actual trend is the use of controlled fermentation with multiple yeast species or strains inoculation in order to overcome complexity lacks, determined in the wines produced by pure yeast mono-cultures (Barrajón, Capece, Arévalo-Villena, Briones, & Romano, 2011). Several studies have already described fermentation with

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mixtures of non-*Saccharomyces* yeasts and *S. cerevisiae* (Garcia et al., 2002; Moreno, Millan, Ortega, & Medina, 1991; Soden, Francis, Oakey, & Henschke, 2000), and mixtures of different strains of *S. cerevisiae* (Cheraiti, Guezenec, & Salmon, 2005; Howell, Cozzolino, Bartowsky, Fleet, & Henschke, 2006), finding chemical differences in the wines produced. When using particular yeast cultures to obtain a special character or style in the final product a dominant growth of the inoculated strain(s) is required. The ability of inoculated starter to compete with native yeasts present in the grape must is a fundamental aspect. Only if this condition is guaranteed, the inoculated starter can produce the desired effect in the final product.

In this study we analyzed *S. cerevisiae* isolates obtained from spontaneous fermentations of grapes collected in one vineyard (same rows) of the Tuscany Region (Italy), during four harvest seasons. A wild strain, resulted dominant among natural yeast microflora for two consecutive years, was tested in mixed fermentation in combination with the other wild strains in order to investigate the "dominance" character of this strain during wine fermentation. The analysis of volatile components in wines produced in single and mixed fermentations was used to evaluate the influence of strain dominance on wine aroma. The strain ability to dominate the fermentation represents an important trait for a starter culture, which has been poorly investigated until now.

2. Material and methods

2.1. Yeasts

One hundred and sixty *S. cerevisiae* isolates were used. The yeasts were previously isolated from spontaneously fermented Sangiovese grapes, which were harvested during four years (2006–2007–2008–2010) in a vineyard, located in the Tuscany region (Italian center). Yeast isolation and identification was performed following the procedure reported by Capece et al. (2010). Samples were not always collected from the same plant, but from plants located in the same row (±1–2 m). The studied isolates were grown on YPD medium (1% (w/v) yeast extract; 2% (w/v) peptone; 2% (w/v) glucose; 2% (w/v) agar) and maintained at 4 °C for further characterization. For each vintage year, forty isolates were chosen.

2.2. Yeast isolate characterization

The isolates were characterized by PCR amplification of inter-δ region and mtDNA restriction analysis (RFLP-mtDNA). PCR reactions were performed on EuroClone One Gradient thermal cycler, using the primers δ2 (5′-GTGGATTTTTATTCCAACA-3′) and δ12 (5′-TCAACAATGGAATCCCAAC-3′), as reported by Le Jeune et al. (2006). The amplification of δ region was performed directly from the colony, without previous DNA extraction, by increasing the time and the temperature of initial denaturation, following the protocol reported by Capece, Romaniello, Siesto, and Romano (2012). RFLP-mtDNA was performed according the protocol described by Capece et al. (2010).

2.3. Killer activity

The isolates were tested to verify the presence/absence of killer phenotype. Killer activity tests were performed on medium, containing malt extract broth (2%), agar (2%), methylene blue (0.0003%), buffered at pH 4.6 with 0.1 M citric acid–phosphate buffer. As sensitive reference strain, *S. cerevisiae* DBVPG 6500 (NCYC 1006; National Collection of Yeast Cultures, Norwich, England) was used, by suspending its cells in sterile water and incorporating a concentration of about 10^6 CFU/ml into the medium. All the studied colonies were inoculated on the plates, by using the killer *S. cerevisiae* strains K1 and K2 (DBVPG 6497and DBVPG 6499, respectively) as positive controls; the plates were incubated at 26 °C for three days. The tested isolates were designated as killer strain when the colony was surrounded by a clear zone in which no growth of the inoculated sensitive strain had occurred. The non-killer yeasts were suspended in sterile water and inoculated in the medium previously described. The killer reference strains were inoculated on the seeded plates, which were incubated at 26 °C for three days. If the killer reference strain was surrounded by a clear zone of growth inhibition, the tested strain was designated as sensitive. If there was no clear zone of growth inhibition, the tested strain was designated as neutral.

2.4. Fermentations at lab scale

Six strains, representative of the different molecular profiles found among the isolates, were tested in fermentation at laboratory-scale. In particular, 6 fermentations were performed with single strains and 5 mixed fermentations were carried out by inoculating one chosen strain in co-cultures with each of the other 5 strains (Fig. 1).

The fermentations were carried out in 130-ml Erlenmeyer flasks filled with 100 ml of sulphited (50 mg/l) Sangiovese grape must (pH 3.24, sugar concentration 258 g/l); the surface of grape must was covered with a thin layer of paraffin oil in order to avoid the contact with air. The flasks were inoculated with 48-h pre-cultures grown in the same must; the co-inoculations were conducted by simultaneously inoculating two yeast strains, each at half the recommended inoculation rate, to achieve approximately 1×10^6 cells/ml. In the case of monoculture fermentations, each strain was inoculated at the same inoculum concentrations of mixed starter (10^6 cells/ml).

The single and mixed fermentations were performed at 26 °C and the fermentative course was monitored by measuring weight loss, determined by carbon dioxide evolution during the process. The process was considered completed when a constant weight of the samples was recorded. All the experiments were performed in duplicate. At the end of mixed fermentations, yeast isolation was carried out on WL medium (Pallmann et al., 2001) and 20 colonies, randomly chosen from each sample, were submitted to amplification of inter-δ region.

2.5. Analysis of volatile compounds

The wines obtained from single and mixed inoculated fermentations were analyzed for the content of secondary compounds.

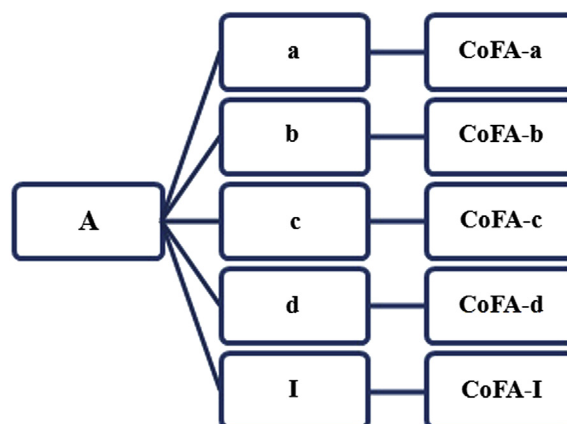

Fig. 1. Formulation of the different co-cultures used in the five mixed fermentations. CoF = co-fermentation; the letters represent the strains included in the co-cultures.

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Furthermore, the same compounds were determined in blended wines, which consisted of equal proportions of monoculture wines obtained by the yeasts inoculated in mixed fermentations. Blended wines corresponding to mixed fermentations CoFA-a, CoFA-b, CoFA-c, CoFA-d, CoFA-I, were named VA-Va, VA-Vb, Va-Vc, Va-Vd, Va-VI. For example, blended wine VA-Va contained equal proportions of monoculture wines prepared with strains “A” and “a” and corresponding to CoFA-a.

Acetaldehyde, *n*-propanol, isobutanol, amyl alcohols, ethyl acetate and acetic acid were determined by direct injection gas chromatography of 1 μl sample into a 180 cm × 2 mm glass column packed with 80/120 Carbopack B/5% Carbowax 20 M (Supelco, Bellefonte, PA). The column was run from 70 to 140 °C, the temperature being ramped up at a rate of 7 °C/min. The carrier gas was helium at a flow rate of 20 ml/min. Levels of the secondary compounds were determined by calibration lines.

Other volatile compounds, such as acetates and ethyl esters, volatile fatty acids, terpenes, were analyzed by SPME-GC-MS. About 20 ml of each sample were placed into a 50 ml amber glass vial containing 3 g of NaCl (saturation level) and 0.5 ml of isooctane as internal standard (IS). The fiber used for the extraction of the volatile components, in headspace condition, was the polydimethylsiloxane (PDMS) 100 μm. The sample vials were equilibrated for 30 min at 40 °C followed by fiber exposure to the headspace for 20 min. The fiber was then inserted into the injection port of the GC apparatus for thermal desorption. The analytes removal from the fiber was carried out in the splitless mode at 240 °C for 5 min. The analyses were performed using a Agilent 6890 GC gaschromatograph coupled with Agilent 5973mass spectrometry (MS) detector and a DB-WAXetr column, 30 m × 0.25 mm i.d., 0.25 nm film thickness (J&W Scientific, Folsom, CA, USA) was employed. Helium was used as the carrier gas with a flow rate of 1.5 ml/min; the injector temperature was 250 °C.

2.6. Statistical analysis

Levels of secondary compounds were submitted to statistical analysis by one-way Analysis of Variance (ANOVA). Tukey's test was used to compare the mean values of secondary compounds between mixed and respective single fermentations. Principal component analysis (PCA) was carried out to compare the secondary compounds detected both in wines by single and mixed fermentations and in wines by mixed fermentation and blending of single-starter wines. The PAST software ver. 1.90 (Hammer, Harper, & Ryan, 2001) was used for all the statistical analyses.

3. Results

3.1. Molecular characterization of wild isolates

The PCR amplification of the δ interspersed sequences was used to identify genomic differences among 160 *S. cerevisiae* strains isolated from the same vineyard (same row) during four years. These yeasts were isolated from spontaneously fermented grapes of Sangiovese cultivar, collected in a vineyard, planted on 2003 and located at a distance of about 1000 m from the winery. Other than the indigenous isolates, the starters used in the wine cellar were included in the study. As shown in Fig. 2a, the amplification of inter-δ region of the 160 isolates generated six different molecular profiles (indicated with I, A, a, b, c, d). Furthermore, these patterns were different from biotypes generated by amplification of inter-δ region of the starters used in the wine cellar (Fig. 2b). The profiles of the isolates seem to be correlated with year of isolation; in fact, all the yeasts isolated in 2006 vintage exhibited the same profile, indicated with I (Fig. 2a), such as the *S. cerevisiae* isolated during 2007 and 2008 vintages, which showed the same biotype A (Fig. 2a). On the contrary, among the yeasts isolated in 2010, 4 different profiles (a, b, c and d, Fig. 2a) were found. The profiles were present with different frequencies among the 40 isolates (data not shown); in particular, the profile “a” was the most diffused, being showed by 29 isolates, followed by the profile “b” (detected in 9 isolates), whereas the “c” and “d” profiles were found only in one isolate. It has be underlined that none of profiles found among 2010 year isolates was exhibited by *S. cerevisiae* isolated in the previous years.

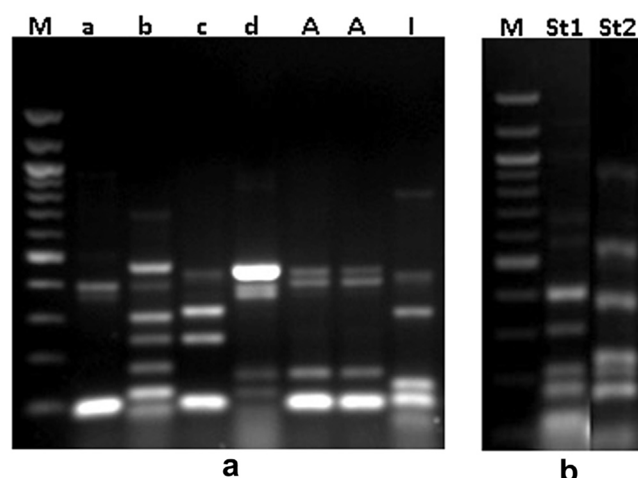

Fig. 2. Molecular profiles obtained by amplification of inter-δ region of wild *S. cerevisiae* isolates (a) and starters St1 and St2 used in the cellar (b) M = 100 bp marker (Biolabs).

By using the RFLP-mtDNA analysis, only 4 different profiles were recorded among the 160 isolates. In particular, the isolates showing “I” delta profile exhibited the same RFLP-mtDNA profile of isolates showing “a” delta profile and isolates showing “b” delta profile exhibited the same RFLP-mtDNA pattern of isolates possessing “A” delta pattern.

After these results, six strains were selected, as representatives of the different biotypes obtained with amplification of inter-δ region. The selected clones were designated with the same letters assigned to the corresponding biotypes (I, A, a, b, c, d), reported in Fig. 2a.

3.2. Killer phenotype of selected strains

The selected clones were tested for the killer activity in order to verify a correlation between the high frequency of a biotype and the killer character. The “I”, “A” and “a” strains exhibited the killer phenotype since these strains inhibited the growth of the killer-sensitive reference strain DBVPG 6500. About the non-killer strains, “c” showed neutral phenotype, whereas “b” and “d” resulted sensitive strains.

3.3. Strain dominance during fermentation at laboratory scale

The six *S. cerevisiae* strains were tested in inoculated fermentations of Sangiovese grape must as single and co-cultures. The strain “A” was selected as the base strain and was used in separate mixed fermentations with strains “I”, “a”, “b”, “c”, “d”. The strain “A” was chosen for the use in all the mixed fermentations because this biotype was the only found in two consecutive vintages (2007–2008).

All single-strain and coinoculated fermentations were completed successfully after 12 days. All the strains in single culture

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exhibited a similar fermentative power, producing about 11.5 g CO_2/100 ml of grape must; as regards the mixed fermentations, the lowest fermentative power (10.7 g CO_2/100 ml must) was found in co-inoculated fermentation A-I, whereas the highest production of CO_2 (11.7) was exhibited by co-culture A-d. The capacity of strain "A" to compete with the other strains in mixed fermentations was tested by analysis of inter-δ region on 100 yeast colonies (20 for each co-fermentations) isolated at the end of the fermentative process. Data related to the proportions of each yeast strain in the five mixed culture fermentations are reported in Table 1. The strain "A" was the better competitor because it was present in higher percentage than the other ones in four co-fermentations (in CoFA-b the strain "A" was present at 90%). Only in mixed fermentation inoculated with strains exhibiting patterns A and c, the strain "c" was predominant against the strain with pattern A.

3.4. Metabolite composition in single, mixed-culture and blended wines

The wines obtained from single and mixed inoculated fermentations were analyzed for the content of volatile compounds correlated to wine flavor. The amounts of metabolites determined were reported in Table 2. One-way ANOVA was applied to evaluate in each co-fermentation the differences between single and mixed fermentations.

As regards the secondary compounds usually present in high concentrations in wines, such as acetaldehyde, n-propanol, isobutanol, amyl alcohols, ethyl acetate and acetic acid, high variability was found mainly for acetaldehyde and acetic acid. The highest producer of acetaldehyde resulted the strain "c", whereas the lowest level of this compound was detected in wine obtained by inoculating the strain "d". The highest level of acetic acid was detected in wine produced by strain "A" (about 320 mg/l), followed by wine obtained with CoFA-c and CoFA-d; however, in all the obtained wines the levels of acetaldehyde and acetic acid were comprised in the desirable range. For these compounds, the differences between wines by single and mixed fermentations are mainly related to acetaldehyde, isobutanol and acetic acid. In each mixed fermentations, no statistically significant differences between wine produced by "A" and mixed starter were found, except in the case of isobutanol for the CoFA-I.

About the volatile compounds present at very low levels, such as terpenic compounds, other alcohols, ethyl acetates and esters, volatile fatty acids, significant differences between wines by single and mixed starters were detected for almost all the compounds analyzed ($P < 0.05$). Generally, the wines obtained by co-fermentations contained higher levels of these metabolites than wines derived by single fermentation.

For the majority of mixed fermentations, the wines obtained by co-fermentations were statistically different from wines by both the single starters. An exception is represented by wine produced by CoFA-I, which for the majority of compounds differed significantly from wine obtained by strain "I", except in the case of isobutanol and geraniol, where the wine by mixed starter was different also by wine obtained by "A".

Table 1
Inter-δ patterns (%) of isolates from mixed fermentations.

Mixed fermentation[a]	Patterns[b]
CoFA-a	A (65), a (35)
CoFA-b	A (90), b (10)
CoFA-c	c (65), A (35)
CoFA-d	A (65), d (35)
CoFA-I	A (60), I (40)

[a] For formulation of mixed fermentations, see Fig. 1.
[b] The percentage of presence of each inter-δ pattern is given in brackets.

Data of secondary compounds determined in experimental wines obtained by single and mixed fermentations were submitted to Principal Component Analysis (PCA). The first two components account about 81% of the total variance. The first principal component (PC1) explained 74% of data variability and was strongly correlated with terpenic compounds, ethyl esters and fatty acids (Fig. 3b), while acetaldehyde, *n*-propanol and amyl alcohols contribute more strongly to the second principal component (PC2). The plot of the 11 wines on the plane defined by these first two components is shown in Fig. 3a. This analysis was able to separate the monoculture fermentations from the mixed culture fermentations. In fact, wines from monoculture fermentations were located in the left side of the PCA plot (Fig. 3a), whereas the wines obtained by co-culture fermentations were distributed in the right side. Only the wine produced by strain with biotype A was located in the same quadrant of mixed-culture wines. Furthermore, the analysis of Fig. 3a revealed that the wine produced by CoFA-I is located very near to the wine obtained by pure culture fermentation with strain "A", whereas it was very far from monoculture wine by strain "I". This result could indicate a high influence of strain "A" in CoFA-I according to ANOVA results, which indicated that CoFA-I differed significantly from wine by "I" for almost all the compounds analyzed (Table 2).

Successively, wines from single fermentations were mixed in equal amounts, obtaining blended wines corresponding to the mixed culture wines (i.e. the VA + VI blended wine corresponded to the A + I mixed culture wine). These blended wines were analyzed for the content of secondary compounds; the PCA analysis on secondary compounds discriminated wines obtained by mixed culture and blended wines (Fig. 4a). In fact, wines from mixed-culture fermentations and the corresponding blended wines were separated into opposite quadrants of PCA plot; the first two components explain the 85% of variance. Only wines produced by mixed fermentation A + c and the corresponding blended wine (VA + Vc) are placed in the same quadrant and the wine obtained by mixed fermentation A + I is located in the same side of blended wines. The variables affecting the first two principal components are reported in Fig. 4b.

4. Discussion

The main objective of the present work was to verify if a native selected strain of *S. cerevisiae*, resulted dominant during spontaneous fermentations for two consecutive years, dominated also during inoculated mixed fermentations. In this study, this strain was selected among yeasts isolated from the same vineyard during four consecutive years. A change in yeast population composition of grapes from year to year was found, individuating six different biotypes among 160 isolates. Probably the different conditions at each harvest (temperature, rainfall,…) could determine which specific strains will develop. The same strains were not found from one year to another, except in the case of yeasts isolated during the 2007 and 2008 vintages. This could indicate that although some of these strains are able to remain in the ecosystem, they are not always able of dominating the natural yeast community of the vineyard. In fact, the populations of *Saccharomyces* on mature healthy grapes is quite low and the isolation of these yeasts from grapes using direct isolation methods is very difficult (Combina et al., 2005; Mercado, Dalcero, Masuelli, & Combina, 2007). Therefore, in our study an enrichment procedure was applied in order to recover the *S. cerevisiae* strains from the grapes. This methodology could give a 'misrepresented' image of the *Saccharomyces* populations present in vineyards, but it is a commonly used strategy for the study of vineyard yeast populations (Martínez, Gac, Lavin, & Ganca, 2004; Schuller & Casal, 2007; Valero, Schuller,

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Table 2
Secondary compounds (mg/l) in wines produced by single and mixed-starter cultures of *S. cerevisiae*.

Compounds	Starter culture										
	A	a	CoFA-a	b	CoFA-B	c	CoFA-C	d	CoFA-d	I	CoFA-I
Acetaldehyde	30.78 ± 3.82	44.94 ± 3.95	31.95 ± 3.40	24.82 ± 1.29	37.03 ± 0.71^{b}	57.26 ± 0.41	41.96 ± 9.26	15.78 ± 4.94	32.24 ± 0.40^{b}	24.44 ± 1.46	26.72 ± 6.04
Ethyl acetate	54.29 ± 1.62	55.46 ± 1.85	56.41 ± 0.56	53.5 ± 1.68	57.23 ± 1.63	54.87 ± 0.33	58.31 ± 1.47	57.61 ± 0.49	55.74 ± 1.24	54.79 ± 0.98	54.41 ± 1.07
n-Propanol	48.82 ± 1.05	48.01 ± 0.56	48.55 ± 0.08	51.61 ± 0.37	50.32 ± 0.63	46.88 ± 0.20	47.65 ± 0.76	48.6 ± 0.3	49.37 ± 0.38	47.45 ± 0.01	47.03 ± 0.18
Isobutanol	35.62 ± 2.45	27.45 ± 1.48	29.98 ± 1.66	21.86 ± 0.57	29.54 ± 0.74^{b}	29.09 ± 1.18	33.92 ± 3.27	28.7 ± 1.64	34.39 ± 3.91	23.03 ± 0.42	25.13 ± 0.15^{a}
d-Amyl alcohol	53.24 ± 4.41	48.35 ± 3.08	53.95 ± 2.86	55.11 ± 0.81	55.38 ± 5.13	47.21 ± 0.35	53.37 ± 5.28	46.31 ± 0.11	51.45 ± 4.99	52.01 ± 1.28	47.45 ± 0.99
Isoamyl alcohol	162.04 ± 13.52	150.82 ± 7.85	170.95 ± 9.50	160.28 ± 5.46	166.39 ± 16.31	165.57 ± 2.18	174.98 ± 20.0	149.99 ± 4.72	160.59 ± 16.97	156.55 ± 1.97	140.18 ± 2.36
Acetic acid	323.27 ± 18.61	195.06 ± 5.14	261.47 ± 22.87^{b}	150.3 ± 14.20	279.55 ± 37.64	234.08 ± 1.65	331.55 ± 38.42	135.57 ± 18.02	310.97 ± 59.73^{b}	217.36 ± 12.88	264.93 ± 16.03
Linalool	10.80 ± 0.30	6.48 ± 0.47	15.69 ± 1.02^{ab}	5.28 ± 0.54	12.87 ± 1.14^{b}	7.74 ± 0.10	17.21 ± 1.48^{ab}	8.86 ± 2.18	16.21 ± 1.92^{b}	3.60 ± 0.26	11.39 ± 1.65^{b}
a-Terpineol	5.85 ± 0.24	5.00 ± 1.03	10.86 ± 0.78^{ab}	3.74 ± 0.02	9.44 ± 0.20^{ab}	5.32 ± 0.015	11.94 ± 0.98^{ab}	6.02 ± 1.36	11.37 ± 1.53^{ab}	2.49 ± 0.18	7.79 ± 0.94^{b}
Geraniol	11.20 ± 0.11	4.89 ± 0.50	11.94 ± 1.16^{b}	3.91 ± 0.37	10.02 ± 0.23^{ab}	5.66 ± 0.11	13.03 ± 1.52^{b}	6.47 ± 1.75	11.88 ± 1.39^{b}	2.66 ± 0.15	8.24 ± 0.98^{ab}
Nerolidol	2.30 ± 0.28	1.21 ± 0.12	2.93 ± 0.25^{b}	1.00 ± 0.04	2.54 ± 0.01^{b}	1.40 ± 0.01	3.17 ± 0.28^{b}	1.64 ± 0.38	3.15 ± 0.56	0.71 ± 0.01	2.08 ± 0.27^{b}
p-Cymene	1.45 ± 0.03	1.11 ± 0.08	2.70 ± 0.15^{ab}	1.45 ± 0.28	1.88 ± 0.11	1.48 ± 0.28	3.03 ± 0.35^{ab}	0.00 ± 0.00	1.37 ± 0.12^{b}	0.62 ± 0.05	1.92 ± 0.38^{b}
Limonene	0.16 ± 0.03	0.09 ± 0.01	0.24 ± 0.01^{ab}	0.08 ± 0.01	0.23 ± 0.04^{b}	0.13 ± 0.03	0.26 ± 0.02^{b}	0.14 ± 0.01	0.24 ± 0.02^{b}	0.05 ± 0.01	0.18 ± 0.04
Butan-1-ol	0.16 ± 0.01	0.12 ± 0.01	0.29 ± 0.01^{ab}	0.10 ± 0.01	0.24 ± 0.02^{ab}	0.14 ± 0.01	0.33 ± 0.03^{ab}	0.17 ± 0.04	0.30 ± 0.03^{ab}	0.07 ± 0.01	0.21 ± 0.02^{b}
Hexan-1-ol	0.12 ± 0.01	0.90 ± 0.05	0.22 ± 0.02^{b}	0.74 ± 0.02	0.19 ± 0.01^{ab}	0.11 ± 0.01	0.24 ± 0.03^{ab}	0.12 ± 0.03	0.23 ± 0.03^{ab}	0.05 ± 0.01	0.16 ± 0.02^{b}
Butan-2,3-diol	0.04 ± 0.01	0.03 ± 0.01	0.07 ± 0.01^{ab}	0.02 ± 0.01	0.06 ± 0.01^{ab}	0.03 ± 0.01	0.08 ± 0.01^{ab}	0.04 ± 0.01	0.07 ± 0.01^{ab}	0.02 ± 0.01	0.05 ± 0.01^{b}
2-Phenylethanol	3.30 ± 0.01	1.86 ± 0.13	4.45 ± 0.35^{ab}	1.54 ± 0.06	3.87 ± 0.10^{ab}	2.20 ± 0.06	4.94 ± 0.39^{ab}	2.49 ± 0.57	4.59 ± 0.45^{b}	1.01 ± 0.06	3.21 ± 0.50^{b}
Benzyl alcohol	5.29 ± 0.19	3.96 ± 0.15	5.09 ± 0.73	3.32 ± 0.10	8.50 ± 0.15^{ab}	4.76 ± 0.20	1.08 ± 0.10^{ab}	5.48 ± 1.44	5.19 ± 0.81	2.23 ± 0.18	6.79 ± 0.87^{b}
Phenylethylacetate	7.20 ± 0.25	4.57 ± 0.35	10.76 ± 1.05^{ab}	3.73 ± 0.22	9.58 ± 0.02^{ab}	5.42 ± 0.08	12.00 ± 1.37^{ab}	6.17 ± 1.63	11.33 ± 1.18^{b}	2.51 ± 0.21	7.89 ± 0.92^{b}
Isoamylacetate	1.04 ± 0.13	6.90 ± 0.50	1.69 ± 0.08^{b}	5.58 ± 0.23	1.42 ± 0.078^{b}	8.16 ± 0.22	1.77 ± 0.07^{ab}	4.43 ± 0.45	1.69 ± 0.81^{b}	3.76 ± 0.36	1.22 ± 0.20^{b}
2-Methylbutylacetate	1.30 ± 0.18	0.81 ± 0.07	1.95 ± 0.10^{ab}	0.70 ± 0.09	1.80 ± 0.06^{b}	0.91 ± 0.01	2.12 ± 0.18^{ab}	1.11 ± 0.29	2.02 ± 0.22	0.44 ± 0.02	1.41 ± 0.21^{b}
Ethyl-hexanoate	2.20 ± 0.25	1.21 ± 0.10	2.95 ± 0.14^{ab}	0.97 ± 0.05	2.54 ± 0.08^{b}	1.43 ± 0.07	3.16 ± 0.24^{ab}	1.62 ± 0.39	2.98 ± 0.32	0.65 ± 0.07	2.11 ± 0.28
Ethyl-octanoate	2.30 ± 0.27	1.25 ± 0.18	2.92 ± 0.10^{b}	0.96 ± 0.03	2.48 ± 0.11^{b}	1.42 ± 0.05	3.13 ± 0.26^{b}	1.59 ± 0.41	2.97 ± 0.31	0.66 ± 0.06	2.06 ± 0.25^{b}
Ethyl-decanoate	0.38 ± 0.07	0.22 ± 0.01	0.56 ± 0.01^{ab}	0.18 ± 0.02	0.47 ± 0.01^{b}	0.28 ± 0.01	0.59 ± 0.05^{ab}	0.31 ± 0.08	0.55 ± 0.06^{ab}	0.13 ± 0.01	4.41 ± 0.06^{b}
Diethylsuccinate	3.00 ± 0.06	1.68 ± 0.17	4.02 ± 0.33^{ab}	1.38 ± 0.06	3.46 ± 0.08^{ab}	1.98 ± 0.01	4.35 ± 0.37^{ab}	2.18 ± 0.54	4.10 ± 0.45^{b}	0.91 ± 0.11	3.06 ± 0.68^{b}
Butanoic acid	5.24 ± 0.21	3.92 ± 0.21	9.82 ± 0.83^{ab}	3.31 ± 0.12	8.39 ± 0.23^{ab}	4.66 ± 0.14	10.56 ± 0.98^{ab}	5.35 ± 1.40	9.90 ± 1.02^{ab}	2.02 ± 0.16	6.83 ± 0.84^{b}
Hexanoic acid	5.41 ± 0.33	4.09 ± 0.20	10.08 ± 0.67^{ab}	3.37 ± 0.15	8.72 ± 0.16^{ab}	4.95 ± 0.13	11.05 ± 0.95^{ab}	5.73 ± 1.39	10.39 ± 1.19^{ab}	2.30 ± 0.15	7.31 ± 1.12^{b}
Octanoic acid	9.50 ± 0.35	5.83 ± 0.49	13.88 ± 1.02^{ab}	4.73 ± 0.11	12.13 ± 0.25^{ab}	6.86 ± 0.18	15.39 ± 1.39^{ab}	7.85 ± 1.92	14.36 ± 1.61^{b}	3.23 ± 0.31	9.97 ± 1.27^{b}
Decanoic acid	1.80 ± 0.17	0.91 ± 0.12	2.09 ± 0.13^{b}	0.70 ± 0.04	1.82 ± 0.04^{b}	1.07 ± 0.10	2.34 ± 0.26^{b}	1.15 ± 0.28	2.14 ± 0.23^{b}	0.53 ± 0.04	1.51 ± 0.29^{b}
Phenylacetic acid	4.60 ± 0.21	2.8 ± 0.21	6.79 ± 0.38^{ab}	2.27 ± 0.09	5.87 ± 0.16^{ab}	3.45 ± 0.01	7.41 ± 0.63^{b}	3.81 ± 0.95	6.96 ± 0.86^{b}	1.55 ± 0.14	4.92 ± 0.52^{b}
Benzaldehyde	0.34 ± 0.10	0.24 ± 0.08	0.47 ± 0.01	0.17 ± 0.02	0.35 ± 0.07	0.22 ± 0.01	0.52 ± 0.05^{b}	0.26 ± 0.06	0.48 ± 0.06	0.11 ± 0.01	0.36 ± 0.03^{b}
Eugenol	7.30 ± 0.38	3.78 ± 0.33	8.98 ± 0.74^{b}	3.05 ± 0.13	7.80 ± 0.21^{b}	4.34 ± 0.22	10.03 ± 1.05^{b}	5.10 ± 1.28	9.32 ± 0.90^{b}	2.08 ± 0.16	6.33 ± 0.66^{b}

Data are means ± standard deviation of two independent experiments; A–I = single starter; CoFA-a,…, CoFA-I = mixed cultures.
For each co-fermentation and each compound, superscript letters indicate significantly different values (Tukey's test, $P < 0.05$) between wines from single and mixed fermentation. i.e., in the case of CoFA-a, it was added the letter a when wine by starter A is statistically different from wine by starter A + a, letter b when wine by starter a is statistically different from wine by starter A + a.

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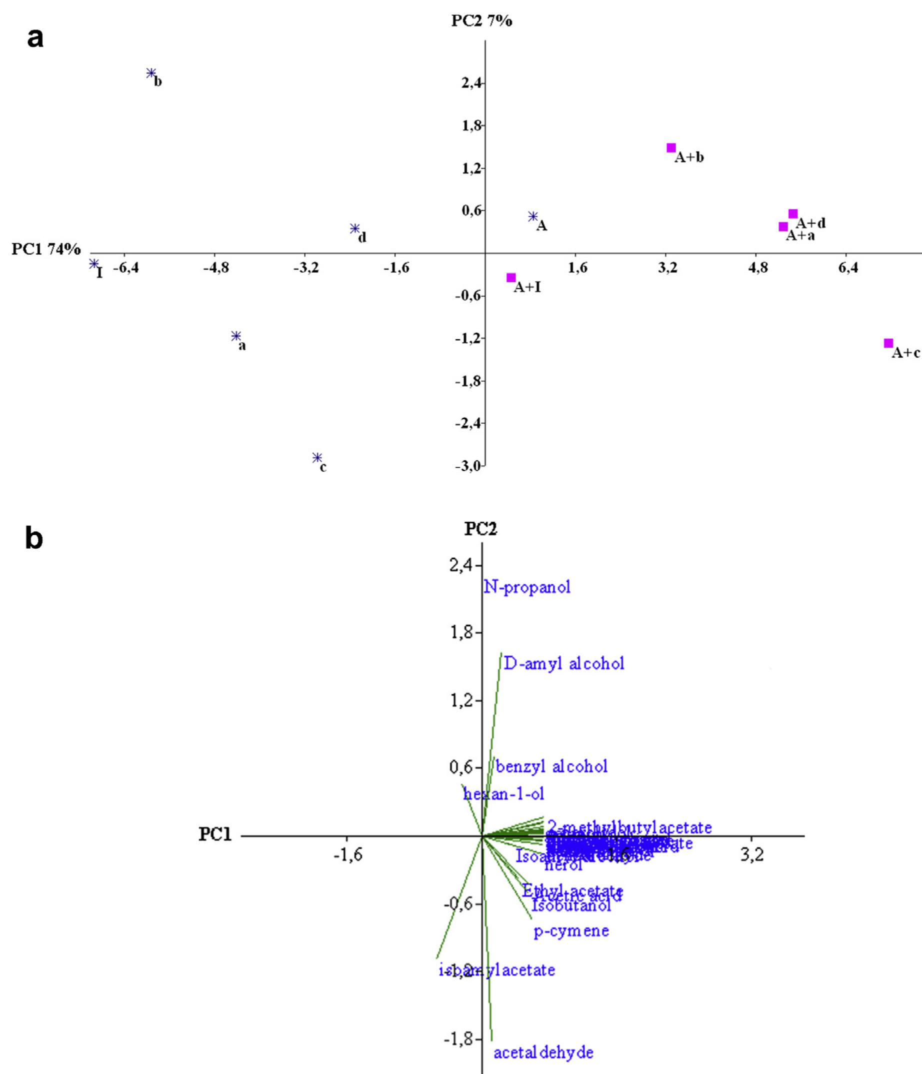

Fig. 3. Principal component analysis (PCA) biplot (a) and loading (b) of mean values of secondary compounds determined in wines obtained by single (*) and mixed fermentations (■).

Cambon, Casal, & Dequin, 2005). Although the *S. cerevisiae* isolates, obtained after enrichment through must fermentation, may not accurately represent vineyard populations and this experimental approach does not provide a precise description in terms of relative strain abundance in nature, the isolates might reflect those yeasts with particular competitive traits, allowing them to survive the fermentation process. In our study, the strain showing the biotype A could possess particular features because it was the only found for two consecutive years.

The strain dominance among native isolates seems to be correlated to killer activity. In fact, "I" and "A", possessing killer activity, represented the only biotypes found among isolates of 2006 and 2007–2008 vintages respectively, whereas "a" (killer native strain) was the profile found with the highest frequency among 2010 isolates. These killer strains could represent the yeasts survived within the wild population of spontaneous fermentations of collected grapes. Different ecological studies report that killer phenomenon confers a competitive edge to the producer strain by excluding other yeasts from its habitat (Yap et al., 2000).

The "dominance" character was tested during mixed fermentation at lab scale. At this purpose, the biotype A, dominant for two consecutive years, was tested in combination with the other five biotypes. The high dominance level, exhibited by this strain in almost all the mixed fermentations (except in the CoFA-c), indicates that the strain "A" possesses characteristics able to confer it a competitive advantage against the other strains. This aptitude seems not to be related with killer activity in all the fermentations. In fact, in the case of CoFA-a (dominance of strain "A") both the strains are characterized by killer activity, whereas in CoFA-c (dominance of strain "c") the strain "c" is a neutral strain. On the other hand, in winery *S. cerevisiae* killer strains don't always dominate at the end of fermentation (Heard & Fleet, 1987). Furthermore, several factors as those related to the cellar operations, among others, can affect the implantation capacity of a yeast starter (Barrajón et al., 2009; Lopes, Rodríguez, Sangorrín, Querol, & Caballero, 2007). In our case, the dominance of strain "A" cannot be related to enological practice because the strain imposition was evaluated during lab-scale fermentations, in which all the conditions were standardized. Therefore, intrinsic strain characteristics can play a determinant role on the behavior of strain "A".

Its "dominance" character is confirmed also by analyzing the metabolic compounds of single and mixed-culture wines. In fact, the wine obtained by strain "A" grouped together with mixed-culture wines (right side, Fig. 3a), whereas the other single-

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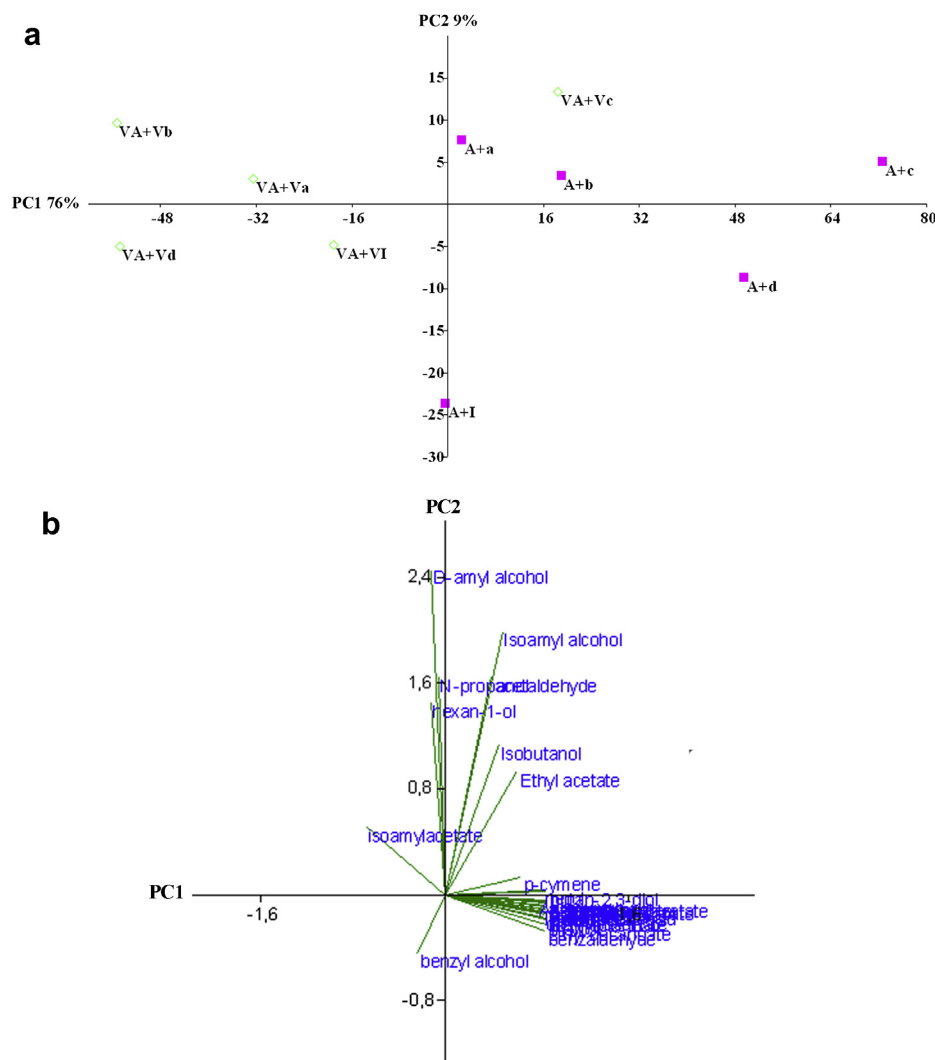

Fig. 4. Principal component analysis (PCA) biplot (a) and loading (b) of mean values of secondary compounds determined in mixed-culture (■) and blended wines (◆).

culture wines were located in the opposite side of PCA plot. This indicates that in mixed-culture wines the influence on wine composition of strain "A" was prevalent in comparison to the other strains included in the mixed starter, because it was included in all the co-cultures. On the other hand, the other strains included in each co-culture affected wine composition because the aromatic composition of mixed-culture wines is significantly different from both the corresponding single-culture wines (Table 2).

A potential metabolic interaction among strains included in mixed-cultures seems to be confirmed by the results of the comparison of mixed-culture and blended wines. During mixed fermentation yeast strains can metabolically interact each other, producing wines characterized by a composition different from the wines obtained by blending single strain wines (Fig. 4a). Therefore, yeasts modify their metabolism during growth in mixed fermentation, where interaction among strains composing mixed starter cultures can determine sharing of some secondary metabolites (Cheraiti et al., 2005; Howell et al., 2006; King et al., 2008). As a consequence, wine flavor deriving from mixed culture fermentations cannot be reproduced by blending mono-culture wines because flavor complexity originates from interactions, largely unknown until now, between strains included in mixed starters.

5. Conclusions

Taking into account that the reproducibility of wine quality is strictly related to starter ability to dominate the fermentation process, our experimental approach allowed to individuate two strains, "A" and "c", as suitable starter candidates for industrial vinification. The strain "A" resulted a good "competitor" not only among natural yeast population from the vineyard, but it was also capable to dominate in mixed fermentations performed at lab scale. By considering that this yeast possesses killer activity, it could be proposed as starter in fermentations in which it is important to assure the total starter dominance on native microbiota. The strain "c", characterized as killer neutral phenotype, was the only able to compete with strain "A"; it could represent a good candidate for fermentations in which the activity of other yeasts is desirable.

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